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2 ## Main Manuscript for

3 **DNA affinity purification sequencing and transcriptional profiling reveals**
4 **new aspects of nitrogen regulation in a filamentous fungus**
5
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45 N.L.G. analyzed data; and L.B.H. and N.L.G. wrote the paper.
46
47 **Supplementary materials for this manuscript include the following:**
48
49 Supplementary Materials and Methods

50 SI Figures S1 to S12
51 SI Tables S1 to S4
52 Legends for Datasets S1 to S4
53 Datasets S1 to S4

ABSTRACT

Sensing available nutrients and efficiently utilizing them is a challenge common to all organisms. The model filamentous fungus *Neurospora crassa* is capable of utilizing a variety of inorganic and organic nitrogen sources. Nitrogen utilization in *N. crassa* is regulated by a network of pathway-specific transcription factors that activate genes necessary to utilize specific nitrogen sources in combination with nitrogen catabolite repression regulatory proteins. We identified an uncharacterized pathway-specific transcription factor, *amn-1*, that is required for utilization of the nonpreferred nitrogen sources proline, branched chain amino acids, and aromatic amino acids. AMN-1 also plays a role in regulating genes involved in responding to the simple sugar mannose, suggesting an integration of nitrogen and carbon metabolism. The utilization of nonpreferred nitrogen sources, which require metabolic processing before being used as a nitrogen source, is also regulated by the nitrogen catabolite regulator NIT-2. Using RNA sequencing combined with DNA affinity purification sequencing, we performed a survey of the role of NIT-2 and the pathway-specific transcription factors NIT-4 and AMN-1 in directly regulating genes involved in nitrogen utilization. Although previous studies suggested promoter binding by both a pathway-specific transcription factor and NIT-2 may be necessary for activation of nitrogen responsive genes, our data show that pathway-specific transcription factors regulate genes involved in the catabolism of specific nitrogen sources, while NIT-2 regulates genes involved in utilization of all nonpreferred nitrogen sources, such as nitrogen transporters. Together, these transcription factors form a nutrient sensing network that allows *N. crassa* cells to regulate nitrogen utilization.

78 **SIGNIFICANCE STATEMENT**

79 Microorganisms have evolved transcriptional networks to prioritize utilization
80 of available nutrient sources. For filamentous fungi, such as *Neurospora crassa*, this
81 entails distinguishing between a variety of organic and inorganic nitrogen sources.
82 Here, we transcriptionally profiled the response of *N. crassa* to a variety of nitrogen
83 sources and used DNA affinity purification sequencing to characterize the role of
84 regulatory genes and their direct downstream targets. We identified a transcription
85 factor responsible for regulating genes involved in amino acid and mannose
86 metabolism. By comparing the genes regulated by transcription factors that
87 regulate specific nitrogen utilization pathways and transcription factors that
88 regulate utilization of all nitrogen sources that require metabolic processing before
89 utilization, we revealed new aspects of the nitrogen regulatory network.

Main text

INTRODUCTION

Sensing available nutrients and efficiently utilizing them is a challenge common to all organisms. In fungi, accurate nutrient sensing is important in the establishment of fungal colonies and in continued, rapid fungal growth for the exploitation of environmental resources. During fungal pathogenesis, mutations in nutrient sensing and utilization pathways cause reduced virulence in plant and human pathogenic fungi (1-3). Saprophytic and plant pathogenic fungi harvest their nutrients from plants, where carbon is abundant. However, nitrogen is often a limiting nutrient source for fungal cells (4).

Fungi are capable of scavenging nitrogen from a variety of sources, including both inorganic and organic nitrogen sources. Utilization of different nitrogen sources requires the activation of a number of different transporters and metabolic enzymes. The fungal cell regulates the production of these proteins to ensure that they are only produced when necessary for nitrogen utilization. The nitrogen sources glutamine, glutamate, and ammonium are preferred in filamentous fungi and are utilized first in a mixture of nitrogen sources (5). Fungi are also capable of utilizing nitrate, nitrite, purines, amides, most amino acids, and proteins as nitrogen sources. Uptake and catabolism of these secondary nitrogen sources is highly regulated and requires the transcription of specific permeases and catabolic enzymes. The repression of these genes when preferred nitrogen sources are present is known as nitrogen catabolite repression (6).

Early work in the model filamentous fungus *Neurospora crassa* identified two genes that are required for nitrogen catabolite repression: *nit-2* (NCU09068) and *nmr* (NCU04158) (Fig. 1). The *nit-2* gene encodes a transcription factor that is

115 required for the expression of genes involved in the utilization of nitrate and a

116 number of amino and nucleic acids (7, 8). NMR represses the activity of NIT-2 when

117 preferred nitrogen sources are available (9-11). Much of the work exploring

118 regulation of this pathway focused on the nitrate reductase gene *nit-3* (NCU05298).

119 Activation of *nit-3* transcription requires two transcription factors: NIT-2 and NIT-4

120 (NCU08294) (12) (Fig. 1). NIT-4 is a pathway-specific transcription factor that

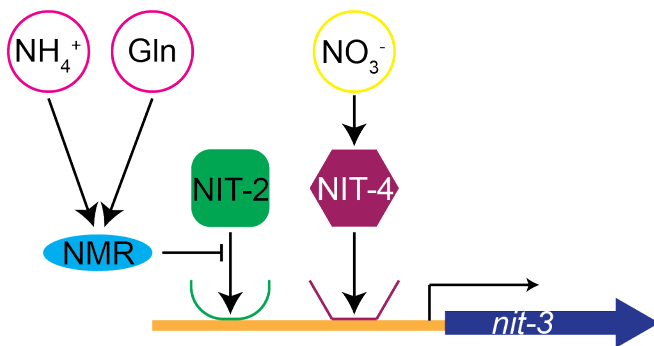
121 specifically activates genes necessary for nitrate utilization (13). In the presence of

122 nitrate and absence of any preferred nitrogen sources, NIT-4 and NIT-2 promote

123 expression of *nit-3* (12). When nitrate is present in combination with a preferred

124 nitrogen source, such as ammonium, NMR represses the activity of NIT-2, and *nit-3*

125 is not expressed (14).



126

127 **Fig. 1. Regulation of *nit-3* expression in *N. crassa*.** Both the transcription

128 factors NIT-2 and NIT-4 are required for *nit-3* expression. In the presence of nitrate,

129 NIT-4 promotes *nit-3* expression. NIT-2 promotes *nit-3* expression in the absence of

130 preferred nitrogen sources, such as ammonium. In the presence of preferred

131 nitrogen sources, NMR represses the activity of NIT-2, and *nit-3* is not expressed (6).

132

133 Utilization of nonpreferred organic nitrogen sources, such as amino and

134 nucleic acids is also thought to be regulated by NIT-2 in concert with pathway-

135 specific transcription factors that activate genes necessary to utilize specific

136 nitrogen sources (6). In *N. crassa* only a small number of these pathway-specific
137 transcription factors have been identified, such as the transcription factor required
138 for purine utilization, *pco-1* (NCU07669) (15). Although NIT-2 works in concert with
139 these pathway-specific transcription factors to promote utilization of nonpreferred
140 nitrogen sources, we do not understand how these transcription factors regulate
141 genes required for nitrogen utilization on a global scale.

142 In nature, *N. crassa* utilizes dead plant material, where carbon and nitrogen
143 sources occur in the matrix of the plant cell wall (16). We hypothesized that the
144 integration of carbon and nitrogen metabolism via nutrient sensing networks must
145 occur for optimal growth on these substrates. By combining RNA sequencing
146 (RNAseq) data from 12 different nitrogen sources with transcriptional profiling of *N.*
147 *crassa* on 40 different carbon sources (17), we identified and characterized a
148 pathway-specific transcription factor, *amn-1* (NCU00445), that is required for the
149 utilization of a number of amino acids, as well as for expression of genes in
150 response to the simple sugar mannose. DNA affinity purification sequencing
151 (DAPseq) (18) of AMN-1, NIT-2, and NIT-4 enabled us to identify the direct targets of
152 these transcription factors. Although previous studies suggested promoter binding
153 by both a pathway-specific transcription factor and NIT-2 may be necessary for
154 activation of nitrogen responsive genes (12, 19), our data suggest a model in which
155 pathway-specific transcription factors activate genes that encode enzymes required
156 for utilization of a specific nitrogen source, while NIT-2 regulates genes that are
157 required for general nitrogen metabolism, such as nitrogen transporters.

158 **RESULTS**

159 ***The zinc binuclear cluster transcription factor NCU00445 regulates genes***
160 ***involved in mannan and mannose utilization***

161 To identify genes involved in nutrient sensing in filamentous fungi, we
162 exposed *N. crassa* to a diverse set of carbon sources and measured the
163 transcriptome using RNAseq (17). Transcription of the conserved zinc binuclear
164 cluster transcription factor NCU00445 was more than 4-fold higher during exposure
165 to 2mM mannose than 2% sucrose and less strongly induced on a number of other
166 hemicellulosic components (17). RNAseq data from wild type cells exposed to
167 mannose showed that the expression of 91 genes was at least 4-fold higher than
168 during exposure to carbon starvation (*SI Appendix*, Fig. S1*A* and Dataset S1) (17). If
169 NCU00445 is a positive regulator of the mannose response, we would expect the
170 expression of these genes to be reduced in cells lacking NCU00445 as compared to
171 wild type cells during exposure to mannose. Indeed, the expression of 83 of these
172 91 genes was at least 4-fold downregulated in ΔNCU00445 cells as compared to
173 wildtype (*SI Appendix*, Fig. S1 and Dataset S1).
174 The transcriptional response of cells lacking NCU00445 during exposure to
175 mannose led us to hypothesize that NCU00445 might play a role in mannose
176 utilization. Mannose-6-phosphate isomerase is the only enzyme necessary for
177 mannose utilization (20, 21). The *N. crassa* genome contains two mannose-6-
178 phosphate isomerase genes, *man-2* (NCU02322) and *man-3* (NCU07165). The
179 expression of *man-2* was reduced 6-fold in ΔNCU00445 cells as compared to
180 wildtype during exposure to mannose; the expression of *man-3* was essentially
181 unchanged (*SI Appendix*, Fig. S2*A*). When wild type and ΔNCU00445 cells were
182 inoculated into media containing mannose as the carbon source, the growth of
183 ΔNCU00445 cells was indistinguishable from that of wild type cells, indicating that
184 NCU00445 was not required for mannose utilization (*SI Appendix*, Fig. S2*B*, S2*C*, and
185 Dataset S2).

186 Mannose is a building block of the complex carbohydrate mannan, which is a
187 component of the fungal cell wall and hemicellulose in the plant cell wall. Although
188 the *N. crassa* genome contains enzymes necessary for mannan degradation, *N.*
189 *crassa* is unable to grow on mannan alone (20, 22). The ability to grow on mannan
190 is enabled by constitutive expression of the transcription factor, CLR-2 (NCU08042),
191 which binds the promoter and regulates the expression of an extracellular endo-β-
192 1,4-mannanase *gh5-7* (NCU08412) and predicted β-mannosidase *gh2-1* (NCU00890)
193 (17, 20, 23). The expression of both *gh5-7* and *gh2-1* was more than 3-fold lower in
194 ΔNCU00445 cells than wild type under mannose conditions. However, unlike with
195 *clr-2*, increasing the expression of NCU00445 by 2.4-fold by placing it under the
196 control of the *gpd-1* (NCU01528) promoter did not allow *N. crassa* cells to grow on
197 mannan (*SI Appendix,* Fig. S2*C*, S2*D*, and Dataset S2).
198 Deletion of NCU00445 caused the expression of 335 genes to be altered by
199 more than 4-fold as compared to wildtype during exposure to mannose (*SI*
200 *Appendix*, Fig. S1 and Dataset S1). However, aside from genes involved in mannose
201 or mannan utilization, only 15 additional carbohydrate active enzymes were
202 regulated by NCU00445 in response to mannose, the majority of which were
203 involved in hemicellulose degradation (Dataset S1).

204 ***NCU00445 is required for utilization of a variety of amino acids as a***
205 ***nitrogen source***

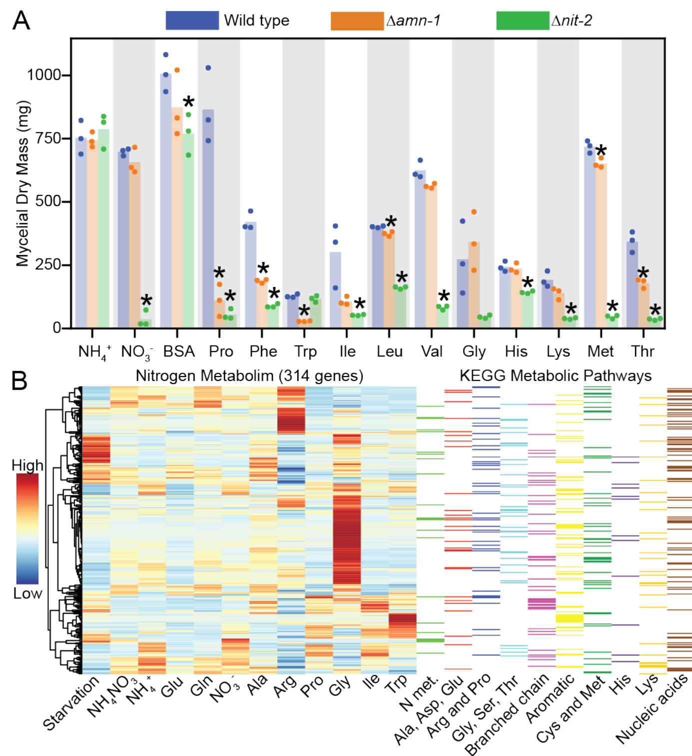
206 To identify direct targets of NCU00445, we performed DAPseq, a technique
207 that uses DNA sequencing to identify target sites in genomic DNA bound by *in vitro*
208 synthesized transcription factors (18). Using DAPseq, we identified 296 NCU00445
209 DNA binding sites within 3000 base pairs upstream of a translational start site of
210 290 genes (Dataset S3). Surprisingly, none of the promoters of genes involved in

211 mannose or mannan utilization were bound by NCU00445. Additionally, KEGG
212 categories associated with carbon metabolism were not enriched in the set of 290
213 genes whose promoters were bound by NCU00445 (*SI Appendix*, Fig. S3*A* and
214 Dataset S3).

215 To identify cellular processes directly regulated by NCU00445, we used
216 functional enrichment analysis (24). Of the 335 genes that were at least 4-fold
217 differentially expressed between wild type and ΔNCU00445 cells during exposure to
218 mannose, 40 had promoters directly bound by NCU00445 (*SI Appendix*, Fig. S4 and
219 Dataset S3). NCU00445 also bound its own promoter. Because we used
220 transcriptional profiling of cells lacking NCU00445 to identify genes that were
221 directly regulated by NCU00445, we were unable to conclusively determine whether
222 NCU00445 regulated its own expression; we included NCU00445 in our list of
223 NCU00445 direct targets. Functional enrichment analysis of the entire set of 335
224 genes (plus NCU00445 itself) showed enrichment for genes involved in valine,
225 leucine, and isoleucine degradation and biosynthesis, alanine, aspartate, and
226 glutamate metabolism, tryptophan metabolism and degradation of aromatic
227 compounds, pentose and glucuronate interconversions, and biosynthesis of
228 secondary metabolites (*SI Appendix*, Fig. S3*B*). Among the 41 genes whose
229 promoters were bound by NCU00445, we observed an enrichment for valine,
230 leucine, and isoleucine degradation and biosynthesis, tyrosine metabolism, and
231 thiamine metabolism (*SI Appendix*, Fig. S3*C*). Because mannose may not be the
232 only condition in which NCU00445 plays a role, we investigated the KEGG pathways
233 that were enriched amongst all 290 genes whose promoters were bound by
234 NCU00445 and found enrichment for branched chain amino acid degradation,
235 tyrosine, phenylalanine, cysteine, methionine, and thiamine metabolism, and

236 ubiquinone and other terpenoid-quinone biosynthesis (*SI Appendix*, Fig. S3*A*). These
237 data indicated a potential role for NCU00445 in directly regulating amino acid
238 metabolism.

239 Since the NCU00445 mutant grew without amino acid supplements (*SI*
240 *Appendix*, Fig. S2*B*, S2*C*, and Dataset S2), we hypothesized that NCU00445 may be
241 required for utilization of amino acids as a nitrogen source. To test this hypothesis,
242 we grew wild type and ΔNCU00445 cells on media containing individual amino acids
243 as the sole nitrogen source. As a control we included Δ*nit-2* cells, which are not able
244 to utilize many amino acids as a nitrogen source, since wild type cells are not able
245 to utilize every nitrogen source equally well (8) (Fig. 2*A*, *SI Appendix*, Fig. S5*A*, S5*B*,
246 and Dataset S2). Biomass measurements of the NCU00445 deletion mutant in 3 ml
247 and 100ml cultures showed ΔNCU00445 cells had growth defects on proline,
248 phenylalanine, tryptophan, isoleucine, leucine, and threonine (Fig. 2*A*, *SI Appendix*,
249 Fig. S5*A*, S5*B*, and Dataset S2). Expressing NCU00445 from the *gpd-1* promoter in
250 cells lacking NCU00445, mitigated growth defects on proline, isoleucine, and
251 tryptophan (*SI Appendix*, Fig. S2*D*, S5*B*, and Dataset S2). Because NCU00445 was
252 required for amino acid catabolism, we named NCU00445 *amn-1* for “amino acid
253 utilization-1”.



254

255 **Fig. 2. AMN-1 is required for utilization of a variety of amino acids. A.**
256 Mycelial dry weight of wild type, Δ*amn-1*, and Δ*nit-2* cells inoculated into 100ml of
257 media containing the indicated nitrogen source. (The concentration of all nitrogen
258 sources was 50mM, except for 1% BSA.) Asterisks indicate mycelial dry weights that
259 are statistically significantly different than that of wild type cells, $*p_{adj}<0.05$. **B.**
260 Hierarchical clustering of the expression level of the set of genes in a KEGG
261 pathway associated with nitrogen metabolism in wild type cells exposed to the
262 indicated nutrient condition (Table S1) (21). Genes in a particular KEGG pathway are
263 indicated with colored bars from left to right: nitrogen metabolism (light green);
264 alanine, aspartic acid, glutamic acid metabolism (red); arginine and proline
265 metabolism (dark blue); glycine, serine, and threonine metabolism (cyan); branched
266 chain amino acid metabolism (magenta); aromatic amino acid metabolism (yellow);
267 cysteine and methionine metabolism (dark green); histidine metabolism (purple);
268 lysine metabolism (orange); and nucleic acid metabolism (brown).

269

270 ***AMN-1 regulates genes encoding enzymes required for the catabolism of***
271 ***amino acids***

272 To further investigate the role of AMN-1 in amino acid utilization, we first
273 transcriptionally profiled the response of wild type cells to a panel of 12 nitrogen
274 conditions to identify genes involved in the utilization of amino acids. These
275 nitrogen conditions included: nitrogen starvation, ammonium nitrate, preferred
276 nitrogen sources (ammonium, glutamate, and glutamine), and non-preferred
277 nitrogen sources (nitrate, alanine, arginine, proline, glycine, isoleucine, and
278 tryptophan) covering a range of metabolic pathways (21).

279 Analysis of the expression of genes involved in nitrogen metabolism and the
280 metabolism of amino acids across the nitrogen panel revealed that many genes
281 involved in the metabolism of a specific nitrogen source were upregulated on that
282 nitrogen source (Fig. 2*B* and Dataset S1). For example, the expression of the nitrate
283 reductase *nit-3*, the nitrite reductase *nit-6* (NCU04720), the nitrate transporter *nit-*
284 *10* (NCU07205), and the FMN-dependent 2-nitropropane dioxygenase *npd-1*
285 (NCU03949) all showed increased expression levels on nitrate. Similarly, the
286 expression of genes involved in tryptophan and quinate metabolism, such as the
287 kynureninase gene *kyn-1* (NCU09183) and the indoleamine 2,3-dioxygenase genes
288 *iad-1* (NCU09184) and *iad-2* (NCU01402), was highest on tryptophan (Dataset S1).

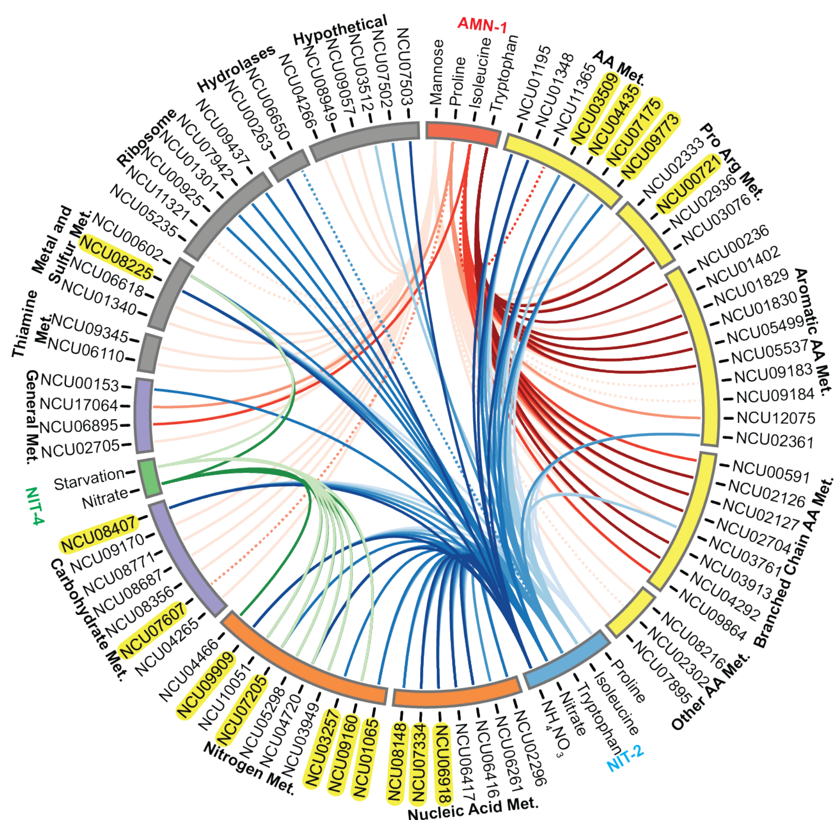
289 Exposure to glycine resulted in the upregulation of a large number of genes.
290 These included genes involved in glycine metabolism, such as *gly-3* (NCU02727),
291 *gyd-1* (NCU02475), *oxD* (NCU06558), and NCU03761, but also genes involved in the
292 metabolism of a variety of other amino acids (Fig. 2*B* and Dataset S1) (25). This
293 result may indicate that since cells rarely see glycine in isolation from other amino

294 acids, the presence of glycine activated expression of genes involved in a variety of
295 amino acid utilization pathways. We asked whether genes from a variety of
296 metabolic pathways were expressed during exposure to other amino acids. Indeed,
297 when the expression pattern of genes in KEGG metabolic pathways was analyzed, it
298 became clear that genes in a KEGG pathway associated with the metabolism of a
299 particular amino acid were not specifically upregulated in response to that nutrient
300 (Fig. 2*B*). Additionally, hierarchical clustering of gene expression across our panel of
301 nitrogen sources did not show discrete clusters of genes in a particular KEGG
302 pathway (Fig. 2*B*).

303 To identify genes specifically regulated by AMN-1, we exposed the Δ*amn-1*
304 mutant to media containing proline and the aromatic and branched chain amino
305 acids for which Δ*amn-1* cells showed the most severe growth defects, tryptophan
306 and isoleucine, respectively (Fig. 2*A*. *SI Appendix*, Fig. S5*A*, S5*B*, and Dataset S2).
307 To control for growth defects of Δ*amn-1* cells under these conditions, we grew wild
308 type and Δ*amn-1* cells in media containing ammonium nitrate as the nitrogen
309 source, washed the mycelial mass in media lacking a nitrogen source, and
310 transferred the mycelial cell mass into media containing the experimental nitrogen
311 source for 4h prior to harvesting for RNAseq.

312 We first investigated mechanisms of AMN-1-mediated regulation of proline
313 catabolism. Functional enrichment analysis of the 57 genes that were at least 4-fold
314 differentially expressed between Δ*amn-1* and wild type cells during exposure to
315 proline showed significant enrichment for valine, leucine, and isoleucine
316 degradation, β-alanine metabolism, tyrosine metabolism, phenylalanine
317 metabolism, and glycerolipid metabolism (*SI Appendix*, Fig. S6*A* and Dataset S1).
318 Eight of the 57 genes had promoters bound by AMN-1, including the homogentisate

319 1,2-dioxygenase NCU05499 and the dimethylallyl tryptophan synthase NCU12075,
320 both predicted to be involved in aromatic amino acid metabolism, and the
321 promoters of three genes predicted to be involved in branched chain amino acid
322 metabolism: the branched-chain α-keto acid dehydrogenase E2 component
323 NCU02704, the isovaleryl-CoA dehydrogenase NCU02126, and the methylcrotonoyl-
324 CoA carboxylase β subunit *mcc-2* (NCU02127) (Fig. 3, 4*A*, *SI Appendix*, Fig. S4, S5*C*,
325 and Dataset S1).



326

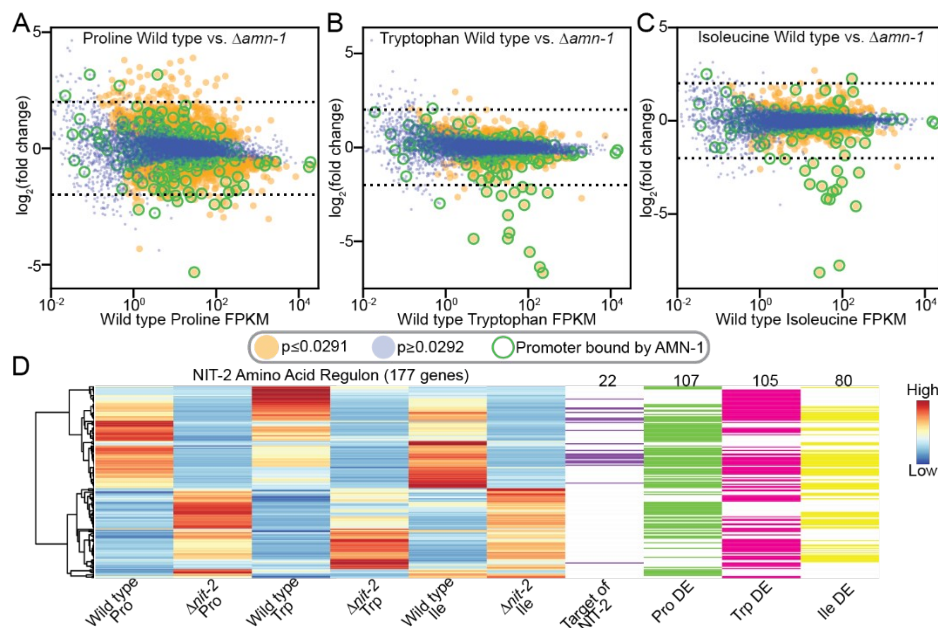
327 **Fig. 3. NIT-2 regulates transporters and genes associated with general**
328 **nitrogen metabolism while pathway specific transcription factors activate**
329 **genes that are required for utilization of a specific nitrogen source.** Plot
330 built with Circos, version 0.69 (26) to display the regulation of genes whose
331 promoters were bound and whose expression was regulated by AMN-1, NIT-2, or
332 NIT-4 when cells were exposed to the indicated conditions. Regulated genes were at

333 least 4-fold differentially expressed between wild type and Δ*amn-1*, Δ*nit-2*, or Δ*nit-4*

334 cells. Genes encoding transporters are highlighted in yellow. Solid lines indicate

335 genes that were activated by AMN-1, NIT-2, or NIT-4. Dotted lines indicate genes

336 that were repressed by AMN-1, NIT-2, or NIT-4.



337

338 **Fig. 4. Regulation of amino acid utilization by AMN-1 and NIT-2 occurs**

339 **through different genetic pathways. A-C.** Differential expression analysis of

340 Δ*amn-1* relative to wild type cells after a shift to (**A**) 50mM proline, (**B**) 50mM

341 tryptophan, and (**C**) 50mM isoleucine. Green circles indicate genes whose

342 promoters were bound by AMN-1. Dotted lines indicate 4-fold change in expression.

343 **D.** Heatmap of the expression level of the 176 genes that were at least 4-fold

344 differentially expressed between wild type and Δ*nit-2* cells exposed to 50mM

345 proline, 50mM isoleucine, or 50mM tryptophan plus *nit-2* itself. Purple bars indicate

346 genes whose promoters were bound by NIT-2. Green bars indicate genes that were

347 differentially expressed between wild type and Δ*nit-2* cells exposed to proline plus

348 *nit-2*. Pink bars indicate genes that were differentially expressed between wild type

349 and Δ*nit-2* cells exposed to tryptophan plus *nit-2*. Yellow bars indicate genes that

350 were differentially expressed between wild type and Δ*nit-2* cells exposed to
351 isoleucine plus *nit-2*. Numbers above each column indicate the total number of
352 genes in each category.
353
354 The remaining genes whose promoters were bound by AMN-1 included the
355 proline oxidase *pro-7* (NCU02936), which is predicted to catalyze the first step in
356 proline degradation. *S. cerevisiae* cells lacking the *pro-7* ortholog, *PUT1* are unable
357 to utilize proline as a nitrogen source (27). In *N. crassa*, cells lacking *pro-7* showed a
358 severe growth defect when provided proline as the sole nitrogen source (*SI*
359 *Appendix*, Fig. S7*A*, S7*B*, and Dataset S2). We hypothesized that the reduced
360 expression of *pro-7* in the *amn-1* deletion mutant might be at least partially
361 responsible for the inability of Δ*amn-1* cells to utilize proline. To test this, we asked
362 whether expression of *pro-7* under the regulation of the constitutive *gpd-1* promoter
363 restored growth in cells lacking *amn-1* (*SI Appendix*, Fig. S7*C* and Dataset S2).
364 Indeed, P_{gpd-1}-*pro-7* Δ*amn-1* cells grew significantly better than the Δ*amn-1* mutant
365 when proline was provided as the sole nitrogen source, indicating that reduced
366 expression of *pro-7* in cells lacking *amn-1* was at least partially responsible for the
367 inability of Δ*amn-1* cells to utilize proline (*SI Appendix*, Fig. S7*A*, S7*B*, and Dataset
368 S2).
369 Although cells lacking *amn-1* had a severe growth defect on media containing
370 tryptophan as the sole nitrogen source, only 16 genes were at least 4-fold
371 differentially expressed in Δ*amn-1* cells as compared to wildtype (Fig. 4*B*, *SI*
372 *Appendix*, Fig. S5*C*, and Dataset S1). Of these 16 genes, the promoters of 12 were
373 bound by AMN-1 (Fig. 3, 4*B*, *SI Appendix*, Fig. S4, and S5*C*). Five of the genes whose
374 promoters were bound by AMN-1 and whose expression was regulated by AMN-1 in

375 response to tryptophan were predicted to be involved in aromatic amino acid
376 metabolism: the flavoprotein oxygenase *fpo-1* (NCU00236), the
377 fumarylacetoacetase *fah-1* (NCU05537), the homogentisate 1,2-dioxygenase
378 NCU05499, the 4-hydroxyphenylpyruvate dioxygenase *hpd-1* (NCU01830), and a
379 hypothetical protein NCU01829 predicted to be involved in tyrosine catabolism. *N.*
380 *crassa* cells lacking *fah-1* had a slight growth defect on tryptophan (*SI Appendix*,
381 Fig. S7*D*, S7*E*, and Dataset S2). However, deletion of NCU05499 did not significantly
382 affect growth on tryptophan, suggesting that the mechanism by which AMN-1
383 regulated tryptophan metabolism may be through a complex combination of genes
384 (*SI Appendix*, Fig. S7*D*, S7*E*, and Dataset S2). The remaining genes whose
385 promoters were bound by AMN-1 and regulated by AMN-1 in response to tryptophan
386 were involved in either proline metabolism: *pro-7* and the pyrroline 5-carboxylate
387 dehydrogenase *pcd-2* (NCU03076); or branched chain amino acid metabolism: the
388 branched chain amino acid aminotransferase *val-1* (NCU04292), the 2-
389 oxoisovalerate dehydrogenase β subunit *ovd-2* (NCU03913), *mcc-2*, NCU02704, and
390 NCU02126 (Fig. 3, 4*B*, *SI Appendix*, Fig. S4, S5*C*, and Dataset S1).
391 Cells lacking *amn-1* also had a growth defect when branched chain amino
392 acids were provided as the nitrogen source (Fig. 2*A*, *SI Appendix*, Fig. S5*A*, S5*B*, and
393 Dataset S2). The expression of 17 genes was at least 4-fold differentially expressed
394 between wild type and Δ*amn-1* cells during exposure to media containing isoleucine
395 as the nitrogen source, and the promoters of 14 of these genes were bound by
396 AMN-1 (Fig. 3, 4*C*, *SI Appendix*, Fig. S4, S5*C*, and Dataset S1). Six of these 14 genes
397 play a role in branched chain amino acid metabolism, including the 2-oxoisovalerate
398 dehydrogenase α and β subunits *ovd-1* (NCU09864) and *ovd-2*; the methylcrotonoyl-
399 CoA carboxylase α and β subunits *mcc-1* (NCU00591) and *mcc-2*; NCU02704; and

400 NCU02126. Additionally, both *pro-7* and *pcd-2*, necessary for proline catabolism,
401 and *fah-1*, *hpd-1*, NCU05499, and NCU01829, involved in aromatic amino acid
402 metabolism were regulated by AMN-1 in response to isoleucine and had promoters
403 bound by AMN-1 (Fig. 3, 4*C*, *SI Appendix*, Fig. S4, S5*C*, and Dataset S1).
404 To comprehensively identify genes regulated directly by AMN-1, we cross
405 referenced the 290 genes with AMN-1 binding sites in the promoter region identified
406 by DAPseq, with genes that were differentially expressed by at least 4-fold across
407 our RNAseq experiments. Although it is likely that AMN-1 regulates additional genes
408 during exposure to conditions or at time points we did not test by RNAseq, we
409 identified 43 genes that were regulated by AMN-1 during exposure to mannose,
410 proline, tryptophan, or isoleucine and whose promoters were bound by AMN-1 (Fig.
411 3, *SI Appendix*, Fig. S4, and Dataset S3). Supporting our hypothesis that AMN-1 is
412 required for utilization of a variety amino acids, functional analysis of these 43
413 genes (plus *amn-1* itself) showed enrichment for genes involved in valine, leucine,
414 and isoleucine degradation and biosynthesis, tyrosine metabolism, and thiamine
415 metabolism (*SI Appendix*, Fig. S3*C*). Using the 45 promoter binding sites found in
416 these 44 genes, we identified a consensus binding motif KCGGYTWKYRKCGGCHWW
417 for AMN-1 (*SI Appendix*, Fig. S8*A*). This motif provided additional specificity to the
418 NNCGGNNNNN motif identified in a broad survey of transcription factor binding
419 motifs (28). As expected, AMN-1 only bound a small subset of the 2189 locations
420 where the KCGGYTWKYRKCGGCHWW motif was identified in *N. crassa* promoters
421 (Dataset S4) (29).

422 ***The nitrogen catabolite repressor NIT-2 regulates genes necessary for***
423 ***nitrogen import and enzymes responsible for amino acid utilization***

424 NIT-2 is a conserved GATA-type transcription factor responsible for regulating
425 genes involved in utilizing nonpreferred nitrogen sources in filamentous fungi (6).
426 Although initially identified for its role in promoting nitrate utilization in *N. crassa*,
427 early studies also found NIT-2 is necessary for growth when a variety of amino and
428 nucleic acids are provided as the nitrogen source (8). Unlike cells lacking *nit-2*, the
429 Δ*amn-1* mutant exhibited normal growth when nitrate was provided as the nitrogen
430 source (Fig. 2*A* and Dataset S2). Similarly, *amn-1* is not required for growth on
431 nucleic acids (*SI Appendix*, Fig. S5*A*, S5*B*, and Dataset S2). When exposed to amino
432 acids, there was significant overlap in the substrates that caused growth defects of
433 Δ*amn-1* cells as compared to Δ*nit-2* cells but also a number of notable differences.
434 In particular, Δ*nit-2* cells had a more severe growth phenotype than Δ*amn-1* cells
435 during exposure to glutamic acid, glycine, isoleucine, leucine, valine, phenylalanine,
436 lysine, methionine, serine, and threonine while the reverse was true for tryptophan
437 ($p_{adj}<0.05$) (Fig. 2*A*, *SI Appendix*, Fig. S5*A*, S5*B*, and Dataset S2). These data
438 indicated that NIT-2 has a broader role in nitrogen metabolism than AMN-1.
439 Because of the expanded role of NIT-2 relative to AMN-1 with respect to
440 nitrogen metabolism, we hypothesized that AMN-1 and NIT-2 may not act through
441 the same pathway to regulate nitrogen metabolism. To test this hypothesis, we
442 performed RNAseq on cells lacking *nit-2* under the same nitrogen conditions that we
443 tested Δ*amn-1* cells (proline, tryptophan, and isoleucine), as well as exposure to
444 nitrate and ammonium nitrate. To complement these transcriptional profiling
445 experiments, we also performed DAPseq on NIT-2 to identify genes whose
446 promoters were bound by NIT-2. Using DAPseq, we identified 354 NIT-2 binding sites
447 in the putative promoter regions of 344 genes (Dataset S3). By comparing these
448 344 genes to the differentially expressed genes (at least 4-fold) between wild type

449 and Δ*nit-2* cells during exposure to nitrate, ammonium nitrate, proline, tryptophan,
450 or isoleucine, we identified the direct regulation of 33 of these genes by NIT-2 (Fig.
451 3, Datasets S1, and S3).

452 The expression of 176 genes differed by at least 4-fold in wildtype as
453 compared to Δ*nit-2* cells during exposure to media containing proline, tryptophan,
454 or isoleucine as the nitrogen source (Fig. 4*D*, *SI Appendix*, Fig. S9*A-C*, and Dataset
455 S1). Functional analysis of these 176 genes showed enrichment for genes involved
456 in the metabolism of a number of amino acids, including glycine, serine, threonine,
457 tyrosine, phenylalanine, arginine, proline, and β-alanine. We also observed an
458 enrichment for glyoxylate and dicarboxylate metabolism as well as for carbon and
459 nitrogen metabolism generally (*SI Appendix*, Fig. S6*C*). Of these 176 genes, the
460 promoters of 22 were bound by NIT-2 (Fig. 3, 4*D*, *SI Appendix*, Fig. S9*A-C*, Datasets
461 S1, and S3). Fully half of these 22 genes encoded transporters, suggesting that NIT-
462 2 may regulate nitrogen metabolism primarily by regulating import of nitrogen
463 substrates (Fig. 3, *SI Appendix*, Fig. S9*A-C*, and Dataset S1).

464 To further explore these data, we first analyzed the 13 genes directly
465 regulated by NIT-2 in response to proline, tryptophan, and isoleucine. Consistent
466 with the role of NIT-2 functioning as a transcriptional activator (6), the expression of
467 all 13 genes was downregulated in the Δ*nit-2* mutant as compared to wildtype (Fig.
468 3, 4D, and Dataset S1). Nine of these 13 genes encoded for transporters, including
469 the general amino acid permease *aap-22* (NCU04435) and the oligopeptide
470 transporter *opt-1* (NCU09773) (Fig. 3 and Dataset S1). The expression of both of
471 these transporters was reduced by more than 80-fold to an FPKM of less than 1 in
472 cells lacking *nit-2* as compared to wild type cells when exposed to proline,
473 tryptophan, or isoleucine (Dataset S1). This dramatic reduction in expression may at

474 least partially explain the inability of Δ*nit-2* cells to grow on many amino acids as
475 the sole nitrogen source. Previously posited to be regulated by NIT-2 (30, 31), the
476 uracil permease *uc-5* (NCU07334) promoter was bound by and transcription of *uc-5*
477 was activated by NIT-2. NIT-2 also directly regulated the purine permease *pup-2*
478 (NCU06918) and the H+/nucleoside cotransporter NCU08148 (Fig. 3 and Dataset
479 S1). The regulation of these three nucleotide transporters may at least partially
480 explain the role of NIT-2 in utilizing nucleotides as a nitrogen source (6). Urea
481 uptake is also under the control of nitrogen catabolite repression (32); the urea
482 transporter *urt* (NCU09909) was directly regulated by NIT-2 (Fig. 3 and Dataset S1).
483 Consistent with its role in regulating the utilization of nonpreferred nitrogen sources
484 in the presence of preferred nitrogen sources such as ammonium (6), the
485 ammonium transporter *tam-4* (NCU01065) was also directly regulated by NIT-2.
486 Additionally, NIT-2 regulated the expression and bound the promoters of the high
487 affinity nickel transporter *trm-34* (NCU08225) and the MFS transporter NCU08407
488 (Fig. 3 and Dataset S1).

489 Four other genes downregulated in Δ*nit-2* cells as compared to wild type cells
490 during exposure to proline, tryptophan, and isoleucine and whose promoters were
491 bound by NIT-2 encoded enzymes important for amino and nucleic acid
492 degradation. Unlike AMN-1, which mainly regulated the expression of enzymes
493 important for utilizing a particular amino acid or class of amino acids, NIT-2
494 regulated the transcription of genes that encoded enzymes involved in more
495 general metabolic pathways (Fig. 3). These included the agmatinase *put-3*
496 (NCU01348), an enzyme involved in the catabolism of amino groups, and the
497 formamidase *fma-1* (NCU02361), which participates in glyoxylate and dicarboxylate
498 metabolism and may play a role in tryptophan metabolism. NIT-2 also directly

499 regulated two genes involved in nucleic acid metabolism: the thymine dioxygenase
500 *uc-3* (NCU06416) and the uracil-5-carboxylate decarboxylase *uc-7* (NCU06417) (33,
501 34) (Fig. 3 and Dataset S1).

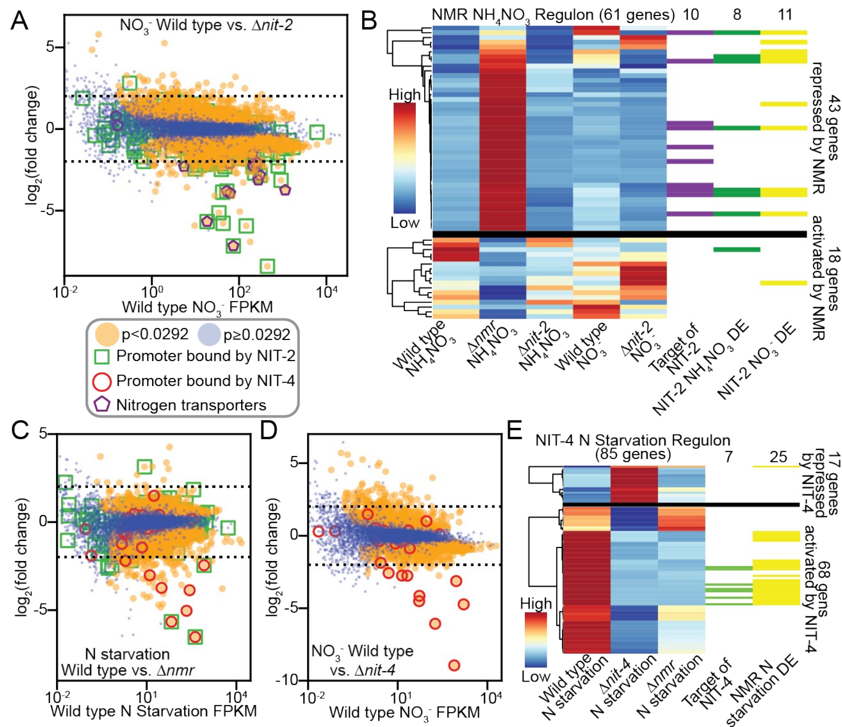
502 Nine genes whose promoters were bound by NIT-2 were differentially
503 expressed in a Δ*nit-2* mutant as compared to wildtype when exposed to one or two
504 amino acids as nitrogen sources (Fig. 3, 4*D*, and Dataset S1). Several of these
505 genes play a general role in amino acid utilization, including the general amino acid
506 permease *pmg* (NCU03509), the APC amino acid permease *aap-9* (NCU07175) (35),
507 and the Glu/Leu/Phe/Val dehydrogenase *am* (NCU01195) (32, 36, 37). Two genes
508 played a role in purine catabolism: the allantoinase *aln-1* (NCU02296) and the uracil
509 phosphoribosyltransferase *uc-8* (NCU06261) (38). Taken together these data
510 indicated that NIT-2 primarily functions to regulate genes, such as transporters, that
511 have a general effect on the metabolism of a variety of amino acids rather than
512 regulating genes directly involved in the utilization of specific amino acids.

513 ***NIT-2 regulation in response to nitrate***

514 A large number of genes that did not have a direct connection to amino acid
515 catabolism were directly regulated by NIT-2 in response to amino acids (Fig. 3 and
516 Dataset S1). As NIT-2 regulates the utilization of nonpreferred inorganic nitrogen
517 sources, such as nitrate (6), we hypothesized that the expression of these genes
518 may also be activated by NIT-2 when cells are exposed to nitrate.

519 To test this hypothesis, we transferred wild type and Δ*nit-2* cells to media
520 containing nitrate and did RNAseq to measure global gene expression. The
521 expression of 113 genes differed by at least 4-fold between wild type and Δ*nit-2*
522 cells 4h post-shift to nitrate (Fig. 5*A*, *SI Appendix*, Fig. S9*D*, and Dataset S1).
523 Functional analysis of these genes showed an enrichment for genes involved in

524 nitrogen metabolism and ribosome biogenesis (*SI Appendix*, Fig. S6*D*). Over 40% of
525 the genes regulated by NIT-2 during exposure to nitrate were also regulated by NIT-
526 2 during exposure to proline, tryptophan, or isoleucine, including 13 genes that
527 encoded for transporters (Dataset S1). To distinguish between genes that NIT-2
528 directly, as opposed to indirectly, regulated in response to nitrate, we compared the
529 genes that were differentially expressed between wild type cells and cells lacking
530 *nit-2* during exposure to nitrate with genes whose promoters were bound by NIT-2.
531 We identified 22 genes directly regulated by NIT-2 in response to nitrate, all of
532 which were downregulated in Δ*nit-2* cells as compared to wildtype (Fig. 3, 5*A*, *SI*
533 *Appendix*, Fig. S9*D*, Datasets S1, and S3). These 22 genes included 12 of the 13
534 genes that were differentially expressed in Δ*nit-2* cells in response to proline,
535 tryptophan, and isoleucine as compared to wild type cells and whose promoters
536 were bound by NIT-2, with only *fma-1* not represented. The promoter of an
537 additional gene, *aln-1* was bound by NIT-2, and *aln-1* was differentially expressed
538 during exposure to nitrate, isoleucine, and tryptophan in Δ*nit-2* as compared to wild
539 type cells (Fig. 3, Datasets S1, and S3).



540

541 **Fig. 5. NMR and NIT-4 activate genes during starvation conditions. A.**

542 Differential expression analysis of Δ*nit-2* relative to wild type cells after a shift to

543 50mM nitrate. Green squares indicate genes whose promoters are bound by NIT-2.

544 Purple pentagons indicate genes predicted to encode nitrogen transporters. Dotted

545 lines indicate 4-fold change in expression. **B.** Heatmap of the expression level of the

546 60 genes that were at least 4-fold differentially expressed between wild type and

547 Δ*nmr* cells exposed to 25mM ammonium nitrate plus *nmr* itself. Upper heatmap

548 includes genes repressed by NMR. Lower heatmap includes genes activated by NMR

549 plus *nmr* itself. Purple bars indicate genes whose promoters were bound by NIT-2.

550 Green bars indicate genes that were differentially expressed between Δ*nit-2* and

551 wild type cells exposed to 25mM ammonium nitrate. Yellow bars indicate genes that

552 were differentially expressed between Δ*nit-2* and wild type cells exposed to 50mM

553 nitrate. Numbers above each column indicate the total number of genes in each

554 category. **C.** Differential expression analysis of Δ*nmr* relative to wild type cells after

555 a shift to nitrogen starvation. Green squares indicate genes whose promoters were
556 bound by NIT-2. Red circles indicate genes whose promoters were bound by NIT-4.
557 Dotted lines indicate 4-fold change in expression. **D.** Differential expression analysis
558 of Δ*nit-4* relative to wild type cells after a shift to 50mM nitrate. Red circles indicate
559 genes whose promoters were bound by NIT-4. Dotted lines indicate 4-fold change in
560 expression. **E.** Heatmap of the expression level of the set of genes that were at
561 least 4-fold differentially expressed between wild type and Δ*nit-4* cells exposed to
562 nitrogen starvation. Upper heatmap includes genes repressed by NIT-4. Lower
563 heatmap includes genes activated by NIT-4. Green bars indicate genes whose
564 promoters were bound by NIT-4. Yellow bars indicate genes differentially expressed
565 between Δ*nmr* and wild type cells exposed to nitrogen starvation. Numbers above
566 each column indicate the total number of genes in each category.
567
568 We investigated the 9 genes whose promoters were bound by NIT-2 and
569 whose expression was regulated by NIT-2 in response to nitrate but not proline,
570 tryptophan, or isoleucine. Two of these genes were specifically involved in nitrate
571 utilization: the nitrite reductase *nit-6* and the nitrate transporter *nit-10* (6). The
572 promoter of the ammonium transporter *tam-1* (NCU03257) was also bound by NIT-
573 2, and *tam-1* expression was regulated by NIT-2 during exposure to nitrate. The
574 majority of the remaining genes were involved in functions relating to the ribosome
575 (Fig. 3 and Dataset S1). To our surprise, although previous studies identified NIT-2
576 binding sites in the *nit-3* promoter, we did not find NIT-2 bound to the *nit-3*
577 promoter using DAPseq (12, 39). Since DAPseq is an *in vitro* method of identifying
578 transcription factor binding sites, this may suggest that DNA modification or
579 additional proteins are required to facilitate NIT-2 binding in some promoter regions.

580 Using the 38 NIT-2 binding sites in the promoters of these 33 directly
581 regulated genes, we identified a consensus binding motif for NIT-2 (HGATAAGV) that
582 had similarity to the binding motif identified in a broad survey of eukaryotic
583 transcription factors (NNGATHNN) as well as other GATA transcription factor motifs,
584 including the binding motif of the NIT-2 *S. cerevisiae* homolog Gln3 (28, 40-42) (*SI*
585 *Appendix*, Fig. S8*B*). Prior work investigating the nucleotide sequence of the NIT-2
586 binding site in specific promoters, rather than globally, suggested a highly similar
587 binding motif (43-45). This motif was present 804 times in the promoter regions of
588 *N. crassa* genes, indicating that NIT-2 did not bind to every HGATAAGV motif in the
589 genome (Dataset S4).

590 ***The transcriptional repressor NMR acts as an activator under nitrogen***
591 ***starvation conditions***

592 Regulation of nitrate utilization is accomplished through the interactions of
593 NIT-2, NIT-4 and NMR (6) (Fig. 1). Previous work characterizing the regulation of the
594 nitrate reductase gene *nit-3* showed that NIT-4 promotes expression of *nit-3* in the
595 presence of nitrate (46). If nitrate is present in the absence of a preferred nitrogen
596 source, NIT-2 also promotes expression of *nit-3*. However, when preferred nitrogen
597 sources are present, the activity of NIT-2 is repressed by NMR (11), and *nit-3* is not
598 expressed (6) (Fig. 1).

599 Our data showed that the expression of three genes required for nitrate
600 utilization, *nit-3*, the nitrite reductase gene *nit-6*, and the nitrate transporter *nit-10*
601 was negligible in wild type cells exposed to media containing the preferred nitrogen
602 source ammonium (*SI Appendix*, Fig. S10*A* and Dataset S1). Exposure of wild type
603 cells to media containing nitrate increased expression of *nit-3*, *nit-6*, and *nit-10* by
604 over 6-fold. Deletion of either *nit-2* or *nit-4* resulted in a more than 4-fold drop in

605 expression of *nit-3*, *nit-6*, and *nit-10* under nitrate conditions. Upon exposure to
606 media containing both ammonium and nitrate, expression of *nit-3*, *nit-6*, and *nit-10*
607 dropped by nearly 2-fold as compared to exposure to nitrate alone in wild type cells
608 in an NMR-dependent fashion (*SI Appendix*, Fig. S10*A* and Dataset S1). These data
609 broadly supported the model of nitrate utilization developed using classical genetics
610 (Fig. 1).

611 To investigate the role of the nitrogen repressor, NMR, we exposed wild type
612 cells and cells lacking *nmr* to media containing both nitrate and ammonium. The
613 expression of 60 genes was at least 4-fold differentially expressed between wild
614 type and Δ*nmr* cells (Fig. 5*B*, *SI Appendix*, Fig. S10*B*, and Dataset S1). Functional
615 analysis showed an enrichment for genes involved in the metabolism of a number of
616 amino acids, including arginine, proline, glycine, serine, threonine, phenylalanine,
617 tyrosine, alanine, aspartate, glutamate, β-alanine, and cyanoamino acids (*SI*
618 *Appendix*, Fig. S10C). As expected, all 10 genes bound by NIT-2 and differentially
619 expressed between wild type and Δ*nmr* cells during exposure to ammonium nitrate
620 were more highly expressed in Δ*nmr* cells, providing confirmation of the role of NMR
621 in repressing NIT-2 activity (Fig. 5*B*, *SI Appendix*, Fig. S10*B*, and Dataset S1).
622 Although NMR is canonically only thought to act through NIT-2, only 11 genes were
623 at least 4-fold differentially expressed between both wild type and Δ*nmr* cells
624 exposed to ammonium nitrate and wild type and Δ*nit-2* cells exposed to nitrate,
625 suggesting that NMR may have another role in transcriptional regulation (Fig. 5*B*
626 and Dataset S1). For example, while many of the 60 genes regulated by NMR were
627 predicted to play a role in nitrogen metabolism, there were also a significant
628 number of genes involved in carbon metabolism, including genes encoding
629 carbohydrate active enzymes and sugar transporters (Dataset S1). These data

630 suggested a role for NMR in regulating carbon utilization as well as nitrogen
631 utilization.

632 Our data supported the role of NMR as a transcriptional repressor. Of the 60
633 genes that were differentially expressed by at least 4-fold between wild type and
634 Δ*nmr* cells, 43 were more highly expressed in cells lacking *nmr* (Fig. 5*B SI Appendix*,
635 Fig. S10*B*, and Dataset S1). However, the carbon catabolite repressor *cre-1*
636 (NCU08807) acts as a repressor when preferred carbon sources are available but as
637 an activator during carbon starvation (47). We hypothesized that *nmr* might
638 similarly function as an activator during nitrogen starvation. To test this hypothesis,
639 we performed RNAseq on wild type and Δ*nmr* cells during exposure to media lacking
640 a nitrogen source. Seventy genes were differentially expressed (at least 4-fold)
641 between wild type and Δ*nmr* cells, and of these, 60 were downregulated in cells
642 lacking *nmr* (Fig. 5*C*, *SI Appendix*, Fig. S10*D*, and Dataset S1). Two of the three
643 most highly downregulated genes were *nit-3* and *nit-6*, which were expressed more
644 than 32-fold higher in wild type cells than Δ*nmr* cells during nitrogen starvation (*SI*
645 *Appendix*, Fig. S10*A* and Dataset S1). Other genes downregulated in the Δ*nmr*
646 mutant during nitrogen starvation included the nitrate transporter *nit-10* and genes
647 involved in amino acid metabolism. Only nine genes were differentially expressed in
648 the Δ*nmr* mutant as compared to wild type during nitrogen starvation and in media
649 containing ammonium nitrate (Dataset S1). This result could be because NMR plays
650 a role in regulating nitrogen metabolism overall, not simply the interplay between
651 nitrate and ammonium.

652 ***NIT-4 directly activates genes necessary for nitrate utilization in response***
653 ***to nitrate and nitrogen starvation***

654 Along with NIT-2, the transcription factor NIT-4 is required for expression of
655 genes necessary to utilize nitrate (Fig. 1) (6). We hypothesized that the interplay of
656 NIT-2 and NIT-4 may be similar to that of NIT-2 and AMN-1, with NIT-2 mainly
657 directly regulating genes involved in general nitrogen metabolism while NIT-4
658 directly regulates the enzymes required for nitrate utilization. To test this
659 hypothesis, we did RNAseq on wild type and Δ*nit-4* cells after a shift to media
660 containing nitrate as the nitrogen source. The expression of 76 genes was at least
661 4-fold differentially expressed in Δ*nit-4* cells as compared with wildtype during
662 exposure to nitrate (Fig. 5*D*, *SI Appendix*, Fig. S11*A*, and Dataset S1). Functional
663 analysis of these 76 genes showed enrichment for genes involved in nitrate and
664 methane metabolism (*SI Appendix*, Fig. S11*B*).

665 NIT-4 is thought to act as a transcriptional activator (Fig. 1). However, only 21
666 of the 76 genes that were differentially expressed between wild type and Δ*nit-4*
667 cells during exposure to nitrate were downregulated in the Δ*nit-4* mutant (Fig. 5*D*, *SI*
668 *Appendix*, Fig. S11*A*, and Dataset S1). To determine whether any of the genes
669 repressed by NIT-4 were directly regulated, we performed DAPseq on NIT-4 and
670 identified 29 NIT-4 binding sites within 3000 base pairs upstream of translational
671 start sites of 29 genes (Dataset S3). We compared the 29 genes with NIT-4 binding
672 sites in their promoters with the list of genes that were differentially expressed in
673 the *nit-4* deletion mutant during exposure to nitrate. Only eight genes whose
674 promoters were bound by NIT-4 were also differentially expressed in Δ*nit-4* cells as
675 compared to wildtype exposed to nitrate (Fig. 3, 5*D*, *SI Appendix*, Fig. S11*A*, and
676 Dataset S1). All eight of these genes were downregulated in Δ*nit-4* cells as
677 compared to wildtype, suggesting that the 54 genes that were upregulated in cells
678 lacking *nit-4* were indirectly regulated, perhaps in response to nitrogen starvation

679 (Fig. 5*D*, *SI Appendix*, Fig. S11*A*). Indeed, many of the genes that were repressed by
680 NIT-4 in response to nitrate, were upregulated in wild type cells exposed to nitrogen
681 starvation (*SI Appendix*, Fig. S11*A*). The eight directly regulated genes included *nit-*
682 *3*, *nit-6*, and *nit-10* as well as the FMN-dependent 2-nitropropane dioxygenase *npd-*
683 *1*, the flavohemoglobin protein *fhb-2* (NCU10051), and the cyanamide hydratase
684 NCU04466, which are all important in nitrogen metabolism. NIT-4 also bound and
685 regulated the HPP family protein NCU09160 (Fig. 3 and Dataset S1); HPP family
686 proteins have been reported to have nitrite transport activity in cyanobacteria (48).
687 We used the NIT-4 binding sites located in these eight genes to identify the
688 consensus binding motif KCCGCGGAGARAG for NIT-4, which shows some similarity
689 to NTCCGCGGVN, the NIT-4 binding motif identified in a survey of eukaryotic
690 transcription factors (28) (*SI Appendix*, Fig. S8*C*). The KCCGCGGAGARAG NIT-4
691 binding motif was present 1912 times in *N. crassa* promoters, indicating that NIT-4
692 did not bind every location in the genome where its binding motif was present
693 (Dataset S4).

694 A comparison of NIT-4- and NIT-2-regulated genes did not show as stark of a
695 contrast as when comparing NIT-2- and AMN-1-regulated genes. Half of the genes
696 that were regulated by NIT-4 in response to nitrate were also regulated by NIT-2
697 under ammonium nitrate, nitrate, proline, tryptophan, or isoleucine conditions
698 (Dataset S1). These coregulated genes included *nit-3* and *nit-6*, as well as the
699 nitrate transporter *nit-10* (Dataset S1). A closer examination showed that many
700 genes whose expression was activated by NIT-2 were repressed by NIT-4, including
701 three amino acid or oligopeptide transporters, suggesting that much of the overlap
702 in regulation may be due to the cell experiencing nitrogen starvation conditions (*SI*
703 *Appendix*, Fig. S9*D*, S11*A*, and Dataset S1).

704 Our data suggested that NMR, which lacks a DNA binding domain, functioned
705 as an activator during nitrogen starvation. However, it was not clear what
706 transcription factor(s) might act downstream of NMR. Since NIT-4 activated the
707 transcription of a number of genes that were upregulated in response to nitrogen
708 starvation, we asked whether NIT-4 might play a role in transcriptional activation
709 during nitrogen starvation by doing RNAseq on wild type and Δ*nit-4* cells. The
710 expression of 85 genes was at least 4-fold differentially expressed between wild
711 type and Δ*nit-4* cells during nitrogen starvation (Fig. 5*E*, *SI Appendix*, S11*C*, and
712 Dataset S1). Functional analysis showed an enrichment for genes involved in the
713 metabolism of a number of amino acids, including branched chain amino acids,
714 aromatic amino acids, glycine, serine, and threonine, as well as other genes
715 involved in nitrogen metabolism (*SI Appendix*, Fig. S11*D*). Over 3 quarters of these
716 genes were downregulated in Δ*nit-4* cells (Fig. 5*E*, *SI Appendix*, S11*C*, and Dataset
717 S1). Notably, all 7 genes whose promoters were bound by NIT-4 and were regulated
718 by NIT-4 in response to nitrogen starvation were downregulated in Δ*nit-4* cells,
719 including 5 of the 6 most highly downregulated genes (Fig. 5*E*, *SI Appendix*, S11*C*,
720 and Dataset S1). All 7 of these genes were also directly regulated by NIT-4 in
721 response to nitrate (Fig. 3). These data suggested that NIT-4 acts as a
722 transcriptional activator and may function downstream of NMR in response to
723 nitrogen starvation.

724 **DISCUSSION**

725 *N. crassa* is a saprotrophic fungus that gets the majority of its nutrients from
726 plant biomass. In this environment, nitrogen is a limiting nutrient (4). Genes
727 involved in nitrogen metabolism are regulated by the interplay of transcription
728 factors that respond to a particular nitrogen source and transcription factors that

729 activate genes involved in the utilization of nonpreferred nitrogen sources when no
730 preferred nitrogen sources are present (6). Through a combination of RNAseq and
731 DAPseq, we compared the genes regulated by pathway-specific transcription factors
732 and those regulated through nitrogen catabolite repression by NIT-2. Our data
733 indicate that pathway-specific transcription factors generally directly regulate genes
734 that encode for enzymes necessary for metabolism of a specific nitrogen source,
735 while NIT-2 directly regulates genes that encode for transporters and genes
736 necessary for the metabolism of a broad range of nitrogen sources.

737 ***amn-1 encodes a zinc binuclear cluster transcription factor that is required***
738 ***for amino acid utilization***

739 Although pathway-specific transcription factors regulating nitrogen utilization
740 have been characterized for a few nitrogen substrates, including nitrate and purine
741 catabolism, regulation of the catabolism of many nonpreferred nitrogen sources is
742 poorly understood in filamentous fungi. Our data indicate that AMN-1 plays a role in
743 directly regulating genes involved in the catabolism of proline, aromatic amino
744 acids, and branched chain amino acids (Fig. 3, *SI Appendix*, Fig. S4). Interestingly,
745 AMN-1 also indirectly regulates the expression of genes in response to mannose,
746 although AMN-1 is not required for mannose utilization (*SI Appendix*, Fig. S1 and
747 S2).

748 AMN-1 is conserved amongst ascomycete fungi (*SI Appendix*, Fig. S12).
749 Although AMN-1 is required for the utilization of a number of amino acids, homologs
750 of AMN-1, such as Aro80 in *S. cerevisiae*, are required only for aromatic amino acid
751 utilization (49) (Fig. 2*A*, *SI Appendix*, Fig. S5*A* and S5*B*). In the human pathogens
752 *Aspergillus fumigatus* and *Penicillium marneffei*, the *amn-1* homolog *hmgR*
753 regulates a gene cluster required for tyrosine catabolism (50, 51). While the

754 homologs of these genes are not contained in a gene cluster in the *N. crassa*
755 genome, AMN-1 binds the promoters of all but 1 of the 6 genes in the *A. fumigatus*
756 cluster. The *P. marneffei* gene cluster contains an additional 2 genes, one of which
757 encodes for a putative α-1,2-mannosidase (51). This observation may indicate a
758 conserved role for AMN-1 regulation of genes involved in mannose or mannan
759 utilization across ascomycete fungi.
760 Unlike Aro80 and HmgR, AMN-1 also plays a role in regulating genes involved
761 in branched chain amino acid and proline metabolism (Fig. 3, *SI Appendix*, Fig. S4).
762 In fungi, both branched chain and aromatic amino acids can be catabolized through
763 the Ehrlich pathway (52). However, proline is catabolized through a separate
764 pathway and regulated by Put3/PrnA in *S. cerevisiae* and *A. nidulans*, respectively
765 (53, 54). The *prnA* gene in *A. nidulans* is part of a proline utilization gene cluster
766 containing 4 genes required for proline utilization (55). AMN-1 binds the promoters
767 of orthologs of 3 of these 4 genes, although they are not clustered in the *N. crassa*
768 genome.
769 The majority of genes whose promoters are bound by AMN-1 are regulated in
770 response to mannose rather than proline, aromatic amino acids, or branched chain
771 amino acids (Fig. 3, *SI Appendix*, Fig. S4). Many of the amino acids available for *N.*
772 *crassa* utilization in the wild are likely part of proteins secreted either from plant or
773 fungal cells and are likely to be glycosylated. We postulate that *N. crassa* may use
774 mannose as a signal to indicate the presence of glycosylated proteins to activate
775 genes involved in amino acid catabolism. Further work will be necessary to
776 investigate the connection and conservation of mannose and amino acid catabolism
777 in filamentous fungi.

778 ***Nitrogen catabolite repression regulates transporters, while pathway-***

779 ***specific transcription factors regulate catabolic enzymes***

780 In *N. crassa*, nitrogen catabolite repression is regulated by NIT-2 (7).

781 Utilization of nonpreferred nitrogen sources requires the activity of both NIT-2 and

782 pathway-specific transcription factors (6). Prior studies suggested that binding of

783 NIT-2 and a pathway-specific transcription factor is required for activation of gene

784 transcription (12, 19). Here, a global analysis of NIT-2 promoter binding sites

785 combined with the promoter binding sites of the pathway-specific transcription

786 factors AMN-1 and NIT-4 indicated that while there are isolated incidences of gene

787 co-regulation by NIT-2 and either NIT-4 or AMN-1, the set of genes whose promoters

788 were bound by NIT-2 was broadly separate from the set of genes whose promoters

789 were bound by the pathway-specific transcription factors NIT-4 and AMN-1 (Fig. 3).

790 The pathway-specific transcription factors AMN-1 and NIT-4 generally

791 regulated genes that encode enzymes involved in the catabolism of a particular

792 nitrogen source, while NIT-2 regulated genes that encode for proteins required for

793 utilization of all nitrogen sources. In particular, NIT-2 directly regulated the

794 expression of a number of transporters that import nitrogen sources, including

795 amino acids, nucleic acids, ammonium, nitrate, and urea, into the cell (Fig. 3). This

796 role is similar to that of the carbon catabolite repressor CRE-1, which binds the

797 promoters of genes encoding a number of transporters involved in importing

798 different carbon sources into the cell (17). Regulation of transporters by major

799 transcriptional regulators of both carbon and nitrogen catabolite repression

800 indicates that controlling import of nutrients into the cell is one of the major

801 mechanisms by which fungi prioritize the utilization of preferred nutrient sources

802 over nonpreferred nutrient sources.

803 ***Nitrogen utilization genes are activated by NMR and NIT-4 in response to***
804 ***starvation***

805 The role of regulators of carbon and nitrogen catabolite repression has mainly
806 been explored in conditions in which nutrients are present (6, 56). However, major
807 regulators of carbon and nitrogen catabolite repression also appear to play a role in
808 gene regulation during starvation conditions. In *A. nidulans*, the NIT-2 ortholog AreA
809 activates the expression of target genes during nitrogen starvation (57). CRE-1,
810 canonically thought of as a transcriptional repressor, activates the expression of
811 genes required for utilization of nonpreferred carbon sources during carbon
812 starvation (47). In a similar fashion, the nitrogen catabolite repressor NMR activated
813 the expression of genes necessary to utilize nonpreferred nitrogen sources during
814 nitrogen starvation (Fig. 5*C*, *SI Appendix*, Fig. S10*D*). Unlike CRE-1, NMR does not
815 have a DNA binding domain, so NMR must work through transcription factors, such
816 as NIT-2, to activate the expression of downstream targets (58). Our work suggests
817 it may also act upstream of NIT-4 during nitrogen starvation. It will be the role of
818 future studies to investigate potential biochemical interactions between NMR, NIT-4,
819 and other downstream transcription factors.

820 Characterizing the regulatory network through which fungal cells control
821 nitrogen utilization is important in understanding how fungi interact with their
822 environment, including how saprophytic fungi recycle nutrients and the
823 mechanisms by which fungi infect both plants and animals (1-3). Transcriptional
824 profiling combined with transcription factor promoter binding data is a powerful tool
825 to help elucidate regulatory networks, as it enabled us to make and test hypotheses
826 addressing the role of transcription factors in nutrient sensing. We used these

827 genomic tools to describe a network of transcription factors that regulate genes in
828 response to changes in nitrogen conditions.

829 Studies in *S. cerevisiae* have shown that additional transcription factors, such
830 as Gcn4, which regulates genes in response to amino acid starvation, are also
831 involved in regulating nitrogen catabolite repression (59). Although we did not see
832 evidence of direct regulation of the *GCN4* ortholog, *cpc-1* (NCU04050), by NIT-2,
833 NIT-4, or AMN-1 in the conditions tested here, we expect future studies to place *cpc-*
834 *1* and other nitrogen regulators in the nitrogen sensing network. Additionally, our
835 data indicating a role for AMN-1 in regulating genes involved in both mannose and
836 amino acid utilization suggest a connection between nitrogen and carbon
837 metabolism (Fig. 4, *SI Appendix*, Fig. S1). Previous studies have suggested that NIT-
838 2 and its ortholog AreA may also play a role in plant cell wall deconstruction (60,
839 61). Future studies dissecting the connections between carbon and nitrogen sensing
840 networks will elucidate how fungal cells respond to and utilize nutrient sources in a
841 variety of environments.

842 **MATERIALS and METHODS**

843 ***N. crassa strains and culturing***

844 Strains used in this study are listed in Table S2 (*SI Appendix*). All strains were
845 derived from the wild type reference strain FGSC 2489 using standard genetic
846 techniques and confirmed by PCR and DNA sequencing (62, 63). *N. crassa* cultures
847 were grown on Vogel's minimal medium (VMM) (64). In all experiments where the
848 nitrogen source was not ammonium nitrate, Vogel's salts (64) were made without
849 ammonium nitrate, and nitrogen sources were added as described in Table S1 (*SI*
850 *Appendix*). Thus, the indicated nitrogen source was the only nitrogen source
851 present in the experiment. Nitrogen starvation indicates that Vogel's salts were

852 made without ammonium nitrate and no nitrogen source was added. Carbon
853 sources were added at 2% weight/volume and nitrogen sources were added at
854 50mM unless otherwise noted. Specifics of the carbon and nitrogen sources used
855 can be found in Table S1 (*SI Appendix*). Cells were grown from freezer stocks on
856 VMM + sucrose + 1.5% agar (Fisher Scientific) slants for 2 days at 30°C in the dark
857 and 4-8 days at 25°C in constant light prior to inoculation into the indicated media
858 at 10^6 conidia/ml. All chemicals were purchased from Sigma-Aldrich unless
859 otherwise noted.

860 ***Gene expression analysis, RNA sequencing, and transcript abundance***

861 RNA extraction, library preparation, and sequencing were modified from Wu
862 *et al* (2020) (17). Quantitative reverse transcription PCR (qRT-PCR) was performed
863 using the EXPRESS One-Step SYBR GreenER kit (Life Technologies). For details see
864 SI Materials and Methods (*SI Appendix*).

865 ***Statistical significance tests***

866 For RNAseq data, experiments had at least 3 biological replicates and
867 statistical significance was determined using Cufflinks v2.2.1 (65). The exact
868 number of replicates for all RNAseq experiments is shown in Dataset S1 and Table
869 S3. For all growth and qRT-PCR experiments at least 3 biological replicates were
870 done. The exact number of replicates for all growth and qRT-PCR experiments is
871 shown in Dataset S2. Biological replicates refer to independent cultures inoculated
872 on the same or independent days. Statistical significance was determined using a 2-
873 tailed homoscedastic (equal variance) Student's *t*-test with a Benjamini-Hochberg
874 multiple hypothesis correction. In bar graphs, bars indicate the mean of biological
875 replicates and dots indicate individual biological replicates.

876 ***DAPseq***

877 DAPseq was done as described in Wu *et al* (2020) (17). For details see SI
878 Materials and Methods (*SI Appendix*).

879 ***DNA binding consensus motif generation***

880 Motif discovery was performed using MEME v5.1.0 (66). Identification of motif
881 locations was performed using FIMO v5.2.0 (67). For details see SI Materials and
882 Methods (*SI Appendix*).

883 ***Mycelial dry weight***

884 10^6 conidia/ml were inoculated in liquid VMM with the indicated carbon or
885 nitrogen source either in 100ml cultures in 250ml flasks or 3ml cultures in round-
886 bottomed 24-well plates. Media in which the carbon source is specified had 25mM
887 ammonium nitrate as the nitrogen source. Media in which the nitrogen source is
888 specified had 2% sucrose as the carbon source. Cells were grown at 25°C in
889 constant light with constant shaking at 200rpm. The mycelial cell mass was
890 harvested onto Whatman Grade 1 filter paper and dried by vacuuming away the
891 media. Mycelial cell masses were then further dried in a 65°C drying oven for 2 days
892 prior to weighing the dried mycelia.

893 ***Functional Enrichment Analysis and Gene Annotation***

894 Functional enrichment analysis was done using the FungiFun2 online
895 resource tool (https://elbe.hki-jena.de/fungifun/) with Kyoto Encyclopedia of Genes
896 and Genomes (KEGG) as the classification ontology (21, 24). The gene to category
897 associations were tested for over-representation using hypergeometric distribution
898 with Benjamini-Hochberg correction for false discovery rate.

899 Gene annotations were pulled from FungiDB (https://fungidb.org) or inferred
900 from homology to characterized genes in related fungi.

901 ***Data availability***

902 RNAseq data used in this study were deposited in the Gene Expression
903 Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) and are
904 accessible through GEO series accession number GSE150256. Processed RNAseq
905 data are available in Dataset S1. DAPseq data used in this study were deposited in
906 the NCBI Sequence Read Archive (SRA) and are accessible through SRA series
907 accession number PRJNA436200. Processed DAPseq data are available in Dataset
908 S3. The numerical values used to generate all mycelial dry weight and qRT-PCR
909 graphs are shown in Dataset S2. Strains constructed in this study are available from
910 the Fungal Genetics Stock Center (www.fgsc.net).

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