DNA affinity purification sequencing and transcriptional profiling reveal new aspects of nitrogen regulation in a filamentous fungus

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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.

transcriptional networks | nitrogen utilization | nutrient sensing | DAPseq | RNAseq

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, in which 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 required for the expression of genes involved in the utilization of nitrate and a number of amino and nucleic acids (7, 8). NMR represses the activity of NIT-2 when preferred nitrogen sources are available (9–11). Much of the work exploring regulation of this pathway focused on the nitrate reductase gene *nit-3* (NCU05298). Activation of *nit-3* transcription requires two transcription factors: NIT-2 and NIT-4 (NCU08294) (12) (Fig. 1). NIT-4 is a pathway-specific transcription factor that specifically activates genes necessary for nitrate utilization (13). In the presence of nitrate and absence of any preferred nitrogen sources, NIT-4 and NIT-2 promote expression of *nit-3* (12). When nitrate is present in combination with a preferred nitrogen source, such as ammonium, NMR represses the activity of NIT-2, and *nit-3* is not expressed (14).

Significance

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

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The authors declare no competing interest.

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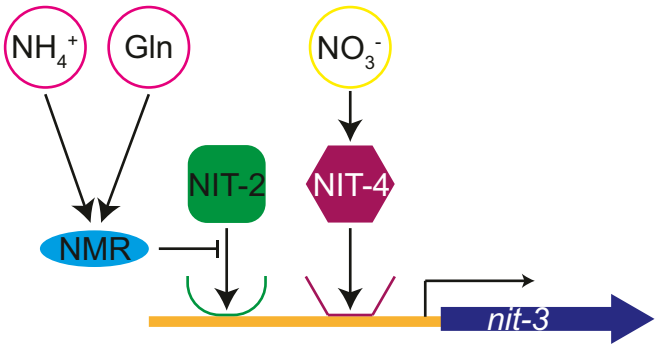

Fig. 1. Regulation of *nit-3* expression in *N. crassa*. Both the transcription factors NIT-2 and NIT-4 are required for *nit-3* expression. In the presence of nitrate, NIT-4 promotes *nit-3* expression. NIT-2 promotes *nit-3* expression in the absence of preferred nitrogen sources such as ammonium. In the presence of preferred nitrogen sources, NMR represses the activity of NIT-2, and *nit-3* is not expressed (6).

Utilization of nonpreferred organic nitrogen sources such as amino and nucleic acids is also thought to be regulated by NIT-2 in concert with pathway-specific transcription factors that activate genes necessary to utilize specific nitrogen sources (6). In *N. crassa*, only a small number of these pathway-specific transcription factors have been identified, such as the transcription factor required for purine utilization, *pco-1* (NCU07669) (15). Although NIT-2 works in concert with these pathway-specific transcription factors to promote utilization of nonpreferred nitrogen sources, we do not understand how these transcription factors regulate genes required for nitrogen utilization on a global scale.

In nature, *N. crassa* utilizes dead plant material, in which carbon and nitrogen sources occur in the matrix of the plant cell wall (16). We hypothesized that the integration of carbon and nitrogen metabolism via nutrient sensing networks must occur for optimal growth on these substrates. By combining RNA sequencing (RNAseq) data from 12 different nitrogen sources with transcriptional profiling of *N. crassa* on 40 different carbon sources (17), we identified and characterized a pathway-specific transcription factor, *amn-1* (NCU00445), that is required for the utilization of a number of amino acids, as well as for expression of genes in response to the simple sugar mannose. DNA affinity purification sequencing (DAPseq) (18) of AMN-1, NIT-2, and NIT-4 enabled us to identify the direct targets of these transcription factors. 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 (12, 19), our data suggest a model in which pathway-specific transcription factors activate genes that encode enzymes required for utilization of a specific nitrogen source, while NIT-2 regulates genes that are required for general nitrogen metabolism, such as nitrogen transporters.

Results

The Zinc Binuclear Cluster Transcription Factor NCU00445 Regulates Genes Involved in Mannan and Mannose Utilization. To identify genes involved in nutrient sensing in filamentous fungi, we exposed *N. crassa* to a diverse set of carbon sources and measured the transcriptome using RNAseq (17, 20). Transcription of the conserved zinc binuclear cluster transcription factor NCU00445 was more than fourfold higher during exposure to 2 mM mannose than 2% sucrose and less strongly induced on a number of other hemicellulosic components (17). RNAseq data from wild-type cells exposed to mannose showed that the expression of 91 genes was at least fourfold higher than during exposure to carbon starvation (*SI Appendix*, Fig. S1*A* and Dataset S1) (17). If NCU00445 is a positive regulator of the mannose response, we would expect the expression of these genes to be reduced in cells lacking NCU00445 as compared to wild-type cells during exposure to mannose. Indeed, the expression of 83 of these 91 genes was at least fourfold down-regulated in ΔNCU00445 cells as compared to wild-type (*SI Appendix*, Fig. S1 and Dataset S1).

The transcriptional response of cells lacking NCU00445 during exposure to mannose led us to hypothesize that NCU00445 might play a role in mannose utilization. Mannose-6-phosphate isomerase is the only enzyme necessary for mannose utilization (21, 22). The *N. crassa* genome contains two mannose-6-phosphate isomerase genes, *man-2* (NCU02322) and *man-3* (NCU07165). The expression of *man-2* was reduced sixfold in ΔNCU00445 cells as compared to wild-type during exposure to mannose; the expression of *man-3* was essentially unchanged (*SI Appendix*, Fig. S2*A*). When wild-type and ΔNCU00445 cells were inoculated into media containing mannose as the carbon source, the growth of ΔNCU00445 cells was indistinguishable from that of wild-type cells, indicating that NCU00445 was not required for mannose utilization (*SI Appendix*, Fig. S2 *B* and *C* and Dataset S2).

Mannose is a building block of the complex carbohydrate mannan, which is a component of the fungal cell wall and hemicellulose in the plant cell wall. Although the *N. crassa* genome contains enzymes necessary for mannan degradation, *N. crassa* is unable to grow on mannan alone (21, 23). The ability to grow on mannan is enabled by constitutive expression of the transcription factor, CLR-2 (NCU08042), which binds the promoter and regulates the expression of an extracellular endo-β-1,4-mannanase *gh5-7* (NCU08412) and predicted β-mannosidase *gh2-1* (NCU00890) (17, 21, 24). The expression of both *gh5-7* and *gh2-1* was more than threefold lower in ΔNCU00445 cells than wild-type under mannose conditions. However, unlike with *clr-2*, increasing the expression of NCU00445 by 2.4-fold by placing it under the control of the *gpd-1* (NCU01528) promoter did not allow *N. crassa* cells to grow on mannan (*SI Appendix*, Fig. S2 *C* and *D* and Dataset S2).

Deletion of NCU00445 caused the expression of 335 genes to be altered by more than fourfold as compared to wild-type during exposure to mannose (*SI Appendix*, Fig. S1 and Dataset S1). However, aside from genes involved in mannose or mannan utilization, only 15 additional carbohydrate active enzymes were regulated by NCU00445 in response to mannose, the majority of which were involved in hemicellulose degradation (Dataset S1).

NCU00445 Is Required for Utilization of a Variety of Amino Acids as a Nitrogen Source. To identify direct targets of NCU00445, we performed DAPseq, a technique that uses DNA sequencing to identify target sites in genomic DNA bound by in vitro synthesized transcription factors (18). Using DAPseq, we identified 296 NCU00445 DNA binding sites within 3,000 base pairs upstream of the translational start site of 290 genes (Dataset S3). Surprisingly, none of the promoters of genes involved in mannose or mannan utilization were bound by NCU00445. Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) categories associated with carbon metabolism were not enriched in the set of 290 genes whose promoters were bound by NCU00445 (*SI Appendix*, Fig. S3*A* and Dataset S3).

To identify cellular processes directly regulated by NCU00445, we used functional enrichment analysis (25). Of the 335 genes that were at least fourfold differentially expressed between wild-type and ΔNCU00445 cells during exposure to mannose, 40 had promoters directly bound by NCU00445 (*SI Appendix*, Fig. S4 and Dataset S3). NCU00445 also bound its own promoter. Because we used transcriptional profiling of cells lacking NCU00445 to identify genes that were directly regulated by NCU00445, we were unable to conclusively determine whether NCU00445 regulated its own expression; we included NCU00445 in our list of NCU00445 direct targets. Functional enrichment analysis of the entire set of 335 genes (plus NCU00445 itself) showed enrichment

for genes involved in valine, leucine, and isoleucine degradation and biosynthesis; alanine, aspartate, and glutamate metabolism; tryptophan metabolism and degradation of aromatic compounds; pentose and glucuronate interconversions; and biosynthesis of secondary metabolites (*SI Appendix*, Fig. S3*B*). Among the 41 genes whose promoters were bound by NCU00445, enrichment for valine, leucine, and isoleucine degradation and biosynthesis; tyrosine metabolism; and thiamine metabolism was observed (*SI Appendix*, Fig. S3*C*). Because mannose may not be the only condition in which NCU00445 plays a role, we investigated the KEGG pathways that were enriched among all 290 genes whose promoters were bound by NCU00445 and found enrichment for branched-chain amino acid degradation; tyrosine, phenylalanine, cysteine, methionine, and thiamine metabolism; and ubiquinone and other terpenoid–quinone biosynthesis (*SI Appendix*, Fig. S3*A*). These data indicated a potential role for NCU00445 in directly regulating amino acid metabolism.

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

AMN-1 Regulates Genes Encoding Enzymes Required for the Catabolism of Amino Acids. To further investigate the role of AMN-1 in amino acid utilization, we first transcriptionally profiled the response of wild-type cells to a panel of 12 nitrogen conditions to identify genes involved in the utilization of amino acids. These nitrogen conditions included the following: nitrogen starvation, ammonium nitrate, preferred nitrogen sources (ammonium, glutamate, and glutamine), and nonpreferred nitrogen sources (nitrate, alanine, arginine, proline, glycine, isoleucine, and tryptophan) covering a range of metabolic pathways (22).

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

Exposure to glycine resulted in the upregulation of a large number of genes. These included genes involved in glycine metabolism such as *gly-3* (NCU02727), *gyd-1* (NCU02475), *oxD* (NCU06558), and NCU03761 but also genes involved in the metabolism of a variety of other amino acids (Fig. 2*B* and Dataset S1) (26). This result may indicate that since cells rarely see glycine in isolation from other amino acids, the presence of glycine activated expression of genes

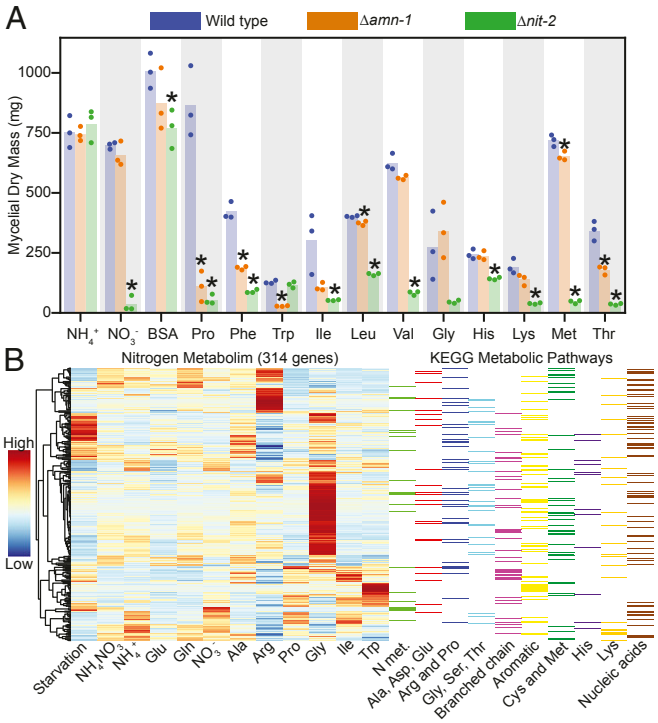

Fig. 2. AMN-1 is required for utilization of a variety of amino acids. (*A*) Mycelial dry weight of wild-type, Δ*amn-1*, and Δ*nit-2* cells inoculated into 100 mL media containing the indicated nitrogen source (the concentration of all nitrogen sources was 50 mM, except for 1% bovine serum albumin [BSA]). Asterisks indicate mycelial dry weights that are statistically significantly different from that of wild-type cells; $*P_{adj} < 0.05$. (*B*) Hierarchical clustering of the expression level of the set of genes in a KEGG pathway associated with nitrogen metabolism in wild-type cells exposed to the indicated nutrient condition (*SI Appendix*, Table S1) (22). Genes in a particular KEGG pathway are indicated with colored bars from left to right: nitrogen metabolism (light green); alanine, aspartic acid, and glutamic acid metabolism (red); arginine and proline metabolism (dark blue); glycine, serine, and threonine metabolism (cyan); branched-chain amino acid metabolism (magenta); aromatic amino acid metabolism (yellow); cysteine and methionine metabolism (dark green); histidine metabolism (purple); lysine metabolism (orange); and nucleic acid metabolism (brown).

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

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

We first investigated mechanisms of AMN-1–mediated regulation of proline catabolism. Functional enrichment analysis of

the 57 genes that were at least fourfold differentially expressed between Δ*amn-1* and wild-type cells during exposure to proline showed significant enrichment for valine, leucine, and isoleucine degradation; β-alanine metabolism; tyrosine metabolism; phenylalanine metabolism; and glycerolipid metabolism (*SI Appendix*, Fig. S6*A* and Dataset S1). Eight of the 57 genes had promoters bound by AMN-1, including the homogentisate 1,2-dioxygenase NCU05499 and the dimethylallyl tryptophan synthase NCU12075, both predicted to be involved in aromatic amino acid metabolism, and the promoters of three genes predicted to be involved in branched-chain amino acid metabolism: the branched-chain α-keto acid dehydrogenase E2 component NCU02704, the isovaleryl-CoA dehydrogenase NCU02126, and the methylcrotonoyl-CoA carboxylase β subunit *mcc-2* (NCU02127) (Figs. 3 and 4*A* and *SI Appendix*, Figs. S4 and S5*C* and Dataset S1).

The remaining genes whose promoters were bound by AMN-1 included the proline oxidase *pro-7* (NCU02936), which is predicted to catalyze the first step in proline degradation. *S. cerevisiae* cells lacking the *pro-7* ortholog, *PUT1* are unable to utilize proline as a nitrogen source (27). In *N. crassa*, cells lacking *pro-7* showed a severe growth defect when provided proline as the sole nitrogen source (*SI Appendix*, Fig. S7 *A* and *B* and Dataset S2). We hypothesized that the reduced expression of *pro-7* in the *amn-1* deletion mutant might be at least partially responsible for the inability of Δ*amn-1* cells to utilize proline. To test this, we asked whether expression of *pro-7* under the regulation of the constitutive *gpd-1* promoter restored growth in cells lacking *amn-1* (*SI Appendix*, Fig. S7*C* and Dataset S2). Indeed, $P_{gpd\text{-}1}$-*pro-7* Δ*amn-1* cells grew significantly better than the Δ*amn-1* mutant when proline was provided as the sole nitrogen source, indicating that reduced expression of *pro-7* in cells lacking *amn-1* was at least partially responsible for the inability of Δ*amn-1* cells to utilize proline (*SI Appendix*, Fig. S7 *A* and *B* and Dataset S2).

Although cells lacking *amn-1* had a severe growth defect on media containing tryptophan as the sole nitrogen source, only 16 genes were at least fourfold differentially expressed in Δ*amn-1* cells as compared to wild-type (Fig. 4*B* and *SI Appendix*, Fig. S5*C* and Dataset S1). Of these 16 genes, the promoters of 12 were bound by AMN-1 (Figs. 3 and 4*B* and *SI Appendix*, Figs. S4 and S5*C*). Five of the genes whose promoters were bound by AMN-1 and whose expression was regulated by AMN-1 in response to tryptophan were predicted to be involved in aromatic amino acid metabolism: the flavoprotein oxygenase *fpo-1* (NCU00236), the fumarylacetoacetase *fah-1* (NCU05537), the homogentisate 1,2-dioxygenase NCU05499, the 4-hydroxyphenylpyruvate dioxygenase *hpd-1* (NCU01830), and a hypothetical protein NCU01829 predicted to be involved in tyrosine catabolism. *N. crassa* cells lacking *fah-1* had a slight growth defect on tryptophan (*SI Appendix*, Fig. S7 *D* and *E* and Dataset S2). However, deletion of NCU05499 did not significantly affect growth on tryptophan, suggesting that the mechanism by which AMN-1 regulated tryptophan metabolism may be through a complex combination of genes (*SI Appendix*, Fig. S7 *D* and *E* and Dataset S2). The remaining genes whose promoters were bound by AMN-1 and regulated by AMN-1 in response to tryptophan were involved in either proline metabolism (*pro-7* and the pyrroline 5-carboxylate dehydrogenase

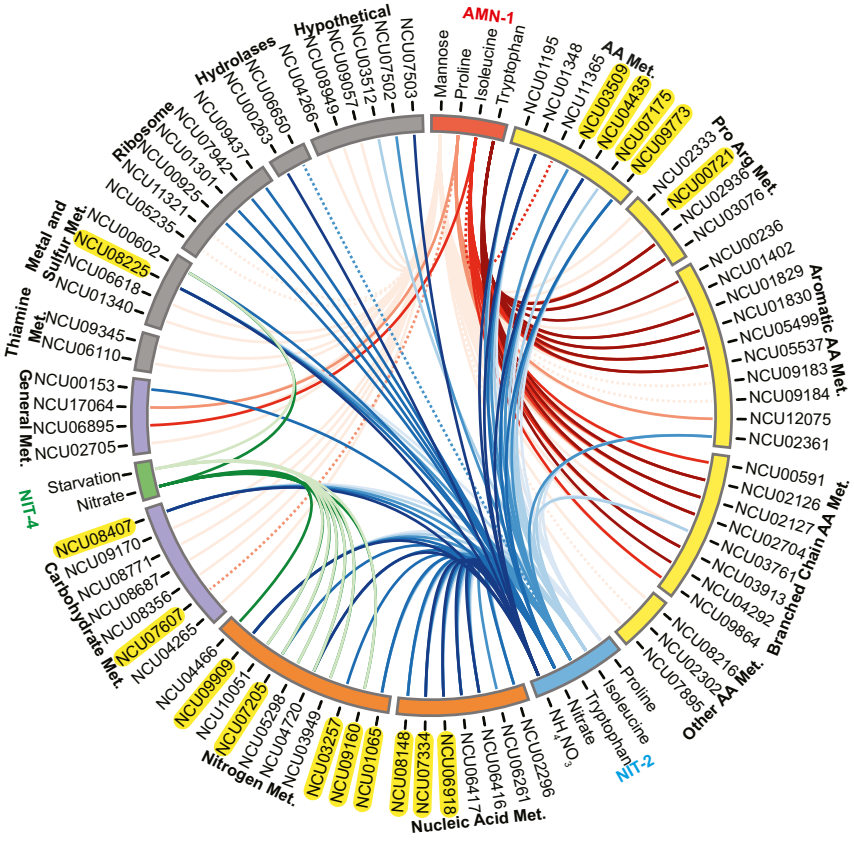

Fig. 3. NIT-2 regulates transporters and genes associated with general nitrogen metabolism, while pathway-specific transcription factors activate genes that are required for utilization of a specific nitrogen source. The plot was built with Circos, version 0.69 (70), to display the regulation of genes whose promoters were bound and whose expression was regulated by AMN-1, NIT-2, or NIT-4 when cells were exposed to the indicated conditions. Regulated genes were at least fourfold differentially expressed between wild-type and Δ*amn-1*, Δ*nit-2*, or Δ*nit-4* cells. Genes encoding transporters are highlighted in yellow. Solid lines indicate genes that were activated by AMN-1, NIT-2, or NIT-4. Dotted lines indicate genes that were repressed by AMN-1, NIT-2, or NIT-4.

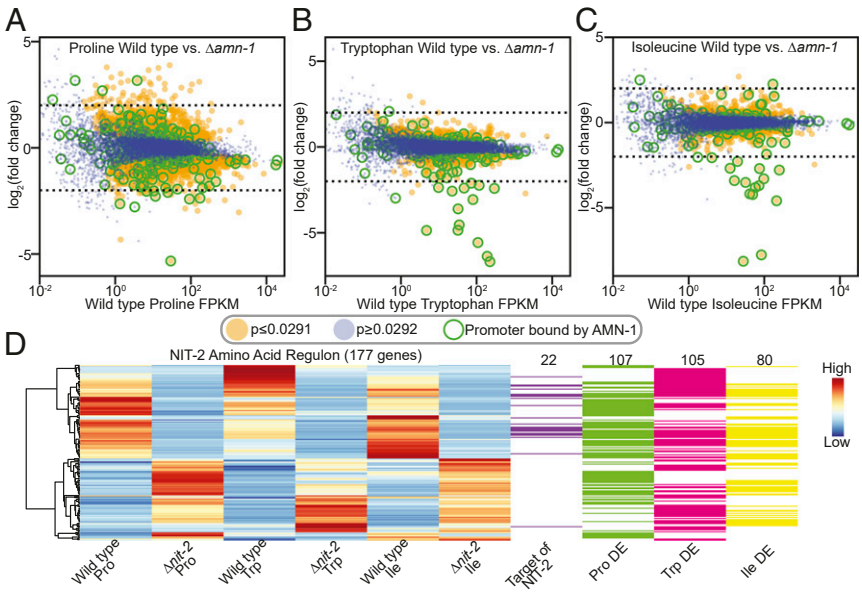

Fig. 4. Regulation of amino acid utilization by AMN-1 and NIT-2 occurs through different genetic pathways. (*A*–*C*) Differential expression analysis of Δ*amn-1* relative to wild-type cells after a shift to (*A*) 50 mM proline, (*B*) 50 mM tryptophan, and (*C*) 50 mM isoleucine. Green circles indicate genes whose promoters were bound by AMN-1. Dotted lines indicate fourfold change in expression. (*D*) Heatmap of the expression level of the 176 genes that were at least fourfold differentially expressed between wild-type and Δ*nit-2* cells exposed to 50 mM proline, 50 mM isoleucine, or 50 mM tryptophan plus *nit-2* itself. Purple bars indicate genes whose promoters were bound by NIT-2. Green bars indicate genes that were differentially expressed between wild-type and Δ*nit-2* cells exposed to proline plus *nit-2*. Pink bars indicate genes that were differentially expressed between wild-type and Δ*nit-2* cells exposed to tryptophan plus *nit-2*. Yellow bars indicate genes that were differentially expressed between wild-type and Δ*nit-2* cells exposed to isoleucine plus *nit-2*. Numbers above each column indicate the total number of genes in each category.

pcd-2 [NCU03076]) or branched-chain amino acid metabolism (the branched-chain amino acid aminotransferase *val-1* [NCU04292], the 2-oxoisovalerate dehydrogenase β subunit *ovd-2* [NCU03913], *mcc-2*, NCU02704, and NCU02126) (Figs. 3 and 4*B* and *SI Appendix*, Figs. S4 and S5*C* and Dataset S1).

Cells lacking *amn-1* also had a growth defect when branched-chain amino acids were provided as the nitrogen source (Fig. 2*A* and *SI Appendix*, Fig. S5 *A* and *B* and Dataset S2). The expression of 17 genes was at least fourfold differentially expressed between wild-type and Δ*amn-1* cells during exposure to media containing isoleucine as the nitrogen source, and the promoters of 14 of these genes were bound by AMN-1 (Figs. 3 and 4*C* and *SI Appendix*, Figs. S4 and S5*C* and Dataset S1). Six of these 14 genes play a role in branched-chain amino acid metabolism, including the 2-oxoisovalerate dehydrogenase α and β subunits *ovd-1* (NCU09864) and *ovd-2*, the methylcrotonoyl-CoA carboxylase α and β subunits *mcc-1* (NCU00591) and *mcc-2*, NCU02704, and NCU02126. Additionally, both *pro-7* and *pcd-2*, necessary for proline catabolism, and *fah-1*, *hpd-1*, NCU05499, and NCU01829, involved in aromatic amino acid metabolism were regulated by AMN-1 in response to isoleucine and had promoters bound by AMN-1 (Figs. 3 and 4*C* and *SI Appendix*, Figs. S4 and S5*C* and Dataset S1).

To comprehensively identify genes regulated directly by AMN-1, we cross referenced the 290 genes with AMN-1 binding sites in the promoter region identified by DAPseq with genes that were differentially expressed by at least fourfold across our RNAseq experiments. Although it is likely that AMN-1 regulates additional genes during exposure to conditions or at time points we did not test by RNAseq, we identified 43 genes that were regulated by AMN-1 during exposure to mannose, proline, tryptophan, or isoleucine and whose promoters were bound by AMN-1 (Fig. 3 and *SI Appendix*, Fig. S4 and Dataset S3). Supporting our hypothesis that AMN-1 is required for utilization of a variety amino acids, functional analysis of these 43 genes (plus *amn-1* itself) showed enrichment for genes involved in valine, leucine, and isoleucine degradation and biosynthesis, tyrosine metabolism, and thiamine metabolism (*SI Appendix*, Fig. S3*C*). Using the 45 promoter binding sites found in these 44 genes, we identified a consensus binding motif KCGGYTWKYRKCGGCHWW for AMN-1 (*SI Appendix*, Fig. S8*A*). This motif provided additional specificity to the NNCGGNNNNN motif identified in a broad survey of transcription factor binding motifs (28). As expected, AMN-1 only bound a small subset of the 2,189 locations where the KCGGYTWKYRKCGGCHWW motif was identified in *N. crassa* promoters (Dataset S4) (29).

The Nitrogen Catabolite Repressor NIT-2 Regulates Genes Necessary for Nitrogen Import and Enzymes Responsible for Amino Acid Utilization. NIT-2 is a conserved GATA-type transcription factor responsible for regulating genes involved in utilizing non-preferred nitrogen sources in filamentous fungi (6). Although initially identified for its role in promoting nitrate utilization in *N. crassa*, early studies also found NIT-2 is necessary for growth when a variety of amino and nucleic acids are provided as the nitrogen source (8). Unlike cells lacking *nit-2*, the Δ*amn-1* mutant exhibited normal growth when nitrate was provided as the nitrogen source (Fig. 2*A* and Dataset S2). Similarly, *amn-1* is not required for growth on nucleic acids (*SI Appendix*, Fig. S5 *A* and *B* and Dataset S2). When exposed to amino acids, there was significant overlap in the substrates that caused growth defects of Δ*amn-1* cells as compared to Δ*nit-2* cells but also a number of notable differences. In particular, Δ*nit-2* cells had a more severe growth phenotype than Δ*amn-1* cells during exposure to glutamic acid, glycine, isoleucine, leucine, valine, phenylalanine, lysine, methionine, serine, and threonine while the reverse was true for tryptophan ($p_{adj} < 0.05$) (Fig. 2*A* and *SI Appendix*, Fig. S5 *A* and *B* and Dataset S2). These data indicated that NIT-2 has a broader role in nitrogen metabolism than AMN-1.

Because of the expanded role of NIT-2 relative to AMN-1 with respect to nitrogen metabolism, we hypothesized that AMN-1 and NIT-2 may not act through the same pathway to regulate nitrogen metabolism. To test this hypothesis, we performed RNAseq on cells lacking *nit-2* under the same nitrogen conditions that we tested Δ*amn-1* cells (proline, tryptophan, and isoleucine) as well as exposure to nitrate and ammonium nitrate. To complement these transcriptional profiling experiments, we also performed DAPseq on NIT-2 to identify genes whose promoters were bound by NIT-2. Using DAPseq, we identified 354 NIT-2 binding sites in the putative promoter regions of 344 genes (Dataset S3). By comparing these 344 genes to the differentially expressed genes (at least fourfold) between wild-type and Δ*nit-2* cells during exposure to nitrate, ammonium nitrate, proline, tryptophan, or isoleucine, we identified the direct regulation of 33 of these genes by NIT-2 (Fig. 3 and Datasets S1 and S3).

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

To further explore these data, we first analyzed the 13 genes directly regulated by NIT-2 in response to proline, tryptophan, and isoleucine. Consistent with the role of NIT-2 functioning as a transcriptional activator (6), the expression of all 13 genes was down-regulated in the Δ*nit-2* mutant as compared to wild-type (Figs. 3 and 4*D* and Dataset S1). Nine of these 13 genes encoded for transporters, including the general amino acid permease *aap-22* (NCU04435) and the oligopeptide transporter *opt-1* (NCU09773) (Fig. 3 and Dataset S1). The expression of both of these transporters was reduced by more than 80-fold to a fragments per kilobase of transcript per million mapped reads value (FPKM) of less than 1 in cells lacking *nit-2* as compared to wild-type cells when exposed to proline, tryptophan, or isoleucine (Dataset S1). This dramatic reduction in expression may at least partially explain the inability of Δ*nit-2* cells to grow on many amino acids as the sole nitrogen source. Previously posited to be regulated by NIT-2 (30, 31), the uracil permease *uc-5* (NCU07334) promoter was bound by and transcription of *uc-5* was activated by NIT-2. NIT-2 also directly regulated the purine permease *pup-2* (NCU06918) and the H+/nucleoside cotransporter NCU08148 (Fig. 3 and Dataset S1). The regulation of these three nucleotide transporters may at least partially explain the role of NIT-2 in utilizing nucleotides as a nitrogen source (6). Urea uptake is also under the control of nitrogen catabolite repression (32); the urea transporter *urt* (NCU09909) was directly regulated by NIT-2 (Fig. 3 and Dataset S1). Consistent with its role in regulating the utilization of nonpreferred nitrogen sources in the presence of preferred nitrogen sources such as ammonium (6), the ammonium transporter *tam-4* (NCU01065) was also directly regulated by NIT-2. Additionally, NIT-2 regulated the expression and bound the promoters of the high-affinity nickel transporter *trm-34* (NCU08225) and the major facilitator superfamily (MFS) transporter NCU08407 (Fig. 3 and Dataset S1).

Four other genes down-regulated in Δ*nit-2* cells as compared to wild-type cells during exposure to proline, tryptophan, and isoleucine and whose promoters were bound by NIT-2 encoded enzymes important for amino and nucleic acid degradation. Unlike AMN-1, which mainly regulated the expression of enzymes important for utilizing a particular amino acid or class of amino acids, NIT-2 regulated the transcription of genes that encoded enzymes involved in more general metabolic pathways (Fig. 3). These included the agmatinase *put-3* (NCU01348), an enzyme involved in the catabolism of amino groups, and the formamidase *fma-1* (NCU02361) which participates in glyoxylate and dicarboxylate metabolism and may play a role in tryptophan metabolism. NIT-2 also directly regulated two genes involved in nucleic acid metabolism: the thymine dioxygenase *uc-3* (NCU06416) and the uracil-5-carboxylate decarboxylase *uc-7* (NCU06417) (33, 34) (Fig. 3 and Dataset S1).

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

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

To test this hypothesis, we transferred wild-type and Δ*nit-2* cells to media containing nitrate and did RNAseq to measure global gene expression. The expression of 113 genes differed by at least fourfold between wild-type and Δ*nit-2* cells 4 h postshift to nitrate (Fig. 5*A* and *SI Appendix*, Fig. S9*D* and Dataset S1). Functional analysis of these genes showed an enrichment for genes involved in nitrogen metabolism and ribosome biogenesis (*SI Appendix*, Fig. S6*D*). Over 40% of the genes regulated by NIT-2 during exposure to nitrate were also regulated by NIT-2 during exposure to proline, tryptophan, or isoleucine, including 13 genes that encoded for transporters (Dataset S1). To distinguish between genes that NIT-2 directly, as opposed to indirectly, regulated in response to nitrate, we compared the genes that were differentially expressed between wild-type cells and cells lacking *nit-2* during exposure to nitrate with genes whose promoters were bound by NIT-2. We identified 22 genes directly regulated by NIT-2 in response to nitrate, all of which were down-regulated in Δ*nit-2* cells as compared to wild-type (Figs. 3 and 5*A* and *SI Appendix*, Fig. S9*D* and Datasets S1 and S3). These 22 genes included 12 of the 13 genes that were differentially expressed in Δ*nit-2* cells in response to proline, tryptophan, and isoleucine as compared to wild-type cells and whose promoters were bound by NIT-2, with only *fma-1* not represented. The promoter of an additional gene, *aln-1*, was bound by NIT-2, and *aln-1* was differentially expressed during exposure to nitrate, isoleucine, and tryptophan in Δ*nit-2* as compared to wild-type cells (Fig. 3 and Datasets S1 and S3).

We investigated the nine genes whose promoters were bound by NIT-2 and whose expression was regulated by NIT-2 in response to nitrate but not proline, tryptophan, or isoleucine. Two of these genes were specifically involved in nitrate utilization: the nitrite reductase *nit-6* and the nitrate transporter *nit-10* (6). The promoter of the ammonium transporter *tam-1* (NCU03257) was also bound by NIT-2, and *tam-1* expression was regulated by NIT-2

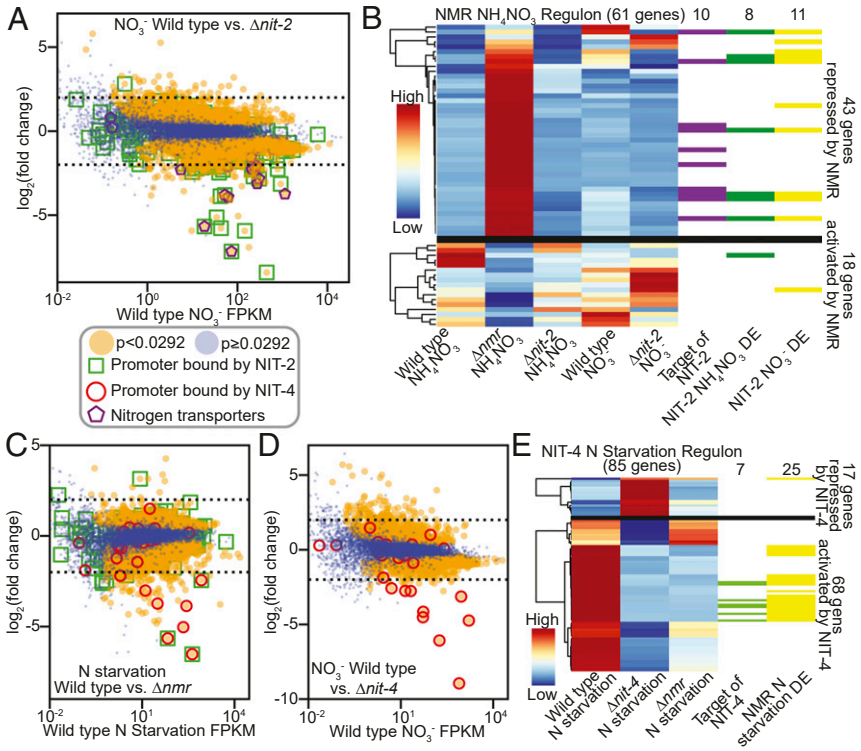

Fig. 5. NMR and NIT-4 activate genes during starvation conditions. (*A*) Differential expression analysis of Δ*nit-2* relative to wild-type cells after a shift to 50 mM nitrate. Green squares indicate genes whose promoters are bound by NIT-2. Purple pentagons indicate genes predicted to encode nitrogen transporters. Dotted lines indicate a fourfold change in expression. (*B*) Heatmap of the expression level of the 60 genes that were at least fourfold differentially expressed between wild-type and Δ*nmr* cells exposed to 25 mM ammonium nitrate plus *nmr* itself. Upper heatmap includes genes repressed by NMR. Lower heatmap includes genes activated by NMR plus *nmr* itself. Purple bars indicate genes whose promoters were bound by NIT-2. Green bars indicate genes that were differentially expressed between Δ*nit-2* and wild-type cells exposed to 25 mM ammonium nitrate. Yellow bars indicate genes that were differentially expressed between Δ*nit-2* and wild-type cells exposed to 50 mM nitrate. Numbers above each column indicate the total number of genes in each category. (*C*) Differential expression analysis of Δ*nmr* relative to wild-type cells after a shift to nitrogen starvation. Green squares indicate genes whose promoters were bound by NIT-2. Red circles indicate genes whose promoters were bound by NIT-4. Dotted lines indicate a fourfold change in expression. (*D*) Differential expression analysis of Δ*nit-4* relative to wild-type cells after a shift to 50 mM nitrate. Red circles indicate genes whose promoters were bound by NIT-4. Dotted lines indicate a fourfold change in expression. (*E*) Heatmap of the expression level of the set of genes that were at least fourfold differentially expressed between wild-type and Δ*nit-4* cells exposed to nitrogen starvation. Upper heatmap includes genes repressed by NIT-4. Lower heatmap includes genes activated by NIT-4. Green bars indicate genes whose promoters were bound by NIT-4. Yellow bars indicate genes differentially expressed between Δ*nmr* and wild-type cells exposed to nitrogen starvation. Numbers above each column indicate the total number of genes in each category.

during exposure to nitrate. The majority of the remaining genes were involved in functions relating to the ribosome (Fig. 3 and Dataset S1). To our surprise, although previous studies identified NIT-2 binding sites in the *nit-3* promoter, we did not find NIT-2 bound to the *nit-3* promoter using DAPseq (12, 39). Since DAPseq is an in vitro method of identifying transcription factor binding sites, this may suggest that DNA modification or additional proteins are required to facilitate NIT-2 binding in some promoter regions.

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

The Transcriptional Repressor NMR Acts as an Activator under Nitrogen Starvation Conditions. Regulation of nitrate utilization is accomplished through the interactions of NIT-2, NIT-4, and NMR (6) (Fig. 1). Previous work characterizing the regulation of the nitrate reductase gene *nit-3* showed that NIT-4 promotes expression of *nit-3* in the presence of nitrate (46). If nitrate is present in the absence of a preferred nitrogen source, NIT-2 also promotes expression of *nit-3*. However, when preferred nitrogen sources are present, the activity of NIT-2 is repressed by NMR (11), and *nit-3* is not expressed (6) (Fig. 1).

Our data showed that the expression of three genes required for nitrate utilization, *nit-3*, the nitrite reductase gene *nit-6*, and the nitrate transporter *nit-10*, was negligible in wild-type cells exposed to media containing the preferred nitrogen source ammonium (*SI Appendix*, Fig. S10*A* and Dataset S1). Exposure of wild-type cells to media containing nitrate increased expression of *nit-3*, *nit-6*, and *nit-10* by over sixfold. Deletion of either *nit-2* or *nit-4* resulted in a more than fourfold drop in expression of *nit-3*, *nit-6*, and *nit-10* under nitrate conditions. Upon exposure to media containing both ammonium and nitrate, expression of *nit-3*, *nit-6*, and *nit-10* dropped by nearly twofold as compared to exposure to nitrate alone in wild-type cells in an NMR-dependent fashion (*SI Appendix*, Fig. S10*A* and Dataset S1). These data broadly supported the model of nitrate utilization developed using classical genetics (Fig. 1).

To investigate the role of the nitrogen repressor, NMR, we exposed wild-type cells and cells lacking *nmr* to media containing

both nitrate and ammonium. The expression of 60 genes was at least fourfold differentially expressed between wild-type and Δ*nmr* cells (Fig. 5*B* and *SI Appendix*, Fig. S10*B* and Dataset S1). Functional analysis showed an enrichment for genes involved in the metabolism of a number of amino acids including arginine, proline, glycine, serine, threonine, phenylalanine, tyrosine, alanine, aspartate, glutamate, β-alanine, and cyanoamino acids (*SI Appendix*, Fig. S10*C*). As expected, all 10 genes bound by NIT-2 and differentially expressed between wild-type and Δ*nmr* cells during exposure to ammonium nitrate were more highly expressed in Δ*nmr* cells, providing confirmation of the role of NMR in repressing NIT-2 activity (Fig. 5*B* and *SI Appendix*, Fig. S10*B* and Dataset S1). Although NMR is canonically only thought to act through NIT-2, only 11 genes were at least fourfold differentially expressed between both wild-type and Δ*nmr* cells exposed to ammonium nitrate and wild-type and Δ*nit-2* cells exposed to nitrate, suggesting that NMR may have another role in transcriptional regulation (Fig. 5*B* and Dataset S1). For example, while many of the 60 genes regulated by NMR were predicted to play a role in nitrogen metabolism, there were also a significant number of genes involved in carbon metabolism, including genes encoding carbohydrate active enzymes and sugar transporters (Dataset S1). These data suggested a role for NMR in regulating carbon utilization as well as nitrogen utilization.

Our data supported the role of NMR as a transcriptional repressor. Of the 60 genes that were differentially expressed by at least fourfold between wild-type and Δ*nmr* cells, 43 were more highly expressed in cells lacking *nmr* (Fig. 5*B* and *SI Appendix*, Fig. S10*B* and Dataset S1). However, the carbon catabolite repressor *cre-1* (NCU08807) acts as a repressor when preferred carbon sources are available but as an activator during carbon starvation (47). We hypothesized that *nmr* might similarly function as an activator during nitrogen starvation. To test this hypothesis, we performed RNAseq on wild-type and Δ*nmr* cells during exposure to media lacking a nitrogen source. In total, 70 genes were differentially expressed (at least fourfold) between wild-type and Δ*nmr* cells, and of these, 60 were down-regulated in cells lacking *nmr* (Fig. 5*C* and *SI Appendix*, Fig. S10*D* and Dataset S1). Two of the three most highly down-regulated genes were *nit-3* and *nit-6*, which were expressed more than 32-fold higher in wild-type cells than Δ*nmr* cells during nitrogen starvation (*SI Appendix*, Fig. S10*A* and Dataset S1). Other genes down-regulated in the Δ*nmr* mutant during nitrogen starvation included the nitrate transporter *nit-10* and genes involved in amino acid metabolism. Only nine genes were differentially expressed in the Δ*nmr* mutant as compared to wild-type during nitrogen starvation and in media containing ammonium nitrate (Dataset S1). This result could be because NMR plays a role in regulating nitrogen metabolism overall, not simply the interplay between nitrate and ammonium.

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

NIT-4 is thought to act as a transcriptional activator (Fig. 1). However, only 21 of the 76 genes that were differentially expressed between wild-type and Δ*nit-4* cells during exposure to nitrate were down-regulated in the Δ*nit-4* mutant (Fig. 5*D* and *SI Appendix*, Fig. S11*A* and Dataset S1). To determine whether any of the genes repressed by NIT-4 were directly regulated, we performed DAPseq on NIT-4 and identified 29 NIT-4 binding sites within 3,000 base pairs upstream of translational start sites of 29 genes (Dataset S3). We compared the 29 genes with NIT-4 binding sites in their promoters with the list of genes that were differentially expressed in the *nit-4* deletion mutant during exposure to nitrate. Only eight genes whose promoters were bound by NIT-4 were also differentially expressed in Δ*nit-4* cells as compared to wild-type exposed to nitrate (Figs. 3 and 5*D* and *SI Appendix*, Fig. S11*A* and Dataset S1). All eight of these genes were down-regulated in Δ*nit-4* cells as compared to wild-type, suggesting that the 54 genes that were up-regulated in cells lacking *nit-4* were indirectly regulated, perhaps in response to nitrogen starvation (Fig. 5*D* and *SI Appendix*, Fig. S11*A*). Indeed, many of the genes that were repressed by NIT-4 in response to nitrate were up-regulated in wild-type cells exposed to nitrogen starvation (*SI Appendix*, Fig. S11*A*). The eight directly regulated genes included *nit-3*, *nit-6*, and *nit-10* as well as the FMN-dependent 2-nitropropane dioxygenase *npd-1*, the flavohemoglobin protein *fhb-2* (NCU10051), and the cyanamide hydratase NCU04466, which are all important in nitrogen metabolism. NIT-4 also bound and regulated the HPP family protein NCU09160 (Fig. 3 and Dataset S1); HPP family proteins have been reported to have nitrite transport activity in cyanobacteria (48). We used the NIT-4 binding sites located in these eight genes to identify the consensus binding motif KCCGCGGAGARAG for NIT-4, which shows some similarity to NTCCGCGGVN, the NIT-4 binding motif identified in a survey of eukaryotic transcription factors (28) (*SI Appendix*, Fig. S8*C*). The KCCGCGGAGARAG NIT-4 binding motif was present 1,912 times in *N. crassa* promoters, indicating that NIT-4 did not bind every location in the genome where its binding motif was present (Dataset S4).

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

Our data suggested that NMR, which lacks a DNA binding domain, functioned as an activator during nitrogen starvation. However, it was not clear what transcription factor(s) might act downstream of NMR. Since NIT-4 activated the transcription of a number of genes that were up-regulated in response to nitrogen starvation, we asked whether NIT-4 might play a role in transcriptional activation during nitrogen starvation by doing RNAseq on wild-type and Δ*nit-4* cells. The expression of 85 genes was at least fourfold differentially expressed between wild-type and Δ*nit-4* cells during nitrogen starvation (Fig. 5*E* and *SI Appendix*, Fig. S11*C* and Dataset S1). Functional analysis showed an enrichment for genes involved in the metabolism of a number of amino acids including branched-chain amino acids, aromatic amino acids, glycine, serine, and threonine as well as other genes involved in nitrogen metabolism (*SI Appendix*, Fig. S11*D*). Over three-quarters of these genes were down-regulated in Δ*nit-4* cells (Fig. 5*E* and *SI Appendix*, Fig. S11*C* and Dataset S1). Notably, all seven genes whose promoters were bound by NIT-4 and were regulated by NIT-4 in response to nitrogen starvation were down-regulated in Δ*nit-4* cells, including five of the six most highly down-regulated genes

(Fig. 5*E* and *SI Appendix*, Fig. S11*C* and Dataset S1). All seven of these genes were also directly regulated by NIT-4 in response to nitrate (Fig. 3). These data suggested that NIT-4 acts as a transcriptional activator and may function downstream of NMR in response to nitrogen starvation.

Discussion

N. crassa is a saprotrophic fungus that gets the majority of its nutrients from plant biomass. In this environment, nitrogen is a limiting nutrient (4). Genes involved in nitrogen metabolism are regulated by the interplay of transcription factors that respond to a particular nitrogen source and transcription factors that activate genes involved in the utilization of nonpreferred nitrogen sources when no preferred nitrogen sources are present (6). Through a combination of RNAseq and DAPseq, we compared the genes regulated by pathway-specific transcription factors and those regulated through nitrogen catabolite repression by NIT-2. Our data indicate that pathway-specific transcription factors generally directly regulate genes that encode for enzymes necessary for metabolism of a specific nitrogen source, while NIT-2 directly regulates genes that encode for transporters and genes necessary for the metabolism of a broad range of nitrogen sources.

AMN-1 Encodes a Zinc Binuclear Cluster Transcription Factor that Is Required for Amino Acid Utilization. Although pathway-specific transcription factors regulating nitrogen utilization have been characterized for a few nitrogen substrates, including nitrate and purine catabolism, regulation of the catabolism of many nonpreferred nitrogen sources is poorly understood in filamentous fungi. Our data indicate that AMN-1 plays a role in directly regulating genes involved in the catabolism of proline, aromatic amino acids, and branched-chain amino acids (Fig. 3 and *SI Appendix*, Fig. S4). Interestingly, AMN-1 also indirectly regulates the expression of genes in response to mannose, although AMN-1 is not required for mannose utilization (*SI Appendix*, Figs. S1 and S2).

AMN-1 is conserved among ascomycete fungi (*SI Appendix*, Fig. S12). Although AMN-1 is required for the utilization of a number of amino acids, homologs of AMN-1, such as Aro80 in *S. cerevisiae*, are required only for aromatic amino acid utilization (49) (Fig. 2*A* and *SI Appendix*, Fig. S5 *A* and *B*). In the human pathogens *Aspergillus fumigatus* and *Penicillium marneffei*, the *amn-1* homolog *hmgR* regulates a gene cluster required for tyrosine catabolism (50, 51). While the homologs of these genes are not contained in a gene cluster in the *N. crassa* genome, AMN-1 binds the promoters of all but one of the six genes in the *A. fumigatus* cluster. The *P. marneffei* gene cluster contains an additional two genes, one of which encodes for a putative α-1,2-mannosidase (51). This observation may indicate a conserved role for AMN-1 regulation of genes involved in mannose or mannan utilization across ascomycete fungi.

Unlike Aro80 and HmgR, AMN-1 also plays a role in regulating genes involved in branched-chain amino acid and proline metabolism (Fig. 3 and *SI Appendix*, Fig. S4). In fungi, both branched chain and aromatic amino acids can be catabolized through the Ehrlich pathway (52). However, proline is catabolized through a separate pathway and regulated by Put3/PrnA in *S. cerevisiae* and *Aspergillus nidulans*, respectively (53, 54). The *prnA* gene in *A. nidulans* is part of a proline utilization gene cluster containing four genes required for proline utilization (55). AMN-1 binds the promoters of orthologs of three of these four genes, although they are not clustered in the *N. crassa* genome.

The majority of genes whose promoters are bound by AMN-1 are regulated in response to mannose rather than proline, aromatic amino acids, or branched-chain amino acids (Fig. 3 and *SI Appendix*, Fig. S4). Many of the amino acids available for *N. crassa* utilization in the wild are likely part of proteins secreted either from plant or fungal cells and are likely to be glycosylated. We postulate that *N. crassa* may use mannose as a signal to indicate the presence of glycosylated proteins to activate genes involved in amino acid catabolism. Further work will be necessary to investigate the connection and conservation of mannose and amino acid catabolism in filamentous fungi.

Nitrogen Catabolite Repression Regulates Transporters, while Pathway-Specific Transcription Factors Regulate Catabolic Enzymes. In *N. crassa*, nitrogen catabolite repression is regulated by NIT-2 (7). Utilization of nonpreferred nitrogen sources requires the activity of both NIT-2 and pathway-specific transcription factors (6). Prior studies suggested that binding of NIT-2 and a pathway-specific transcription factor is required for activation of gene transcription (12, 19). Here, a global analysis of NIT-2 promoter binding sites combined with the promoter binding sites of the pathway-specific transcription factors AMN-1 and NIT-4 indicated that while there are isolated incidences of gene coregulation by NIT-2 and either NIT-4 or AMN-1, the set of genes whose promoters were bound by NIT-2 was broadly separate from the set of genes whose promoters were bound by the pathway-specific transcription factors NIT-4 and AMN-1 (Fig. 3).

The pathway-specific transcription factors AMN-1 and NIT-4 generally regulated genes that encode enzymes involved in the catabolism of a particular nitrogen source, while NIT-2 regulated genes that encode for proteins required for utilization of all nitrogen sources. In particular, NIT-2 directly regulated the expression of a number of transporters that import nitrogen sources, including amino acids, nucleic acids, ammonium, nitrate, and urea, into the cell (Fig. 3). This role is similar to that of the carbon catabolite repressor CRE-1, which binds the promoters of genes encoding a number of transporters involved in importing different carbon sources into the cell (17). Regulation of transporters by major transcriptional regulators of both carbon and nitrogen catabolite repression indicates that controlling import of nutrients into the cell is one of the major mechanisms by which fungi prioritize the utilization of preferred nutrient sources over nonpreferred nutrient sources.

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

Characterizing the regulatory network through which fungal cells control nitrogen utilization is important in understanding how fungi interact with their environment, including how saprophytic fungi recycle nutrients and the mechanisms by which fungi infect both plants and animals (1–3). Transcriptional profiling combined with transcription factor promoter binding data are a powerful tool to help elucidate regulatory networks, as it enabled us to make and test hypotheses addressing the role of

transcription factors in nutrient sensing. We used these genomic tools to describe a network of transcription factors that regulate genes in response to changes in nitrogen conditions.

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

Materials and Methods

***N. crassa* Strains and Culturing.** Strains used in this study are listed in *SI Appendix*, Table S2. All strains were derived from the wild-type reference strain FGSC 2489 using standard genetic techniques and confirmed by PCR and DNA sequencing (62, 63). *N. crassa* cultures were grown on Vogel's minimal medium (VMM) (64). In all experiments in which the nitrogen source was not ammonium nitrate, Vogel's salts (64) were made without ammonium nitrate, and nitrogen sources were added as described in *SI Appendix*, Table S1. Thus, the indicated nitrogen source was the only nitrogen source present in the experiment. Nitrogen starvation indicates that Vogel's salts were made without ammonium nitrate, and no nitrogen source was added. Carbon sources were added at 2% wt/vol, and nitrogen sources were added at 50 mM unless otherwise noted. Specifics of the carbon and nitrogen sources used can be found in *SI Appendix*, Table S1. Cells were grown from freezer stocks on VMM + sucrose + 1.5% agar (Thermo Fisher Scientific) slants for 2 d at 30 °C in the dark and 4 to 8 d at 25 °C in constant light prior to inoculation into the indicated media at 10^6 conidia/mL. All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

Gene Expression Analysis, RNAseq, and Transcript Abundance. RNA extraction, library preparation, and sequencing were modified from Wu et al. (2020) (17) qRT-PCR was performed using the EXPRESS One-Step SYBR GreenER kit (Life Technologies). For details, see *SI Appendix*, *SI Materials and Methods*.

Statistical Significance Tests. For RNAseq data, experiments had at least three biological replicates, and statistical significance was determined using Cufflinks version 2.2.1 (65). The exact number of replicates for all RNAseq experiments is shown in Dataset S1 and *SI Appendix*, Table S3. For all growth and qRT-PCR experiments, at least three biological replicates were done. The exact number of replicates for all growth and qRT-PCR experiments is shown in Dataset S2. Biological replicates refer to independent cultures inoculated on the same or independent days. Statistical significance was determined using a two-tailed homoscedastic (equal variance) Student's *t* test with a Benjamini–Hochberg multiple hypothesis correction. In bar graphs, bars indicate the mean of biological replicates and dots indicate individual biological replicates.

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

DNA Binding Consensus Motif Generation. Motif discovery was performed using Multiple Expectation maximizations for Motif Elicitation (MEME) version 5.1.0 (66). Identification of motif locations was performed using Find Individual Motif Occurrences (FIMO) v5.2.0 (67) For details, see *SI Appendix*, *SI Materials and Methods*.

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

Functional Enrichment Analysis and Gene Annotation. Functional enrichment analysis was done using the FungiFun2 online resource tool (https://elbe.hki-jena.de/fungifun/) with KEGG as the classification ontology (22, 25). The gene to category associations were tested for overrepresentation using hypergeometric distribution with Benjamini–Hochberg correction for false discovery rate.

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

Data Availability. RNAseq data used in this study were deposited in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) and are accessible through GEO series accession number GSE150256 (68). Processed RNAseq data are available in Dataset S1. DAPseq data used in this study were deposited in the NCBI Sequence Read Archive (SRA) and are accessible through SRA series accession number PRJNA436200 (69). Processed DAPseq data are available in Dataset S3. The numerical values used to generate all mycelial dry weight and qRT-PCR graphs are shown in Dataset S2. Strains constructed in this study are available from the Fungal Genetics Stock Center (www.fgsc.net).

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Supplementary Information for

DNA affinity purification sequencing and transcriptional profiling reveal new aspects of nitrogen regulation in a filamentous fungus

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SI References

Other supplementary materials for this manuscript include the following:

Datasets S1 to S4

Supplementary Information Text

SI MATERIALS and METHODS

Gene expression analysis

Conidia ($3x10^6$) were inoculated into 3ml of Vogel's minimal medium (VMM) (1) in round-bottomed 24-well plates and incubated at 25^{o}C in constant light with constant shaking at 200 rpm for 24h. The media was then vacuumed out and mycelia was washed three times with VMM without a carbon source (for a transfer to VMM, VMM + mannose, or VMM + mannan) or VMM without a nitrogen source (for all other experiments). Mycelia were then resuspended in 3ml of the indicated medium and grown as above for 4h.

Mycelia were harvested by filtering on Whatman paper no. 1 and flash frozen in liquid nitrogen. Total RNA was extracted using TRIzol (Life Technologies) and cleaned up with the RNeasy kit (Qiagen). The EXPRESS One-Step SYBR GreenER kit (Life Technologies) was then used to determine relative transcript abundance by qRT-PCR. Primer sequences used for qRT-PCR are shown in Table S4.

RNA sequencing and transcript abundance

RNA extraction and library preparation were done as described in Wu *et al* (2020) (2). Briefly, total RNA was harvested from mycelia flash frozen in liquid nitrogen 4h post-transfer to the indicated media using a TRIzol (Life Technologies) extraction and cleaned up with the RNeasy kit (Qiagen). Libraries were prepared from total RNA using poly(A) enrichment and standard Illumina protocols. The location of library preparation, sequencing machine, and length of sequencing runs can be found in Table S3. The RNA sequences were aligned to the predicted transcripts from the *N. crassa* OR74A genome (v12) (3) with HiSat2 v2.0.5 (4) with the maximum intron length set to 5000bp. Transcript abundance (fragments per kilobase of transcript per million mapped reads, FPKM) and differential expression were determined using Cufflinks v2.2.1 (5) with the options --library-type fr-unstranded, --max-bundle-frags=10000000,

and --compatible-hits-norm. Differential expression was only called between samples sequenced at the same center. Genes were denoted as differentially expressed between two conditions if there was at least a 4-fold change in expression and the FPKM of the gene was at least 10 in either of the 2 conditions.

Hierarchical clustering was performed using the clustermap function of the Python visualization library Seaborn (https://seaborn.pydata.org/) using the average linkage method with Pearson Correlation as the similarity metric.

RNAseq data used in this study were deposited in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) and are accessible through GEO series accession number GSE150256. Processed RNAseq data are available in Dataset S1.

DAPseq

DAPseq was done as described in Wu *et al* (2020) (2). Briefly, the open reading frames for each transcription factor were amplified from cDNA using RNA to cDNA EcoDry premix (Clontech) and inserted into an expression vector containing T7 and SP6 promoters upstream of a HALO tag as previously described (6). The transcription factors were transcribed and translated *in vitro* using Promega TnT T7 Rabbit Reticulocyte Quick Coupled Transcription/Translation System and expression was verified by Western blot with the Promega Anti-HaloTag monoclonal antibody. The transcription factors were then bound to Promega Magne HaloTag Beads.

Genomic DNA was harvested from wild type *N. crassa* mycelia grown in liquid VMM for 25h at 25^{o}C using the DNeasy Blood & Tissue Kit (Qiagen). DNA was sheared to a 300bp peak and size selected using AMPure XP beads (Beckman Coulter). The *in vitro* translated transcription factors were bound to Promega Magne HaloTag Beads and incubated with genomic DNA libraries on a rotator for 1h at room temperature. Transcription factors and bound genomic DNA that were bound to the beads were washed and then heated to 98^{o}C for 10 min to release DNA fragments into solution. The KAPA library kit for Illumina sequencing was used to

prepare the DAPseq libraries for sequencing from these DNA fragments. A final DAPseq DNA library was generated in the same conditions with no plasmid added to the TnT Master Mix as a negative control. DAPseq libraries were sequenced with paired end 150bp reads on either an Illumina MiSeq or NextSeq 500.

DAPseq data used in this study were deposited in the NCBI Sequence Read Archive (SRA) and are accessible through SRA series accession number PRJNA436200. Processed DAPseq data are available in Dataset S3.

DNA binding consensus motif generation

Motif discovery was performed using MEME v5.1.0 (7). The input for MEME motif discovery was DAPseq binding peak sequences with a maximum motif width of 20bp, a minimum motif width of 6bp, any number of motif sites in the sequences, the classic objective function, and a 0^{th} order Markov model for sequences. For AMN-1, binding peak sequences were included in motif generation if they were within 3kbp upstream of a translated gene that was at least 4-fold differentially expressed between wild type and Δ*amn-1* cells during exposure to mannose, proline, isoleucine, or tryptophan and had an FPKM of at least 10 in any one of these conditions. For NIT-2, binding peak sequences were included in motif generation if they were within 3kbp upstream of a translated gene that was at least 4-fold differentially expressed between wild type and Δ*nit-2* cells during exposure to nitrate, ammonium nitrate, proline, isoleucine, or tryptophan and had an FPKM of at least 10 in any one of these conditions. For NIT-4, binding peak sequences were included in motif generation if they were within 3kbp upstream of a translated gene that was at least 4-fold differentially expressed between wild type and Δ*nit-4* cells during exposure to nitrate or starvation and had an FPKM of at least 10 in either of these conditions.

Identification of motif locations was performed using FIMO v5.2.0. The input for FIMO motif identification was KCGGYTWKYRKCGGCHWW for AMN-1, HGATAAGV for NIT-2, and

KCCGCGGAGARAG for NIT-4 with a p-value cutoff of p<0.0001. Motifs were scanned against the DNA sequences upstream of each *N. crassa* gene (-1000 to 200 basepairs) found in the "Upstream Sequences: Fungal" *Neurospora crassa* GCF 000182925.2 NC12 database on the http://meme-suite.org/tools/fimo website.

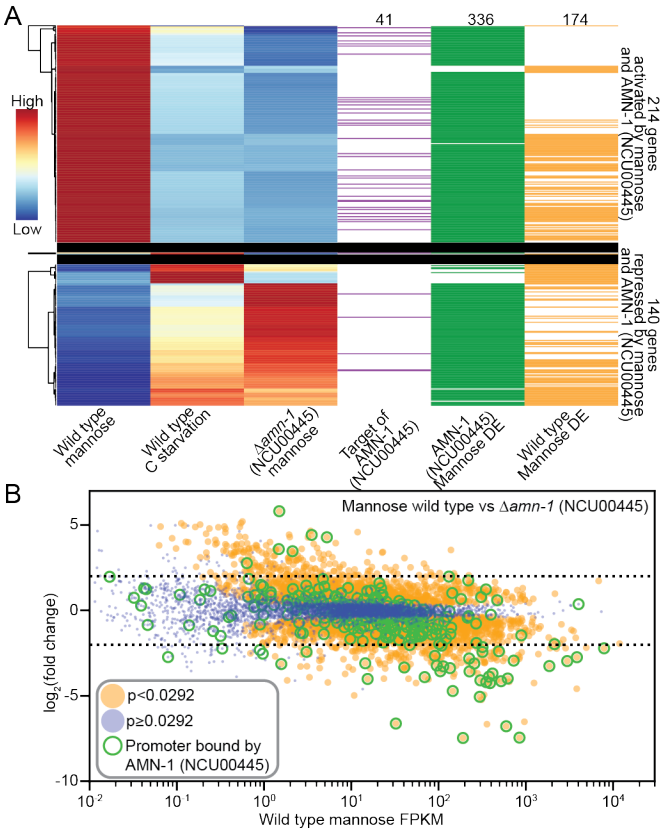

Fig. S1. NCU00445 regulates genes involved in mannan and mannose utilization. A. Heatmap of the expression level of the set of genes that were at least 4-fold differentially expressed between wild type cells exposed to 2mM mannose and carbon starvation or between Δ*amn-1* (NCU00445) and wild type cells exposed to 2mM mannose plus *amn-1* itself. Top heatmap shows genes activated by mannose and AMN-1 (includes *amn-1*). Bottom heatmap shows genes repressed by mannose and AMN-1. Middle heatmap (separated by 2 black bars) includes 2 genes with mixed phenotypes. Purple bars indicate genes whose promoters were bound by AMN-1. Green bars indicate genes that were at least 4-fold differentially expressed between Δ*amn-1* and wild type cells exposed to 2mM mannose plus *amn-1* itself. Orange bars indicate genes that were at least 4-fold differentially expressed between wild type cells exposed to 2mM mannose and carbon starvation. Numbers above each column indicate the total number of genes in each category. **B.** Differential expression analysis of Δ*amn-1* relative to wild type

cells after a shift to 2mM mannose. Green circles indicate genes whose promoters were bound by AMN-1. Dotted lines indicate 4-fold change in expression.

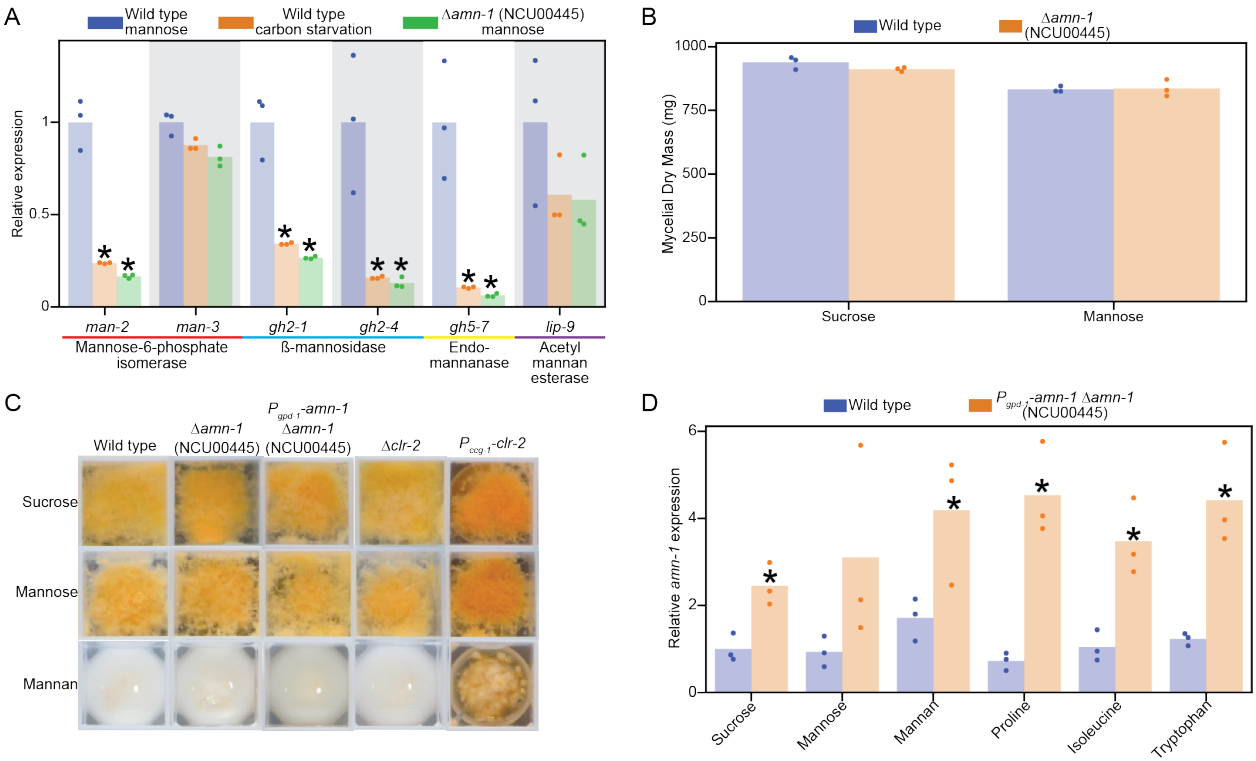

Fig. S2. NCU00445 is not required for growth on mannose or mannan. A. Relative expression (relative FPKM) of the indicated genes in wild type and Δ*amn-1* cells 4h post-transfer to carbon starvation or 2mM mannose. Asterisks indicate expression values that were statistically significantly different than in wild type cells exposed to 2mM mannose as determined using cuffdiff (5), *$p=5x10^{-5}$. **B.** Mycelial dry weight of wild type and Δ*amn-1* cells inoculated into 100ml of media containing 2% sucrose or 2% mannose. **C.** Pictures of wild type and mutant cells inoculated into 3ml of liquid media with either 2% sucrose, 2% mannose, or 1% mannan. The white substance in wells containing mannan is unconsumed mannan. Representative image of at least 3 biological replicates. **D.** Transcript abundance of *amn-1* expression relative to *act* in wild type cells versus P_{gpd-1}-*amn-1* Δ*amn-1* cells 4h post-transfer to 2% sucrose, 2% mannose, 1% mannan, 50mM proline, 50mM isoleucine, or 50mM tryptophan as measured by qRT-PCR. Asterisks indicate expression values that were statistically significantly different than wild type cells exposed to the indicated medium, *$p_{adj}<0.1$.

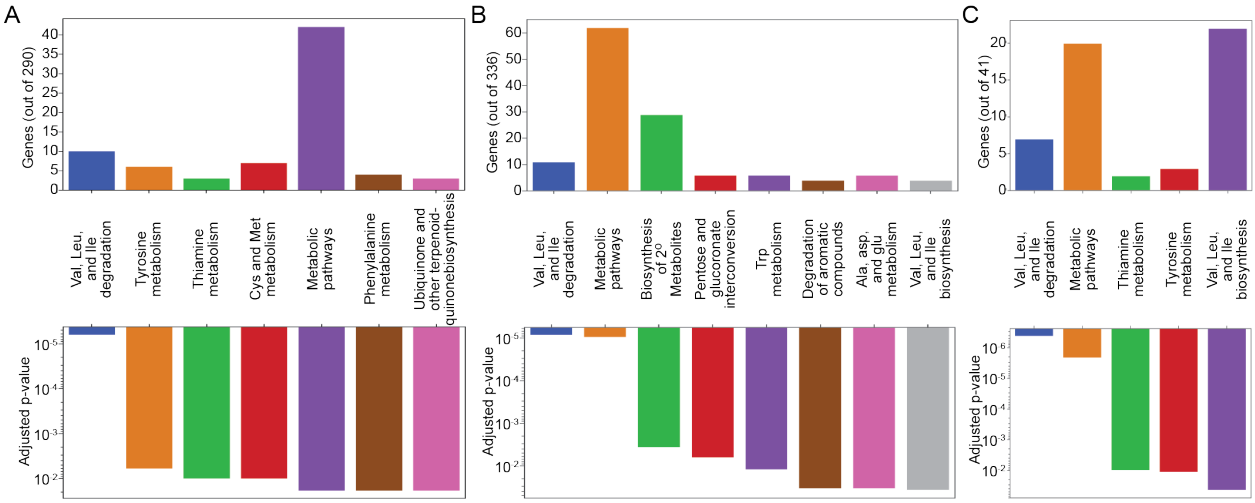

Fig. S3. KEGG category analysis. Distribution of genes in KEGG categories (8) and p-values reflecting the significance of enrichment of genes assigned to a given KEGG category as calculated using FungiFun 2.2.8 (9) of the set of genes **A.** whose promoters were bound by AMN-1, **B.** at least 4-fold differentially expressed between wild type and Δ*amn-1* cells exposed to 2mM mannose, and **C.** whose promoters were bound by AMN-1 and were at least 4-fold differentially expressed between wild type and Δ*amn-1* cells exposed to 2mM mannose. (The sets of genes include AMN-1 itself, which bound its own promoter.)

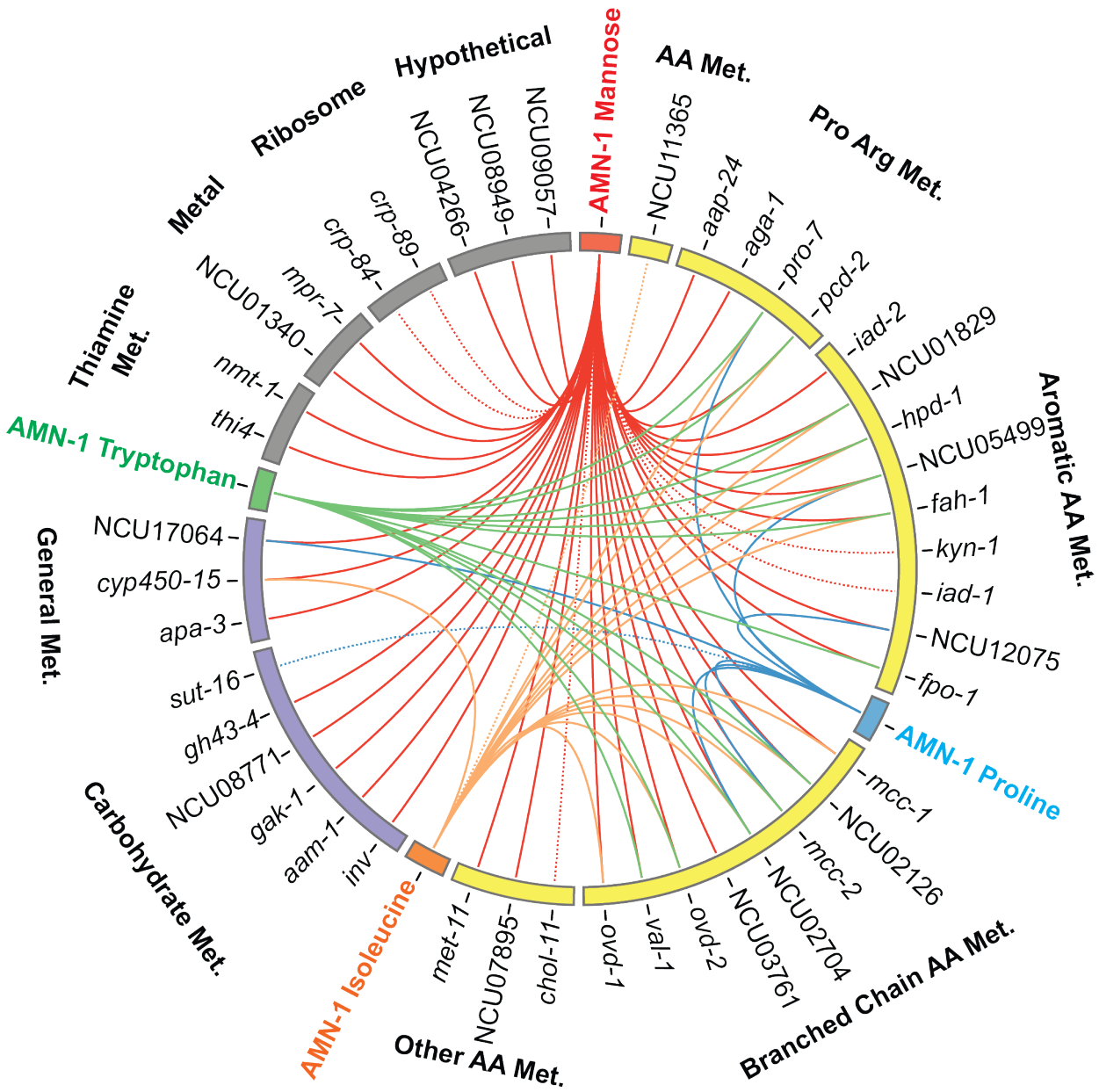

Fig. S4. AMN-1 is required for utilization of a variety of amino acids. Plot built with Circos, version 0.69 (10) to display the regulation of genes whose promoters were bound and whose expression was regulated by AMN-1 when cells were exposed to either 2mM mannose as the carbon source or 50mM proline, 50mM isoleucine, or 50mM tryptophan as the nitrogen source. Regulated genes were at least 4-fold differentially expressed between wild type and Δ*amn-1* cells during exposure to the indicated nutrient source. Activation by AMN-1 is indicated by a solid line. Repression by AMN-1 is indicated by a dotted line.

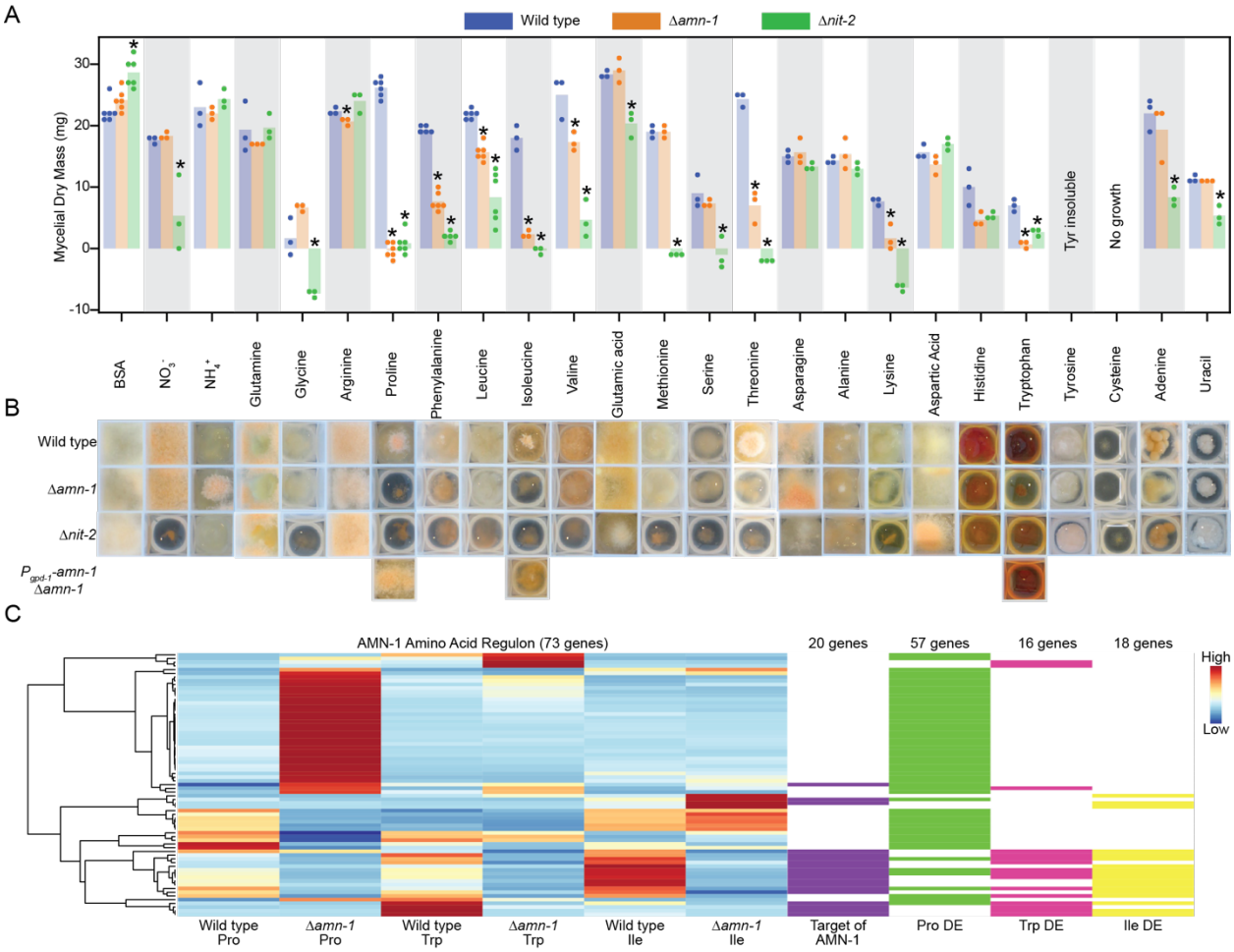

Fig. S5. AMN-1 is required for utilization of a variety of amino acids. A. Mycelial dry weight of wild type, Δ*amn-1*, or Δ*nit-2* cells inoculated into 3ml of media containing the indicated nitrogen source. The concentration of all nitrogen sources was 50mM, except for 1% BSA. Asterisks indicate mycelial dry weights that were statistically significantly different than that of wild type cells, *p_{adj}<0.05. Negative measurements sometimes occurred when the total accumulated biomass was less than the accuracy of the scale. **B.** Pictures of wild type and mutant cells inoculated into 3ml of liquid media with the indicated nitrogen source. The concentration of all nitrogen sources was 50mM, except for 1% BSA. Representative image of at least 3 biological replicates. (Note: Tyrosine is not soluble at 50mM, but *N. crassa* is still capable of utilizing the tyrosine as a nitrogen source. *N. crassa* is not capable of utilizing cysteine as the sole nitrogen source.) **C.** Heatmap of the expression level of the 72 genes that

were at least 4-fold differentially expressed between wild type and Δ*amn-1* cells exposed to 50mM proline, 50mM isoleucine, or 50mM tryptophan plus *amn-1* itself. Purple bars indicate genes whose promoters were bound by AMN-1 (includes *amn-1*). Green bars indicate genes that were differentially expressed between wild type and Δ*amn-1* cells exposed to proline. Pink bars indicate genes that were differentially expressed between wild type and Δ*amn-1* cells exposed to tryptophan. Yellow bars indicate genes that were differentially expressed between wild type and Δ*amn-1* cells exposed to isoleucine plus *amn-1* itself. Numbers above each column indicate the total number of genes in each category.

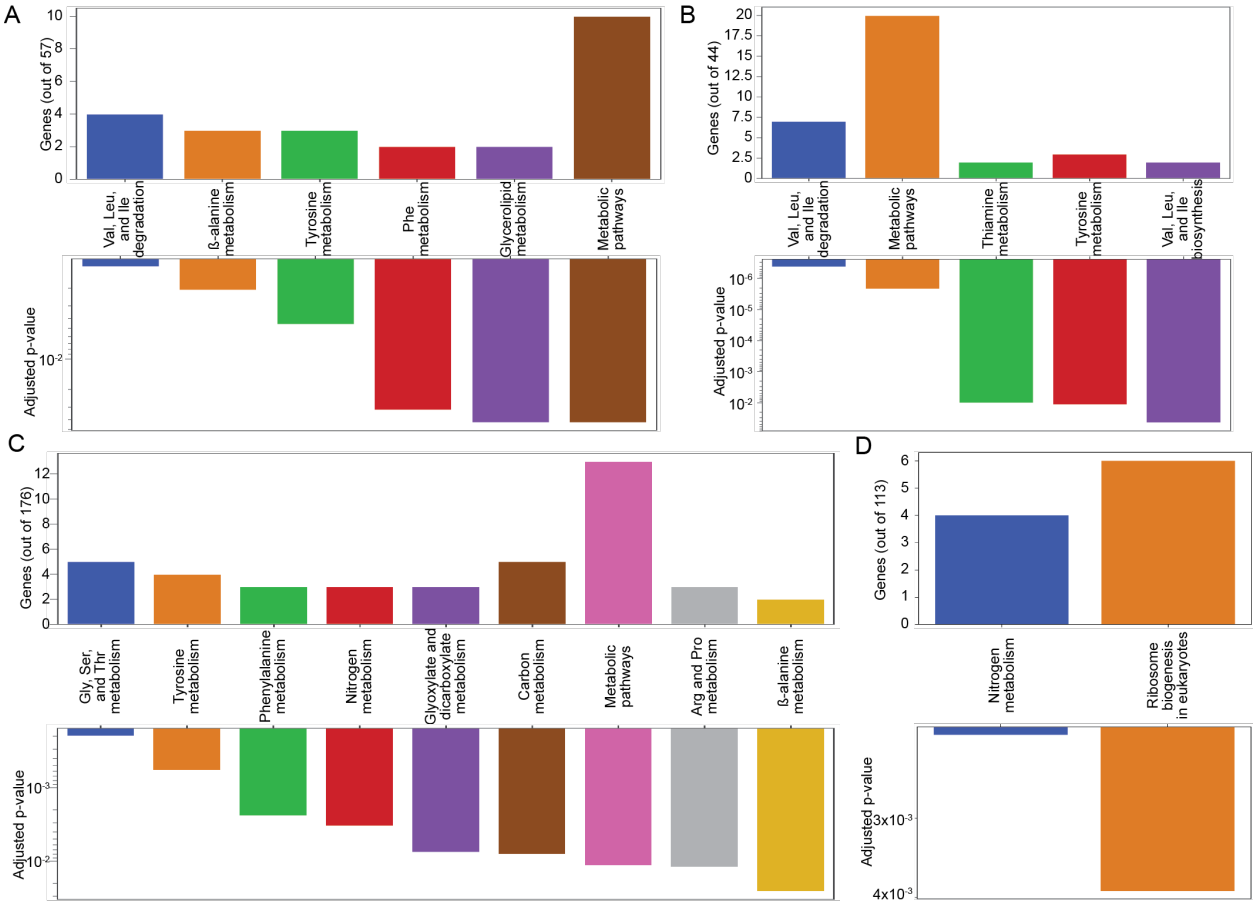

Fig. S6. KEGG category analysis. Distribution of genes in KEGG categories (8) and p-values reflecting the significance of enrichment of genes assigned to a given KEGG category as calculated using FungiFun 2.2.8 (9). **A.** Set of genes at least 4-fold differentially expressed between wild type and Δ*amn-1* cells exposed to 50mM proline. **B.** Set of genes whose promoters were bound by AMN-1 and were at least 4-fold differentially expressed between wild type and Δ*amn-1* cells exposed to 50mM proline, 50mM isoleucine, 50mM tryptophan, or 2mM mannose. **C.** Set of genes at least 4-fold differentially expressed between wild type and Δ*nit-2* cells exposed to 50mM proline, 50mM isoleucine, or 50mM tryptophan. **D.** Set of genes at least 4-fold differentially expressed between wild type and Δ*nit-2* cells exposed to 50mM nitrate.

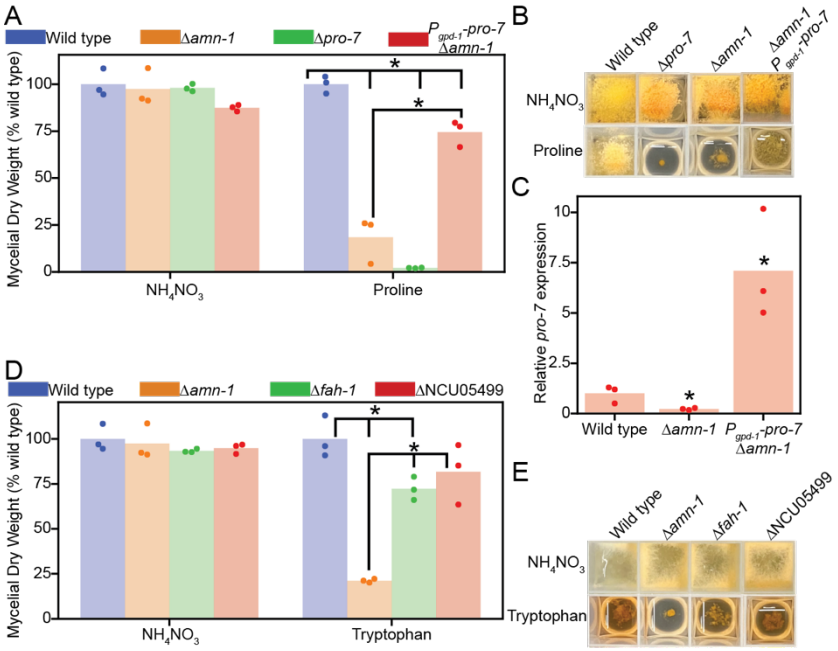

Fig. S7. AMN-1 acts through *pro-7* to regulate proline utilization. A. Mycelial dry weight relative to wild type of wild type and mutant cells inoculated into 100ml of media containing 25mM ammonium nitrate or 50mM proline, *p_{adj}<0.05. **B.** Pictures of wild type and mutant cells inoculated into 3ml of liquid media with 25mM ammonium nitrate or 50mM proline. Representative image of at least 3 biological replicates. **C.** Transcript abundance of *pro-7* relative to *act* in wild type versus mutant cells 4h post-transfer to 50mM proline as measured by qRT-PCR. Asterisks indicate expression values that were statistically significantly different than wild type cells, *p_{adj}<0.05. **D.** Mycelial dry weight relative to wild type of wild type and mutant cells inoculated into 100ml of media containing 25mM ammonium nitrate or 50mM tryptophan, *p_{adj}<0.05. **E.** Pictures of wild type and mutant cells inoculated into 3ml of liquid media with 25mM ammonium nitrate or 50mM tryptophan. Representative image of at least 3 biological replicates.

Fig. S8. Transcription factor DNA consensus binding motifs. Consensus DNA binding motifs of **A.** AMN-1, E-value = 2.1×10^{-26}, **B.** NIT-2, E-value = 3.3×10^{-37}, and **C.** NIT-4, E-value = 5.9×10^{-6} built using MEME version 5.1.0 (7).

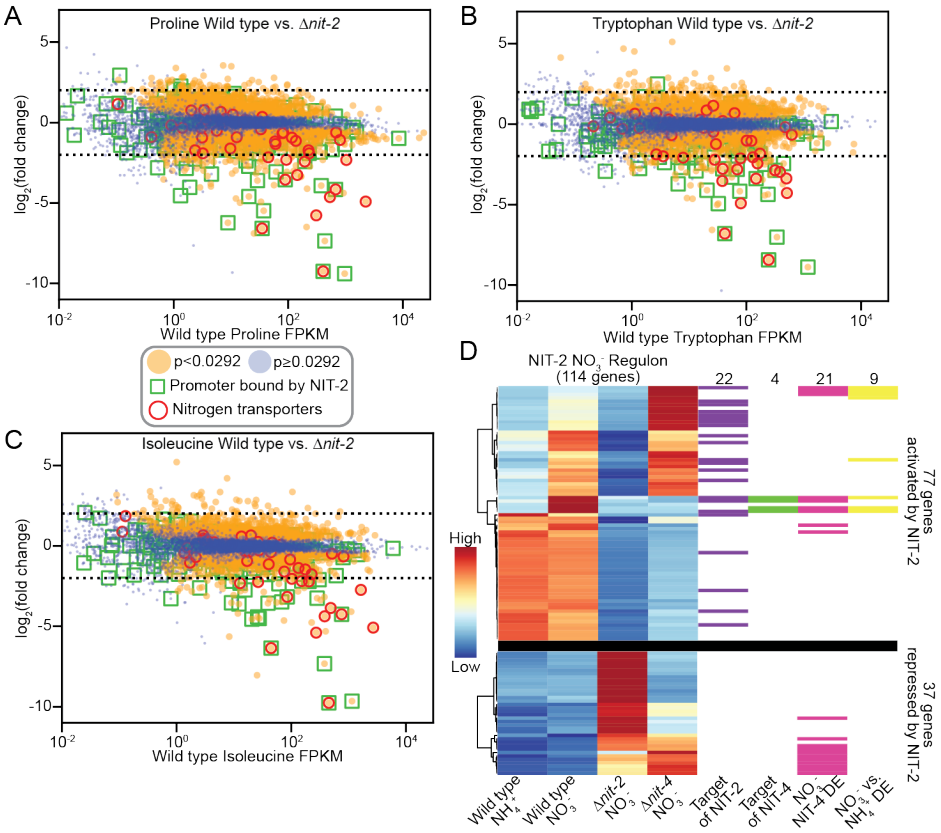

Fig. S9. NIT-2 regulates genes necessary for nitrogen import and enzymes responsible for utilization of all amino and nucleic acids. A-C. Differential expression analysis of Δ*nit-2* relative to wild type cells after a shift to (**A**) 50mM proline, (**B**) 50mM tryptophan, and (**C**) 50mM isoleucine. Green squares indicate genes whose promoters were bound by NIT-2. Red circles indicate genes predicted to encode nitrogen transporters. Dotted lines indicate 4-fold change in expression. **D.** Heatmap of the expression level of the 113 genes at least 4-fold differentially expressed between wild type and Δ*nit-2* cells exposed to 50mM nitrate plus *nit-2* itself. Upper heatmap includes genes activated by NIT-2 plus *nit-2* itself. Lower heatmap includes genes repressed by NIT-2. Purple bars indicate genes whose promoters were bound by NIT-2. Green bars indicate genes whose promoters were bound by NIT-4. Pink bars indicate genes differentially expressed between Δ*nit-4* and wild type cells exposed to 50mM nitrate. Yellow bars indicate genes differentially expressed between wild type cells exposed to 50mM nitrate and

50mM ammonium. Numbers above each column indicate the total number of genes in each category.

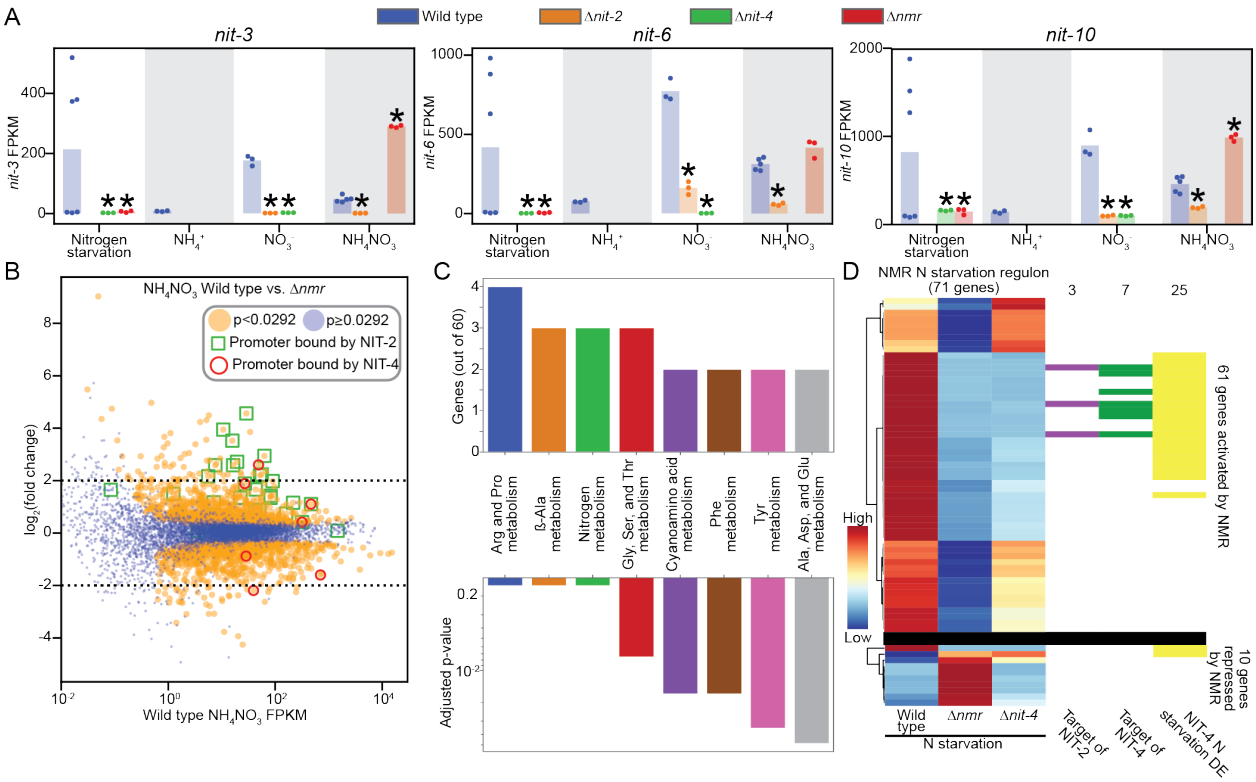

Fig. S10. NIT-4 and NMR regulate genes involved in nitrate and amino acid metabolism.

A. FPKMs of the indicated genes in wild type and mutant strains 4h post-transfer to nitrogen starvation, 50mM ammonium, 50mM nitrate, or 25mM ammonium nitrate. Asterisks indicate expression values that were statistically significantly different than in wild type cells as determined using cuffdiff (5), *$p<10^{-4}$. **B.** Differential expression analysis of Δ*nmr* relative to wild type cells after a shift to 25mM ammonium nitrate. Green squares indicate genes whose promoters were bound by NIT-2. Red circles indicate genes whose promoters were bound by NIT-4. Dotted lines indicate 4-fold change in expression. **C.** Distribution of genes in KEGG categories (8) and p-values reflecting the significance of enrichment of genes assigned to a given KEGG category as calculated using FungiFun 2.2.8 (9) of the set of genes that were at least 4-fold differentially expressed between wild type and Δ*nmr* cells exposed to 25mM ammonium nitrate. **D.** Heatmap of the expression level of the 70 genes at least 4-fold differentially expressed between wild type and Δ*nmr* cells exposed to nitrogen starvation plus *nmr* itself. Upper heatmap includes genes activated by NMR plus *nmr* itself. Lower heatmap

includes genes repressed by NMR. Purple bars indicate genes whose promoters were bound by NIT-2. Green bars indicate genes whose promoters were bound by NIT-4. Yellow bars indicate genes differentially expressed between Δ*nit-4* and wild type cells exposed to nitrogen starvation. Numbers above each column indicate the total number of genes in each category.

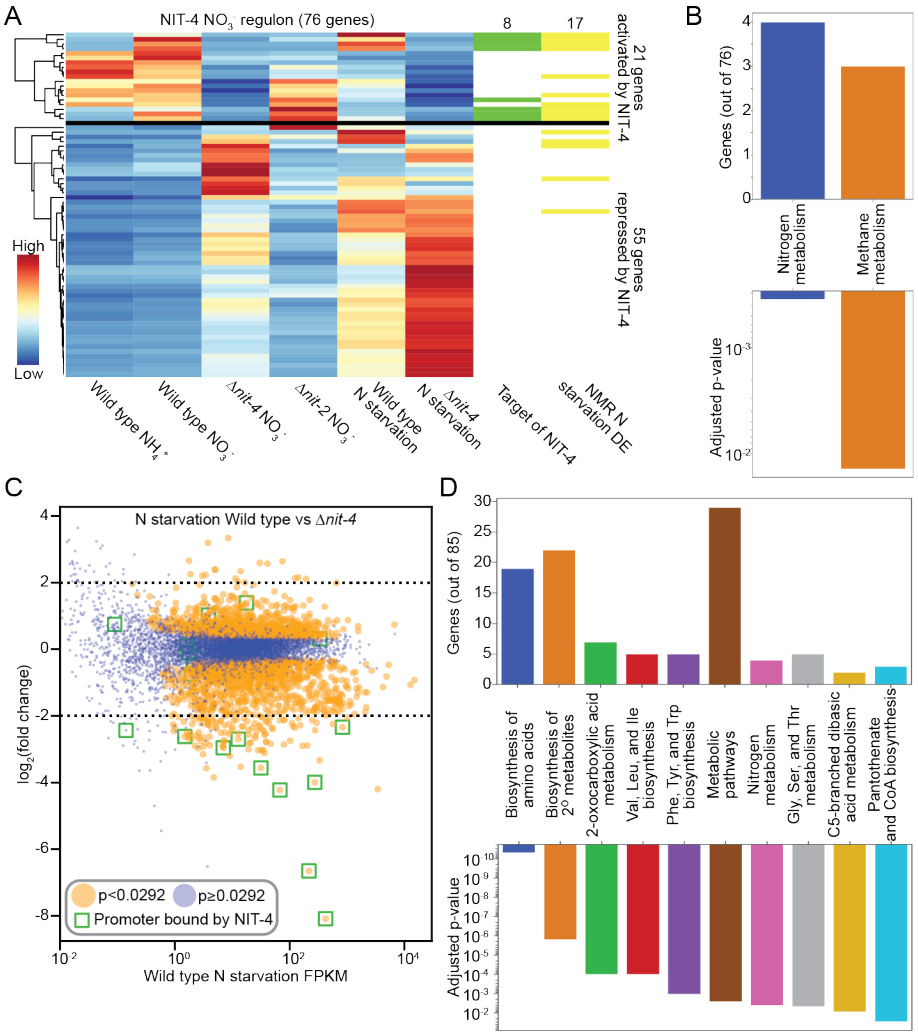

Fig. S11. NIT-4 activates genes during exposure to nitrate and nitrogen starvation. A. Heatmap of the expression level of the set of genes at least 4-fold differentially expressed between wild type and Δ*nit-4* cells exposed to 50mM nitrate. Upper heatmap includes genes activated by NIT-4. Lower heatmap includes genes repressed by NIT-4. Green bars indicate genes whose promoters were bound by NIT-4. Yellow bars indicate genes differentially expressed between Δ*nmr* and wild type cells exposed to nitrogen starvation. Numbers above each column indicate the total number of genes in each category. **B.** Distribution of genes in KEGG categories (8) and p-values reflecting the significance of enrichment of genes assigned to a given KEGG category as calculated using FungiFun 2.2.8 (9) of the set of genes at least 4-fold differentially expressed between wild type and Δ*nit-4* cells exposed to 50mM nitrate. **C.**

Differential expression analysis of Δ*nit-4* relative to wild type cells after a shift to nitrogen starvation. Green squares indicate genes whose promoters were bound by NIT-4. Dotted lines indicate 4-fold change in expression. **D.** Distribution of genes in KEGG categories (8) and p-values reflecting the significance of enrichment of genes assigned to a given KEGG category as calculated using FungiFun 2.2.8 (9) of the set of genes at least 4-fold differentially expressed between wild type and Δ*nit-4* cells exposed to nitrogen starvation.

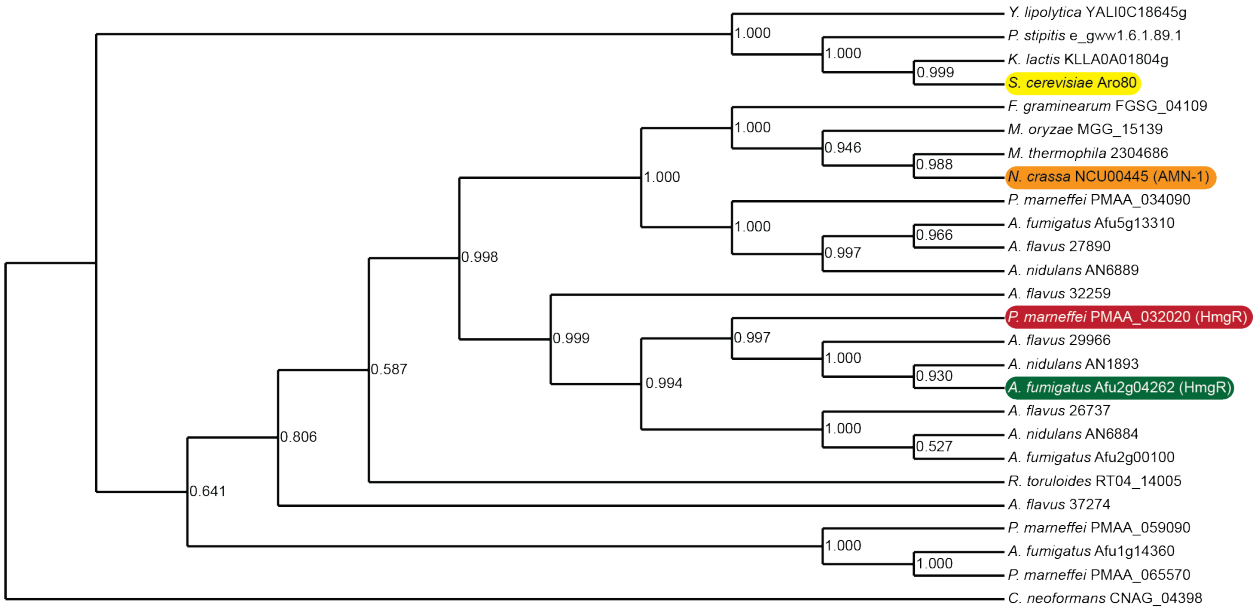

Fig. S12. NCU00445 is conserved in ascomycete fungi. Protein sequences of AMN-1 homologs were used to build a phylogenetic tree using the maximum likelihood method based on the Jones-Taylor-Thornton matrix-based model using FastTree with *Cryptococcus neoformans* as an outgroup (11). The reliability of each split in the tree was computed using the Shimodaira-Hasegawa test on three alternate topologies around that split. *N. crassa* AMN-1 is highlighted in orange. *S. cerevisiae* Aro80 is highlighted in yellow. *P. marneffei* HmgR is highlighted in red. *A. fumigatus* HmgR is highlighted in green. Full species names: *Yarrowia lipolytica*, *Pichia stipitis*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Fusarium graminearum*, *Magnaporthe oryzae*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium marneffei*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus nidulans*, *Rhodosporidium toruloides*, *Cryptococcus neoformans*.

SI TABLES

Table S1. Nutrient sources used in this study.

Nutrient source	Concentration
Ammonium nitrate (VMM)[1]	25mM
Bovine serum albumin (BSA)	1%
Nitrate ($NaNO_3$)[2]	50mM
Ammonium (NH_4Cl)	50mM
Alanine (RNAseq*)	10mM (12)
Alanine (growth experiment)	50mM
Arginine (RNAseq)	3.4mM
Arginine (growth experiment)	50mM
Asparagine	50mM
Aspartic acid	50mM
Cysteine	50mM
Glutamate (RNAseq)	5mM (13)
Glutamate (growth experiment)	50mM
Glutamic acid	50mM
Glutamine (RNAseq)	5mM (13)
Glutamine (growth experiment)	50mM
Glycine (RNAseq)[2]	20mM
Glycine (growth experiment)[2]	50mM
Histidine	50mM
Isoleucine	50mM
Leucine[2]	50mM
Lysine	50mM
Methionine	50mM
Phenylalanine	50mM
Proline	50mM
Serine	50mM
Threonine	50mM
Tryptophan[2]	50mM
Tyrosine[3]	50mM (not soluble)
Valine[2]	50mM
Adenine	50mM
Uracil	50mM
Mannose (growth experiment†)[4]	2%
Mannose (RNAseq†)[3]	2mM
Sucrose (VMM)[4]	2%

Mannan[5]	1%

[1]Purchased from Ward's Science

[2]Purchased from Fisher Scientific

[3]Purchased from Acros

[4]Purchased from Research Products International

[5]Purchased from Megazyme (ivory nut)

* Nitrogen sources were added at 50mM to match the concentration of nitrogen in Vogel's minimal medium (1), except where noted.

† Sugar concentrations of 2% were used to measure cell growth. However, in order to reduce the effects of carbon catabolite repression, 2mM mannose was used for transcriptional profiling (2, 14).

Table S2. Strains used in this study.

Name	**Genotype**	**Source**
Wild type	Wild-type *mat A*	FGSC 2489 (15)
Δ*amn-1*	Δ*amn-1::hyg*^{R} *mat A*	FGSC 16386 (16)
Δ*nit-2*	Δ*nit-2::hyg*^{R} *mat A*	This study (FGSC 26743)
Δ*nit-2*	Δ*nit-2::hyg*^{R} *mat a*	FGSC 11392 (16)
Δ*nit-4*	Δ*nit-4::hyg*^{R} *mat A*	FGSC 11007 (16)
Δ*nmr*	Δ*nmr::hyg*^{R} *mat A*	FGSC 14031 (16)
Δ*clr-2*	Δ*clr-2::hyg*^{R} *mat A*	FGSC 15834 (16)
Δ*pro-7*	Δ*pro-7::hyg*^{R} *mat A*	FGSC 16324 (16)
Δ*fah-1*	Δ*fah-1::hyg*^{R} *mat A*	FGSC 12107 (16)
ΔNCU05499	ΔNCU05499::*hyg*^{R} *mat A*	FGSC 13584 (16)
P_{ccg-1}-*clr-2*	P_{ccg-1}-*clr-2::his-3* Δ*sad-1::hyg*^{R} *rid-1-;* Δ*clr-2::hyg*^{R} *mat A*	Coradetti *et al* (2013) (17)
P_{gpd-1}-*amn-1* Δ*amn-1*	P_{gpd-1}-*amn-1::csr-1;* Δ*amn-1::hyg*^{R} *mat A*	This study (FGSC 26744)
P_{gpd-1}-*pro-7* Δ*amn-1*	P_{gpd-1}-*pro-7::csr-1;* Δ*amn-1::hyg*^{R} *mat A*	This study (FGSC 26803)

Table S3. RNAseq library construction and sequencing.

Strain	Condition	Location	Sequencer	Read length	Replicates
Wild type	VMM	Joint Genome Institute	HiSeq 2000	1x100	5
Wild type	Alanine	Joint Genome Institute	HiSeq 2000	1x100	3
Wild type	Arginine	Joint Genome Institute	HiSeq 2000	1x100	3
Wild type	Glutamate	Joint Genome Institute	HiSeq 2000	1x100	3
Wild type	Glutamine	Joint Genome Institute	HiSeq 2000	1x100	3
Wild type	Glycine	Joint Genome Institute	HiSeq 2000	1x100	3
Wild type	Isoleucine	UC Davis Genome Center	HiSeq 4000	1x90	3
Wild type	Proline	UC Davis Genome Center	HiSeq 4000	1x90	3
Wild type	Tryptophan	UC Davis Genome Center	HiSeq 4000	1x90	3
Wild type	Nitrate	UC Davis Genome Center	HiSeq 4000	1x90	3
Wild type	Ammonium	UC Davis Genome Center	HiSeq 4000	1x90	3
Wild type	Nitrogen starvation	Joint Genome Institute	HiSeq 2000	1x100 (3 samples) 1x101 (3 samples)	6
Wild type	Mannose	Joint Genome Institute	HiSeq 2000	1x100	3
Wild type	Carbon starvation	Joint Genome Institute	HiSeq 2000	1x100	3
Δ*amn-1*	Mannose	Joint Genome Institute	HiSeq 2000	1x100	3
Δ*amn-1*	Isoleucine	UC Davis Genome Center	HiSeq 4000	1x90	3
Δ*amn-1*	Proline	UC Davis Genome Center	HiSeq 4000	1x90	3
Δ*amn-1*	Tryptophan	UC Davis Genome Center	HiSeq 4000	1x90	3
Δ*nit-2*	Nitrate	UC Davis Genome Center	HiSeq 4000	1x90	3
Δ*nit-2*	Isoleucine	UC Davis Genome Center	HiSeq 4000	1x90	3
Δ*nit-2*	Proline	UC Davis Genome Center	HiSeq 4000	1x90	3
Δ*nit-2*	Tryptophan	UC Davis Genome Center	HiSeq 4000	1x90	3
Δ*nit-2*	VMM	Joint Genome Institute	HiSeq 2000	1x101	3
Δ*nit-4*	Nitrate	UC Davis Genome Center	HiSeq 4000	1x90	3
Δ*nit-4*	Nitrogen starvation	Joint Genome Institute	HiSeq 2000	1x101	3
Δ*nmr*	VMM	Joint Genome Institute	HiSeq 2000	1x101	3
Δ*nmr*	Nitrogen starvation	Joint Genome Institute	HiSeq 2000	1x101	3

Table S4. qRT-PCR primer sequences.

Gene	Forward primer 5'→3'	Reverse primer 5'→3'
act	TGATCTTACCGACTACCT	CAGAGCTTCTCCTTGATG
amn-1	CTTGGTGAGTTATCCTGGCCT	TTGTAGGATTGCCGCGTTCT
pro-7	GGCGACTTCGTAGCTCTCAA	CGATGGCAGAACCTAGAGCC

Dataset S1. Normalized FPKM counts and differential expression analysis of wild type, Δ*amn-1*, Δ*nit-2*, Δ*nit-4*, and Δ*nmr* cells exposed to various nitrogen and carbon sources.

Dataset S2. Mycelial dry weights and qRT-PCR data from wild type and mutant cells exposed to various nitrogen and carbon sources.

Dataset S3. DAPseq data for AMN-1, NIT-2, and NIT-4.

Dataset S4. *N. crassa* genes containing AMN-1, NIT-2, and NIT-4 DNA consensus binding motifs in their promoter regions.

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