RCO-3 and COL-26 form an External-to-Internal Module that Regulates the Dual-Affinity Glucose Transport System in Neurospora Crassa

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Research

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Abstract

Background Low- and high-affinity glucose transport system is a conserved strategy of microorganism to cope with environmental glucose fluctuation for their growth and competitiveness. In *Neurospora crassa*, the dual-affinity glucose transport system consists of a low-affinity glucose transporter GLT-1 and two high-affinity glucose transporters HGT-1/HGT-2, which play diverse roles in glucose transport, carbon metabolism, and cellulase expression regulation. However, the regulation of this dual-transporter system in response to environmental glucose fluctuation is not yet clear.

Results In this study, we report that a regulation module consisting of a downstream transcription factor COL-26 and an upstream non-transporting glucose sensor RCO-3 regulates the dual-affinity glucose transport system in *N. crassa*. COL-26 directly binds to the promoter regions of *glt-1*, *hgt-1*, and *hgt-2*, whereas RCO-3 is an upstream factor of the module whose deletion mutant resembles the Δcol-26 mutant phenotypically. Transcriptional profiling analysis revealed that Δcol-26 and Δrco-3 mutants had similar transcriptional profiles, and both mutants had impaired response to a glucose gradient. We also showed that the AMP-activated protein kinase (AMPK) complex is involved in regulation of the glucose transporters. AMPK is required for repression of *glt-1* expression in starvation conditions by inhibiting the activity of RCO-3.

Conclusions RCO-3 and COL-26 form an external-to-internal module that regulates the glucose dual-affinity transport system. Transcription factor COL-26 was identified as the key regulator. AMPK was also involved in the regulation of the dual transporter system. Our findings provide novel insight into the molecular basis of glucose uptake and signaling in filamentous fungi, which may aid in the rational design of fungal strains for industrial purposes.

Background Glucose is the preferred carbon source for most microorganisms as well as a signaling molecule that regulates physiological and pathological processes (1). The sensing and uptake of glucose triggers a cellular regulatory network that influences multiple biological processes including sugar transporter expression, carbon catabolism, and biomass accumulation (2). In cellulolytic filamentous fungi, the detection of glucose triggers the repression of genes encoding lignocellulose-degrading enzymes, a mechanism known as carbon catabolite repression (CCR), which is mediated by the transcription factor CreA/CRE1 (3, 4). Although CreA/CRE1-mediated transcriptional repression has been extensively studied (3, 5–9), sensing of extracellular glucose concentrations and subsequent transport are barely characterized in filamentous fungi.

In *Saccharomyces cerevisiae*, two transporter-like glucose sensors, Rgt2p and Snf3p, mediate a glucose signaling pathway (10, 11). Upon glucose detection, Rgt2p/Snf3p-associated casein kinase I (Yck1p/Yck2p) phosphorylates Mth1p and Std1p (12), leading to their degradation by SCF^Grr1^-mediated proteolysis (13), which triggers the release of Rgt1p from hexose transporter (HXT) promoter regions and
derepresses the HXT genes (14). Extracellular glucose concentrations are also detected by a G-protein coupled receptor, Gpr1p. Binding of glucose to Gpr1p activates the downstream heterotrimeric Ga protein Gpa2p (15). The Gpr1/Gpa2 pathway works in parallel with Ras to activate adenylate cyclase Cyr1p, which increases cAMP levels, thereby activating protein kinase A (PKA). The cAMP/PKA pathway is important for spore germination, hyphal growth, cell wall homeostasis, conidiation, and secondary metabolite production (16–19). PKA catalyzes phosphorylation of Rgt1p and regulates its function (20, 21), suggesting some crosstalk between these two sensing pathways.

The signaling pathway required to adapt to nutrient limitation and to use alternative carbon sources centers on the kinase Snf1p, a component of the S. cerevisiae SNF1 complex (22, 23). The SNF1 complex is homologous to AMP-activated protein kinase (AMPK) in higher eukaryotes, which acts as a regulator of cellular energy homeostasis (24). Like AMPK, SNF1 is a heterotrimer consisting of a catalytic α-subunit (Snf1p), a regulatory γ-subunit (Snf4p), and one of three β-subunits (Sip1p, Sip2p, or Gal83p) (25, 26). In low glucose conditions, Snf1p is activated by the phosphorylation of residue Thr210 by one of three upstream kinases (Sak1p, Tos3p, or Elm1p) (27–29). In response to high glucose concentrations, Snf1p is inactivated by dephosphorylation of Thr210 by phosphatase Glc7p (30). ADP association with Snf4p protects Thr210 of Snf1p from dephosphorylation (31). Active Snf1p phosphorylates the inhibitor Mig1p, the homolog of CreA/CRE1 in filamentous fungi, to relieve CCR through its translocation to the cytoplasm (32), promoting the use of alternative carbon sources such as ethanol and acetate (33). Snf1p also phosphorylates Cyr1p and negatively regulates PKA-dependent transcription (34), whereas PKA phosphorylates the Snf1-activating kinase Sak1p (35) and the β-subunit Sip1p (36), suggesting a crosstalk between the Snf1p and PKA pathways. Snf1/Mig1, Rgt2/Snf3, and cAMP/PKA pathways, as well as their crosstalk, enable yeast cells to sense a wide range of glucose concentrations and subsequently express corresponding transporters such as low-affinity glucose transporters Hxt1p and Hxt3p or high-affinity glucose transporters Hxt6p and Hxt7p (37, 38).

The low-affinity and high-affinity glucose uptake systems in filamentous fungi were described a long time ago (39), but the corresponding genes were characterized at the molecular level much later (39–41). In Aspergillus niger, MstA, MstF, MstG, and MstH were determined to be high-affinity glucose transporters, whereas MstC is a low-affinity glucose transporter (42–44). In A. nidulans, MstE is a low-affinity glucose transporter, whereas HxtA, MstA (HxtD), and MstC (HxtB) were described as high-affinity glucose transporters (45–48), and HxtC and HxtE are glucose transporters with unknown affinity (47). MstC (HxtB) is also involved in glucose signaling and metabolism in A. nidulans (49). In Neurospora crassa, GLT1 was characterized as a low-affinity glucose transporter, and HGT-1 and HGT-2 are two high-affinity glucose transporters (50, 51). GLT-1 and HGT-1/-2 were identified as the major components of the dual-affinity glucose transport system of N. crassa with HGT-1/-2 also involved in glucose signaling and CCR (51). In Colletotrichum graminicola, CgHXT1 and CgHXT3 are glucose transporters with high-affinity, whereas the A. nidulans MstE and N. crassa GLT1 ortholog CgHXT5 is a low-affinity glucose transporter (52). Other characterized high-affinity glucose transporters include GTT1 from Trichoderma harzianum (53); UfHXT1p from Uromyces fabae (54); the N. crassa RCO-3 ortholog AmMst1 from Amanita muscaria (55, 56); RiMST2, RiMST5, and RiMST6 from Rhizophagus irregularis (57, 58); and GpMST1 from...
Geosiphon pyriformis (59). Glucose transporters with unknown affinity include Stp1 and TrHxt1 from Trichoderma reesei (60, 61), and MSF1 from Colletotrichum lindemuthianum, whose deletion mutant resembles to some extent the Δrco-3 mutant of N. crassa in growth and conidiation (62).

However, in contrast to the comprehensive analysis of hexose transport in S. cerevisiae, much less is known about glucose sensing and the subsequent transcriptional regulation of glucose transporter-encoding genes in filamentous fungi. In N. crassa, the S. cerevisiae Gpr1p ortholog GPR-4 (NCU06312) functions as a carbon source receptor. Ligand binding to GPR-4 activates the downstream Ga protein GNA-1 (NCU06493), leading to an increase in cAMP level produced by the activated adenylate cyclase CR-1 (NCU08377) (63). Similarly, in A. nidulans, the G-protein coupled receptor GprH and Ga subunit GanB have been shown to be involved in glucose sensing (64, 65). During early conidial germination events, GanB activates cAMP synthesis and subsequent PKA activity in the presence of glucose (64). In Ustilago maydis, the S. cerevisiae Rgt2p/Snf3p ortholog Hxt1 was characterized as a high-affinity glucose transporter and sensor involved in glucose signaling (66). Similarly in N. crassa, the yeast Rgt2p/Snf3p ortholog RCO-3 was shown to be involved in glucose sensing (67). Mutation of this non-transporting glucose sensor leads to complete dysfunction of the low-affinity glucose transport system and partial impairment of the high-affinity system (67). However, orthologs of S. cerevisiae Mth1p, Sth1p, and Rgt1p have not been found in filamentous fungi, suggesting the presence of a different signaling pathway from the S. cerevisiae Rgt2p/Snf3p pathway.

In this study, we identified a module including transcription factor COL-26, the ortholog of the Zn(II)$_2$Cys$_6$ transcription factor AmyR in Aspergillus (68, 69), and RCO-3, a non-transporting glucose sensor, that regulate the dual-affinity glucose transport system in the model fungus N. crassa. COL-26 binds to the promoter regions of glt-1 and hgt-1/-2 to promote their expression, and RCO-3 is also essential for glt-1 expression in both glucose-rich and starvation conditions, and regulates the pathway, possibly by indirectly affecting the expression of COL-26. Transcriptomic analysis showed that the response of Δcol-26 and Δrco-3 mutants to a glucose gradient was greatly impaired. In addition, AMPK is also involved in the pathway by inhibiting the activity of RCO-3 in starvation conditions. Since the dual-affinity glucose transport system is widely conserved among fungal species, knowledge about its regulation will provide a foundation for further investigation into the molecular basis of nutrient transport and signaling as well as plant cell wall degradation in fungi.

Results

Identification of COL-26 as a key transcription factor to promote glt-1 expression in response to external glucose

To search for transcription factors essential for expression of glt-1, deletion mutants of 36 transcription factors of N. crassa, based on their expression in adequate glucose conditions (Additional file 1: Table S1) (51), were chosen and screened through batch culture with glucose as the sole carbon source followed by qRT-PCR assay. Most of the mutants showed no significant change in glt-1 expression
compared with the WT strain. However, the ΔNCU07788 (Δcol-26) mutant showed dramatically decreased expression of *glt-1* (Fig. 1A). COL-26 is a zinc binuclear cluster [Zn(II)$_2$Cys$_6$] DNA-binding protein that is essential for starch utilization (69). Expression of *hgt-1/-2* was significantly upregulated in the Δcol-26 mutant in response to glucose, whereas the expression of *hgt-1/-2* and *glt-1* was downregulated in response to starvation (Fig. 1B). This suggested that COL-26 is involved in regulating the dual-affinity glucose transport system in *N. crassa*. The nuclear localization of COL-26 was independent of glucose concentration (Fig. 1C).

To further investigate whether COL-26 directly regulates *glt-1* and *hgt-1/-2*, EMSAs were performed. GST-fused DNA binding domain of COL-26 was expressed in and purified from *E. coli* (Additional file 2: Fig. S1A). EMSA results showed that the recombinant COL-26 bound to the promoter regions of all three genes in a typical protein concentration-dependent manner (Fig. 2A–C). Retardation occurred upon addition of 16 nM recombinant COL-26. The downregulation of *hgt-1/-2* expression in a Δcol-26 mutant in response to starch has been reported (69). In addition, several other transporter genes were reported to be regulated by COL-26, including xylose transporter-1 *xyt-1* (NCU05627), a predicted sugar transporter (NCU04537), a predicted ammonium transporter *tam-1* (NCU03257), and a uracil permease *uc-5* (NCU07334), as well as the starch-active lytic polysaccharide monooxygenase (NCU08746) (69). EMSAs showed that COL-26 also binds to the promoter regions of *xyt-1* and NCU08746 but not NCU04537, *tam-1*, or *uc-5* (Additional file 2: Fig. S1B–F), suggesting the involvement of COL-26 in multiple biological processes beyond regulation of sugar transporter expression.

**Δ col-26 mutant phenotypically and transcriptionally resembles Δrco-3 mutant**

In *N. crassa*, RCO-3 acts as a non-transporting glucose sensor (67). Expression of *glt-1* is dramatically downregulated in a Δrco-3 mutant, whereas the expression of *hgt-1/-2* is significantly upregulated in glucose-rich conditions (51), which is similar to the Δcol-26 mutant (Fig. 1A, 1B). In addition, both mutants are defective in glucose uptake and biomass accumulation in the presence of high glucose levels, and are resistant to 2-deoxyglucose (2-DG, which cannot be catalyzed during glycolysis and is a drug often used for glucose repression analysis in filamentous fungi) (4, 67). The Δcol-26 mutant showed severe defects in using other simple sugars including sucrose, fructose, mannose, and maltose, although the growth defect on cellobiose was minor (4, 69). So, we used medium containing cellobiose to assess the effect of stress on the growth of the Δcol-26 and Δrco-3 mutants. Both mutants were sensitive to osmotic stress and H$_2$O$_2$-induced oxidative stress compared with the WT strain, as shown by plate growth assays (Additional file 3: Fig. S2) and the corresponding mycelial diameter of the Δcol-26 and Δrco-3 mutants (Fig. 3A). We assumed that COL-26 and RCO-3 are probably in a common regulatory cascade, in which membrane-located RCO-3 transduces a glucose signal to nuclear-located COL-26 in the presence of glucose.

To test this hypothesis and also obtain a broad view of the mode of expression, we conducted high-throughput sequencing (RNA-Seq) of wild-type, Δcol-26, and Δrco-3 mycelia exposed to a gradient of glucose (0%, 0.05%, 0.5%, 2.0%) for 1 h. Pearson and Spearman correlation analysis demonstrated that
the biological replicates were reliable for all tested samples (Additional file 4: Fig. S3A). RNA-Seq data (Additional file 1: Table S2) from the WT, Δcol-26, and Δrco-3 biological replicates were subjected to principal component analysis and data from the same strain grown in the same growth conditions clustered together. Compared with the WT strain, data from the Δcol-26 mutant and the Δrco-3 mutant exposed to glucose (0.05%, 0.5% and 2.0%) clustered together (Fig. 3B). This indicated that both mutants had impaired transcriptomic responses to 0.05%, 0.5%, and 2% glucose and had similar expression profiles, which was in accordance with sample-to-sample clustering (Additional file 4: Fig. S3B).

Consistent with these observations, the number of differentially expressed genes (DEGs) in Δrco-3 vs. Δcol-26 was much lower than that in Δrco-3 vs. WT and Δcol-26 vs. WT exposed to glucose. At 2%, 0.5%, and 0.05% glucose, there were only 197, 99, and 120 DEGs, respectively, in Δrco-3 vs. Δcol-26 (Fig. 3C, Additional file 1: Table S3), whereas the numbers were 665, 533, and 1024 for Δrco-3 vs. WT and 745, 566, and 788 for Δcol-26 vs. WT, respectively (Fig. 3E, Additional file 1: Table S3). Gene Ontology (GO) enrichment analysis showed that DEGs in Δrco-3 vs. WT and Δcol-26 vs. WT in each condition were mainly enriched in oxidation-reduction processes (GO: 0055114) and metabolic processes (GO: 0008152) (Additional file 1: Table S4). In addition, the number of DEGs in the Δrco-3 mutant and Δcol-26 mutant comparing 0.05% glucose with 0.5% glucose was dramatically lower than the number in the WT (Fig. 3D, Additional file 1: Table S3). Further investigation showed that in the presence of glucose, rco-3 and col-26 regulate a large proportion of DEGs in common as described above, whereas there were far fewer DEGs in Δrco-3 vs. WT than in Δcol-26 vs. WT in carbon-free conditions, or in Δrco-3 vs. WT in glucose conditions (Fig. 3E, Additional file 1: Table S3). This indicated that COL-26 functions in both glucose and carbon-free conditions, whereas RCO-3 mainly functions in the presence of glucose.

Next, the effect of COL-26 and RCO-3 on the sugar uptake system was investigated. Among the 39 putative sugar transporters present in the genome of N. crassa (70), 26 showed robust expression levels (FPKM > 20) in at least one condition (Additional file 5: Fig. S4A). In the WT strain, the transcriptional responses of these sugar transporters to a glucose gradient were in good accordance with previously published data (51). Some genes displayed a strong or moderate response to external glucose changes, including glt-1, hgt-1/-2, xyt-1, cdt-1/-2, NCU05897, NCU00821, xat-1, lat-1, gat-1, clp-1, and NCU09287. However, the responses of these transporter genes to a glucose gradient (2%, 0.5%, and 0.05%) were impaired in Δrco-3 and Δcol-26 mutants (Additional file 5: Fig. S4A). This is consistent with the observations that both mutants had similar transcriptional profiles and impaired transcriptomic response to glucose fluctuation (Fig. 3B–E). As for glt-1, hgt-1/-2, and xyt-1, which displayed the strongest response to external glucose changes in the WT strain and whose promoters were bound by COL-26 (Fig. 2 and Additional file 2: Fig. S1B), their changed expression was probably due to the absence of COL-26 in the Δcol-26 mutant or an inactivated form of COL-26 in the Δrco-3 mutant. Significantly downregulated expression of glt-1 was observed in both the Δcol-26 and Δrco-3 mutants at all glucose concentrations, even though the expression level of col-26 in the Δrco-3 mutant was almost three times that in the WT strain in the presence of 2% and 0.5% glucose (Additional file 1: Table S2), indicating that both genes are essential for glt-1 expression and that RCO-3 acts upstream of COL-26. Notably, hgt-1 and hgt-2 had very
similar expression profiles (Additional file 5: Fig. S4A), indicating the synergetic regulation of the two major components of the high-affinity glucose transport system. (51).

Since glucose transport is the first step of glycolysis and glucose uptake is defective in the Δrco-3 and Δcol-26 mutants, we focused on genes involved in central metabolism. The gene encoding phosphofructokinase (NCU00629) was downregulated whereas the gene encoding fructose-1,6-bisphosphatase (NCU04797) was upregulated in the Δrco-3 and Δcol-26 mutants in the presence of glucose (2%, 0.5%, and 0.05%). Other downregulated genes in both mutants in the presence of glucose included those encoding phosphoglycerate kinase (NCU07914), phosphoglycerate mutase (NCU02252), enolase (NCU10042), pyruvate kinase (NCU06075), and pyruvate decarboxylase (NCU02193) (Additional file 5: Fig. S4B), indicating that glycolysis in the Δrco-3 and Δcol-26 mutants in the presence of glucose is downregulated, probably because of the defect in glucose uptake.

Phosphoproteome of the WT strain grown on glucose vs. starvation conditions

Given that expression of col-26 at the transcriptional level is not affected by a gradient of glucose (0–10% w/v) (51), we wondered if a post-translational modification, such as the phosphorylation level of COL-26, explains the significant differentially expression of glt-1 between carbon-rich (2% glucose) and starvation (no-carbon, NC) conditions. Phosphoproteome profiling of the WT strain grown on glucose (Glu) compared with starvation conditions was performed. The coefficient of variation showed that the phosphopeptide abundance correlated well between the three replicates in each condition (Additional file 6: Fig. S5). We identified 11992 phosphopeptides, mapped to 2508 proteins (Additional file 1: Table S5). Of these phosphopeptides, 661 (representing 360 proteins) increased in abundance, and 709 (representing 410 proteins) decreased in abundance in the NC vs. Glu comparison (Fig. 4A, Additional file 1: Table S6).

There are 43 proteins highly phosphorylated in one or more regions but dephosphorylated in other regions in the NC vs. Glu comparison (Fig. 4B), including protein phosphatase regulator REG1 (NCU09310), nitrate nonutilizer-2 NIT-2 (NCU09068), eukaryotic peptide chain release factor ERF2 (NCU04790), chromatin remodeling factor CRF4-3 (NCU02684), and the S/T protein kinases STK-10 (NCU03200), STK-30 (NCU04335), and STK-31 (NCU04747). A similar phenomenon was also observed by Xiong et al. (71). GO analysis showed that proteins highly phosphorylated on starvation vs. Glu were over-represented in the categories cytoplasm (GO: 0005737) (30), intracellular transport (GO:0046907) (3), and carbohydrate phosphorylation (GO:0046835) (4) (Fig. 4C, Additional file 1: Table S7), and included some glycolytic proteins such as two hexokinases (NCU02542 and NCU00575), two 6-phosphofructo-2-kinases (NCU01178 and NCU01728), glyceraldehyde 3-phosphate dehydrogenase (NCU01528), and alcohol dehydrogenase-1 (NCU01754). Highly phosphorylated proteins not belonging to these GO terms included NCU06482 (pyruvate dehydrogenase E1 component α subunit), NCU01328 (transketolase), GLT-1, and NCU03100 (6-phosphogluconate dehydrogenase) (Additional file 1: Table S6), indicating that the glycolytic pathway and pentose phosphate pathway are regulated by post-translational modifications.
Proteins highly dephosphorylated in the NC vs. Glu comparison were over-represented in various categories mainly associated with the membrane, transport, and ATP metabolism (Fig. 4D, Additional file 1: Table S7), suggesting active metabolism in the presence of glucose. Pathway enrichment analysis of the highly dephosphorylated proteins using the Kyoto Encyclopedia of Genes and Genomes identified only one pathway—the MAPK signaling pathway-yeast (ko04011)—including protoperithecium-1 (PP-1, NCU00340), osmotic sensitive-4 (OS-4, NCU03071), MAPKK kinase NRC-1 (NCU06182), an uncharacterized protein (NCU06252), WSC-1 (NCU06910), osmotic sensitive-2 (OS-2, NCU07024), and mitogen-activated protein kinase-2 (MAK-2, NCU02393). NRC-1 (MAPKKK) and MAK-2 (MAPK) are core components of the conserved \textit{N. crassa} MAK-2 pathway (72) that mediates cell fusion and activates transcription factor PP-1 required for the activation of genes that play a role during the cell fusion (73). However, another core component, MEK-2 (MAPKK, NCU04612), was highly phosphorylated in the NC vs. Glu comparison (Additional file 1: Table S6). Other highly dephosphorylated proteins in dataset NC vs. Glu included a scaffold protein HAM-5 (NCU01789) of the MAK-2 pathway, HAM-8 (NCU02811), HAM-9 (NCU07389), CSP-6 (NCU08380), RCO-1 (NCU06205), ADA-3 (NCU02896), and PRK1 (NCU00506), which all relate to the NRC-1/MEK-2/MAK-2 signaling pathway and are required for cell-to-cell fusion (74, 75). OS-4 (MAPKKK) and OS-2 (MAPK) are components of the hyperosmotic response (OS) MAP kinase pathway involved in carbon sensing (76). Other highly phosphorylated peptides in the NC vs. Glu comparison were from CK-1b (NCU04005), which is involved in growth and developmental processes (77); ASD-4 (NCU15829), which functions in ascus and ascospore development (78); CEL-2 (NCU07307), which is involved in fatty acid biosynthesis (79); an actin-binding protein FIM (NCU03992) (80); and COL-26. Three phosphopeptides from COL-26 showed S79 and S83 phosphorylation decreased in abundance in the NC vs. Glu comparison and no other phosphorylated sites were found in COL-26 (Additional file 1: Table S6).

The function of COL-26 itself may be regulated at the protein level rather than the phosphorylation level.

Previous studies have identified four phosphorylation sites (S79, S83, S674, and S676) in COL-26 (71, 72, 81), among which S79 and S83 were also identified in this study and showed decreased abundance in the NC vs. Glu comparison (Additional file 1: Table S6). To dissect potential functions of these phosphorylation sites in the expression of the dual-transporter system, we constructed plasmids harboring \textit{col-26-egfp} without or with site-directed mutations under the control of the promoter of the glyceraldehyde-3-phosphate dehydrogenase-1 gene (\textit{gpd-1}, NCU01528). Nuclear localization of WT COL-26 and protein with simultaneous mutations at S79 and S83 (S79A, S83A), S674 and S676 (S674A, S676A), or all four sites (S4A) (Fig. 5A), as well as their recovered biomass relative to \textit{\Delta col-26} mutant on culture grown with sucrose (Fig. 5B), indicated the successful expression and correct function of these analogs. Expression of \textit{glt-1} in \textit{\Delta col-26::P\textit{gpd-col-26} (S79A, S83A), \Delta col-26::P\textit{gpd-col-26} (S674A, S676A), and \Delta col-26::P\textit{gpd-col-26} (S4A) in glucose and NC conditions was not different from that in \textit{\Delta col-26::P\textit{gpd-col-26} (WT) (Fig. 5C), suggesting that these phosphorylation sites of COL-26 are not involved in the regulation of glucose transporter expression in \textit{N. crassa}.}
Notably, *glt-1* expression in the Δ*col-26* mutant complemented with WT and mutated COL-26 grown in starvation conditions was much higher than that in the WT strain (Fig. 5C), probably because of the high expression level of *col-26* driven by the *gpd-1* promoter which leads to a high level of COL-26. So, we constructed the complemented strain Δ*col-26*:Pn-*col-26* expressing *col-26-egfp* under the control of its native promoter. The protein level of COL-26 in the presence of different concentrations of glucose was determined by western blotting using anti-GFP antibody. The protein level of COL-26 in the presence of adequate glucose (0.5% and 2%) was higher than that in starvation conditions (0.05%, or no glucose) (Fig. 6), suggesting that the function of COL-26 itself might be regulated at the protein level rather than by phosphorylation.

### AMPK represses *glt-1* expression, possibly by inhibiting RCO-3 activity, in starvation conditions

Previous study showed that the OS MAP kinase pathway is involved in carbon sensing (76). Two of its components (OS-2 and OS-4) were highly phosphorylated in the WT strain in the Glu vs. NC comparison (Additional file 1: Table S6). Thus, the roles of this pathway in regulation of the dual-affinity glucose transport system were investigated by characterization of deletion mutants of *os-1* and *os-2*, two essential components of this pathway. Expression of *glt-1* and *hgt-1* in Δ*os-1* and Δ*os-2* mutants showed no difference from that in the WT strain in either glucose-rich or starvation conditions. *glt-1* and *hgt-1/-2* expression in Δ*rco-3;Δos-1* and Δ*rco-3;Δos-2* double mutants was identical to that in the Δ*rco-3* mutant in both conditions (Additional file 7: Fig. S6). These results indicate that *os-1* and *os-2* are not involved in regulation of the dual-affinity glucose transport system.

The resistance of Δ*rco-3*, Δ*col-26*, and Δ*hgt-1;Δhgt-2* mutants to 2-DG (4, 51, 67) indicates that genes whose deletion leads to resistance to 2-DG might be regulatory elements of the dual-affinity glucose transport system. Considering that phosphorylation events are the most common and important mechanism underpinning regulation of glucose transport in *S. cerevisiae* (23, 38), multiple serine/threonine protein kinase mutants of *N. crassa* were screened for 2-DG resistance. Three mutants—ΔNCU00914, ΔNCU01940, and ΔNCU01498—showed high resistance to 2-DG (Additional file 9: Fig. S7A). However, the *glt-1* expression in these mutants showed no difference from that in the WT strain in either glucose-rich or NC conditions (Additional file 8: Fig. S7B), indicating that resistance to 2-DG is not necessarily connected with glucose transport and signaling.

In *S. cerevisiae*, the SNF1 complex plays an important role in regulation of glucose transport (33, 38). The effect of AMPK, which is homologous to SNF1, on expression of *glt-1* was investigated in *N. crassa*, in which *prk-10* (NCU04566, the ortholog of *snf1*) and NCU01471 (here named *snf4*) encode the α-subunit and γ-subunit of the AMPK complex, respectively. Expression of *glt-1* in Δ*prk-10* and Δ*snf4* mutants was the same as that in the WT strain in the presence of glucose, but significantly upregulated in starvation conditions. Expression of *glt-1* in Δ*rco-3;Δprk-10* and Δ*rco-3;Δsnf4* double mutants was the same as that in the Δ*rco-3* mutant (Fig. 7A), indicating that AMPK-repressed expression of *glt-1* might occur via inhibition of RCO-3 activity in starvation conditions. This conclusion was supported by transcriptomic data, which showed that *rco-3* mainly functions in the presence of glucose (Fig. 3E). Notably, the lower
number of DEGs in the Δrco-3 vs. WT comparison in starvation conditions compared with glucose condition (Fig. 3E) and the significantly reduced expression level of glt-1 in the Δrco-3 mutant (Fig. 7A) indicated that RCO-3 activity was not totally inhibited in starvation condition. Though deletion of prk-10 or snf4 had no effect on glt-1 expression in glucose-rich conditions, expression of hgt-1 was upregulated when the Δprk-10 and Δsnf4 mutants were grown on glucose (Fig. 7B). Besides, deletion of prk-10 or snf4 in the Δrco-3 background further decreased hgt-1 expression in starvation conditions (Fig. 7B), indicating that other regulatory component(s) are also involved in regulation of hgt-1 expression.

The glucose transport system shows conserved regulation by COL-26-like transcription factors in ascomycete species

Despite some minor differences in use of some kinds of sugars, conserved roles of COL-26/AmyR homologs have been reported in various fungi, including Magnaportha oryzae (82), Fusarium graminearum and F. verticillioides (83), A. nidulans (84), A. oryzae (68), A. niger (85), T. reesei (86), Penicillium oxalicum (87), Talaromyces pinophilus (88), and Myceliophthora thermophila (89). In P. oxalicum, the N. crassa HGT-1 ortholog PDE_03475 showed a high expression level on cellulose and a decreased expression level in a ΔamyR mutant compared with the WT strain (87). In A. niger, the A. nidulans MstE ortholog An02g03540 showed a high expression level on glucose and maltose, and its expression in a ΔamyR mutant was significantly downregulated (90). To test the hypothesis that the dual-affinity glucose transport system and its regulation by COL-26 homologs are conserved in filamentous fungi, the effect of deletion of M. thermophila AmyR (Mycth_2301920), the closest homolog to N. crassa COL-26, on expression of the putative dual-affinity glucose transport system was investigated. The M. thermophila ΔamyR mutant exhibited significantly reduced growth on glucose, fructose, sucrose, maltose, trehalose, xylose, and soluble starch, but grew well on cellobiose and cellulose (89), which is similar to the N. crassa Δcol-26 mutant. Alignment showed that Mycth_112491 is the closest ortholog of N. crassa GLT-1. However, Mycth_112491 is only 352 amino acids long with six transmembrane helices (TMHs), compared with the typical 12 TMHs for glucose transporters predicted by TMHMM Server v2.0. The second closest GLT-1 ortholog is Mycth_108924, which has 12 predicted TMHs. Both Mycth_112491 and Mycth_108924 showed an elevated expression level on glucose compared with NC and cellulose conditions (89, 91). Mycth_2308157 and Mycth_2295230 are the closest orthologs of N. crassa HGT-1 and HGT-2, respectively. Both Mycth_2308157 and Mycth_2295230 showed increased expression on cellulose compared with glucose (91), while Mycth_2295230 also showed significantly higher expression in NC conditions than in glucose-rich conditions (89), consistent with the expression pattern of high-affinity glucose transporters. These data were supported by qRT-PCR (Additional file 9: Fig. S8A). We determined the expression levels of these glucose transporter-encoding genes in a ΔamyR mutant of M. thermophila. In glucose-rich conditions, both Mycth_112491 and Mycth_108924 showed significantly decreased expression, whereas Mycth_2295230 was upregulated in the ΔamyR mutant, compared with the WT strain (Fig. 8A). In NC conditions, Mycth_108924 and Mycth_2308157 showed decreased expression levels in the ΔamyR mutant compared with WT strain (Fig. 8B). This indicates that AmyR is a key transcription factor involved in regulation of the dual-affinity glucose transport system in M. thermophila, like COL-26 in N. crassa. In addition, like the Δcol-26 mutant of N. crassa, the ΔamyR mutant
M. thermophila was sensitive to osmotic stress and H$_2$O$_2$-induced oxidative stress compared with the WT strain, as shown by plate growth assays (Additional file 9: Fig. S8B) and the corresponding mycelial diameter (Fig. 8C). Since COL-26 orthologs and the dual-affinity glucose transporter system are ubiquitous in ascomycete species based on phylogenetic analysis (51, 69), the regulatory role of col-26 orthologs may also be conserved in many other filamentous fungal species.

Discussion

Glucose uptake is the first and rate-limiting step of glucose metabolism. To cope with environmental changes in glucose availability, fungi express low-affinity glucose transporters when glucose levels are high and high-affinity glucose transporters when glucose levels are low. The N. crassa dual-affinity glucose transport system is the best-studied example, and consists of a low-affinity glucose transporter GLT-1 and two high-affinity glucose transporters HGT-1 and HGT-2 (51). This dual-transporter system is conserved in filamentous fungi and many glucose transporters have been characterized. However, the mechanism underpinning the regulation of the dual-affinity glucose transport system in response to environmental changes remains elusive. Here, using N. crassa as a model fungus, we identified a glucose signaling pathway consisting of multiple components, including the major transcription factor COL-26, non-transporting glucose sensor RCO-3, and a cellular energy sensor AMPK. COL-26 regulates the dual-affinity glucose transport system at the transcriptional level. Deletion of col-26 or rco-3 leads to a significantly reduced expression level of glt-1 in both glucose-rich and starvation conditions (Fig. 1A and Fig. 7A), indicating that the basal level of glt-1 in starvation conditions is also maintained by COL-26 and RCO-3. Interestingly, we found that COL-26-dependent regulation of the dual-transporter system might depend on the regulation of its protein homeostasis, rather than on post-translational modification such as phosphorylation. The expression level of col-26 in the WT strain remains the same in glucose-rich and carbon-free conditions, and it is only induced by starch, trehalose and maltose (51, 69) (Additional file 1: Table S2). This could reduce the transcriptional and translational investment when N. crassa growing in nutrition-deficient conditions encounters nutrient-rich conditions. Upon nutrition shift, the basal level glt-1 mRNA can be immediately translated, and there is no need to synthesize col-26 mRNA de novo for translation. Once COL-26 is synthesized from existing mRNA, it promotes expression of glt-1 in a COL-26 concentration-dependent manner at the protein level (Fig. 6) rather than in a phosphorylation-dependent manner (Fig. 5). However, overexpression of col-26 did not further promote expression of glt-1 in the presence of glucose (Fig. 5C), indicating that COL-26 does not function independently. Other components of the pathway that control COL-26 synthesis/degradation or dissociation/association with other protein(s) are worth investigating in the future. Moreover, as only four phosphorylation sites (S79, S83, S674, and S676) have so far been identified in COL-26 by various studies (71, 72, 81) (Additional file 1: Table S6), whether other phosphorylation site(s) are involved in the regulation of the dual-affinity glucose transport system requires investigation. As for the high-affinity glucose transport system, the high expression level of hgt-1/-2 in glucose-limited conditions is also dependent on COL-26 (Fig. 1B), indicating that COL-26 acts as an activator of hgt-1/-2. However, expression of hgt-1/-2 in the presence of adequate glucose was repressed by CCR mediated by CRE-1 (51), even though COL-26 remains at a
relatively high level (Fig. 6), indicating that CRE-1 is antagonistic to COL-26. Therefore, the high expression level of hgt-1/-2 in glucose-limited conditions is due to a combination of derepression by CRE-1 and activation by COL-26 (Fig. 9).

Another finding of this study is that RCO-3 is deeply involved in the regulation of the glucose transport system, since the expression of low-affinity glucose transporter gene glt-1 under starvation was repressed by AMPK by partially inhibiting the activity of RCO-3 (Fig. 7A). The similarity of multiple phenotypes between the Δcol-26 mutant and Δrco-3 mutant indicates that RCO-3 and COL-26 are in the same signaling pathway, in which RCO-3 acts as a glucose sensor and transduces a glucose signal to nuclear COL-26. Supporting this, the expression level of col-26 in the Δrco-3 mutant was almost three times that in the WT strain in the presence of a high glucose concentration (Additional file 1: Table S2), but expression of glt-1 was still significantly downregulated due to the absence of RCO-3 (Fig. 7A). Indeed, RCO-3 and COL-26 regulate a large portion of genes in common (Fig. 3E), including several genes involved in sugar uptake and glycolysis (Additional file 1: Fig. S4A, B). Lowered glycolysis in Δcol-26 and Δrco-3 mutants might be attributed to the defect in glucose transport, which leads to starvation even in the presence of adequate glucose. Notably, however, there is a small portion of genes that are exclusively regulated by RCO-3 or COL-26, respectively (Fig. 3E). Besides, components downstream of RCO-3 and upstream of COL-26, but not yet identified, probably regulate not only the dual-affinity glucose transport system but also other biological processes. Identification of the missing part(s) of the RCO-3/COL-26 signaling pathway will help us to understand the architecture of nutrient signaling regulation in filamentous fungi.

Methods

Strains

*Escherichia coli* strains DH5α (Invitrogen, Shanghai, China) and BL21 (DE3; Gibco BRL, Rockville, MD, USA) were used for plasmid propagation and gene expression, respectively. *Myceliophthora thermophila* ATCC 42464 was obtained from the American Type Culture Collection. A ΔamyR mutant of *M. thermophila* was constructed by our laboratory in a previous study (89). Strains of *N. crassa* were obtained from the Fungal Genetics Stock Center (FGSC, http://www.fgsc.net), including the wild-type (WT) reference strain (FGSC 2489), Δcol-26 (FGSC11030, mat a), Δrco-3 (FGSC17928, mat a), Δprk-10 (FGSC12421, mat A), and Δsnf4 (FGSC13236, mat A). The double-deletion strains Δrco-3Δsnf4 and Δrco-3Δprk-10 were generated by performing sexual crosses as previously described (http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm). The mis-expression strains were constructed by transforming the Δcol-26 mutant with linearized plasmid pCSR1 (92) harboring the promoter of the glyceraldehyde-3-phosphate dehydrogenase-1 gene (gpd-1), various gene-coding sequences or point-mutated analogs, and flanking regions of the csr-1 gene sequence. Transformants were selected for resistance to cyclosporin A and tested for genotypes by diagnostic PCR.

Culture conditions
**E. coli** was grown at 37°C in Luria-Bertani medium supplemented with 100 µg·ml⁻¹ kanamycin or ampicillin when necessary. **M. thermophila** strains were cultured on Vogel’s minimal medium (VMM) (93) supplemented with 2% sucrose at 45°C for 7–10 days to obtain conidia. **N. crassa** strains were inoculated on slants containing 3 mL VMM with 2.0% (w/v) sucrose as the sole carbon source and grown at 28°C in the dark for 2 days, then at room temperature in constant light for 6–10 days to stimulate conidia production. Conidia were inoculated into 100 mL liquid VMM with various carbon sources at 10⁶ conidia·mL⁻¹ and grown at 25°C in constant light with shaking (200 rpm). For plate growth assays, 1 µL of conidia suspension (1×10⁶ conidia·mL⁻¹) was plated on VMM supplemented with 2% cellobiose and cultured at 28°C for 30 h for **N. crassa**, or 37°C for 4 d for **M. thermophila**. NaCl and H₂O₂ were added to media to a final concentration of 0.5 M and 2 mM, respectively, for **N. crassa**. For **M. thermophila**, H₂O₂ was added to a final concentration of 1 mM.

**Medium shift experiments**

Conidia were inoculated into 100 mL liquid VMM supplemented with 2.0% sucrose and grown at 28°C and 200 rpm in constant light for 16 h. The mycelial biomass was washed with sterilized water at least five times and then transferred to 100 mL VMM with 2% glucose or with no carbon source added for 1 h before RNA extraction.

**Plasmid construction and transformation**

A fragment containing 5'- and 3'-flanking regions of **csr-1** (NCU00726) was amplified with pLC-5-F/pLC-3-R from pCSR1 (92). The promoter of **gpd-1** (NCU01528) was amplified from genomic DNA using primers Pgpd-NC-F/Pgpd-NC-R. The open reading frame of **col-26** was amplified using Col26-ORF-F/Col26-ORF-R from cDNA which was synthesized from total RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Japan). The coding sequence of **gfp** was amplified using primers GFP-F/GFP-R from pMF272. These four fragments were assembled using the NEB Gibson Assembly Kit (New England Biolabs, USA) to give pCSR-COL-26-GFP. The variants **col-26** (S79A, S83A), **col-26** (S674A, S676A), and **col-26** (S4A) were generated by site-directed mutagenesis using PCR with high-fidelity polymerase. Transformation by electroporation was performed as described previously (92). Transformants resistant to cyclosporin A were further confirmed by PCR and green fluorescent protein (GFP) fluorescence.

**Quantitative real-time qPCR (qRT-PCR)**

qRT-PCR was performed as previously described (94). The **actin** gene NCU04173 was used as an endogenous control for **N. crassa**, and Mycth_2314852 was used as an endogenous control for **M. thermophila**. All primers used in this study are listed in **Additional file 1: Table S8**. The transcript level of each gene was estimated using the 2⁻ΔΔCt method (95). The ratio of each gene transcript in each mutant to that in the WT strain was calculated as the relative transcript level.

**RNA sequencing and transcription expression analysis**
After harvesting via vacuum filtration, mycelia were immediately homogenized in liquid nitrogen for total RNA extraction. Total RNA was isolated from frozen samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA), treated with DNase I, and purified using a Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA integrity was checked by agarose gel electrophoresis and using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Qualified RNA with OD\textunderscore{260}/OD\textunderscore{280} > 2.0 and RNA Integrity Number > 8.0 was used for RNA-Seq, which was performed using the BGI SEQ-500 platform at Beijing Genomics Institute (BGI) (Shenzhen, China). All data were generated by sequencing two independent duplicate samples. Prior to read mapping, adaptors and low-quality reads were trimmed using Trimmomatic v0.36 (96). Filtered clean reads were aligned against predicted transcripts from the 
\textit{N. crassa} OR74A genome v12 (97) using Bowtie2 v2.2.5 (98). The read counts were determined using RSEM v1.2.8 (99). The abundance of each transcript was calculated from fragments per kilobase of transcript per million mapped reads (FPKM) values (\textit{Additional file 1: Table S2}). We used fuzzy c-means clustering to group genes based on similarity between concentration-specific gene expression patterns. Fuzzy clustering was conducted using Mfuzz v2.34.0 (100). Differential gene expression analysis was performed using the DESeq package (v1.5.1). Genes with fold-change > 2.0 (|log2 ratio| ≥ 1) and DESeq \( P_{adj} \)-value (Q-value) < 0.001 were considered significantly differentially expressed between different conditions or strains. To discover significantly up- and downregulated genes, only genes with relatively high transcript abundance (FPKM-value > 20 in at least one strain) were considered for further analysis. RNA-Seq data are available at the Gene Expression Omnibus under accession number GSE157186.

**Protein gel electrophoresis**

Culture supernatants were mixed with 4×SDS loading buffer and boiled for 10 min before loading onto Criterion 4%–15% Tris-HCl Precast Gels (Bio-Rad). GelCode Blue Stain Reagent (Thermo Scientific) was used for gel staining.

**Expression and purification of DNA-binding domain of COL-26**

A fragment encoding the DNA-binding domain of COL-26 was amplified with primers Ecol26-F/Ecol26-R using cDNA as template. After digestion with \textit{Bam}HI and \textit{Xho}I, this fragment was ligated into the corresponding sites of pGEX-4T-1 (GE Healthcare) to give pGEX-col-26. The plasmid was subsequently introduced into \textit{E. coli} BL21 (DE3) for protein expression. \textit{E. coli} BL21 (DE3) harboring pGEX-col-26 was grown at 37°C in 100 mL LB medium supplemented with 100 μg/mL ampicillin to an \( OD_{600} \) of 0.6. Isopropyl \( \beta \)-D-1-thiogalactopyranoside was then added to a final concentration of 0.5 mM, and the cultures were incubated for an additional 3 h at 37°C. The cells were harvested by centrifugation and resuspended in phosphate-buffered saline followed by sonication, after which the insoluble material was removed by centrifugation at 8000 × \( g \) for 10 min. The glutathione S-transferase (GST)-fused protein was purified using a BeaverBeads™ GST-tag Protein Purification Kit (Beaver, China) according to the manufacturer’s manual. Protein purity was determined by Coomassie Blue staining after 12% SDS-PAGE, and protein concentration was measured by BCA assay (Thermo Scientific, Waltham, MA, US).
Electrophoretic mobility shift assays (EMSAs)

Different DNA fragments were used as probes in gel-shift experiments. For COL-26-binding experiments, the promoter regions of *glt-1* (P1, −1906 to −1442; P2, −1461 to −1032; P3, −1049 to −625; P4, −682 to −233); *hgt-1* (P1, −992 to −557; P2, −575 to −142); *hgt-2* (P1, −1532 to −1094; P2, −1114 to −698; P3, −720 to −284); *xyt-1* (NCU05627) (P1, −1394 to −963; P2, −982 to −561; P3, −579 to −156); *lpmo* (NCU08746) (P1, −559 to −427; P2, −448 to −315; P3, −333 to −192; P4, −210 to −25); NCU04537 (P1, −827 to −406; P2, −424 to +18); *tam-1* (NCU03257) (P1, −1971 to −1496; P2, −1519 to −1064; P3, −1094 to −660); and *uc-5* (NCU07334) (P1, −2427 to −1969; P2, −1987 to −1519; P3, −1537 to −1073) were obtained by PCR from the genomic DNA of WT *N. crassa* using primers shown in **Additional file 1: Table S8.** The PCR products were purified by electrophoresis and quantified using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). The subsequent binding experiments were performed using a modified gel mobility shift assay as described previously (101). In each EMSA, different quantities of recombinant protein were incubated with a constant amount (10 ng) of the DNA probes individually at 25°C for 30 min. The experiments were performed at least three times.

Phosphopeptide identification by mass-spectrometry (MS)-based analysis

Proteins were reduced with 10 mM dithiothreitol for 1 h at 56°C and subsequently alkylated with 55 mM iodoacetamide. Samples were digested with trypsin at 1:20 enzyme-to-substrate ratio. Digested samples were desalted using C18 solid phase extraction tubes. The resulting peptide samples were concentrated and a BCA assay was performed to determine the peptide concentration and samples were diluted with nanopure water for MS analysis. Desalted peptides were labeled with 8-plex iTRAQ reagents (AB SCIEX) according to the manufacturer’s instructions. Peptide aliquots for each sample (200 mg) were dried for TiO₂ enrichment, and used for phosphoproteome analysis; TiO₂ enrichment of phosphopeptides followed a previously established protocol (102). Phosphopeptide samples were analyzed using a nanoESI system (Waters NanoAcquity LC, Waters Corporation) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Proteomics data were analyzed using a combination of Proteome Discoverer (v1.4) and Mascot (v2.3) software. The MS results were filtered based on a 5% false discovery rate and phosphoRS probability ≥ 0.75. Phosphopeptide abundance changes > 1.5-fold were considered and subjected to downstream analysis.

Western blot analysis

Western blotting was performed as previously described (103). Anti-GFP or anti-actin rabbit antibody and anti-rabbit IgG horseradish peroxidase-conjugated antibody at a dilution of 1:3000 were used as the primary and secondary antibodies (Abmart, Shanghai, China), respectively.

Microscopy and imaging

To localize GFP fusion proteins using microscopy, all strains were inoculated into liquid VMM supplemented with 2% sucrose and grown for 16 h at 25°C. The hyphae were harvested, washed several
times with Vogel's salts, transferred into media containing different concentrations of glucose, and cultured for another 1 h. Microscopic observations were performed using an Olympus BX51 fluorescence microscopy system and images were processed using ImageJ software.

**Declarations**

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**Authors’ contributions**

CT designed the research, supervised the project, and wrote the manuscript. JYL designed the research and contributed to the writing of the manuscript. QL, LL, XL and YZ performed experiments and analyzed the data. JGL aided in interpreting the results and provided critical revision. All authors read and approved the final manuscript.

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**Availability of data and material**

All data generated or analyzed during this study are included in this published article and its additional files.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Figures**

**Figure 1**

![Graph](image-url)

(a) Relative expression of *glt-1* (log2)

(b) Relative expression level of WT (log2)

(c) Images showing the effect of different glucose concentrations and no carbon on gene expression.
COL-26 is a key transcription factor that regulates the dual-affinity glucose transport system in Neurospora crassa. 

**a** Relative expression level of glt-1 in 36 transcription factor mutants of N. crassa grown in Vogel’s minimal medium (VMM) supplemented with glucose as the sole carbon source. Detailed information about these transcription factors is shown in Additional file 1: Table S1. 

**b** Relative expression of glt-1 and hgt-1/-2 in the presence of glucose and in carbon-free conditions in the wild-type (WT) strain and Δcol-26 mutant. 

**c** Subcellular location of COL-26 in N. crassa exposed to a gradient of glucose. Each scale bar represents 10 µm.

### Figure 2

Electrophoretic mobility shift assays of binding of COL-26 to upstream DNA regions of glt-1 (a), hgt-1 (b), and hgt-2 (c). Each lane contained 10 ng probe and the indicated amount of purified COL-26 binding domain (in nM).

### Figure 3

The Δcol-26 mutant phenotypically resembles a Δrco-3 mutant of N. crassa. 

**a** Mycelial diameter of the WT strain, and Δcol-26 and Δrco-3 mutants grown on VMM plus 2% cellobiose at 28°C for 30 h with or without 0.5 M NaCl or 2 mM H2O2. 

**b** Principal component analysis of RNA-Seq data from the WT, Δrco-3,
and Δcol-26 strains grown in different concentrations of glucose. c Number of differentially expressed genes between Δrco-3 and Δcol-26 strains in each glucose condition. d Number of differentially expressed genes between 0.05% glucose and 0.5% glucose for the WT, Δrco-3, and Δcol-26 strains. e Venn diagram of differentially expressed genes in the Δrco-3 mutant and Δcol-26 mutant compared with the WT in different concentrations of glucose.
Phosphoproteomic analysis of the WT strain of *N. crassa* grown on glucose or in no-carbon (NC) conditions for 1 h. 

a Differential phosphopeptides between NC and glucose (Glu) conditions. 
b Venn diagram of proteins whose peptides showed differential phosphorylation levels. 
c Gene Ontology (GO) enrichment analysis of the 317 proteins highly phosphorylated in the comparison NC vs. Glu. 
d GO enrichment analysis of the 367 proteins highly dephosphorylated in the comparison NC vs. Glu.

### Figure 5

Determination of the role of residues S79, S83, S674, and S676 of *N. crassa* COL-26 in regulation of the dual-affinity glucose transport system. 

a Subcellular location of WT and point-mutated COL-26. Strains were grown in liquid VMM supplemented with 2% sucrose for 16 h at 28°C. Each scale bar represents 10 µm. 
b Biomass of different strains grown in liquid VMM containing 2% glucose for 16 h. 
c Relative expression levels of glt-1 in different strains in glucose and NC conditions. Mycelia were grown in VMM.
plus 2% sucrose for 16 h, then transferred to VMM with or without 2% glucose. After additional cultivation for 1 h, mycelia were harvested and mRNA was extracted, after which quantitative real-time qPCR was performed to determine glt-1 expression.

Figure 6

Western blotting analysis of COL-26 in the WT strain of N. crassa. Mycelia were cultivated in VMM supplemented with 2% sucrose for 16 h, then transferred to VMM supplemented with a gradient of glucose for an additional 1 h. The same amount of total protein was loaded into each lane.

Figure 7
Relative expression levels of glt-1 (a) and hgt-1 (b) in WT, Δprk-10, Δsnf4, Δrco-3, Δrco-3;Δprk-10, and Δrco-3;Δsnf4 strains of N. crassa in the presence of glucose and in no-carbon conditions. Mycelia were grown in VMM supplemented with 2% sucrose for 16 h, then transferred to VMM with or without 2% glucose. After additional cultivation for 1 h, mycelia were harvested and the glt-1 expression level was determined by qRT-PCR.

Figure 8

Phenotype of Myceliophthora thermophila ΔamyR mutant. a Expression of glucose transporter genes in the presence of glucose. b Expression of glucose transporter genes in no-carbon conditions. c Mycelial diameter of the WT strain and ΔamyR mutant grown on VMM plus 2% cellobiose at 37°C for 4 d with or without 0.5 M NaCl or 1 mM H2O2.
Figure 9

Model for the role of COL-26 in regulating the dual-affinity glucose transport system in filamentous fungi. Under high levels of glucose, RCO-3 transduces a glucose signal to COL-26 to promote expression of glt-1 for nutrient assimilation. Adequate glucose stimulates CRE-1-mediated carbon catabolite repression (CCR) to repress expression of hgt-1/-2. When external glucose is depleted or limited, hgt-1 and hgt-2 are rapidly derepressed by the lifting of CCR and their expression is activated by COL-26. This process synergistically activates AMP-activated protein kinase (AMPK), which inhibits activity of RCO-3, leading to low expression of glt-1.

Supplementary Files

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