Internalization of Heterologous Sugar Transporters by Endogenous α-Arrestins in the Yeast Saccharomyces cerevisiae

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ABSTRACT

When expressed in Saccharomyces cerevisiae using either of two constitutive yeast promoters (PGK1prom and CCW12prom), the transporters CDT-1 and CDT-2 from the filamentous fungus Neurospora crassa are able to catalyze, respectively, active transport and facilitated diffusion of cellobiose (and, for CDT-2, also xylan and its derivatives). In S. cerevisiae, endogenous per- meases are removed from the plasma membrane by clathrin-mediated endocytosis and are marked for internalization through ubiquitylation catalyzed by Rsp5, a HECT class ubiquitin-protein ligase (E3). Recruitment of Rsp5 to specific targets is mediated by a 14-member family of endocytic adaptor proteins, termed α-arrestins. Here we demonstrate that CDT-1 and CDT-2 are subject to α-arrestin-mediated endocytosis, that four α-arrestins (Rod1, Rog3, Aly1, and Aly2) are primarily responsible for this internalization, that the presence of the transport substrate promotes transporter endocytosis, and that, at least for CDT-2, residues located in its C-terminal cytosolic domain are necessary for its efficient endocytosis. Both α-arrestin-deficient cells expressing CDT-2 and otherwise wild-type cells expressing CDT-2 mutants unresponsive to α-arrestin-driven internalization exhibit an increased level of plasma membrane-localized transporter compared to that of wild-type cells, and they grow, utilize the transport substrate, and generate ethanol anaerobically better than control cells.

IMPORTANCE

Ethanolic fermentation of the breakdown products of plant biomass by budding yeast Saccharomyces cerevisiae remains an attractive biofuel source. To achieve this end, genes for heterologous sugar transporters and the requisite enzyme(s) for subsequent metabolism have been successfully expressed in this yeast. For one of the heterologous transporters examined in this study, we found that the amount of this protein residing in the plasma membrane was the rate-limiting factor for utilization of the cognate carbon source (cellobiose) and its conversion to ethanol.

Ethanol is a widely used, environmentally clean, and renewable biofuel produced by microbial fermentation of sugar sources derived from food-related crop plants, such as corn and sugar cane, referred to as “first-generation” ethanol (1, 2). An alternative source of ethanol that avoids the “food versus fuel” ethical conflict is sugar derived from non-crop plant biomass, referred to as “second-generation” ethanol (2, 3). Plant biomass is composed of lignocellulosic material, which consists of cellulose (the most abundant fraction), hemicellulose, and lignin (4). For its fermentation to occur, lignocellulosic biomass is first pretreated to make its components more accessible to breakdown and then hydrolyzed either enzymatically or chemically to release fermentable sugars (5). The principal sugars liberated by hydrolysis of cellulose consist of cellobextrins and glucose, whereas hydrolysis of hemicelluloses releases primarily xyloans and xylose.

To produce ethanol as a biofuel, industrial strains of budding yeast Saccharomyces cerevisiae are primarily used (6, 7). Native S. cerevisiae, although unable to efficiently utilize xylose (8, 9), is proficient in the utilization and fermentation of glucose. However, large-scale enzymatic degradation of cellulose into glucose is expensive, requiring, first, hydrolysis of cellulose by cellulases to generate the β(1→4)-linked disaccharide cellobiose (and higher cellobextrins) and then subsequent cleavage of cellobiose into glucose by β-glucosidases. Aside from the expense, complete enzymatic conversion of cellulose to glucose is problematic because high glucose concentrations inhibit both cellulases and β-glucosidases (10, 11). One approach that reduces cost, eliminates glucose-mediated inhibition of enzymes, and facilitates cofermentation of nonglucose sugars is based on the successful uptake of cellobiose, which is subsequently broken down to glucose after its transport into the cell. This end was achieved by ectopic coexpression of three yeast genes for a cellobiose/cellodextrin transporter, either CDT-1 (NCU00801) or CDT-2 (NCU08114), and the gene for an intracellular β-glucosidase (ghI-1, NCU00130) from the filamentous fungus Neurospora crassa (12). CDT-1 catalyzes active transport of cellobiose, and CDT-2 mediates entry of cellobiose (as well as xyloans) by facilitated diffusion (12, 13). Cellobiose fermentation, like fermentation of other nonglucose sugars in S. cerevisiae, occurs at a substantially lower rate than glucose fermentation.
Endocytosis of *N. crassa* CDT-1 and CDT-2 in *S. cerevisiae*

**TABLE 1 Yeast strains used in this study**

| Strain | Description | Reference or source |
|--------|-------------|---------------------|
| BY4741 | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 | Yeast deletion collection (Open Biosystems, Inc.) |
| 9arrΔ (EN60) derivative | MATα ccm21Δ::KANMX csr2Δ::KANMX bsl2Δ rol1Δ rog3Δ::NATMX ygr068cΔ ldb19Δ aly1Δ aly2Δ ypl392cΔ::HIS3 his3 ura3Δ0 len2Δ0 | This study |
| 4arrΔ derivative | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 aly1Δ::KANMX ally2Δ::KANMX rol1Δ::HYG rog3Δ::NATMX | This study |
| rim1Δ art5Δ mutant | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 rim1Δ::KANMX art5Δ::KANMX | 22 |
| rodlΔ rog3Δ mutant | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 rodl1Δ::KANMX rog3Δ::KANMX | 22 |
| rodl1Δ rog3Δ ldb19Δ mutant | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 rodl1Δ::HYG rog3Δ::NATMX ldb19Δ::NATMX | 22 |
| ecm21Δ csr2Δ mutant | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 ecm21Δ::KANMX csr2Δ::KANMX | 22 |
| aly1Δ aly2Δ mutant | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 aly1Δ::KANMX aly2Δ::KANMX | 22 |
| art10Δ mutant | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 art10Δ::KANMX | Yeast deletion collection (Open Biosystems, Inc.) |
| ecm21Δ csr2Δ rodl1Δ rog3Δ mutant | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 ecm21Δ::KANMX csr2Δ::KANMX rodl1Δ::HYG rog3Δ::NATMX | This study |
| ecm21Δ csr2Δ aly1Δ aly2Δ mutant | MATα len2Δ0 ura3Δ0 his3Δ1 met15Δ0 ecm21Δ::KANMX csr2Δ::KANMX aly1Δ::HYG aly2Δ::NATMX | This study |

**TABLE 2 Plasmids used in this study**

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| CDT-1–GFP | PGK1prom CDT-1–GFP–His6 CEN URA3 | This study |
| CDT-2–GFP | PGK1prom CDT-2–GFP–His6 CEN URA3 | This study |
| GH1-1 | CCW12prom GH1-1 CEN LEU2 | This study |
| CDT-2 WT–GFP | CCW12prom CDT-2–GFP–His6 CEN URA3 | This study |
| CDT-2 Nfr–GFP | CCW12prom CDT-2(K68R K79R K31R K32R K34R)–GFP–His6 CEN URA3 | This study |
| CDT-2 MidKR–GFP | CCW12prom CDT-2(K231R K233R K243R K263R K274R K283R)–GFP–His6 CEN URA3 | This study |
| CDT-2 Ctr–GFP | CCW12prom CDT-2 Ctr(K484R K493R K512R K522R)–GFP–His6 CEN URA3 | This study |
| CDT-2 Trm–GFP | CCW12prom CDT-2 Trm–GFP–His6 CEN URA3 | This study (see Fig. S5) |

In *S. cerevisiae*, endogenous nutrient permeases in the plasma membrane (PM), including sugar transporters, are marked for endocytosis through ubiquitylation by the ubiquitin:protein ligase (E3) Rsp5 (18–20). Rsp5 associates with the PM via its N-terminal phospholipid-binding C2 domain and binds to potential targets via three internal WW domains that recognize the motif PPXY (and variants thereof, such as VPXY) (21, 22). However, many cargo proteins that undergo Rsp5-dependent ubiquitylation do not contain the PPXY consensus (19). Indeed, endocytosis of most integral PM proteins is brought about through recruitment of Rsp5 via a family of endocytic adaptor proteins called α-arrestins (also referred to as arrestin-related trafficking adaptors or ARTs) (23, 24). In *S. cerevisiae*, this family comprises 14 members, which are characterized by an N-terminal cargo-binding arrestin fold domain and a C-terminal Rsp5-binding extension containing multiple PPXY motifs (23–25). The products of several members of the HXT family of hexose transporter genes in *S. cerevisiae* are endocytosed upon exposure to and transport of extracellular glucose (or glucose analogs) via their interaction with specific α-arrestins and subsequent ubiquitylation by Rsp5 (20, 25, 26). *N. crassa* CDT-1 and CDT-2 belong to the same transporter family as the HXT transporters, namely, the sugar porter (SP) subfamily (Transporter Classification Database identifier 2.A.1.1; http://www.tcdb.org) of major facilitator transporters (27). Thus, it seemed plausible that α-arrestin-mediated downregulation of CDT-1 and/or CDT-2 might remove them from the cell surface, thereby imposing a limitation on the efficacy of cellobiose utilization and ethanol production from this carbon source.

Hence, in this study, we explored whether CDT-1 and CDT-2 are subject to internalization mediated via the endogenous α-arrestins in *S. cerevisiae*. If so, we sought to determine which of the 14 α-arrestins is responsible for the downregulation, what other factors may influence this process, how ubiquitylation may be involved, and whether the rate of transporter removal from the cell surface is sufficiently rapid to negatively affect the efficiency of cellobiose utilization and the generation of ethanol from it.

**MATERIALS AND METHODS**

**Strains and plasmids.** Yeast strains (Table 1) and plasmids (Table 2) were constructed using standard genetic methods (28, 29). DNA amplification by PCR employed Phusion DNA polymerase (New England BioLabs, Ipswich, MA), and all constructs were verified by sequencing. CDT-1 and CDT-2 were PCR amplified from cDNA synthesized from mRNA isolated from *N. crassa* (FGSC 2489) grown on minimal medium plus Avicel (microcrystalline cellulose) as the sole carbon source (12). The *N. crassa cdt-1*
and cdt-2 genes were cloned into the pRS316 plasmid (CEN URA3) using the In-Fusion HD cloning kit (Clontech Laboratories, Inc., Mountain View, CA). These transformants were expressed under the control of the S. cerevisiae PGK1 promoter (PGK1prom) and the CYC1 terminator; all transformants were tagged with enhanced green fluorescent protein (eGFP) at the C terminus. For construction of the CDT-2KR mutants, double-stranded gene fragments spanning regions encompassing each set of mutations were synthesized as gBlocks by Integrated DNA Technologies (Corvalle, IA). These gene fragments (Table 3) were cloned into the CDT-2 coding sequence in a linearized pRS316 plasmid under the control of the S. cerevisiae CCW12prom and a CYC1 terminator by using the InFusion HD cloning kit (Clontech Laboratories, Inc., Mountain View, CA). The codon-optimized version of GHI-1 was expressed in pRS315 plasmid (CEN LEU2) under the control of the CCW12prom and the CYC1 terminator. Codon optimization of this gene has been described elsewhere (17).

**Growth conditions.** Strains were grown at 30°C in either rich (yeast extract-peptone [YP]) or synthetic (S) medium (30) containing 2% cellobiose (unless otherwise specified) with appropriate nutrient supplements to support growth and with certain nutrients omitted to maintain selection for plasmids. For the anaerobic growth assays and fermentation experiments, we used optimized minimal medium (oMM) lacking appropriate nutrients for plasmid selection (17); oMM contained 10 g/liter (NH4)2SO4, 1 g/liter MgSO4·7H2O, 6 g/liter KH2PO4, 100 mg/liter adenine hemisulfate, 1.7 g/liter yeast nitrogen base (YNB; Sigma-Aldrich, St. Louis, MO), 2% recommended CSM—Ura—Leu (complete supplement mixture lacking Ura and Leu) dropout mix (MP Biomedicals, Santa Ana, CA), 10 mg/liter inositol, 100 mg/liter glutamic acid, 20 mg/liter lysine, 375 mg/liter serine, 100 mM morpholinethesulfonic acid (MES), pH 6. Glucose or cellobiose was added to this stock recipe depending on the experiment. Cellobiose and xylose from beechwood were obtained from Sigma-Aldrich (St. Louis, MO). Due to the difficulty of forming >5% stock solution of xylan, it was dissolved directly in YP (2%, wt/vol) with constant heating.

**Fluorescence microscopy.** Images were acquired using an Olympus BH2 microscope equipped with a charge-coupled-device (CCD) camera. For live imaging of cells expressing fluorescently tagged proteins (CDT-1–GFP and CDT-2–GFP), cell cultures were grown overnight at 30°C in synthetic (S) medium (30) containing the indicated carbon source (2%) and the appropriate nutrients for plasmid selection (17); oMM contained 10 g/liter (NH4)2SO4, 1 g/liter MgSO4·7H2O, 6 g/liter KH2PO4, 100 mg/liter adenine hemisulfate, 1.7 g/liter yeast nitrogen base (YNB; Sigma-Aldrich, St. Louis, MO), 2% recommended CSM—Ura—Leu (complete supplement mixture lacking Ura and Leu) dropout mix (MP Biomedicals, Santa Ana, CA), 10 mg/liter inositol, 100 mg/liter glutamic acid, 20 mg/liter lysine, 375 mg/liter serine, 100 mM morpholinethesulfonic acid (MES), pH 6. Glucose or cellobiose was added to this stock recipe depending on the experiment. Cellobiose and xylose from beechwood were obtained from Sigma-Aldrich (St. Louis, MO). Due to the difficulty of forming >5% stock solution of xylan, it was dissolved directly in YP (2%, wt/vol) with constant heating.

**Results.** Ectopically expressed cellulose transporters CDT-1 and CDT-2 are internalized in an α-arrestin-dependent manner. The re-
First, to quantify these observations, we scored a total of 200 cells in random fields of each culture for two features of every cell: its level of PM fluorescence and its level of vacuolar fluorescence. In agreement with the representative images shown in Fig. 1A, we found that a significant fraction (31%) of WT cells expressing CDT-1–GFP exhibited readily detectable vacuole-associated fluorescence (and concomitantly reduced PM fluorescence), whereas in the 9arrΔ strain, the majority of the cells (96%) displayed very bright PM fluorescence and no discernible vacuolar fluorescence. Similarly, the majority (80%) of WT cells expressing CDT-2–GFP exhibited very robust vacuolar fluorescence (and concomitantly reduced PM fluorescence), and conversely, in the 9arrΔ strain, 86% of the cells displayed bright PM fluorescence and very little or no detectable fluorescence in the vacuole. Second, we confirmed that the internal GFP fluorescence indeed represented a signal within the lumen of the vacuole by costaining with 7-amino-4-chloromethyl-coumarin (CMAC), a known marker for this compartment (20) (see Fig. S2 in the supplemental material).

Somewhat unexpectedly, we found that CDT-1 and CDT-2 internalization was promoted by the presence of their transport substrate, cellobiose, because when glucose was the carbon source, both transporters were PM localized and little vacuole-associated fluorescence was detected in either WT cells or the 9arrΔ strain (Fig. 1B). Out of 200 total cells in random fields of each culture, we found that only 2% of WT cells expressing CDT-1–GFP in glucose medium exhibited robust vacuolar fluorescence and the majority (98%) exhibited very bright PM fluorescence and that, in the 9arrΔ strain, all of the cells (100%) displayed only very weak vacuolar fluorescence and very bright PM fluorescence. Likewise, only 3% of WT cells expressing CDT-2–GFP in glucose medium exhibited readily detectable robust vacuolar fluorescence and the majority (97%) exhibited very bright PM fluorescence and, in the 9arrΔ strain, only a very small fraction (1%) displayed readily detectable vacuolar fluorescence and the majority (99%) exhibited very bright PM fluorescence.

As has been observed for other heterologous integral membrane proteins expressed in yeast (32, 33), and indicative of some degree of misfolding or other kinetic delay in the trafficking of these transporters through the secretory pathway, we noted for both CDT-1–GFP and CDT-2–GFP a faint perinuclear fluorescence signal, which is the hallmark of some degree of accumulation of unfolded protein-chaperone complexes in the lumen of the endoplasmic reticulum (ER) (34, 35).

Internalization of CDT-1 and CDT-2 requires joint action of the α-arrestins Rod1, Rog3, Aly1, and Aly2. To determine whether the observed cellobiose-dependent endocytosis of CDT-1 or CDT-2 depends on any specific α-arrestin(s), we examined CDT-1 and CDT-2 localization in strains in which the genes for either a single α-arrestin or for paralogous pairs of α-arrestins were deleted. Among the 14 known S. cerevisiae α-arrestins, the following eight are apparent paralogs on the basis of sequence relatedness and overlapping function: Aly1/Art6 and Aly2/Art3, Csr2/Art8 and Ecm21/Art2, Art5 and Rim8/Art9, and Rod1/Art4 and Rog3/Art7 (23, 25). Hence, we examined CDT-1–eGFP and CDT2–eGFP localization in αly1Δ aly2Δ, csr2Δ ecm21Δ, rim8Δ art5Δ, and rod1Δ rog3Δ double mutant cells. We also examined the localization of these transporters in single mutant cells lacking either the α-arrestin Ldb19 or the α-arrestin Art10. However, we eliminated from our analysis the Art10 paralog Spo23, because it is expressed only in meiotic cells (36). Likewise, we did not exam-
ine the most distantly related α-arrestin-like proteins, Bul1 and Bul2, which have been implicated mainly in intracellular trafficking and sorting of permeases, such as the general amino acid permease Gap1 (37–39), as well as in their removal from the cell surface (40, 41).

We reasoned that if a specific α-arrestin(s) is important for CDT-1 and CDT-2 internalization, then in the absence of that α-arrestin(s), CDT-1 and CDT-2 should phenocopy the enhanced cell membrane localization and reduced vacuolar localization observed in the 9arrΔ strain. However, in all of the α-arrestin-deficient strains tested, CDT-1 and CDT-2 localization was observed in the vacuole as well as at the cell membrane (Fig. 2A), suggesting that multiple sets of α-arrestins contribute to mediating the internalization of these two cellulose transporters.

In this regard, we did note a modest decrease in vacuolar localization in both the rod1Δ rog3Δ cells and the aly1Δ aly2Δ cells. For 200 total cells in random fields of each culture expressing CDT-1–GFP, we observed the following percentages of cells with a robust vacuolar signal: 9arrΔ cells, 4%; WT, 31%; art10Δ cells, 32%; ldb19Δ cells, 37%; csr2Δ ecm21Δ cells, 31%; art5Δ rim8Δ cells, 38%; aly1Δ aly2Δ cells, 26%; and rod1Δ rog3Δ cells, 29%. Similarly, for 200 total cells in random fields of each culture expressing CDT-2–GFP, we observed the following percentages of cells with a robust vacuolar signal: 9arrΔ cells, 14%; WT, 80%; art10Δ cells, 82%; ldb19Δ cells, 82%; csr2Δ ecm21Δ cells, 83%; art5Δ rim8Δ cells, 84%; aly1Δ aly2Δ cells, 78%; and rod1Δ rog3Δ cells, 74%.

For this reason, we constructed an aly1Δ aly2Δ rod1Δ rog3Δ quadruple mutant of BY4741 (here called the 4arrΔ strain). Reassuringly, localization of CDT-1 and CDT-2 in the 4arrΔ strain displayed prominent PM fluorescence and markedly reduced vacuolar fluorescence, phenocopying the localization observed in the 9arrΔ strain (Fig. 2B). Moreover, this result was not simply the cumulative effect of deleting the genes for a total of four α-arrestins, because the same effect was not observed when either aly1Δ aly2Δ or rod1Δ rog3Δ were combined with deletions of other pairs of α-arrestin genes.

**FIG 2** The α-arrestins Rod1, Rog3, Aly1, and Aly2 cooperate to drive the endocytosis of CDT-1 and CDT-2. (A) Cultures of BY4741 (WT), the 9arrΔ strain, and otherwise isogenic strains with paralogous pairs (or more) of α-arrestin genes deleted were grown and imaged in the presence of 2% cellulose as described in Materials and Methods. Each strain was cotransformed with the intracellular β-glucosidase (GH1-1) along with either CDT-1–GFP or CDT-2–GFP. (B) The same experiment as that described for panel A was performed, except the cells harbor quadruple deletions of α-arrestin genes.
of paralogous α-arrestin genes (Fig. 2B). For 200 total cells in random fields of each culture expressing CDT-1–GFP, we observed the following percentages of cells with a robust vacuolar signal: aly1Δ aly2 rod1Δ rog3Δ cells, 7%; rod1Δ rog3Δ csr2Δ ecm21Δ cells, 37%; and aly1Δ aly2 csr2Δ ecm21Δ cells, 35%. Similarly, for 200 total cells in random fields of each culture expressing CDT-2–GFP, we observed the following percentages of cells with a robust vacuolar signal: aly1Δ aly2 rod1Δ rog3Δ cells, 18%; rod1Δ rog3Δ csr2Δ ecm21Δ cells, 69%; and aly1Δ aly2 csr2Δ ecm21Δ cells, 70%. Thus, the joint actions of Aly1, Aly2, Rod1, and Rog3 are specifically and primarily responsible for the α-arrestin-dependent internalization of CDT-1 and CDT-2.

Because industrial-scale fermentations are typically carried out anaerobically, we also examined the localization of CDT-1 and CDT-2 under anoxic conditions (as described in Materials and Methods). The distribution of both CDT-1 and CDT-2 observed in WT, the 9arrΔ strain, and 4arrΔ cells grown in cellobiose medium under anaerobic conditions was quite similar to that observed under aerobic conditions (see Fig. S3 in the supplemental material).

An anaerobic growth and fermentation of cellobiose by CDT-2-expressing cells is improved when the relevant α-arrestins are absent. We found that the cellobiose transporters were localized almost exclusively at the PM in both the 9arrΔ and 4arrΔ cells. This observation raised the possibility that a higher steady-state level of these transporters might increase the transmembrane flux of cellobiose and, if the rate of entry of the disaccharide is a factor limiting its utilization, might result in more efficient growth and fermentation of cellobiose to ethanol. Because it has been observed before that the 9arrΔ strain is somewhat compromised for growth (25), presumably because of the cumulative deleterious effects arising from the simultaneous absence of nine α-arrestins, we were most interested in the 4arrΔ cells. Indeed, when growth assays were performed in cellobiose medium under anaerobic conditions, 4arrΔ cells expressing either CDT-1 or CDT-2 exhibited both a faster doubling time and a higher final growth yield than those of otherwise isogenic WT cells (or the 9arrΔ strain) (Fig. 3A).

We also examined whether the elevated PM localization of the cellobiose transporters would enhance cellobiose utilization and, concomitantly, the efficiency of ethanol production. For 4arrΔ cells expressing CDT-1, the rate and extent of cellobiose consumption and the rate and extent of ethanol production were not detectably different from those of otherwise isogenic WT cells expressing CDT-1 (Fig. 3B). In marked contrast, we observed that 4arrΔ cells expressing CDT-2 exhibited a rate and extent of cellobiose consumption and a rate and extent of ethanol production that were reproducibly better than those of otherwise isogenic WT cells (or the 9arrΔ strain) expressing CDT-2 (Fig. 3C). One consideration that may explain why an effect of removing the relevant α-arrestins was detectable for CDT-2-expressing cells, but not for CDT-1-expressing cells, is that the reported maximum rate of metabolism (Vmax) for cellobiose uptake catalyzed by CDT-1 is more than 2-fold higher than that mediated by CDT-2 (12). Thus, removal of the relevant α-arrestins may result in a greater incremental increase in cellobiose entry in cells expressing CDT-2 than in cells expressing CDT-1.

C-terminal lysine residues of CDT-2 are important for its internalization. CDT-1, an ATP-driven proton symporter, supports a higher rate of cellobiose entry than CDT-2, which mediates cellobiose entry by facilitated diffusion (12, 42). Because cellobiose uptake by CDT-1 is coupled to ATP consumption, less of the cellobiose can, in principle, be converted to ethanol. Thus, CDT-2 is a potentially more attractive alternative for industrial-scale conversion of cellobiose to ethanol, despite the fact that it is less efficient in supporting cellobiose fermentation (43). However, as we have demonstrated here, removal of the cognate α-arrestins that mediate the ubiquitinylation-dependent internalization of
CDT-2 provided significant improvement in cellobiose utilization and ethanol production. Given those findings, we reasoned that an alternative strategy to enhance the amount of CDT-2 in the PM and thereby increase cellobiose entry would be to identify and eliminate the Lys residues that are the targets of its α-arrestin-dependent modification (as long as the residues substituted for Lys did not compromise the folding, trafficking, and/or transport functions of CDT-2).

There is no crystal structure available for the CDT-2 transporter. Therefore, we used the I-TASSER Protein Structure Prediction server (44) to model this transporter against crystal structures of homologous members of the SP subgroup of the major facilitator superfamily (see Fig. S4 in the supplemental material). Rather than bias the modeling to conform to any specific known structures, we allowed the I-TASSER algorithm to find the closest match and build the homology model. Based on both hydrophathy plots (45) and the homology model, CDT-2 contains 12 transmembrane helices organized into two domains with N- and C-terminal extensions that project into the cytosol (Fig. 4A; see Fig. S4). These cytosolic “tails,” along with the five interconnecting loops that face the cytosol, contain multiple Lys residues, any or all of which might serve as sites for the covalent attachment of ubiquitin mediated by the Aly1-, Aly2-, Rod1-, and Rog3-dependent recruitment of the E3 Rsp5. To determine whether any of the three most prominent cytosolic segments of CDT-2 (its N-terminal extension, its C-terminal extension, and the large, predicted interdomain loop between transmembrane helices 6 and 7) represent sites that contribute to its internalization, all of the Lys residues in each of these regions were mutated to Arg, and these CDT-2 mutants are here called the NtKR mutant, the CtKR mutant, and the MidKR mutant. Strikingly, the CtKR mutant displayed the enhanced PM localization and markedly reduced vacuolar localization also observed in 9arrΔ and 4arrΔ cells, whereas the NtKR and MidKR mutants exhibited internalization very similar to that seen in WT cells (Fig. 4B). For 200 total cells in random fields of each culture expressing WT CDT-2–GFP or the indicated CDT-2–GFP mutants, we observed the following percentages of cells with a robust vacuolar signal: WT CDT-2–GFP in WT cells, 83%; WT CDT-2–GFP in 9arrΔ cells, 11%; WT CDT-2–GFP in 4arrΔ cells, 19%; CDT-2 NtKR–GFP in WT cells, 80%; CDT-2 NtKR–GFP in 9arrΔ cells, 9%; CDT-2 NtKR–GFP in 4arrΔ cells, 14%; CDT-2 MidKR–GFP in WT cells, 89%; CDT-2 MidKR–GFP in 9arrΔ cells, 19%; CDT-2 MidKR–GFP in 4arrΔ cells, 24%; CDT-2 CtKR–GFP in WT cells, 11%; CDT-2 CtKR–GFP in 9arrΔ cells, 8%; CDT-2 CtKR–GFP in 4arrΔ cells, 10%. These observations suggested that it is the Lys residues in the C-terminal tail of CDT-2 that must be ubiquitylated as a prerequisite to its endocytosis. Consistent with that conclusion, a version of CDT-2 with a truncated carboxy terminus (CDT-2Trunc; arising from a frameshift resulting in a stop codon that causes premature termination) (see Fig. S5) also exhibited enhanced PM localization and markedly reduced vacuolar localization (Fig. 4B). For 200 total cells in random fields of each culture, we observed the following percentages of cells with a robust vacuolar signal: CDT-2Trunc–GFP in WT cells, 25%; CDT-2Trunc–GFP in 9arrΔ cells, 11%; CDT-2Trunc–GFP in 4arrΔ cells, 15%. Although these latter constructs were expressed under the CCW12 promoter instead of the formerly used PGK1 promoter, we confirmed that the levels of protein expression driven by either promoter are quite similar (see Fig. S6).

Interestingly, in the context of this work, the truncation mutant (CDT-2Trunc) was initially generated in a separate project in which CDT-2 orthologs in other filamentous fungi were identified that likely had similar transporter activity. The ortholog chosen from Fusarium graminearum, here transporter FG, was subjected to random mutagenesis followed by directed evolution, where the selective pressure was the ability to grow in cellobiose-containing medium. This procedure resulted in the isolation of an FG mutant with a single base deletion (A1547) resulting in a frameshift after residue 515, thereby changing the C-terminal sequence to 18RLK KRPFK and causing premature termination of this otherwise 544-residue protein (see Fig. S5 in the supplemental material) (unpublished results). The C-terminal region of FG has high sequence identity to CDT-2 (see Fig. S5). Hence, the CDT-2Trunc mutant was created using the same frameshift mutation to assess whether this alteration would increase its proficiency in cellobiose uptake. In light of our current findings, the apparent reason that FGTrunc and CDT-2Trunc are able to support improved cellobiose utilization is that both are able to escape from their α-arrestin-dependent and Rsp5-mediated ubiquitylation, just like the CDT-2 CtKR mutant. In this regard, it is noteworthy that, compared to WT CDT-2, CDT-2Trunc lacks only a single Lys residue (K522), and, similarly, compared to WT FG, FGTrunc lacks only two Lys residues (K528 and K543), suggesting that it is the most C-terminally situated Lys residues that are the primary sites for Rsp5-mediated ubiquitylation of these two proteins.

Anaerobic fermentation of cellobiose is improved when CDT-2 cannot undergo α-arrestin-dependent internalization. Given that both the CDT-2 CtKR and CDT-2Trunc mutants increased PM localization and decreased internalization of this transporter, and are apparently “immune” to α-arrestin-dependent endocytosis, we reasoned that they should support more efficient cellobiose consumption and ethanol production even in WT cells. Indeed, as predicted, even though the overall growth rate was not significantly affected (Fig. 4C), WT cells expressing either CDT-2 CtKR or CDT-2Trunc displayed markedly improved cellobiose consumption and increased ethanol production compared to the same cells expressing wild-type CDT-2 (Fig. 4D).

CDT-2 is internalized in an α-arrestin-dependent manner in response to xylan. In N. crassa, CDT-2, and not CDT-1, permits utilization of xylan (13). If, as we observed for cellobiose, substrate transport serves as a trigger for transporter endocytosis, we then reasoned that for yeast ectopically expressing CDT-1 and CDT-2, the presence of xylan should promote internalization of the latter but not the former. In this regard, and in contrast to the experiments carried out with cellobiose, which were conducted in minimal medium, the experiments performed with xylan were conducted in rich medium (YP), which was necessary to provide sufficient alternative carbon sources for cell survival (because these S. cerevisiae cells do not possess the enzymatic machinery for subsequent xylan utilization).

In agreement with the conclusion that the presence of a transport substrate promotes transporter endocytosis, we found that internalization of CDT-2, but not CDT-1, was stimulated in xylan-containing medium (Fig. 5A). For 200 total cells in random fields of each culture propagated in yeast extract-peptone-dextrose (YPD) medium containing xylan, we observed the following percentages of cells with a robust vacuolar signal: WT CDT-1–GFP in WT cells, 4%; WT CDT-1–GFP in 9arrΔ cells, 3%; WT CDT-1–GFP in 4arrΔ cells, 3%; WT CDT-2–GFP in WT cells,
Thus, internalization of CDT-2, but not CDT-1, was stimulated in xylan-containing medium, and, as for cellobiose, CDT-2 internalization in response to xylan was eliminated in both the 9arrΔ/H9004 strain and the 4arrΔ cells. Likewise, compared to wild-type CDT-2, the CDT-2 CtKR mutant abrogated xylan-stimulated endocytosis almost completely, and the CDT-2 Trunc mutant significantly reduced internalization (Fig. 5B). For 200 total cells in random fields of each culture propagated in YPD medium containing xylan, we observed the following percentages of cells with a robust vacuolar signal: WT cells expressing WT CDT-2–GFP, 88%; WT cells expressing CDT-2 NtKR–GFP, 81%; WT cells expressing CDT-2 MidKR–GFP, 89%; WT cells expressing CDT-2 CtKR–GFP, 6%; WT cells expressing CDT-2 Trunc–GFP, 21%.

FIG 4 C-terminal mutants of CDT-2 are deficient in endocytosis and show increased cellobiose fermentation. (A) Schematic representation of the topology of CDT-2 on PM. (B) Representative images of Lys-to-Arg mutants of CDT-2 (Nt, N-terminal; Mid, large loop interconnecting the 6th and 7th transmembrane regions; Ct, C-terminal; Trunc, truncation) expressed in WT, 9arrΔ, and 4arrΔ cells. It should be noted that the CDT-2 constructs are expressed under the CCW12 promoter. (C) Anaerobic growth curves of CDT-2 and the derivative mutants expressed in WT cells. (D) Cellobiose consumption and ethanol production for CDT-2 and its mutants expressed in WT cells.
DISCUSSION

CDT-1 and CDT-2 are cellobiose transporters encoded in the *Neurospora crassa* genome. When heterologously expressed in *S. cerevisiae*, along with an intracellular β-glucosidase (GH1-1), this yeast becomes capable of directly transporting and utilizing cellobiose without the need for its prior hydrolysis into glucose (12).

In *S. cerevisiae*, a 14-member family of endocytic adaptors, the α-arrestins, mediates the Rsp5-dependent ubiquitinylation and subsequent internalization of specific integral membrane proteins in response to specific stimuli. For example, α-arrestin Ldb19 mediates internalization of the methionine transporter Mup1 in response to excess exogenous Met (23), α-arrestin Aly2 promotes internalization of the acidic amino acid transporter Dip5 in the presence of surplus Asp (25, 46, 47), and α-arrestin Art5 triggers internalization of the inositol transporter Itr1 when inositol is supplied (25, 48). Similarly, α-arrestin-dependent endocytosis of the glucose transporters Hxt1 and Hxt3 is stimulated by addition to the medium of 2-deoxy-glucose, a nonmetabolizable glucose analog (20), and the general amino acid permease Gap1 and the arginine-specific permease Can1 are internalized in response to their cognate transport substrates (49).

Here we demonstrated that the ectopically expressed cellobiose transporters CDT-1 and CDT-2 are also subject to transport substrate-induced and α-arrestin-dependent endocytosis. Although it appears to be counterintuitive that the presence of the cognate transport substrate (cellobiose in the case of CDT-1 and cellobiose or xylan in the case of CDT-2) triggers internalization of these transporters, it should be recalled that these molecules evolved in the different PM milieu of another organism. It seems likely, therefore, that, when expressed in yeast, the conformational dynamics required for the transport process occasionally causes partial unfolding that exposes epitopes in the transporters that permit their capture by the *S. cerevisiae* quality control machinery, which includes surveillance of the status of integral PM proteins by the endogenous yeast α-arrestins.

We found that in *S. cerevisiae*, CDT-1 and CDT-2 internalization is primarily mediated by just four members of the α-arrestin family: Aly1, Aly2, Rod1, and Rog3. It seems that CDT-1 and CDT-2 must possess sequence and/or structural features that are able to be recognized by each of the these pairs of α-arrestin paralogs, thereby recruiting the ubiquitin ligase Rsp5, which then ubiquitinylates the cellobiose transporters (Fig. 6). Conversely, cells deficient in these four α-arrestins elevate the PM content of these transporters and, as a consequence, allow for increased uptake of cellobiose. Indeed, under anoxic conditions, 4arrΔ cells expressing either CDT-1 or CDT-2 grow better and, in case of CDT-2, allow for improved conversion of cellobiose to ethanol.

For CDT-2, Lys residues within its C-terminal tail are the most critical for its efficient endocytosis, suggesting that these are the sites where α-arrestin-dependent and Rsp5-mediated ubiquitinylation occurs (Fig. 6). Consistent with α-arrestin-mediated internalization limiting the rate of cellobiose utilization, the CDT-2
from the PM in response to changes in carbon source. These transporters may be necessary to sense and transport celloolodextrins at low levels of extracellular cellulobiose, but once the full cellulolytic response has been induced and cellulolytic enzymes are actively degrading cellulose to glucose, their presence in the membrane may no longer be necessary.

Taken together, our findings have important implications for the engineering of production strains for the generation of cellulolodextrin biofuels, because they demonstrate that transporter endocytosis can exert a rate-limiting effect on cellulobiose utilization.

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REFERENCES

1. Nielsen J, Larsson C, van Maris A, Prønk J. 2013. Metabolic engineering of yeast for production of fuels and chemicals. Curr Opin Biotechnol 24:398–404. http://dx.doi.org/10.1016/j.copbio.2013.03.023.
2. Kang Q, Appels L, Tan T, Dewir L. 2014. Bioethanol from lignocellulosic biomass: current findings determine research priorities. ScientificWorldJournal 2014:298153. http://dx.doi.org/10.1155/2014/298153.
3. Zaldivar J, Nielsen J, Olsson L. 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34. http://dx.doi.org/10.1007/s002530100624.
4. McKendry P. 2002. Energy production from biomass (part 1): overview of biomass. Biosci Res Technol 83:37–46. http://dx.doi.org/10.1016/S0960-8524(01)00118-3.
5. Alvira P, Tomas-Pejo E, Ballesteros M, Negro MJ. 2010 Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol 101:4851–4861. http://dx.doi.org/10.1016/j.biortech.2009.11.093.
6. Bujs NA, Siewers V, Nielsen J. 2013. Advanced biofuel production by the yeast Saccharomyces cerevisiae. Curr Opin Chem Biol 17:480–488. http://dx.doi.org/10.1016/j.copbio.2013.03.036.
7. Zabed H, Faruq G, Sahu JN, Azirun MS, Hashim R, Boyce AN. 2014. Bioethanol production from fermentable sugar juice. ScientificWorldJournal 2014:957102. http://dx.doi.org/10.1155/2014/957102.
8. Toivari MH, Aristidou A, Ruohonien L, Penttila M. 2001. Conversion of xylose to ethanol by recombinant Saccharomyces cerevisiae: importance of xylosekinase (XKS1) and oxygen availability. Metab Eng 3:236–249. http://dx.doi.org/10.1006/mben.2000.0191.
9. Toivari MH, Salusjarvi L, Ruohonien L, Penttila M. 2004. Endogenous xylose pathway in Saccharomyces cerevisiae. Appl Environ Microb 70:3681–3686. http://dx.doi.org/10.1128/AEM.70.6.3681-3686.2004.
10. Lee SB, Shin HS, Ryu DD, Mandels M. 1982. Adsorption of cellulose from cellulosic plant material: The effectiveness of a cellulose-adsorption potential and the cellulases. J Biotechnol 13:291–303. http://dx.doi.org/10.1016/0168-1656(87)90093-8.
11. Philippidis GP, Smith TK, Wyman CE. 1993. Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. Biotechnol Bioeng 41:846–853. http://dx.doi.org/10.1002/bit.260410903.
12. Galazka JM, Tian C, Beeon WT, Martinez B, Glass NL, Cate JH. 2010. Celloexetrin transport in yeast for improved biofuel production. Science 308:84–86. http://dx.doi.org/10.1126/science.1192838.
13. Cai P, Gu R, Wang B, Li J, Wan L, Tian C, Ma Y. 2014. Evidence of a 874 – 876.http://dx.doi.org/10.1126/science.1192838.
critical role for cellodextrin transport 2 (CDT-2) in both cellulose and hemicellulose degradation and utilization in \textit{Neurospora crassa}. \textit{PLoS One} 9:e89330. http://dx.doi.org/10.1371/journal.pone.0089330.

14. Hahn-Hagedorn B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF. 2007. Towards endothelial pentose-fermenting yeast strains. Appl Environ Microbiol 74:937–953. http://dx.doi.org/10.1128 /AEM.00605-07 82–7.

15. Wisselink HW, Toirkens MJ, Wu Q, Pronk JT, van Maris AJ. 2009. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered \textit{Saccharomyces cerevisiae} strains. Appl Environ Microbiol 75:907–914. http://dx.doi.org/10.1128/AEM.02268-08.

16. Garcia Sanchez R, Hahn-Hagedorn B, Gorwa-Grauslund MF. 2010. Cross-reactions between engineered xylose and galactose pathways in re- combinant \textit{Saccharomyces cerevisiae}. Biotechnol Biofuels 3:19. http://dx.doi.org/10.1186/1754-6834-3-19.

17. Lin Y, Chomvong K, Acosta-Sampson I, Estrella R, Galazka JM, Kim SR, Jin YS, Cate JH. 2014. Leveraging transcription factors to speed cellobiose fermentation by \textit{Saccharomyces cerevisiae}. Biotechnol Biofuels 7:126. http://dx.doi.org/10.1186/1754-688X-7-126.

18. Hicke L. 1997. Ubiquitin-dependent internalization and down-regulation of plasma membrane protein factors. \textit{FASEB J} 11:1215–1226.

19. Belgareh-Touze N, Leon S, Erpapazoglou Z, Stawiacka-Mirota M, Ur- ban-Grimal D, Hagenauer-Tsapis R. 2008. Versatile role of the yeast ubiquitin ligase Rsp5p in intracellular trafficking. Biochem Soc Trans 36: 70:9–70:9. http://dx.doi.org/10.1042/BST0360791.

20. O’Donnell AF, McCartney RR, Chandrashekarappa DG, Zhang BB, Torner J, Schmidt MC. 2015. 2-Deoxyglucose impairs \textit{Saccharomyces cerevisiae} growth by stimulating Snf1-regulated and alpha-arrestin-cerevisiae ubiquitination and endocytosis of the yeast metal transporter Smf1. http://dx.doi.org/10.1093/mcb/MTE007.

21. Sen et al. 2015. Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). \textit{J Biochem} 157:551–555. http://dx.doi.org/10.1093/jb/mv068.

22. Tevzadze GG, Pierce JV, Espostio RE. 2007. Genetic evidence for a SPO1-dependent signaling pathway controlling meiotic progression in yeast. Genetics 173:1213–1227.

23. Helliwell SB, Losko S, Kaiser CA. 2005. Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. \textit{J Cell Biol} 155:649–662. http://dx.doi.org/10.1083/jcb.153.4.649.

24. Sen et al. 2015. Internal amino acids promote Gap1 permease ubiquitylation and down-regulation via the arrestin-like Bul and Aly proteins. \textit{J Biol Chem} 289:22103–22116. http://dx.doi.org/10.1074/jbc.M115.582230.

25. Kim H, Lee WH, Galazka JM, Cate JH, Jin YS. 2014. Analysis of cellodextrin transporters from \textit{Neurospora crassa} in \textit{Saccharomyces cerevisiae} for cellulosome fermentation. \textit{Appl Microbiol Biotechnol} 98:1087– 1094. http://dx.doi.org/10.1007/s00253-013-5332-9.

26. Lian J, Li Y, HamediRad M, Zhao H. 2014. Directed evolution of a cellodextrin transporter for improved biofuel production under anaerobic conditions in \textit{Saccharomyces cerevisiae}. Biotechnol Bioeng Bioi 111:1521– 1531. http://dx.doi.org/10.1002/bit.25214.

27. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. 2015. The I-TASSER hierarchical protein structure and function prediction. Nat Methods 12:7–8. http://dx.doi.org/10.1038/nmeth.3213.

28. Kall L, Krogh A, Sonnhammer EL. 2004. A combined transmembrane topology and signal peptide prediction method. \textit{J Mol Biol} 348:1027–1036. http://dx.doi.org/10.1016/j.jmb.2004.03.016.

29. Hatakeyama R, Kamiya M, Takahara T, Maeda T. 2010. Endocytosis of the aspartic acid/glutamic acid transporter Dip5 is triggered by substrate-induced ubiquitylation of the Dip5 ubiquitin ligase via the arrestin-like protein Aly2. Mol Cell Biol 30:5598–5607. http://dx.doi.org/10.1128/mcb.00464-10.

30. O’Donnell AF, Huang L, Torner J, Cyert MS. 2010. Endocytosis of a general amino acid permease. \textit{J Cell Biol} 1215–1226. http://dx.doi.org/10.1083/jcb.153.4.649.

31. Sen et al. 2015. Three ubiquitin ligase adaptors regulate endocytosis and protein turnover at the cell surface. \textit{Cell} 135:714–725. http://dx.doi.org/10.1016/j.cell.2008.09.025.

32. Nikko E, Sullivan JA, Pelham HR. 2008. Arrestin-like proteins mediate ubiquitination and endocytosis of the yeast metal transporters Smf1. J Biol Chem 283:16047–16051. http://dx.doi.org/10.1074/jbc.mc1083097.

33. Nishikawa S, Brodsky JL, Nikkaku S. 2003. Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). \textit{J Biochem} 137:551–555. http://dx.doi.org/10.1093/jb/mv068.

34. Tevzadze GG, Pierce JV, Espostio RE. 2007. Genetic evidence for a SPO1-dependent signaling pathway controlling meiotic progression in yeast. Genetics 173:1213–1227.

35. Helliwell SB, Losko S, Kaiser CA. 2005. Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. \textit{J Cell Biol} 155:649–662. http://dx.doi.org/10.1083/jcb.153.4.649.

36. Sen et al. 2015. Internal amino acids promote Gap1 permease ubiquitylation and down-regulation via the arrestin-like Bul and Aly proteins. \textit{J Biol Chem} 289:22103–22116. http://dx.doi.org/10.1074/jbc.M115.582230.

37. Kim H, Lee WH, Galazka JM, Cate JH, Jin YS. 2014. Analysis of cellodextrin transporters from \textit{Neurospora crassa} in \textit{Saccharomyces cerevisiae} for cellulosome fermentation. \textit{Appl Microbiol Biotechnol} 98:1087– 1094. http://dx.doi.org/10.1007/s00253-013-5332-9.

38. Lian J, Li Y, HamediRad M, Zhao H. 2014. Directed evolution of a cellodextrin transporter for improved biofuel production under anaerobic conditions in \textit{Saccharomyces cerevisiae}. Biotechnol Bioeng Bioi 111:1521– 1531. http://dx.doi.org/10.1002/bit.25214.

39. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. 2015. The I-TASSER hierarchical protein structure and function prediction. Nat Methods 12:7–8. http://dx.doi.org/10.1038/nmeth.3213.

40. Kall L, Krogh A, Sonnhammer EL. 2004. A combined transmembrane topology and signal peptide prediction method. \textit{J Mol Biol} 348:1027–1036. http://dx.doi.org/10.1016/j.jmb.2004.03.016.

41. Hatakeyama R, Kamiya M, Takahara T, Maeda T. 2010. Endocytosis of the aspartic acid/glutamic acid transporter Dip5 is triggered by substrate-induced ubiquitylation of the Dip5 ubiquitin ligase via the arrestin-like protein Aly2. Mol Cell Biol 30:5598–5607. http://dx.doi.org/10.1128/mcb.00464-10.

42. O’Donnell AF, Huang L, Torner J, Cyert MS. 2010. Endocytosis of a general amino acid permease. \textit{J Cell Biol} 1215–1226. http://dx.doi.org/10.1083/jcb.153.4.649.

43. Nishikawa S, Brodsky JL, Nikkaku S. 2003. Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). \textit{J Biochem} 137:551–555. http://dx.doi.org/10.1093/jb/mv068.
53. Znameroski EA, Coradetti ST, Roche CM, Tsai JC, Iavarone AT, Cate JH, Glass NL. 2012. Induction of lignocellulose-degrading enzymes in Neurospora crassa by cellodextrins. Proc Natl Acad Sci U S A 109:6012–6017. http://dx.doi.org/10.1073/pnas.1118440109.
54. Sun J, Glass NL. 2011. Identification of the CRE-1 cellulolytic regulon in Neurospora crassa. PLoS One 6:e25654. http://dx.doi.org/10.1371/journal.pone.0025654.
55. Turcotte B, Liang XB, Robert F, Soontorngun N. 2010. Transcriptional regulation of non-fermentable carbon utilization in budding yeast. FEMS Yeast Res 10:2–13. http://dx.doi.org/10.1111/j.1567-1364.2009.00555.x.
56. Hynes MJ, Kelly JM. 1977. Pleiotropic mutants of Aspergillus nidulans altered in carbon metabolism. Mol Gen Genet 150:193–204. http://dx.doi.org/10.1007/BF00695399.
57. Boase NA, Kelly JM. 2004. A role for creD, a carbon catabolite repression gene from Aspergillus nidulans, in ubiquitination. Mol Microbiol 53:929–940. http://dx.doi.org/10.1111/j.1365-2958.2004.04172.x.