Interconnected regulation of carbon catabolite repression and carbon source utilization in an oleaginous yeast

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ABSTRACT

Cells must sense and respond to available nutrients to survive. Microbes evolved mechanisms to activate genes necessary to utilize a carbon source specifically when it is present. To efficiently grow in mixed carbon source environments, microbes repress genes necessary to utilize carbon sources that require substantial resources to catabolize when a simpler carbon source is present, known as carbon catabolite repression. We investigated nutrient utilization in the basidiomycete, oleaginous yeast *Rhodotorula (Rhodosporidium) toruloides*. *R. toruloides* is a saprophytic fungus that utilizes the building blocks of complex polysaccharides found in plant cell walls. A transcription factor homologous to the cellulose degradation regulator CLR-2/ClrB in Ascomycete filamentous fungi, which we named Cbr1, is required for cellobiose utilization in *R. toruloides*. Although the role of CLR-2/ClrB is limited to regulating expression of genes involved in cellulose and hemicellulose utilization, Cbr1 is also involved in fucose and tricarboxylic acid cycle intermediate utilization. Additionally, Cbr1 inhibits carbon catabolite repression specifically during utilization of glucose-glucose disaccharides, which may have evolved to limit the glucose-mediated repression of genes encoding proteins that cleave disaccharides into glucose. In fungi, it is thought carbon source-specific transcription factors activate genes necessary for carbon source utilization, while carbon catabolite repression is regulated by transcription factors that broadly repress all nonpreferred carbon source utilization genes when a preferred carbon source is present. Our data provide evidence for a noncanonical carbon catabolite repression mechanism and suggest transcriptional mechanisms regulating carbon catabolite repression may be less broadly conserved than previously thought.

SIGNIFICANCE STATEMENT

Microbes evolved transcriptional networks regulating nutrient utilization. Carbon sources requiring substantial resources to catabolize are less preferred than carbon sources requiring

less energy. Canonically, a preferred carbon source represses all nonpreferred carbon source utilization genes, termed carbon catabolite repression. We identified a transcription factor in the yeast *Rhodotorula* (*Rhodosporidium*) *toruloides* that specifically inhibits glucose-mediated repression of glucose-glucose disaccharide utilization and is required for cellobiose, carboxylic acid, and fucose utilization. Carbon catabolite repression only of specific carbon sources and co-regulation of carbon catabolite repression with nutrient utilization gene activation establishes a noncanonical carbon catabolite repression mechanism. Characterization of diverse carbon catabolite repression mechanisms is important for engineering industrial fungi and in fungal pathogenesis, where carbon catabolite repression is important for virulence.

53 **INTRODUCTION**

54 Cells must sense and respond to nutrients to successfully grow and divide using the
55 resources available. For fungi, nutrient sensing is critical to establish fungal colonies, compete
56 with other microbes, and establish pathogenic and symbiotic relationships with plants and
57 animals. Saprophytic fungi acquire nutrients from dead plants and other organic matter. The
58 carbon sources found in plant biomass range from simple sugars, like sucrose and fructose, to
59 complex polysaccharides, such as cellulose, found in plant cell walls.

60 Utilization of complex polysaccharides requires extracellular degradation to break down
61 the insoluble molecules into soluble building blocks that can be imported into fungal cells (1).
62 Many yeast species can utilize the soluble building blocks of polysaccharides found in plant
63 biomass but frequently cannot degrade the insoluble polysaccharides (2). These yeast species
64 are excellent choices for growth and engineering in industrial settings because of their
65 uninucleate, single cellular lifestyle and wide carbon utilization repertoire. The basidiomycete,
66 oleaginous yeast *Rhodotorula toruloides* (previously known as *Rhodosporidium toruloides*) can
67 eat most soluble breakdown products of the carbohydrates found in plant cell walls (3, 4). *R.*
68 *toruloides* can also accumulate up to 70% of its biomass as fatty acids in conditions in which
69 carbon is abundant but other nutrients are limiting and produces useful secondary metabolites,
70 such as carotenoids (5-11). This makes *R. toruloides* an excellent choice for green
71 biotechnology in which products that might otherwise be sourced from petroleum or other
72 environmentally unsustainable sources are produced in microbes fed by agricultural waste or
73 plant matter grown on marginal land (12).

74 Most of the work characterizing the genetic pathways regulating utilization of carbon
75 sources has been performed in Ascomycete fungi (1, 13). In these fungi, a network of activating
76 transcription factors activates the genes necessary for utilization of available carbon (14). Plant
77 biomass contains a wide array of carbon sources. These varied carbon sources have differing
78 levels of preference for the fungal cell. When a more preferred carbon source is available, a

79 process known as carbon catabolite repression downregulates the expression of genes required
80 for utilization of any less preferred carbon sources present in the environment (1, 15). In
81 Ascomycete fungi, one of the major regulators of carbon catabolite repression is the
82 transcription factor CRE-1/CreA/Mig1, which broadly represses the expression of genes
83 necessary for utilization of any nonpreferred carbon source in the presence of a more preferred
84 carbon source (15-21). However, the genetic mechanisms through which Basidiomycete fungi
85 activate genes necessary to utilize nonpreferred carbon sources and repress these genes in the
86 presence of more preferred carbon sources is significantly less well studied.

87 We investigated the genetic mechanisms regulating carbon utilization in the
88 basidiomycete yeast *R. toruloides*. Cellulose is the most abundant component of plant biomass
89 and is composed of long chains of glucose linked by β-1,4-glycosidic bonds (22). *R. toruloides*
90 can utilize the disaccharide building block of cellulose, cellobiose (23). We took a phylogenetic
91 approach to identify the regulator of cellobiose utilization in *R. toruloides*. This led to the
92 identification of a transcription factor homologous to the cellulose degradation regulator CLR-
93 2/ClrB in Ascomycete fungi (24) that is required for utilization of cellobiose in *R. toruloides*,
94 which we named Cbr1.

95 Characterization of Cbr1 revealed that the role of this transcription factor is significantly
96 expanded in *R. toruloides*, where it is also required for utilization of tricarboxylic acid (TCA)
97 cycle intermediates and the deoxy hexose sugar fucose. We used transcriptomic analyses to
98 identify genes regulated by Cbr1. These genes included two genes encoding secreted β-
99 glucosidases and two genes encoding transporters. One of these β-glucosidase genes is
100 necessary for cellobiose utilization, while one of the identified transporters is required for
101 utilizing the tricarboxylic acid citrate. Cellobiose, fucose, and TCA cycle intermediates are not
102 utilized through similar pathways, suggesting that *R. toruloides* may inhabit an ecological niche
103 in which these carbon sources are frequently found together. Further investigation into the role

104 of Cbr1 demonstrated that this transcription factor inhibits carbon catabolite repression
105 specifically when a glucose-glucose disaccharide is present as a nonpreferred carbon source.
106 This role for Cbr1 contrasts with previously identified genetic mechanisms of carbon catabolite
107 repression regulation, which are involved in the regulation of genes responsible for the
108 utilization of any nonpreferred carbon source. Taken together, the varied roles of Cbr1 in *R.*
109 *toruloides* metabolic regulation present a novel mechanism of carbon catabolite repression in
110 fungi and may provide insights into the ecological niche of this oleaginous yeast.
111

112 **RESULTS**

113 *Cbr1 is required for wild-type utilization of cellobiose and TCA cycle intermediates*

114 Cellulose is the most abundant component in the plant cell wall. Although *R. toruloides*
115 cannot degrade cellulose, it can utilize the disaccharide breakdown product of cellulose,
116 cellobiose (23). Several transcription factors are known to regulate cellulose and cellobiose
117 utilization in Ascomycete filamentous fungi, including CLR-1/ClrA, CLR-2/ClrB, and XlnR (1, 24-
118 27). To investigate the genetic mechanisms regulating *R. toruloides* cellobiose utilization, we
119 searched for transcription factors in the *R. toruloides* genome with homology to these regulators
120 of cellulose and cellobiose utilization. No clear homolog of CLR-1/ClrA or XlnR existed in the *R.*
121 *toruloides* genome (Fig S1). However, we identified a single homolog of CLR-2/ClrB,
122 RTO4_13588 (Fig 1A and Fig S1).

123 We hypothesized that RTO4_13588 may play a role in regulating cellobiose utilization in
124 *R. toruloides*. To test this hypothesis, we deleted RTO4_13588 and grew the resulting
125 RTO4_13588Δ strain with cellobiose as the sole carbon source. Growth of cells lacking
126 RTO4_13588 with glucose as the sole carbon source was indistinguishable from that of wild
127 type cells (Fig 1B). However, deletion of RTO4_13588 resulted in a complete lack of growth
128 when cellobiose was provided as the sole carbon source, which could be complemented by
129 exogenous expression of RTO4_13588 (Fig 1B). The closest homolog to RTO4_13588 in the

130 model yeast *Saccharomyces cerevisiae* is the transcription factor Gal4, which regulates
131 galactose utilization in *S. cerevisiae* (Fig 1A) (28). To test whether RTO4_13588 also played a
132 role in regulating galactose utilization in *R. toruloides*, we grew wild type and RTO4_13588Δ
133 cells with galactose as the sole carbon source. There was no detectable difference in growth
134 between wild type cells and cells lacking RTO4_13588 on galactose (Fig S2A). Thus, we named
135 RTO4_13588, *CBR1* for cellobiose regulator 1.

136 *R. toruloides* is capable of growth on a wide variety of soluble carbon sources (3, 23).
137 We asked whether *CBR1* was also required for growth on any additional carbon sources. Cells
138 lacking *CBR1* were unable to grow as well as wild type cells on the deoxy hexose sugar fucose,
139 which could be complemented by exogenous expression of *CBR1* (Fig 1B). The growth of *cbr1*Δ
140 cells was indistinguishable from that of wild type cells on all other sugars tested (fructose,
141 xylose, arabinose, galacturonic acid, sucrose, maltose, trehalose, and raffinose) and during
142 growth on the lignin phenolic building blocks, coumaric and ferulic acid (Fig S2A-S2B). *CBR1*
143 was not required for utilization of the lipid mimic Tween-80, the fermentation product acetate, or
144 the amino acids L-alanine, proline, glutamate, or glycine, suggesting *cbr1*Δ cells are capable of
145 respiration (Fig S2C-S2D). Thus, we hypothesized that *cbr1*Δ cells would be able to grow with
146 TCA cycle intermediates provided as the sole carbon source. To our surprise, when we grew
147 wild type, *cbr1*Δ, and *cbr1*Δ + *CBR1* cells on media containing citrate, α-ketoglutarate,
148 succinate, fumarate, or malate as the sole carbon source, the *cbr1*Δ cells had significant growth
149 defects relative to wild type and *cbr1*Δ + *CBR1* cells (Fig 1B).

150 The role for Cbr1 in TCA cycle intermediate and fucose utilization could either be an
151 expanded role for Cbr1 in *R. toruloides* relative to CLR-2/ClrB or a previously uncharacterized
152 role for CLR-2/ClrB. To distinguish between these two possibilities, we assessed growth of the
153 model filamentous fungus *Neurospora crassa* wild type and Δ*clr-2* cells in media containing
154 succinate, α-ketoglutarate, malate, or fucose as the sole carbon source. Wild type *N. crassa*

155 was only capable of limited growth with TCA cycle intermediates or fucose as the sole carbon
156 source, and deletion of *clr-2* caused no detectable difference in growth (Fig S3). These data
157 suggest the role for *CBR1* in regulating TCA cycle intermediate and fucose utilization is an
158 expanded role for this transcription factor, relative to the role of Cbr1 orthologs in Ascomycete
159 filamentous fungi.
160
161 *Cbr1 activates a core regulon of five genes in response to cellobiose, TCA cycle intermediates,*
162 *and fucose*
163 We had three possible hypotheses for the genetic mechanism through which Cbr1 could
164 regulate utilization of cellobiose, TCA cycle intermediates, and fucose: (1) Cbr1 regulates genes
165 necessary to utilize all three classes of carbon sources in response to any of these carbon
166 sources, (2) Cbr1 regulates genes necessary to utilize each carbon source only in response to
167 that specific carbon source, and (3) *R. toruloides* utilization of cellobiose, fucose, and/or TCA
168 cycle intermediates proceeds through atypical metabolic pathways such that they require the
169 same transporters and/or catabolic enzymes. To distinguish between these three hypotheses,
170 we used transcriptional profiling. We exposed exponentially growing wild type and *cbr1*Δ cells to
171 yeast nitrogen base (YNB) medium lacking a carbon source (no carbon) or supplemented with
172 1% cellobiose, fucose, or succinate as the sole carbon source for 8 h prior to harvesting the
173 cells for RNA sequencing (RNAseq). The cellobiose, succinate, and no carbon samples were
174 submitted for whole transcript RNAseq, and the fucose samples were submitted for 3' RNAseq.
175 Given the limited characterization of *R. toruloides* gene expression on cellobiose or
176 succinate, we first examined the transcriptional profile of wild type cells in these conditions
177 relative to carbon starvation. The expression of 1168 genes was at least four-fold differentially
178 expressed in wild type cells exposed to cellobiose compared to carbon starvation, with 155
179 genes increased in expression and 1013 genes decreased. For wild type cells exposed to
180 succinate as a sole carbon source, 525 genes were at least four-fold differentially expressed

181 relative to carbon starved cells with 142 genes increased in expression and 383 genes
182 decreased in expression. Intriguingly, over 75% of genes differentially expressed on succinate
183 (405/525) were also differentially expressed on cellobiose, supporting the hypothesis that similar
184 sets of genes are regulated in response to cellobiose and succinate (Fig S4A-C and Dataset
185 S1-S2).

186 Cells lacking *CBR1* also showed substantial transcriptional responses to cellobiose and
187 succinate in comparison to carbon starvation. Five hundred seventy-nine genes were at least
188 four-fold differentially expressed in cellobiose compared to carbon starvation. Eighty-one of the
189 179 genes (45%) with higher expression on cellobiose in *cbr1*Δ cells were also higher in
190 expression in wild type cells exposed to cellobiose, and 319 of the 400 genes (80%) with
191 decreased expression on cellobiose in *cbr1*Δ cells had the same expression pattern in wild type
192 cells (Fig S4A, S4D, S4F, and Dataset S1-S2). Similarly, 742 genes were differentially
193 expressed on succinate compared to carbon starvation in *cbr1*Δ cells. Eighty-six of the 296
194 genes (29%) with higher expression on succinate in *cbr1*Δ cells were also higher in expression
195 in wild type cells exposed to succinate, and 256 of 446 genes (57%) with lower expression on
196 succinate in *cbr1*Δ cells were also decreased in expression in wild type cells exposed to
197 succinate (Fig S4B, S4E, S4G, and Dataset S1-S2). The significant transcriptional response to
198 cellobiose and succinate combined with the high degree of transcriptional similarity exhibited by
199 the *cbr1*Δ and wild type cells suggests that despite not being able to utilize cellobiose or
200 succinate at wild type levels, *cbr1*Δ cells are still able to respond to the presence of these
201 carbon sources to some extent.

202 Although the expression of many fungal transcription factor genes is upregulated in the
203 condition in which the transcription factor plays a role, *CBR1* expression was not induced in
204 response to either cellobiose or succinate compared to carbon starvation (Fig S5A and Dataset
205 S1-S2). To directly regulate gene expression, Cbr1 must be present in the nucleus. We

206 hypothesized Cbr1 might be transported into the nucleus under conditions where it plays a role.
207 To test this hypothesis, we tagged Cbr1 with green fluorescent protein (mEGFP) and the histone
208 H2A subunit *HTA1* (RTO4_12090) with mRuby2 and exposed these cells to carbon starvation or
209 glucose, cellobiose, or succinate as the sole carbon source to identify the localization pattern of
210 Cbr1. Cbr1 colocalized with the histone H2A subunit under all four conditions, suggesting that
211 Cbr1 is expressed and present in the nucleus during exposure to glucose, cellobiose, succinate,
212 and carbon starvation (Fig S5B).

213 To gain insight into the mechanism behind the growth defects of cells lacking *CBR1* we
214 examined genes that were decreased in expression in *cbr1*Δ relative to wild type cells. Using a
215 four-fold differential expression cutoff, 284, 18, 25, and 25 genes were lower in expression in
216 *cbr1*Δ compared to wild type cells on cellobiose, succinate, carbon starvation, and fucose,
217 respectively (Fig 2A-D and Dataset S1-S2). In concordance with the hypothesis that cellobiose,
218 carboxylic acid, and fucose utilization are, at least in part, transcriptionally linked via *CBR1*,
219 genes annotated as part of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (29) 2-
220 oxocarboxylic acid metabolism category were enriched in the *CBR1* regulon on cellobiose (Fig
221 2E). Additionally, carbohydrate metabolism KEGG (29) and gene ontology (GO) (30, 31) related
222 categories were enriched on succinate, no carbon, and fucose. Further analysis revealed the
223 carbohydrate-related enrichments primarily consisted of genes annotated as having putative β-
224 glucosidase activity, which is responsible for cleaving cellobiose into two glucose molecules (Fig
225 2E).

226 Given the limited number of genes lower in expression in *cbr1*Δ cells relative to wild type
227 cells for all conditions except cellobiose and the substantial overlap in transcriptional responses
228 between cellobiose and succinate, we hypothesized that genes necessary for utilization of the
229 individual carbon sources would be differentially expressed in a *CBR1*-dependent manner
230 across multiple carbon sources. Only five genes were differentially expressed by at least four-

231 fold in at least three of the four conditions: two encoding putative β-glucosidases (RTO4_16716
232 and RTO4_16717) that are adjacent in the *R. toruloides* genome, two encoding putative major
233 facilitator superfamily transporters (RTO4_10339 and RTO4_13825), and one encoding a
234 putative phosphoglycerate mutase (RTO4_11229) (Fig 2A-D and Dataset S1-S2).
235
236 *Cbr1-regulated β-glucosidases are necessary for cellobiose utilization*
237 The expression of two predicted β-glucosidase genes in a *CBR1*-dependent fashion, led
238 us to hypothesize that one or both β-glucosidase genes is essential for cellobiose utilization. An
239 amino acid alignment using LALIGN revealed 72% identity and 90% similarity between the two
240 proteins, suggesting a potential for redundancy, so to test this hypothesis we deleted
241 RTO4_16716 and RTO4_16717, together and individually (32). RTO4_16716Δ RTO4_16717Δ
242 cells grew like wild type on glucose, citrate, succinate, and fucose, but were unable to grow on
243 cellobiose (Fig 3A and S6A). Analysis of the individual deletion mutants revealed that deletion of
244 RTO4_16717 alone was sufficient to eliminate growth on cellobiose and complementation
245 exogenously with RTO4_16717 under its native promoter restored the ability to grow on
246 cellobiose (Fig 3B). Cells lacking RTO4_16716 alone grew as well as wild type cells on
247 cellobiose (Fig 3B). These data indicate RTO4_16717 encodes the primary β-glucosidase for
248 cellobiose utilization. Thus, we named RTO4_16717 and RTO4_16716, *BGL1* and *BGL2*,
249 respectively, for β-glucosidase 1 and 2.
250 Both *BGL1* and *BGL2* have predicted secretion signals, leading us to hypothesize that
251 cleavage of cellobiose by the β-glucosidases may occur extracellularly. To address this
252 hypothesis, we exposed exponentially growing wild type, *cbr1*Δ, and *cbr1*Δ + *CBR1* cells to
253 media containing glucose, cellobiose, or succinate. After 20 h of incubation, we harvested
254 supernatants to measure secreted β-glucosidase activity. β-glucosidase activity was detected in
255 the supernatants of wild type cells exposed to cellobiose indicating that β-glucosidases are

256 indeed secreted by *R. toruloides* (Fig 3C). Consistent with our transcriptomics data, and despite
257 the lack of a role for *BGL1* and *BGL2* in carboxylic acid utilization, β-glucosidase activity was
258 also detectable in the supernatant of wild type cells grown on succinate. Deletion of *CBR1*
259 eliminated β-glucosidase activity in the supernatant of cellobiose and succinate cultures. This
260 activity was restored in the supernatant of cultures of *cbr1*Δ + *CBR1* cells, indicating the
261 secreted β-glucosidase activity was dependent on *CBR1* (Fig 3C).

262 β-glucosidase activity was not constitutively present in *R. toruloides* supernatants, as
263 glucose cultures had no detectable β-glucosidase activity (Fig 3C). A lack of β-glucosidase
264 activity in glucose culture supernatants could either be due to reduced expression of β-
265 glucosidase genes or reduced secretion of β-glucosidase proteins relative to growth on
266 cellobiose and succinate. To distinguish between these possibilities, we performed reverse
267 transcription quantitative PCR (RT-qPCR) on *BGL1* in wild type cells exposed to cellobiose,
268 succinate, glucose, and carbon starvation. Corroborating our RNAseq data, *BGL1* expression
269 was highest on cellobiose, followed by succinate, and then carbon starvation (Fig 3D). During
270 exposure to glucose, *BGL1* expression was nearly undetectable, indicating the lack of β-
271 glucosidase activity in the glucose culture supernatant was due to reduced expression of β-
272 glucosidase genes (Fig 3C and 3D). These data may indicate β-glucosidase expression is
273 regulated by carbon catabolite repression and that β-glucosidases potentially act as "scout"
274 enzymes during carbon starvation similar to some polysaccharide degrading enzymes in some
275 filamentous fungi (1, 14).

276 Given that *bgl1*Δ cells cannot grow when cellobiose is the sole carbon source (Fig 3B),
277 we hypothesized Bgl1 was the source of the secreted β-glucosidase activity. To test this
278 hypothesis, we incubated exponentially growing *bgl1*Δ cells in media containing cellobiose,
279 succinate, or glucose for 20 h. While the β-glucosidase activity present in the cellobiose *bgl1*Δ
280 culture supernatant was 22-fold lower than that of wild type culture supernatants, it was

281 detectable, and β-glucosidase activity of succinate *bgl1*Δ cultures was even more pronounced
282 (Fig 3C and S6C). This reduction in β-glucosidase secretion was mitigated in *bgl1*Δ + *BGL1*
283 cells (Fig 3C). The low level of β-glucosidase activity remaining in *bgl1*Δ cells could either result
284 from Bgl2 secretion or secretion of an unidentified protein. To distinguish between these
285 possibilities, we exposed *bgl1*Δ *bgl2*Δ and *bgl1*Δ *bgl2*Δ + *BGL1 BGL2* cells to cellobiose,
286 succinate, or glucose for 20 h. Cells lacking both *BGL1* and *BGL2* had no detectable β-
287 glucosidase activity, and this could be rescued by complementation with wild type copies of
288 *BGL1* and *BGL2* (Fig 3C and S6C). These data suggested Bgl2 does contribute to the β-
289 glucosidase activity present in cellobiose and succinate culture supernatants. To test this
290 hypothesis, we measured β-glucosidase activity secreted by *bgl2*Δ cells. Although, *BGL2* was
291 dispensable for growth on cellobiose, deletion of *bgl2* resulted in a 2.8-fold reduction of β-
292 glucosidase activity on both cellobiose and succinate (Fig 3C). These data show that both Bgl1
293 and Bgl2 are responsible for the secreted β-glucosidase activity of *R. toruloides*.
294 It was possible either that β-glucosidase genes were the only Cbr1-regulated genes
295 necessary for cellobiose utilization or that other genes were also required. To distinguish
296 between these two possibilities, we expressed *BGL1* under the constitutive *AAC1*
297 (RTO4_12704) promoter (33) at the *YKU70* (RTO4_11920) locus in a *cbr1*Δ background (*cbr1*Δ
298 + P_{AAC1}-*BGL1*). Constitutive expression of *BGL1* in *cbr1*Δ cells resulted in wild type levels of
299 growth on cellobiose when cells were inoculated at very low densities (Fig 3B). However, when
300 cells were inoculated at higher starting densities, similar to those used to quantify extracellular
301 β-glucosidase activity, *cbr1*Δ + P_{AAC1}-*BGL1* cells had a growth advantage on cellobiose relative
302 to wild type cells (Fig S6D-S6E). Correspondingly, *cbr1*Δ + P_{AAC1}-*BGL1* cellobiose culture
303 supernatants had 3.2-fold higher β-glucosidase activity than wild type culture supernatants (Fig
304 S6F). These data suggest *BGL1* is the only Cbr1-regulated gene required for cellobiose

305 utilization and constitutive expression of *BGL1* yields a density-dependent growth advantage
306 when cellobiose is the sole carbon source.

307 Given that *cbr1*Δ + P_{AAC1}-*BGL1* cells are capable of growth on cellobiose, suggesting
308 that β-glucosidase activity is the only Cbr1-regulated requirement for cellobiose utilization, we
309 hypothesized that the growth defect of *cbr1*Δ and *bgl1*Δ *bgl2*Δ cells on cellobiose could be
310 rescued by β-glucosidases secreted into the media by wild type cells. To test this hypothesis, we
311 inoculated wild type, *cbr1*Δ, and *bgl1*Δ *bgl2*Δ cells into YNB 1% cellobiose supplemented with
312 filtered, spent supernatant from wild type cells grown on cellobiose, succinate, or glucose. As a
313 control for growth on residual carbon in the spent supernatant, we also inoculated these strains
314 into YNB without a carbon source supplemented with spent supernatant (Fig S7). Cultures of
315 *cbr1*Δ and *bgl1*Δ *bgl2*Δ cells grown in cellobiose supplemented with spent supernatants from
316 wild type cellobiose and succinate cultures grew significantly more than when *cbr1*Δ and *bgl1*Δ
317 *bgl2*Δ cells were grown on cellobiose alone (Fig 3E-3F). Taken together, our data demonstrate
318 that Cbr1 regulates genes encoding secreted β-glucosidases in response to cellobiose and TCA
319 cycle intermediates and that extracellular degradation of cellobiose by β-glucosidases is
320 required for cellobiose utilization by *R. toruloides*.

321

322 *CBR1-mediated transcriptional response to cellobiose is dependent on extracellular β-*
323 *glucosidases*

324 Rescue of the growth of *cbr1*Δ cells on cellobiose by constitutively expressed *BGL1* or
325 spent supernatant containing β-glucosidase activity provoked the question of whether cellobiose
326 is the signaling molecule activating the *CBR1*-dependent transcriptional response to cellobiose
327 or if the signal is a catabolic derivative of cellobiose. We differentiated between these
328 possibilities in two ways.

329 We first compared the transcriptional profile of *bgl1*Δ *bgl2*Δ cells exposed to cellobiose,
330 which have no extracellular β-glucosidase activity but contain wild type copies of *CBR1* and any
331 unknown cellobiose receptor(s) or transporter(s), to that of wild type and *cbr1*Δ cells using 3'
332 RNAseq. The transcriptional profiles of *bgl1*Δ *bgl2*Δ cells were more similar to that of *cbr1*Δ cells
333 than to wild type cells (Fig 4A-C, S8, and Dataset S3-S4). Thirty-five genes were at least four-
334 fold differentially expressed in *bgl1*Δ *bgl2*Δ cells relative to wild type cells (12 increased and 23
335 decreased in expression) (Fig 4A and Dataset S3-S4). Twenty-three of these genes were also at
336 least four-fold differentially expressed in *cbr1*Δ cells compared to wild type cells (Fig 4B and
337 Dataset S3-S4). In contrast, only five genes were at least four-fold differentially expressed in
338 *bgl1*Δ *bgl2*Δ relative to *cbr1*Δ cells, which included a 38-fold upregulation of the *CBR1*-regulated
339 predicted transporter gene RTO4_13825 (Fig 4C and Dataset S3-S4). The increased
340 expression of RTO4_13825 in *bgl1*Δ *bgl2*Δ cells relative to both *cbr1*Δ and wild type cells, raised
341 the question of whether any of the other genes in the core Cbr1 regulon were involved in
342 cellobiose sensing or utilization. Cells lacking RTO4_13825, RTO4_10339, or RTO4_11229 all
343 grew as well as wild type on cellobiose (Fig S9). These data indicate the signal for the *CBR1*-
344 dependent cellobiose response requires secreted β-glucosidases, suggesting cellobiose is likely
345 not the signaling molecule for Cbr1 activation.
346 The activity of β-glucosidases cleaves cellobiose into two glucose molecules. Thus, a
347 second way we answered the question of whether cellobiose or a catabolic derivative of
348 cellobiose acted as a signaling molecule was to ask whether low glucose concentrations would
349 activate expression of Cbr1-regulated genes. To mitigate changes in glucose concentration due
350 to metabolism, we used the non-metabolizable glucose analog 2-deoxyglucose. We exposed
351 wild type cells to 10-fold dilutions of 2-deoxyglucose (100 μM to 0.1 μM), 1% 2-deoxyglucose,
352 1% cellobiose, or carbon starvation for 1 h and 4 h and measured transcription of *BGL1* using
353 RT-qPCR. As expected, cells exposed to cellobiose expressed *BGL1* at high levels and this

354 expression was repressed during exposure to 1% 2-deoxyglucose (Fig 4D). Exposure to 1 μM
355 2-deoxyglucose resulted in a two to three-fold increase in *BGL1* expression relative to carbon
356 starvation at both timepoints (Fig 4D). While this *BGL1* activation did not rise to the same
357 magnitude as exposure to cellobiose itself, perhaps due to differences in the precise glucose
358 concentration, the rate at which the glucose enters the cell, or a lack of resources due to
359 starvation when 2-deoxyglucose is the sole carbon source, activation of *BGL1* expression in 1
360 μM 2-deoxyglucose suggests a low glucose concentration acts as a signal to activate β-
361 glucosidase expression.
362
363 *A Cbr1-regulated transporter gene is necessary for citrate utilization*
364 Two genes encoding major facilitator superfamily transporters (RTO4_13825 and
365 RTO4_10339) and a gene encoding a predicted phosphoglycerate mutase (RTO4_11229) were
366 also members of the *CBR1* core regulon. We hypothesized that these genes may be necessary
367 for growth on TCA cycle intermediates and/or fucose. To test this hypothesis, we grew
368 RTO4_13825Δ, RTO4_10339Δ, and RTO4_11229Δ cells on media containing glucose, fucose,
369 the tricarboxylic acid citrate, or the dicarboxylic acids succinate, α-ketoglutarate, malate, or
370 fumarate as the sole carbon source. The growth of cells lacking RTO4_10339 or RTO4_11229
371 was indistinguishable from that of wild type cells under all conditions tested (Fig S9). In contrast,
372 cells lacking RTO4_13825 were unable to grow on citrate but grew as well as wild type cells on
373 all other carbon sources tested (Fig 5A and S9A). Thus, we named RTO4_13825 *TCT1* for
374 tricarboxylic acid transporter.
375 To gain further insight into the roles of *TCT1* and *CBR1* in citrate utilization we
376 conducted RNAseq on wild type, *cbr1*Δ, and *tct1*Δ exponentially growing cells exposed to citrate
377 as the sole carbon source for 24 h. Only 20 genes were at least four-fold differentially expressed
378 in *cbr1*Δ relative to wild type cells (Fig 5B and Dataset S5-S6). Surprisingly, these 20 genes did

379 not include *TCT1* or genes predicted to play a role in citrate metabolism. Of the other genes in
380 the *CBR1* core regulon, *BGL1* and *BGL2* were decreased in expression by 6.7 and 3.2-fold,
381 respectively, in *cbr1*Δ relative to wild type cells, but RTO4_10339 and RTO4_11229 were not
382 differentially expressed in this comparison (Fig 5B and Dataset S5-S6). Only one gene,
383 RTO4_15140, had decreased expression in *tct1*Δ cells relative to wild type cells. The expression
384 of RTO4_15140 was also decreased in *cbr1*Δ relative to wild type cells (Fig 5B and Dataset S5-
385 S6). RTO4_15140 is predicted to encode a P-type ATPase transduction domain A containing
386 protein with a single transmembrane domain, and its role in citrate utilization, if any, is unclear.
387 The similarity of the transcriptional profiles of *tct1*Δ and wild type cells may suggest that *tct1*Δ
388 cells are able to sense citrate, despite not being able to grow.
389
390 *Cbr1 inhibits carbon catabolite repression during glucose-glucose disaccharide utilization*
391 Our data show that *R. toruloides* β-glucosidases are secreted into the media where they
392 cleave the cellobiose β-1,4-glycosidic bond, releasing two glucose molecules (Fig 3C). Glucose
393 is a highly preferred carbon source in most fungi. Thus, we hypothesized glucose import would
394 repress genes necessary for cellobiose utilization via carbon catabolite repression (1, 14, 15).
395 *BGL1* expression is almost undetectable when wild type cells are exposed to 1% glucose or at
396 least 100 μM 2-deoxyglucose (Fig 3D and 4D). If *BGL1* expression is repressed by higher levels
397 of glucose, we hypothesized it would be possible to inhibit growth of wild type cells on cellobiose
398 when the non-hydrolyzable glucose analog 2-deoxyglucose was added. Indeed, addition of 61
399 μM 2-deoxyglucose to 1% cellobiose fully inhibited *R. toruloides* growth (Fig 6A). Thus, glucose
400 is preferred over cellobiose by *R. toruloides*.
401 This relative carbon preference represents a biological challenge during utilization of
402 substrates in which glucose is released during degradation; a negative feedback loop could
403 repress genes necessary to utilize the substrate in response to the released glucose, slowing

404 growth on the carbon source. We hypothesized that *CBR1* may play a role in inhibiting carbon
405 catabolite repression to bypass this negative feedback loop. To test this hypothesis, we grew
406 wild type, *cbr1*Δ, and *cbr1*Δ + *CBR1* cells in media with a nonpreferred carbon source and 2-
407 deoxyglucose. Cells lacking *CBR1* were significantly more sensitive to 2-deoxyglucose than wild
408 type or *cbr1*Δ + *CBR1* cells when the glucose-glucose disaccharides maltose or trehalose were
409 the carbon source, suggesting Cbr1 was acting as a negative regulator of carbon catabolite
410 repression (Fig 6B).

411 Canonically, regulators of carbon catabolite repression act to regulate a broad range of
412 nonpreferred carbon source utilization genes (1, 14-21). We hypothesized *CBR1* would play a
413 similar role in *R. toruloides*. However, the growth of *cbr1*Δ cells exposed to 2-deoxyglucose in
414 combination with the glucose-fructose disaccharide sucrose, the hexose monosaccharide
415 fructose, the pentose monosaccharide xylose, the amino acid proline, or the lipid mimic Tween-
416 80 was indistinguishable from wild type cells (Fig 6C-6E). To measure the role of *CBR1* in
417 regulating genes involved in alcohol utilization, we used allyl alcohol, which is converted to the
418 toxic molecule acrolein by alcohol dehydrogenases. The expression of these alcohol
419 dehydrogenases is repressed when glucose is present (34). Wild type and *cbr1*Δ cells were
420 equally sensitive to allyl alcohol when glucose was present, indicating that glucose-mediated
421 repression of alcohol dehydrogenases is also not dependent on Cbr1 (Fig 6F). To determine
422 whether any of the genes in the *CBR1* core regulon play a role in carbon catabolite repression,
423 we tested the 2-deoxyglucose sensitivity of *bgl1*Δ *bgl2*Δ, *tct1*Δ, RTO4_10339Δ, and
424 RTO4_11229Δ cells. The sensitivity to 2-deoxyglucose of these strains during exposure to
425 trehalose, maltose, sucrose, proline, xylose, fructose, and tween-80 was indistinguishable from
426 wild type cells (Fig S10). These data indicate that Cbr1 inhibits carbon catabolite repression of
427 glucose-glucose disaccharides either by directly regulating gene expression or through genes
428 outside of the *CBR1* core regulon.

429 A role for Cbr1 Ascomycete homologs in carbon catabolite repression regulation has not
430 been identified, so we asked whether the *N. crassa* Cbr1 homolog CLR-2 plays a similar role.
431 The sensitivity of wild type and Δ*clr-2* cells to 2-deoxyglucose was indistinguishable during
432 growth on the glucose-glucose disaccharide trehalose or the monosaccharide xylose (Fig S11).
433 These data indicate the role for Cbr1 in *R. toruloides* in inhibiting carbon catabolite repression of
434 glucose-glucose disaccharides is an expanded role for Cbr1 relative to the model Ascomycete
435 ortholog CLR-2.
436
437 **DISCUSSION**
438 The regulation of gene expression in response to nutrient availability requires cells to
439 integrate information from activating and repressing environmental nutrient signals. There have
440 been limited investigations of the genetic regulators of cellulose and cellobiose utilization in
441 Basidiomycetes. A recent study identified Roc1 as a regulator of cellulase-encoding genes in the
442 basidiomycete wood-decaying fungus *Schizophyllum commune* (35). However, homologs of
443 Roc1 do not exist outside of the Agaricomycetes (35). In *Ganoderma lucidum*, the R2R3-type
444 MYB transcription factor *Gl*Myb activates cellulase gene expression (36). In Ascomycete
445 filamentous fungi, CLR-2/ClrB regulates cellulose degradation and utilization through the direct
446 activation of genes encoding cellulases (24, 37). We identified the CLR-2/ClrB ortholog Cbr1 in
447 the oleaginous, basidiomycete yeast *R. toruloides* (Fig 1). Although Ascomycete and
448 Basidiomycete fungi are separated by approximately 400 million years of evolution (38), our
449 study shows that Cbr1 has a role in utilization of the cellulose breakdown product cellobiose (Fig
450 1). *N. crassa* CLR-2 does not directly regulate the expression of genes encoding β-
451 glucosidases, which are regulated by CLR-1 (NCU07705) in *N. crassa* (24, 37). However, a role
452 for Cbr1 in cellobiose utilization suggests that there is conservation in the role of CLR-
453 2/ClrB/Cbr1 between Ascomycete filamentous fungi and Basidiomycete yeast.

454 Our characterization of Cbr1 showed that Cbr1 regulates utilization of citrate through the
455 regulation of the predicted transporter gene *TCT1* and regulates cellobiose utilization through
456 the regulation of the β-glucosidase genes *BGL1* and *BGL2* (Fig 7). Cbr1 is also necessary for
457 wild type levels of growth on fucose and the dicarboxylic acid TCA cycle intermediates, but the
458 genetic mechanisms through which Cbr1 regulates utilization of these carbon sources remains
459 unclear. It is possible that the two uncharacterized genes in the *CBR1* core regulation, the
460 predicted transporter RTO4_10339 and the predicted phosphoglycerate mutase RTO4_11229,
461 play roles in fucose and/or dicarboxylic acid utilization, but that their action is redundant with
462 other genes in the *R. toruloides* genome. Utilization of these substrates could also, at least
463 partially, occur through a mechanism outside of the *CBR1* core regulon, potentially including
464 genes regulated by *CBR1* that did not meet our strict criteria to be part of the *CBR1* core
465 regulon (Fig 2 and S4). It will be the role of future studies to characterize these genetic
466 mechanisms and roles for RTO4_10339 and RTO4_11229 in the *CBR1* pathway.
467 In other fungal species, genes necessary for utilization of multiple carbon sources are
468 also sometimes regulated by a single transcription factor. For example, CLR-2 in *N. crassa*
469 regulates cellulose utilization, mannan utilization, and a small number of genes involved in
470 hemicellulose utilization (14, 24, 37, 39). In *Trichoderma reesei*, *Penicillium oxalicum*, and some
471 Aspergilli, the transcription factor Xyr1/XlnR regulates utilization of both cellulose and
472 hemicellulose (25-27). ARA-1/Ara1 regulates both arabinan and galactose utilization in *N.*
473 *crassa* and *T. reesei* (14, 40), and FarA regulates utilization of both fatty acids and the lignin
474 component ferulic acid (41, 42). In *Candida albicans*, Adr1 is required for growth on citrate and
475 substrates that feed into the TCA cycle, like glutamate and malate (43). However, in these
476 examples linkage of these carbon sources can easily be explained either by the presence of
477 both carbon sources in the plant cell wall (i.e, cellulose and hemicellulose or arabinan and
478 galactose) or catabolism through linked metabolic pathways (i.e., fatty acids and ferulic acid or
479 citrate, glutamate, and malate).

480 Cellobiose, fucose, and TCA cycle intermediates are not linked by any known metabolic
481 pathways, and our data do not support the hypothesis that they are catabolized through the
482 same noncanonical metabolic pathway in *R. toruloides*. The Cbr1-regulated β-glucosidase
483 genes are critical for cellobiose utilization but not fucose or TCA cycle intermediate utilization
484 (Fig 3 and S6A), while Tct1 is critical for citrate utilization but not cellobiose or fucose utilization
485 (Fig 5A and Fig S9A). Aside from *CBR1* itself, we did not identify any genes that were necessary
486 for utilization of all the carbon sources regulated by *CBR1*: cellobiose, fucose, and TCA cycle
487 intermediates. Thus, we speculate the coregulation of cellobiose, fucose, and TCA cycle
488 intermediates may suggest that in nature *R. toruloides* frequently encounters cellobiose, fucose,
489 and TCA cycle intermediates in close proximity. One possible explanation for this is that *R.*
490 *toruloides* scavenges cellobiose and fucose during plant cell wall degradation by filamentous
491 fungi, which also secrete organic acids, such as citrate or succinate, to acidify their environment
492 and may contain fucose in their cell walls (44-47). Activation of β-glucosidase genes prior to
493 encountering cellobiose released during cellulose degradation via a response to organic acids
494 secreted by filamentous fungi or fucose present in fungal cell walls could confer a growth
495 advantage to *R. toruloides* over microbes that only activate β-glucosidase genes in response to
496 cellobiose itself. Indeed, transferring a large population of cells constitutively expressing *BGL1*
497 from glucose to cellobiose that do not require time to activate β-glucosidase genes resulted in a
498 reduced lag phase compared to wild type cells that must activate β-glucosidase genes in
499 response to cellobiose (Fig S6D).
500 Carbon catabolite repression is the genetic mechanism through which fungal cells
501 prioritize utilization of carbon sources that require less energy to metabolize over carbon
502 sources that require more energy to metabolize (1). Known genetic mechanisms of regulating
503 carbon catabolite repression regulate the expression of genes required for utilization of all less
504 preferred carbon sources when a more preferred carbon source is present (14-21). Cbr1

505 demonstrates a regulatory mechanism through which cells inhibit carbon catabolite repression
506 specifically during utilization of glucose-glucose disaccharides (Fig 6). We speculate that this
507 mechanism may allow cells to continue expressing genes necessary for hydrolyzing
508 disaccharide sugar bonds even when this hydrolysis produces preferred monosaccharides like
509 glucose (Fig 7). Genetic linkage of carbon catabolite repression inhibition with the activation of
510 genes necessary to utilize a nonpreferred carbon source has not previously been described in
511 fungi. Additionally, regulation of genes involved in utilization of a specific class of nonpreferred
512 carbon sources in response to a preferred carbon source represents a novel form of carbon
513 catabolite repression regulation. A more complete understanding of mechanisms of carbon
514 catabolite repression in fungi is important for improving rational engineering of fungi for
515 industrial fermentation of plant-derived substrates. Additionally, fungi that do not regulate carbon
516 catabolite repression like wild type cells have reduced virulence (19, 48-50), so understanding
517 diverse mechanisms of carbon catabolite repression present throughout the fungal kingdom is
518 critical in controlling fungal disease.
519

520 **MATERIALS and METHODS**

521 *Strains and culturing*

522 *R. toruloides* strains used in this study are listed in Table S1. All strains were derived
523 from the wild type reference strain IFO 0880 (also called NBRC 0880, obtained from Biological
524 Resource Center, NITE (NBRC), Japan). The starting strain for genetic manipulations was the
525 non-homologous end-joining deficient *yku70*Δ strain (51). *R. toruloides* was transformed using
526 *Agrobacterium tumefaciens*-mediated transformation as previously described (11), using *A.*
527 *tumefaciens* EHA 105 and plasmids derived from pGI2 (52). All strains were confirmed by PCR
528 and/or DNA sequencing of transformed loci. For details see SI Materials and Methods. *R.*
529 *toruloides* strains were grown from freezer stocks on YPD (VWR) + 2% agar (BD Difco™

530 Bacto™) plates at 28°C for 2 d to 3 d prior to inoculation into liquid YPD to grow strains at 28°C
531 with constant shaking at 250 rpm to exponential phase growth prior to starting experiments.
532 *N. crassa* strains used in this study are listed in Table S2. These strains were derived
533 from the wild type reference strain FGSC 2489 and obtained from the Fungal Genetics Stock
534 Center (53, 54). *N. crassa* cells were grown from freezer stocks on Vogel's minimal medium (55)
535 + 2% sucrose + 1.5% agar (BD Difco™ Bacto™) slants for 2 d at 28°C in the dark and 4 d at
536 28°C in constant light. *N. crassa* conidia were harvested from slants with sterile double distilled
537 H_2O for inoculation.
538 All *R. toruloides* experiments were performed with yeast nitrogen base (YNB) with
539 NH_4SO_4 without amino acids (MP Biomedicals) + 12 mM K_2HPO_4 (Fisher) + 38 mM KH_2PO_4
540 (Fisher) + 100 nM Fe(II)SO_4 pH 6.2 with the indicated carbon source at 1% w/v with the
541 exception of coumaric acid and ferulic acid, which were added at a concentration of 0.2% w/v,
542 and citrate which was added at a concentration of 8.5 mM. All *N. crassa* experiments were
543 performed using Vogel's minimal medium (55) with 50 mM NH_4Cl instead of 25 mM NH_4NO_3
544 (VMM NH_4Cl) and the indicated carbon source at 1% w/v. Carbon sources used are listed in
545 Table S3.
546
547 *R. toruloides and N. crassa growth experiments*
548 *R. toruloides* colonies were inoculated into liquid YPD and grown at 28°C with constant
549 shaking at 250 rpm to exponential phase. Cells were then washed and inoculated into 750 μL of
550 the indicated media in 48-well plates at 0.01 OD600/mL or 0.1 OD600/mL, except for the growth
551 curve in Fig S6D, which was inoculated at 0.5 OD600/mL as measured by an Eppendorf
552 BioPhotometer with a 10 mm path length. Plates were incubated at 28°C with constant shaking
553 (3 mm orbit, 600 rpm for individual time points and 3 mm orbit, 425 rpm for kinetic growth
554 experiments). Growth was measured as OD600 on an Agilent BioTek Synergy HTX for

555 individual time points or an Agilent BioTek Epoch2 for kinetic growth experiments. For details
556 see SI Materials and Methods.
557 *N. crassa* conidia were inoculated into either 100 mL of liquid VMM NH_4Cl with the
558 indicated carbon source in 250 mL flasks or 3 mL of liquid VMM NH_4Cl with the indicated carbon
559 source in round-bottomed, deep-well 24-well plates. After 24 h (flasks) or 7-10 d (24-well plates)
560 biomass was harvested, dried for 24 h in a 60°C drying oven, and weighed. For details see SI
561 Materials and Methods.
562
563 *Phylogenetic tree generation*
564 The Basic Local Alignment Search Tool for proteins (BLASTP) was used to identify
565 homologs of CLR-1, CLR-2, and XLR-1 from *N. crassa* (OR74A) and ClrA, ClrB, and XlnR from
566 *Aspergillus nidulans* (A4) in the proteomes of *N. crassa*, *A. nidulans*, *S. cerevisiae* (S288C), and
567 *R. toruloides* (IFO 0880). CLR-1, CLR-2, and XLR-1 from *N. crassa* (OR74A) and ClrA, ClrB,
568 and XlnR from *A. nidulans* (A4) protein sequences were obtained from Mycocosm (56).
569 Homologs with $E < 10^{-10}$ were aligned via MUSCLE (57) in MEGA11 (58) and the phylogenetic
570 tree was generated using FastTree (59) using the maximum likelihood method based on the
571 Jones-Taylor-Thornton matrix-based model and visualized in MEGA11. The reliability of each
572 split in the tree was computed using the Shimodaira-Hasegawa test on three alternate
573 topologies around that split. Trees were visualized in MEGA11 (58) and rooted on the midpoint.
574
575 *Microscopy*
576 Fluorescent microscopy was performed using a Nikon Eclipse Ti2-E inverted microscope
577 equipped with a prime BSI express CMOS camera using a CFI Plan Apochromat 100X oil
578 objective. For details see SI Materials and Methods.
579
580 *RNA sequencing and transcript abundance*

581 Flasks containing 100 mL of YNB without a carbon source or YNB with the indicated
582 carbon source were inoculated with the indicated exponentially growing cells at 0.1 OD600/mL
583 (measured by an Eppendorf BioPhotometer). Culture conditions and sequencing methods for
584 each dataset are summarized in Table S4. Cells were harvested and flash frozen in liquid N_2.
585 RNA was extracted using the Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo), followed by
586 either treatment with the TURBO DNA-free kit (Invitrogen) for standard RNAseq or DNase I
587 treatment (NEB) and purification with the Monarch® Spin RNA Cleanup Kit (NEB) for 3'RNAseq.
588 Standard RNAseq was performed at the California Institute for Quantitative Biosciences at the
589 University of California Berkeley (QB3-Berkeley) on an Illumina NextSeq 2000 with 150 bp
590 paired end reads. 3'RNAseq was performed at the Biotechnology Resource Center at Cornell
591 University on an Illumina NovaSeqX with 150 bp paired end reads.
592 Reads were aligned to the *R. toruloides* IFO 0880 genome (v4) (11). For standard
593 RNAseq, transcript abundance (transcripts per million, TPM) was quantified using Salmon v.
594 1.10.0 (60). For 3'RNAseq, reads were aligned using STAR v. 2.7.11b (61), counted with HTSeq
595 v. 2.0.9 (62), and counts per million (CPM) values were calculated by dividing counts for a gene
596 by total counts for the sample and multiplying by 10^6. The GFF file used to analyze 3'RNAseq
597 data is available in Dataset S7. Differential expression was determined using DESeq2 v. 1.50.1
598 (63). RNAseq data were deposited in the Gene Expression Omnibus (GEO) at the National
599 Center for Biotechnology Information (NCBI) and are accessible through GEO series accession
600 numbers GSE293943 (64) (standard RNAseq) and GSE313844 (65) (3'RNAseq). For details
601 see SI Materials and Methods.
602
603 *Functional enrichment analysis*
604 KEGG and GO enrichment analyses were performed using the enricher function of the
605 clusterProfiler R package v. 4.18.1 with Benjamini-Hochberg multiple hypothesis testing
606 correction (66). For details see SI Materials and Methods.

607

608 *Reverse transcription quantitative PCR*

609 To measure *BGL1* expression, wild type cells were exposed to the indicated carbon
610 source in 100 mL of the indicated media in 250 mL flasks and incubated at 28ºC with shaking at
611 200 rpm for the indicated length of time. Cells were harvested, flash frozen in liquid N_2, and RNA
612 was purified as described above. RT-qPCR was performed using the Luna® Universal One-Step
613 RT-qPCR Kit (NEB) on a CFX96™ Real-Time System (Bio-Rad). For details see SI Materials
614 and Methods.

615

616 *β-glucosidase enzyme activity assays*

617 β-glucosidase activity was measured using p-nitrophenyl-β-D-glucopyranoside (EMD
618 Millipore Corp.). For details see SI Materials and Methods.

619

620 *Spent media growth assays*

621 Filtered supernatants of wild type cells growing in YNB 1% glucose, YNB 1% cellobiose,
622 or YNB 1% succinate were added to YNB lacking a carbon source or YNB 1% cellobiose at the
623 indicated concentration. The indicated strains were inoculated into this media at 0.01 OD600/mL
624 (measured by an Eppendorf BioPhotometer) in 750 μL in 48-well plates and incubated at 28ºC
625 with shaking at 600 rpm (3 mm orbit). For details see SI Materials and Methods.

626

627 *Statistical significance tests*

628 All statistical analyses were performed in Rstudio (v2025.09.2+418; R v4.5.2) using
629 either a one-way or two-way ANOVA with a Tukey post-hoc test (bar graphs), a Welch's two-
630 sample *t*-test (bar graphs), or a general linear model with post-hoc analysis using the emmeans
631 function of the emmeans R package (67) with a holm multiple comparison correction (line

632 graphs). Statistical significance for differential expression of RNAseq data was determined using
633 DESeq2 (63). All experiments were performed with a minimum of three biological replicates.
634
635 *Data availability*
636 Raw RNAseq data is available in GEO at the NCBI through accession numbers
637 GSE293943 (64) (standard RNAseq) and GSE313844 (65) (3'RNAseq). Processed RNAseq
638 data is available in Datasets S1-S6. The numerical values used to generate all other graphs are
639 in Dataset S9. Strains constructed in this study are available upon request.
640
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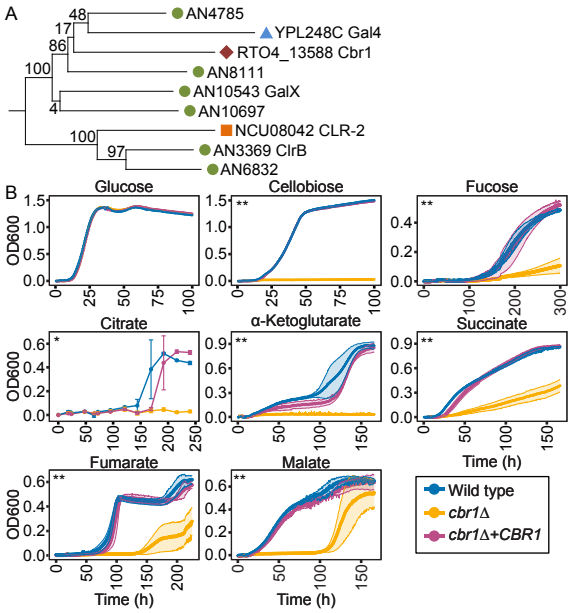
812 **FIGURES**



813

814 **Fig 1. *CBR1* (RTO4_13588) is involved in utilization of cellobiose, TCA cycle**

815 **intermediates, and fucose. (A)** Protein sequences of *N. crassa* CLR-2 and *Aspergillus*

816 *nidulans* ClrB homologs were used to build a phylogenetic tree using the maximum likelihood

817 method based on the Jones-Taylor-Thornton matrix-based model using FastTree (59). Numbers

818 at nodes indicate the reliability of each split in the tree as computed using the Shimodaira-

819 Hasegawa test on three alternate topologies around that split with 1,000 resamples. *R.*

820 *toruloides* proteins are indicated with maroon diamonds. *N. crassa* proteins are indicated with

821 orange squares. *A. nidulans* proteins are indicated with green circles. *S. cerevisiae* proteins are

822 indicated with blue triangles. **(B)** Growth curves (OD600) of the indicated strains grown in YNB

823 with the indicated carbon source at 1% except for citrate which was at 8.5 mM. Lines are the

824 average and bars or colored bands are the standard deviation of three biological replicates. *p_{adj}

825 $< 10^{-4}$ and **$p_{adj} < 10^{-10}$ for *cbr1*Δ vs wild type (WT) and *cbr1*Δ vs *cbr1*Δ + *CBR1* as determined

826 by pairwise comparisons of estimated marginal means with a holm multiple comparison

827 correction.

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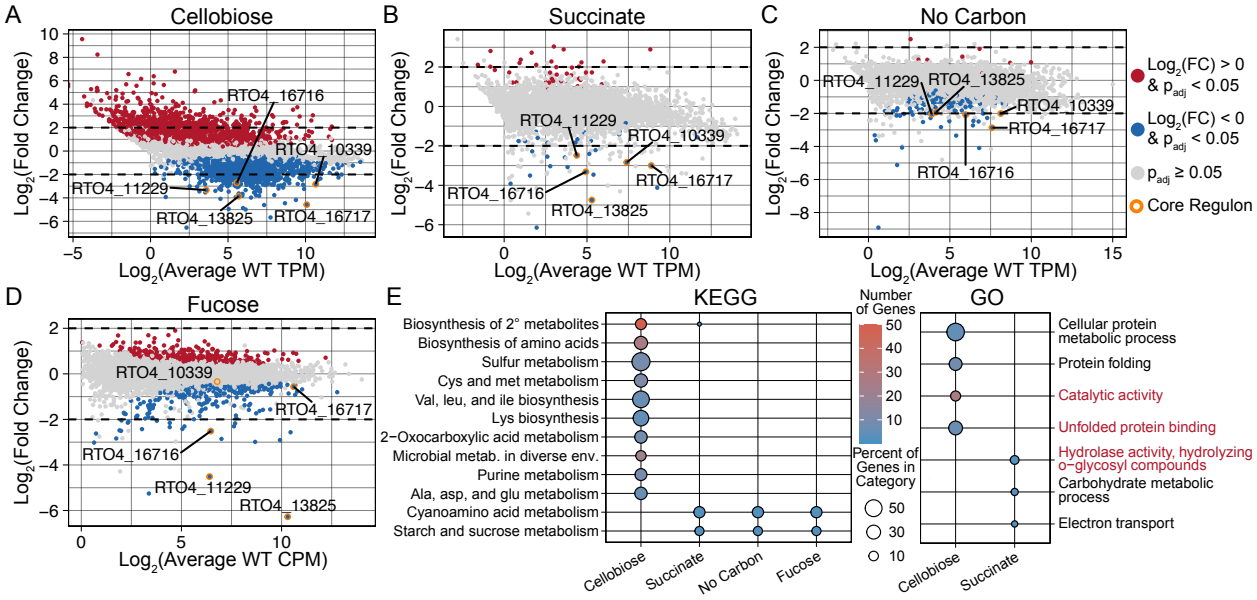
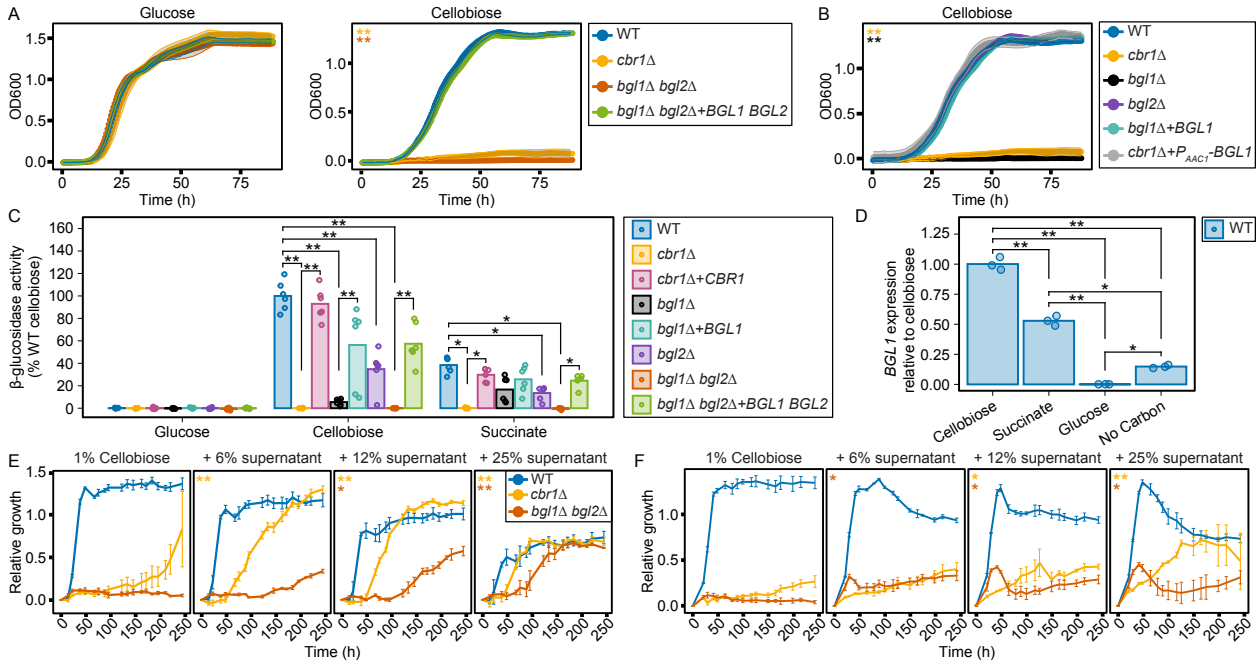


Fig 2. Cbr1 regulates a core regulon of five genes. (**A-D**) Scatterplots of the $\log_2$(average expression) of all genes in wild type (WT) cells versus the $\log_2$(fold change) in gene expression in *cbr1*Δ relative to wild type cells when cells were exposed to (**A**) cellobiose, (**B**) succinate, (**C**) media lacking a carbon source, or (**D**) fucose. Genes with $p_{adj} < 0.05$ are indicated in red or blue if they had higher or lower expression in *cbr1*Δ cells than wild type cells, respectively. Genes differentially expressed by a least four-fold in at least three of the four conditions (*CBR1* core regulon) are circled in orange and labeled with their protein IDs. Genes in grey were not significantly differentially expressed in *cbr1*Δ relative to wild type cells. Black dotted lines indicate four-fold differential expression. Standard RNAseq (**A-C**) was used to determine gene expression in transcripts per million (TPM) and fold change during exposure to cellobiose, succinate, and carbon starvation. 3'RNAseq (**D**) was used to determine gene expression in counts per million (CPM) and fold change during exposure to fucose. (**E**) Genes expressed at least four-fold lower in *cbr1*Δ cells for each carbon source were tested for Kyoto Encyclopedia of Genes and Genomes (KEGG) (29) (left) and gene ontology (GO) (30, 31) (right) functional category enrichment. Comparisons were only displayed if they had at least one statistically significantly enriched category for each analysis. Dot color represents the number of genes

846 differentially expressed belonging to a given category. Dot size represents the percent of genes
847 belonging to a given category that were differentially expressed relative to all genes in that
848 category. The text of GO biological process and molecular function categories are colored black
849 and red, respectively. The expression of *CBR1* was left out of the scatterplots, KEGG, and GO
850 enrichment analyses, since the change in expression of this gene was due to its deletion in the
851 *cbr1*Δ strain.
852



853

854 **Fig 3. Cbr1-dependent expression of secreted β-glucosidase genes *BGL1* (RTO4_16717)**
855 **and *BGL2* (RTO4_16716) is necessary for cellobiose utilization.** (**A-B**) Growth (OD600) of
856 the indicated strains in the indicated carbon sources. (**C**) β-glucosidase activity secreted by the
857 indicated strains into culture supernatants after 20 h growth on the indicated carbon sources. β-
858 glucosidase activity is normalized to the β-glucosidase activity of wild type (WT) cells grown on
859 cellobiose. Corresponding growth (OD600) at time of supernatant harvest is in Fig S6B. (**D**)
860 *BGL1* expression of wild type cells exposed to the indicated carbon source for 4 h relative to
861 *BGL1* expression during exposure to cellobiose for 4 h. (**E-F**) Normalized growth (OD600) of the
862 indicated strains in 1% cellobiose supplemented with the indicated percentage of spent
863 supernatant from wild type cells grown in (**E**) 1% cellobiose, or (**F**) 1% succinate. To account for
864 growth due to nutrients remaining in the supplemented supernatant, growth was normalized by
865 subtracting the growth of each replicate on media lacking a carbon source supplemented with
866 the same concentration of spent supernatant. Growth curves without normalization, the growth
867 of cells in media without a carbon source supplemented with spent supernatants, and the β-
868 glucosidase activity present in the spent supernatants are in Fig S7. Lines are the average and

869 bars or colored bands are the standard deviation of at least three biological replicates in **A**, **B**, **E**,
870 and **F**. Bars are the average and dots are the individual data points of at least three biological
871 replicates in **C** and **D**. In **A** and **B**, colored stars indicate a significant difference in growth of the
872 respectively colored strain compared to wild type cells and, when applicable, the respective
873 complement strain. In **E** and **F**, colored stars indicate a significant difference in growth of the
874 respectively colored strain compared to growth of that strain with no supplemented supernatant.
875 *$p_{adj} < 0.05$ and **$p_{adj} < 10^{-7}$ as determined by either (**A**, **B**, **E**, and **F**) pairwise comparisons of
876 estimated marginal means with a holm multiple comparison correction, (**C**) a two-way ANOVA
877 with a TukeyHSD post-hoc test, or (**D**) a one-way ANOVA with a TukeyHSD post-hoc test.

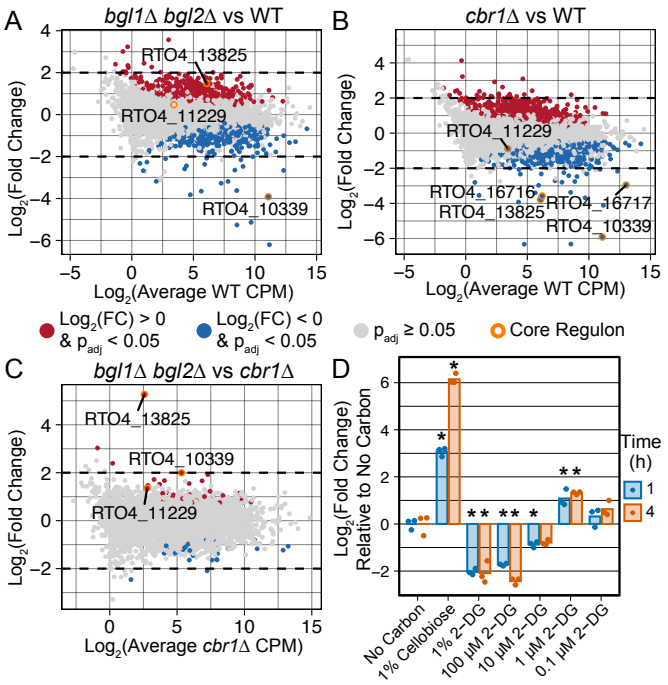


Fig 4. *CBR1*-mediated transcriptional response to cellobiose is dependent on secreted β-glucosidases. (**A-C**) Scatterplots of the $\log_2$(average expression) of all genes in (**A-B**) wild type (WT) or (**C**) *cbr1*Δ cells versus the $\log_2$(fold change) in gene expression for the indicated comparison when cells were exposed to cellobiose as measured by 3'RNAseq. Genes with p_{adj} < 0.05 are indicated in red or blue if they had higher or lower expression, respectively, in (**A** and **C**) *bgl1*Δ *bgl2*Δ cells or (**B**) *cbr1*Δ cells. The genes in the *CBR1* core regulon are circled in orange and labeled with their respective protein IDs. The expression of *CBR1*, *BGL1,* and *BGL2* were left out of the scatterplots involving their respective deletion strains. (**D**) *BGL1* expression in wild type cells exposed to carbon starvation for 1 h followed by exposure to the indicated concentrations of cellobiose or 2-deoxyglucose (2-DG) as the sole carbon source for 1 h or 4 h. Expression values were normalized to the average expression of *BGL1* on no carbon for each timepoint and $\log_2$ transformed. Bars are the average and dots are the values of three biological replicates. *p_{adj} < 0.05 relative to the no carbon condition for the respective time point, as determined by two-way ANOVA with a TukeyHSD post-hoc test.

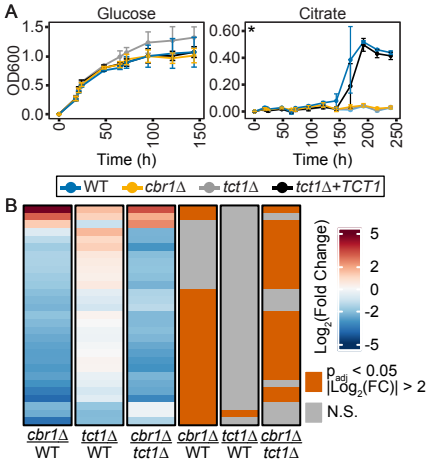


Fig 5. *TCT1* (RTO4_13825) is required for citrate utilization. (**A**) Growth (OD600) of the indicated strains in YNB 1% glucose or 8.5 mM citrate. Lines are the average and bars are the standard deviation of at least three biological replicates. *$p_{adj} < 10^{-7}$ of *cbr1*Δ vs wild type (WT), *tct1*Δ vs WT, and *tct1*Δ vs *tct1*Δ + *TCT1* as determined by pairwise comparisons of estimated marginal means with a holm multiple comparison correction. (**B**) Heatmap of the log_2(fold change) in the expression of genes at least four-fold differentially expressed in one or more of the indicated comparisons when cells were exposed to citrate as the sole carbon source, as measured by 3'RNAseq. Orange bars to the right of the heatmap indicate significance using the criteria of $p_{adj} < 0.05$ and at least four-fold change in expression, while grey bars indicate genes that do not meet those criteria in the indicated comparison (N.S., not significant). The expression of *CBR1* and *TCT1* were left out of the heatmaps since changes in expression were due to their respective deletions in the *cbr1*Δ and *tct1*Δ strains.

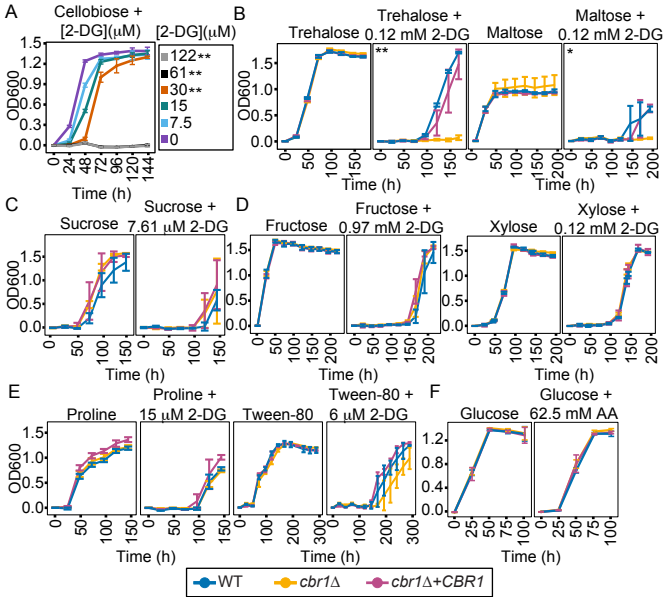


Fig 6. Cbr1 inhibits carbon catabolite repression during glucose-glucose disaccharide utilization. (**A**) Growth (OD600) of wild type (WT) cells in 1% cellobiose supplemented with the indicated concentration of 2-deoxyglucose (2-DG). (**B-F**) Growth of the indicated strains on the indicated carbon sources with the indicated concentration of 2-DG or allyl alcohol (AA). Lines are the average and bars are the standard deviation of at least three biological replicates. *p_{adj} < 0.01 and **p_{adj} < 10^{-4} for (**A**) 1% cellobiose supplemented with the indicated 2-DG concentration vs 1% cellobiose or (**B-F**) *cbr1*Δ vs WT and *cbr1*Δ vs *cbr1*Δ + *CBR1* as determined by pairwise comparisons of estimated marginal means with a holm multiple comparison correction.

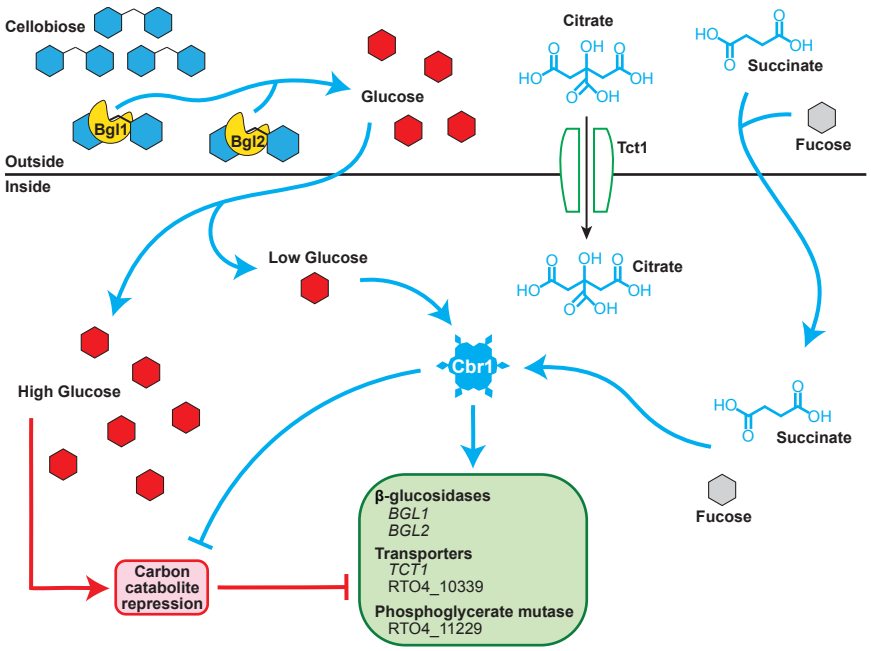


Fig 7. Model for Cbr1-mediated regulation of cellobiose, TCA cycle intermediate, and fucose utilization and carbon catabolite repression. Cbr1 is activated by the presence of a low glucose concentration, fucose, and TCA cycle intermediates or their metabolic derivatives. Activated Cbr1 induces the expression of genes encoding β-glucosidases, predicted transporters, and a predicted phosphoglycerate mutase. The Cbr1-regulated predicted transporter gene *TCT1* is required for citrate utilization. Tct1 is placed at the plasma membrane in this model, but the localization of Tct1 is currently unknown and should be the subject of future studies. Transporters for other TCA cycle intermediates and fucose are unknown. The β-glucosidase genes *BGL1* and *BGL2* encode secreted β-glucosidases that cleave cellobiose into two glucose molecules. *BGL1* is required for growth on cellobiose. A high glucose concentration activates carbon catabolite repression, which represses β-glucosidase gene expression and likely represses the expression of genes necessary to utilize fucose and TCA cycle intermediates. Cbr1 plays a role in inhibiting carbon catabolite repression, specifically when a glucose-glucose disaccharide is present as the nonpreferred carbon source.