Transcription factor reveals interconnected regulation of carbon source utilization and carbon catabolite repression in an oleaginous yeast

Brandon Reyes-Chavez[1,2,*], Joshua D. Kerkaert[1,*], and Lori B. Huberman[1,#]

[1]Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853

[2]Microbiology Department, Cornell University, Ithaca, NY 14853

*These authors contributed equally to this work.

[#]To whom correspondence may be addressed: huberman@cornell.edu

ABSTRACT

Cells must sense and respond to available nutrients to utilize resources and establish fungal colonies. Saprophytic microbes harvest carbon from plant biomass, which contains insoluble, complex carbohydrates that must be degraded extracellularly prior to import into the cell. Filamentous fungi can degrade these complex carbohydrates. However, while many single cellular microbes can utilize the building blocks of these insoluble polysaccharides, they frequently are unable to degrade insoluble carbohydrates. Cellobiose is a disaccharide breakdown product of cellulose, the most abundant component of plant biomass. We investigated the genetic regulation of cellobiose utilization in the oleaginous, basidiomycete yeast *Rhodotorula (Rhodosporidium) toruloides* using a combination of phylogenetic, genetic, and genomic approaches. We tested the closest *R. toruloides* homolog of the cellulose degradation regulator CLR-2/ClrB for a role in cellobiose utilization. Deletion of this transcription factor, which we named Cbr1, eliminated the ability of cells to grow on media containing cellobiose as the sole carbon source. Although the role of CLR-2/ClrB is limited to regulating the expression of genes involved in the utilization of cellulose and hemicellulose, Cbr1 plays a role not only in cellobiose utilization, but also utilization of tricarboxylic acid (TCA) cycle intermediates. Transcriptional profiling revealed that the genes activated by Cbr1 included genes encoding β-glucosidases and transporters. These β-glucosidases are secreted and are necessary for cellobiose utilization, while one of the transporters is required for utilization of the TCA cycle intermediate, citrate. Additionally, Cbr1 inhibits carbon catabolite repression specifically during utilization of disaccharides, which may have evolved to limit the repression of genes encoding secreted proteins that cleave disaccharides into glucose extracellularly. The co-regulation of cellobiose and TCA cycle utilization with carbon catabolite repression may shed light on alternative methods of the regulation of carbon catabolite repression in fungi.

38 **INTRODUCTION**

39 Cells must sense and respond to nutrients to successfully grow and divide using the
40 resources available. For fungi, nutrient sensing is critical to establish fungal colonies, compete
41 with other microbes, and establish pathogenic and symbiotic relationships with plants and
42 animals. Saprophytic fungi acquire nutrients from dead plant matter. The carbon sources found
43 in plant biomass range from simple sugars, like sucrose and fructose, to complex
44 carbohydrates, such as cellulose, found in plant cell walls.

45 Utilization of complex polysaccharides requires extracellular degradation to break down
46 the insoluble molecules into soluble building blocks that can be imported into fungal cells. Many
47 yeast species can utilize the soluble building blocks of polysaccharides found in plant biomass
48 (1, 2). These yeast species are excellent choices for growth and engineering in industrial
49 settings because of their uninucleate, single cellular lifestyle and wide carbon utilization
50 repertoire. The basidiomycete, oleaginous yeast *Rhodotorula toruloides* (previously known as
51 *Rhodosporidium toruloides*) can eat the vast majority of soluble breakdown products in
52 lignocellulose (3, 4). *R. toruloides* can also accumulate up to 70% of its biomass as fatty acids in
53 conditions in which carbon is abundant but other nutrients are limiting and produces useful
54 secondary metabolites, such as carotenoids (5–11). This makes *R. toruloides* an excellent
55 choice as a potential industrial fungus for green biotechnology in which products that might
56 otherwise be sourced from petroleum or other environmentally unsustainable sources are
57 produced in microbes fed by agricultural waste or plant matter grown on marginal land (12).

58 Most of the work to characterize the genetic pathways regulating utilization of carbon
59 sources has been done in Ascomycete fungi (1, 13). In these fungi, a network of activating
60 transcription factors activates the genes necessary for utilization of the carbohydrates found in
61 plant biomass (14). Plant biomass contains a wide array of carbon sources. These varied
62 carbon sources have differing levels of preference for the fungal cell. When a more preferred
63 carbon source is available a process known as carbon catabolite repression downregulates the

64 expression of genes required for utilization of any less preferred carbon sources present in the
65 environment (1, 15). In Ascomycete fungi, one of the major regulators of carbon catabolite
66 repression is the transcription factor CRE-1/CreA/Mig1, which broadly represses the expression
67 of genes necessary for utilization of any nonpreferred carbon source in the presence of any
68 more preferred carbon source (15–21). However, the genetic mechanisms through which
69 Basidiomycete fungi activate genes necessary to utilize nonpreferred carbon sources and
70 repress these genes in the presence of more preferred carbon sources is significantly less well
71 studied.

72 We investigated the genetic mechanisms regulating carbon utilization in the
73 basidiomycete yeast *R. toruloides*. Cellulose is the most abundant component of plant biomass
74 and is composed of long chains of glucose linked by β-1,4-glycosidic bonds (22). *R. toruloides*
75 can utilize the disaccharide building block of cellulose, cellobiose (23). We took a phylogenetic
76 approach to identify the regulator of cellobiose utilization in *R. toruloides* and identified a
77 transcription factor homologous to the cellulose degradation regulator CLR-2/ClrB in
78 Ascomycete fungi (24) that is required for utilization of cellobiose in *R. toruloides*, which we
79 named Cbr1.

80 Characterization of Cbr1 revealed that the role of this transcription factor is significantly
81 expanded in *R. toruloides*, where it is also required for utilization of tricarboxylic acid (TCA)
82 cycle intermediates. We used transcriptomic analysis to identify genes regulated by Cbr1. These
83 genes included two genes encoding secreted β-glucosidases and two genes encoding
84 transporters. These β-glucosidase genes are necessary for cellobiose utilization, while one of
85 the transporters is required for utilization of the TCA cycle intermediate citrate. Cellobiose and
86 TCA cycle intermediates are not utilized through similar pathways, suggesting that *R. toruloides*
87 may inhabit an ecological niche in which these carbon sources are frequently found together.
88 Further investigation into the role of Cbr1 demonstrated that this transcription factor inhibits

89 carbon catabolite repression specifically when a disaccharide is present as a nonpreferred
90 carbon source. This role for Cbr1 contrasts with previously identified genetic mechanisms of
91 carbon catabolite repression regulation, which are involved in the regulation of genes
92 responsible for the utilization of any nonpreferred carbon source. Taken together, the varied
93 roles of Cbr1 in *R. toruloides* metabolic regulation presents a novel mechanism of carbon
94 catabolite repression in fungi and may provide insights into the ecological niche of this
95 oleaginous yeast.
96
97 **RESULTS**
98 *Cbr1 is required for wild type utilization of cellobiose and TCA cycle intermediates*
99 Cellulose is the most abundant component of lignocellulose. Although *R. toruloides*
100 cannot degrade cellulose, it is capable of utilizing the simple sugar breakdown product of
101 cellulose, cellobiose (23). Several transcription factors are known to regulate cellulose and
102 cellobiose utilization in filamentous fungi, including CLR-1/ClrA, CLR-2/ClrB, and XlnR (1, 24–
103 27). To investigate the genetic mechanisms regulating *R. toruloides* cellobiose utilization, we
104 searched for transcription factors in the *R. toruloides* genome with homology to these regulators
105 of cellulose and cellobiose utilization. No clear homolog of CLR-1/ClrA or XlnR existed in the *R.*
106 *toruloides* genome (Fig S1). However, we did identify a single homolog of CLR-2/ClrB,
107 RTO4_13588 (Fig 1A and Fig S1).
108 We hypothesized that RTO4_13588 may play a role in regulating cellobiose utilization in
109 *R. toruloides*. To test this hypothesis, we deleted RTO4_13588 and grew the resulting
110 RTO4_13588Δ strain with cellobiose as the sole carbon source. Growth of cells lacking
111 RTO4_13588 with glucose as the sole carbon source was indistinguishable from that of wild
112 type cells (Fig 1B). However, deletion of RTO4_13588 resulted in a complete lack of growth
113 when cellobiose was provided as the sole carbon source (Fig 1B). Expression of RTO4_13588
114 under the native promoter at an exogenous location (the *YKU70* locus) to complement the

115 RTO4_13588 deletion, restored growth of this strain to wild type levels (Fig 1B). The closest
116 homolog to RTO4_13588 in the model yeast *Saccharomyces cerevisiae* is the transcription
117 factor Gal4, which regulates galactose utilization in *S. cerevisiae* (Fig 1A) (28). To test whether
118 RTO4_13588 also plays a role in regulating galactose utilization in *R. toruloides*, we grew wild
119 type cells and the RTO4_13588Δ strain with galactose as the sole carbon source. There was no
120 detectable difference in growth between wild type cells and cells lacking RTO4_13588 (Fig
121 S2A). Thus, we named RTO4_13588, *CBR1* for cellobiose regulator 1.

122 *R. toruloides* is capable of growth on a wide variety of soluble carbon sources (3, 23).
123 We asked whether *CBR1* is also required for growth on any additional carbon sources. The
124 growth of *cbr1*Δ cells was indistinguishable from that of wild type on all the other sugars we
125 tested (arabinose, fructose, galacturonic acid, maltose, sucrose, and xylose) (Fig S2A). *R.*
126 *toruloides* can use amino acids as a carbon source. However, *CBR1* was not required for
127 utilization of glutamate, glycine, or L-alanine (Fig S2B). Cells lacking *CBR1* were also capable of
128 growing as well as wild type cells on media containing acetate and the lipid Tween-80 as the
129 sole carbon source (Fig S2C). Additionally, both wild type and *cbr1*Δ cells could grow on the
130 phenolic breakdown products of lignin, coumaric acid and ferulic acid (Fig S2D).

131 Cells lacking *CBR1* are capable of respiration, as evidenced by their ability to grow with
132 acetate, lipids, and amino acids as the sole carbon source. Thus, we hypothesized that *cbr1*Δ
133 cells would be able to grow with TCA cycle intermediates provided as the sole carbon source. To
134 our surprise, when we grew wild type and *cbr1*Δ cells on media containing citrate, α-
135 ketoglutarate, succinate, fumarate, and malate as the sole carbon source, the *cbr1*Δ cells had
136 significant growth defects relative to wild type (Fig 1B). Complementation of the *cbr1* deletion
137 with *CBR1* expressed under the native promoter at the *YKU70* locus restored growth of the
138 *cbr1*Δ cells to a level indistinguishable from wild type.

139 The role for Cbr1 in TCA cycle intermediate utilization could either be an expanded role
140 for Cbr1 in *R. toruloides* relative to CLR-2/ClrB or a previously uncharacterized role for CLR-
141 2/ClrB. To distinguish between these two possibilities, we tested whether *clr-2* is required for
142 TCA cycle intermediate utilization in the model filamentous fungus *Neurospora crassa*. We
143 directly inoculated *N. crassa* wild type and Δ*clr-2* cells into media containing succinate, α-
144 ketoglutarate, or malate as the sole carbon source. Wild type *N. crassa* was only capable of
145 limited growth with TCA cycle intermediates as the sole carbon source, and deletion of *clr-2*
146 caused no detectable difference in *N. crassa* growth on these carbon sources (Fig S3). This
147 data suggests that the role for *CBR1* in regulating TCA cycle intermediate utilization is an
148 expanded role for this transcription factor, relative to the role of Cbr1 orthologs in Ascomycete
149 filamentous fungi.
150
151 *Cbr1 activates five genes in response to cellobiose and TCA cycle intermediates*
152 We had three possible hypotheses for the genetic mechanism through which Cbr1 could
153 regulate utilization of both cellobiose and TCA cycle intermediates: (1) Cbr1 regulates genes
154 necessary to utilize both cellobiose and TCA cycle intermediates in response to all of these
155 carbon sources, (2) Cbr1 regulates genes necessary to utilize cellobiose in response to
156 cellobiose and genes necessary to utilize TCA cycle intermediates in response to TCA cycle
157 intermediates, and (3) *R. toruloides* utilization of cellobiose and/or TCA cycle intermediates
158 proceeds through atypical metabolic pathways such that they require the same catabolic
159 enzymes. To distinguish between these three hypotheses, we used transcriptional profiling. We
160 grew wild type cells and *cbr1*Δ cells in yeast extract peptone dextrose (YPD) medium overnight
161 so that the cells were in exponential growth phase, washed the cells three times in yeast
162 nitrogen base (YNB) medium without a carbon source, and then shifted the cells to YNB

163 containing 1% cellobiose as the sole carbon source, 1% succinate as the sole carbon source, or
164 lacking a carbon source for 8 h prior to harvesting the cells for RNA sequencing.

165 The expression of 776 genes was at least 4-fold differentially expressed between wild
166 type cells exposed to carbon starvation and cellobiose as the sole carbon source, with 139
167 genes upregulated and 637 genes downregulated in cells exposed to cellobiose as compared to
168 carbon starvation. Genes upregulated during exposure to cellobiose were enriched for genes
169 associated with carbohydrate metabolism, nucleosome assembly, and sulfur metabolism via
170 selenoamino acid metabolism. Genes downregulated during exposure to cellobiose compared
171 to carbon starvation were enriched for general metabolic processes, including regulation of
172 nitrogen utilization, amino acid metabolism, and transport associated genes (Fig 2B-C).

173 Far fewer genes (373) were at least 4-fold differentially expressed between wild type
174 cells exposed to carbon starvation and succinate, with 116 upregulated and 257 downregulated
175 in cells exposed to succinate as compared to carbon starvation. While only selenoamino acid
176 metabolism was enriched for genes upregulated on succinate, genes associated with nitrogen
177 utilization, electron transport, and carbohydrate transport were downregulated in succinate
178 compared to carbon starvation (Fig 2B-C). The majority of genes (289 out of 373) differentially
179 expressed by at least 4-fold during exposure to succinate as compared to carbon starvation
180 were also differentially expressed by at least 4-fold during exposure to cellobiose as compared
181 to carbon starvation. Fifty-seven genes were upregulated in both cellobiose and succinate as
182 compared to carbon starvation by at least 4-fold. Of the 257 genes downregulated by at least 4-
183 fold in response to succinate, 228 were also downregulated by at least 4-fold in response to
184 cellobiose. This supports the hypothesis that similar sets of genes are regulated in response to
185 both cellobiose and succinate.

186 Although the expression of many fungal transcription factor genes is upregulated in the
187 condition in which the transcription factor plays a role, *CBR1* expression was not activated in
188 response to either cellobiose or succinate as compared to carbon starvation (Fig S4A). To

189 directly regulate gene expression, Cbr1 must be present in the nucleus. We tagged Cbr1 with
190 green fluorescent protein (mEGFP) and the histone H2A subunit *HTA1* (RTO4_12090) with
191 mRuby2 and exposed these cells to glucose, cellobiose, or succinate as the sole carbon source
192 to identify the localization pattern of Cbr1. Cbr1 colocalized with the histone H2A subunit under
193 all conditions, suggesting that Cbr1 is expressed and present in the nucleus during exposure to
194 glucose, cellobiose, and succinate (Fig S4B).

195 To test our hypotheses for potential connections in the regulation of cellobiose and
196 succinate utilization, we examined the genes differentially expressed in wild type as compared
197 to *cbr1*Δ cells. The expression of 635 genes was at least 4-fold differentially expressed in *cbr1*Δ
198 cells relative to wild type cells during exposure to cellobiose, with 270 genes downregulated and
199 365 genes upregulated in *cbr1*Δ cells as compared to wild type. Similar to WT cells on
200 cellobiose compared to carbon starvation, *cbr1*Δ cells on cellobiose had higher levels of
201 transcripts associated with transport than WT cells. Additionally, genes associated with protein
202 folding, amino acid and protein metabolism, and sulfur metabolism were downregulated in *cbr1*Δ
203 cells as compared to wild type cells when exposed to cellobiose (Fig 2B-C). Nearly half of these
204 genes (281) were also at least 4-fold differentially expressed in wild type cells exposed to
205 cellobiose as compared to carbon starvation.

206 Only 22 genes were at least 4-fold differentially expressed between cells lacking *cbr1*
207 and wild type cells exposed to succinate. The 10 genes downregulated in *cbr1*Δ cells as
208 compared to wild type cells during exposure to succinate were enriched for several KEGG
209 categories, including starch and sucrose metabolism, that upon closer inspection were largely
210 driven by a decrease in expression of two putative β-glucosidases. Similarly, the KEGG
211 categories enriched in genes downregulated in *cbr1*Δ cells relative to wild type cells in carbon
212 starvation were largely driven by expression of the two putative β-glucosidase genes (Fig 2B-C).

213 Thirteen genes were at least 4-fold differentially expressed in *cbr1*Δ cells relative to wild
214 type cells during exposure to both cellobiose and succinate. Of these 13 genes, 5 were
215 downregulated and 8 were upregulated in *cbr1*Δ cells relative to wild type during exposure to
216 both cellobiose and succinate. To distinguish between our possible hypotheses for Cbr1 co-
217 regulation of cellobiose and succinate, we focused on genes downregulated in cells lacking *cbr1*
218 as compared to wild type. These genes included two genes encoding predicted β-glucosidases
219 (RTO4_16716 and RTO4_16717), two genes encoding predicted major facilitator superfamily
220 (MFS) transporters (RTO4_13825 and RTO4_10339), and a gene encoding a predicted
221 phosphoglycerate mutase (RTO4_11229). The downregulation of genes encoding β-
222 glucosidases, which may cleave cellobiose into its constituent glucose molecules, in *cbr1*Δ cells
223 relative to wild type cells during exposure to both cellobiose and succinate may support our
224 hypothesis that Cbr1 regulates genes necessary to utilize both cellobiose and TCA cycle
225 intermediates in response to all of these carbon sources. The eight genes upregulated in *cbr1*Δ
226 cells relative to wild type during exposure to both cellobiose and succinate, included a gene
227 encoding a predicted metallo-β-lactamase superfamily protein (RTO4_11764), a gene encoding
228 a predicted peptidoglycan-N-acetylmuramic acid deacetylase (RTO4_9785), a gene encoding a
229 predicted cyclin (RTO4_15596), a gene encoding a predicted methyltransferase (RTO4_14040),
230 and four hypothetical proteins (RTO4_8662, RTO4_9846, RTO4_10117, and RTO4_13502).
231
232 *β-glucosidase genes expressed in a CBR1-dependent fashion in response to cellobiose and*
233 *TCA cycle intermediates are necessary for cellobiose utilization*
234 The expression of predicted β-glucosidase genes in a *CBR1*-dependent fashion in
235 response to both cellobiose and succinate, led us to hypothesize that Cbr1 regulates genes
236 necessary to utilize both cellobiose and TCA cycle intermediates in response to all of these
237 carbon sources. To test this hypothesis, we deleted RTO4_16716 and RTO4_16717, which both

238 encode predicted β-glucosidases and are adjacent on chromosome 9 of the *R. toruloides*
239 genome. Deletion of RTO4_16716 and RTO4_16717 had no effect on growth on glucose but
240 eliminated the ability of *R. toruloides* to grow when cellobiose was provided as the sole carbon
241 source (Fig 3A). Canonical TCA cycle intermediate utilization pathways do not require β-
242 glucosidases. Thus, we hypothesized that even though RTO4_16716 and RTO4_16717 were
243 expressed in a *CBR1*-dependent fashion during succinate exposure, they would not actually be
244 required for TCA cycle intermediate utilization. Indeed, growth of wild type cells and
245 RTO4_16716Δ RTO4_16717Δ cells was indistinguishable when succinate or citrate were
246 provided as the sole carbon source (Fig 3A). This data suggests that RTO4_16716 and
247 RTO4_16717 are necessary for growth on cellobiose but not the TCA cycle intermediates
248 succinate or citrate.
249 Both RTO4_16716 and RTO4_16717 had predicted secretion signals (Fig S5). We
250 asked whether it was possible to detect β-glucosidase activity in the supernatant of *R. toruloides*
251 cultures. We grew wild type cells, *cbr1*Δ cells, and RTO4_16716Δ RTO4_16717Δ cells in YNB
252 with glucose as the sole carbon source overnight to exponential growth phase, back diluted the
253 cultures into YNB containing glucose as the sole carbon source for 8 doublings, washed them
254 twice in YNB lacking a carbon source, shifted them to YNB with either glucose, cellobiose, or
255 succinate as the sole carbon source, and then harvested culture supernatants after 20 hours of
256 incubation to measure β-glucosidase activity. Wild type cells had no detectable β-glucosidase
257 activity during growth on glucose (Fig 3B). When wild type cells were grown on cellobiose, we
258 could detect substantial β-glucosidase activity in the supernatant. However, deletion of *CBR1* or
259 RTO4_16716 and RTO4_16717 resulted in a complete abolition of β-glucosidase activity (Fig
260 3B). β-glucosidases are not required for TCA cycle intermediate utilization. However, β-
261 glucosidase activity was present in wild type succinate cultures at a level that was 30% of wild

262 type cellobiose supernatants (Fig 3B). Thus, we named RTO4_16716 and RTO4_16717, *BGL1*
263 and *BGL2*, respectively, for β-glucosidase 1 and 2.
264 If *BGL1* and *BGL2* are sufficient for growth on cellobiose, we hypothesized that it would
265 be possible to grow cells lacking *CBR1* or *BGL1* and *BGL2* with cellobiose as the sole carbon
266 source if the media was supplemented with spent supernatant from wild type cells grown on
267 cellobiose or succinate. To test this hypothesis, we grew wild type cells in media containing
268 either glucose, cellobiose, or succinate as the sole carbon source. We harvested supernatant
269 from these cultures and then supplemented media containing cellobiose as the sole carbon
270 source with either 6%, 12%, or 25% of the filtered spent supernatant. As expected, supernatants
271 from wild type cells grown in cellobiose and succinate exhibited β-glucosidase activity, while
272 supernatant from wild type cells grown in glucose did not (Fig S6A). Wild type cells, *cbr1*Δ cells,
273 and *bgl1*Δ *bgl2*Δ cells were then inoculated into this cellobiose media supplemented with spent
274 supernatant. As a control for growth on any of the nutrients present in the spent supernatant, we
275 also supplemented media lacking a carbon source with either 6%, 12%, or 25% of the filtered
276 spent supernatant and subtracted any growth seen in this condition from the growth in the spent
277 supernatant supplemented cellobiose media (Fig. 3C-E and Fig S6B-G).
278 To detect growth of the *cbr1*Δ and *bgl1*Δ *bgl2*Δ cells, we allowed the strains to grow for
279 250 hours. Minimal growth of cells lacking *CBR1* in media containing cellobiose as the sole
280 carbon source was detected after approximately one week (Fig 3C-E). The *bgl1*Δ *bgl2*Δ cells
281 were never able to grow when cellobiose was provided as the sole carbon source (Fig 3C-E).
282 When spent supernatant from wild type cells grown on cellobiose was added at 6%, 12%, or
283 25% the growth of wild type cells was unchanged. However, the *cbr1*Δ cells on cellobiose were
284 able to grow exponentially when cellobiose spent supernatant was added, while growth of the
285 *bgl1*Δ *bgl2*Δ cells began after approximately 5 days (Fig 3C and Fig S6B-C). Spent supernatant
286 from wildtype grown on succinate did not impact wildtype growth on cellobiose. However, the

287 growth of both the *cbr1*Δ and *bgl1*Δ *bgl2*Δ cells improved significantly upon addition of spent
288 succinate supernatant (Fig 3D and Fig S6D-E). The addition of 6%, 12%, or 25% spent
289 supernatant from wild type cells grown on glucose did not improve the growth of *cbr1*Δ with 6%
290 or 12% supernatant or *bgl1*Δ *bgl2*Δ cells with any percent supernatant on cellobiose media (Fig
291 3E and Fig S6F-G). Taken together, our data show that genes necessary for growth on
292 cellobiose but not TCA cycle intermediates are expressed in a *CBR1*-dependent fashion on both
293 cellobiose and TCA cycle intermediates.
294
295 *Transporter genes expressed in a CBR1-dependent fashion in response to cellobiose and TCA*
296 *cycle intermediates are necessary for citrate utilization*
297 Two genes encoding MFS transporters were expressed in a *CBR1*-dependent fashion in
298 response to both cellobiose and succinate: RTO4_13825 and RTO4_10339. We hypothesized
299 that these transporters may be responsible for growth on cellobiose and/or TCA cycle
300 intermediates. To test this hypothesis, we deleted RTO4_13825 and RTO4_10339 individually
301 and grew these strains on media containing glucose, cellobiose, citrate, succinate, α-
302 ketoglutarate, malate, and fumarate. The growth of cells lacking RTO4_13825 or RTO4_10339
303 was indistinguishable from that of wild type cells when glucose or cellobiose was provided as
304 the sole carbon source (Fig 4A). These data suggest that RTO4_13825 and RTO4_10339 are
305 not necessary for growth when cellobiose is provided as the sole carbon source.
306 Cells lacking RTO4_13825 were unable to grow when citrate was provided as the sole
307 carbon source (Fig 4B). However, RTO4_13825Δ cells were able to grow as well as wild type
308 cells when any of the dicarboxylic acid TCA cycle intermediates, succinate, α-ketoglutarate,
309 malate, or fumarate, were provided as the sole carbon source (Fig 4B). Thus, we named
310 RTO4_13825 *TCT1* for tricarboxylic acid transporter 1. In contrast, RTO4_10339 had no
311 significant growth phenotype on any of the carbon sources we tested.

312 A gene encoding a predicted phosphoglycerate mutase (RTO4_11229) was expressed in
313 a *CBR1*-dependent fashion in response to cellobiose and succinate. We hypothesized that it
314 may play a role in utilization of cellobiose or TCA cycle intermediates. However, the growth of
315 cells lacking RTO4_11229 in media containing glucose, cellobiose, citrate, succinate, α-
316 ketoglutarate, malate, and fumarate was indistinguishable from wild type cells (Fig S7).
317
318 *Cbr1 inhibits carbon catabolite repression during disaccharide utilization*
319 Our data suggest that *R. toruloides* β-glucosidases are secreted into the media where
320 they cleave the cellobiose β-1,4-glycosidic bond, releasing two glucose molecules (Fig 3). If this
321 is the case, cellobiose is likely imported into the cell as glucose molecules rather than cellobiose
322 molecules. Glucose is a preferred carbon source for most fungi and represses genes necessary
323 for utilization of nonpreferred carbon sources (1, 14, 15). We asked whether glucose was
324 preferred over cellobiose in *R. toruloides* by growing wild type cells in media with cellobiose as
325 the sole carbon source and adding the nonhydrolyzable glucose analog 2-deoxyglucose to the
326 media. Because 2-deoxyglucose cannot be hydrolyzed, it cannot act as a carbon source, but it
327 is still sensed as glucose. It was possible to completely repress the growth of wild type cells on
328 cellobiose by adding 0.001% 2-deoxyglucose (Fig 5A).
329 The extracellular release of glucose by the extracellular β-glucosidase activity of Bgl1
330 and Bgl2 begs the question of how *R. toruloides* avoids carbon catabolite repression during
331 cellobiose utilization. We hypothesized that *CBR1* may play a role in inhibiting carbon catabolite
332 repression. To test this hypothesis, we grew wild type cells and *cbr1*Δ cells in media with a
333 nonpreferred carbon source and 2-deoxyglucose. We first investigated the role of *CBR1* in
334 responding to the presence of 2-deoxyglucose in combination with the disaccharide maltose.
335 When 2-deoxyglucose was added at 0.002%, wild type cells were still able to grow but the
336 growth of cells lacking *CBR1* were completely inhibited (Fig 5B). A similar phenomenon was

337 seen when 2-deoxyglucose was added to media containing the disaccharide trehalose. When
338 0.01% 2-deoxyglucose was added to 1% trehalose the growth of *cbr1*Δ cells was reduced by
339 40% relative to wild type cells and when 0.02% 2-deoxyglucose was added, *cbr1*Δ cells were
340 unable to grow, while the growth of wild type cells was only moderately inhibited (Fig 5B).
341 Complementation of the *cbr1* deletion with *CBR1* placed under its native promoter at the *YKU70*
342 locus, restored the sensitivity to 2-deoxyglucose to levels indistinguishable from that of wild type
343 cells (Fig 5B).
344 In Ascomycete fungi, known regulators of carbon catabolite repression, such as CRE-
345 1/CreA/Mig1 regulate expression of genes responsible for utilization of all nonpreferred carbon
346 sources in response to preferred carbon sources (15–21). We hypothesized that carbon
347 catabolite repression would function similarly in Basidiomycete cells, and thus, *CBR1* would
348 inhibit carbon catabolite repression for all nonpreferred carbon sources. To test this hypothesis,
349 we tested the sensitivity of wild type and *cbr1*Δ cells in media containing other classes of
350 carbohydrates. To our surprise, wild type and *cbr1*Δ cells were equally sensitive to 2-
351 deoxyglucose when exposed to the monosaccharides fructose and xylose, the amino acid
352 proline, and the lipid Tween-80 (Fig 5D-G).
353 To test the role of *CBR1* in regulating carbon catabolite repression in response to
354 alcohols, we grew wild type and *cbr1*Δ cells in media containing glucose and allyl alcohol, which
355 is oxidized to the toxic compound acrolein by alcohol dehydrogenases. If carbon catabolite
356 repression is functioning normally, the presence of glucose should inhibit expression of alcohol
357 dehydrogenases in the presence of glucose (29). Wild type and *cbr1*Δ cells were equally
358 sensitive to allyl alcohol when glucose was present (Fig 5H). These data suggest that *CBR1*
359 inhibits carbon catabolite repression specifically when the available nonpreferred carbon source
360 is a disaccharide. We asked whether the *N. crassa* Cbr1 ortholog, CLR-2, also played a role in
361 inhibiting carbon catabolite repression by exposing wild type cells and Δ*clr-2* cells to trehalose

362 or xylose with the addition of 2-deoxyglucose. The response to 2-deoxyglucose in wild type and
363 Δ*clr-2* cells was indistinguishable (Fig S8), indicating that the role of Cbr1 in inhibiting carbon
364 catabolite repression during exposure to disaccharides is an expanded role for Cbr1 relative to
365 the filamentous fungal ortholog CLR-2.
366
367 **DISCUSSION**
368 The regulation of gene expression in response to nutrient availability requires cells to
369 integrate information from activating and repressing environmental nutrient signals. There have
370 been limited investigations of the genetic regulators of cellulose and cellobiose utilization in
371 Basidiomycetes. A recent study identified Roc1, a regulator of cellulase-encoding genes in the
372 basidiomycete wood-decaying fungus *Schizophyllum commune* (30). However, homologs of
373 Roc1 do not exist outside of the Agaricomycetes (30). In Ascomycete filamentous fungi, CLR-
374 2/ClrB regulates cellulose degradation and utilization through the direct activation of genes
375 encoding cellulases (24, 31). We identified the CLR-2/ClrB ortholog Cbr1 in the oleaginous,
376 basidiomycete yeast *R. toruloides* (Fig 1A). Although Ascomycete and Basidiomycete fungi are
377 separated by approximately 400 million years of evolution (32), our study showed that Cbr1 has
378 a role in utilization of the cellulose breakdown product cellobiose (Fig 1). *N. crassa* CLR-2 does
379 not directly regulate the expression of genes encoding β-glucosidases, which are regulated by
380 CLR-1 (NCU07705) in *N. crassa* (24, 31). However, a role for Cbr1 in cellobiose utilization
381 suggests that there is conservation in the role of CLR-2/ClrB/Cbr1 between Ascomycete
382 filamentous fungi and Basidiomycete yeast.
383 Our characterization of Cbr1 showed that Cbr1 regulates utilization of TCA cycle
384 intermediates through the regulation of a gene encoding a transporter and regulates cellobiose
385 utilization through the regulation of genes encoding β-glucosidases (Fig 6). In other fungal
386 species, genes necessary for utilization of multiple carbon sources are also sometimes

387 regulated by a single transcription factor. For example, CLR-2 in *N. crassa* regulates cellulose
388 utilization, mannan utilization, and a small number of genes involved in hemicellulose utilization
389 (14, 31, 33). In *Trichoderma reesei*, *Penicillium oxalicum*, and some Aspergilli, the transcription
390 factor Xyr1/XlnR regulates utilization of both cellulose and hemicellulose (25–27). ARA-1/Ara1
391 regulates both arabinan and galactose utilization in *N. crassa* and *T. reesei* (14, 34), and FarA
392 regulates utilization of both fatty acids and the lignin component ferulic acid (35, 36). However,
393 in these examples linkage of these carbon sources can easily be explained either by the
394 presence of both carbon sources in the plant cell wall (i.e, cellulose and hemicellulose or
395 arabinan and galactose) or catabolism through the same metabolic pathways (i.e., fatty acids
396 and ferulic acid).

397 Our data does not support the hypothesis that cellobiose and TCA cycle intermediates
398 are catabolized through the same metabolic pathway in *R. toruloides*. The Cbr1-regulated β-
399 glucosidase genes are critical for cellobiose utilization but not TCA cycle intermediate utilization
400 (Fig 3), while one of the Cbr1-regulated transporters is critical for citrate utilization but not
401 cellobiose utilization (Fig 4). Aside from *CBR1* itself, we did not identify any genes that were
402 necessary for utilization of both cellobiose and TCA cycle intermediates. Thus, we speculate
403 that the coregulation of cellobiose and TCA cycle intermediates may suggest that *R. toruloides*
404 frequently encounters cellobiose and TCA cycle intermediates in close proximity. One possible
405 explanation for this is that *R. toruloides* scavenges cellobiose during cellulose degradation by
406 filamentous fungi, which also secrete organic acids, such as citrate, to acidify their environment
407 (37).

408 Carbon catabolite repression is the genetic mechanism through which fungal cells
409 prioritize utilization of carbon sources that require less energy to metabolize over carbon
410 sources that require more energy to metabolize (1). Known genetic mechanisms of regulating
411 carbon catabolite repression regulate the expression of genes required for utilization of all less
412 preferred carbon sources when a more preferred carbon source is present (15–21). Cbr1

413 demonstrates a mechanism through which cells inhibit carbon catabolite repression specifically
414 during utilization of disaccharides (Fig 5). We speculate that this may allow cells to continue
415 expressing genes necessary for hydrolyzing disaccharide sugar bonds even when this
416 hydrolysis produces preferred monosaccharides like glucose (Fig 6). Genetic linkage of carbon
417 catabolite repression inhibition with the activation of genes necessary to utilize a nonpreferred
418 carbon source has not previously been described. Additionally, regulation of genes involved in
419 utilization of a specific class of nonpreferred carbon sources in response to a preferred carbon
420 source represents a novel form of carbon catabolite repression regulation.
421

422 **MATERIALS and METHODS**

423 *Strains and culturing*

424 *R. toruloides* strains used in this study are listed in Table S1. All strains were derived
425 from the wild type reference strain IFO 0880 (also called NBRC 0880, obtained from Biological
426 Resource Center, NITE (NBRC), Japan). The starting strain for genetic manipulations was in a
427 non-homologous end-joining deficient *yku70*Δ background (38). *R. toruloides* was transformed
428 using *Agrobacterium tumefaciens*-mediated transformation using *A. tumefaciens* EHA 105 and
429 plasmids derived from pGI2 (39) that were previously used for *R. toruloides A. tumefaciens*-
430 mediated transformation (11). *A. tumefaciens*-mediated transformation was performed as
431 previously described (11). Gene deletions, complementations, and tags were performed using
432 homologous recombination of a nourseothricin or G418 resistance cassette with flanking arms
433 of ~1000 bp. All strains were confirmed by PCR. Complement and tagged strains were further
434 confirmed by DNA sequencing. We fluorescently tagged the histone H2A subunit gene *HTA1*
435 (RTO4_12090) with the mRuby2 fluorescent protein at the native locus. The Hta1-mRuby2
436 tagged strains were generated using a combination of G418 resistance and a 5-
437 fluorodeoxyuridine (FUDR) sensitive thymidine kinase counterselection marker. The Hta1-
438 mRuby2 construct was transformed into the *yku70*Δ *CBR1-GFP* backgrounds using G418 to

439 select for transformants. Transformed colonies were passaged one time on liquid YPD and
440 plated onto YNB + 1% glucose + FUDR plates to select for loop-outs of the G418 and thymidine
441 kinase markers. Colonies were screened by microscopy, PCR, and locus sequencing for correct
442 integration of the mRuby2 tag and successful removal of the G418 and thymidine kinase
443 markers. As RTO4_16716 and RTO4_16717 are adjacent in the genome, the *bgl1*Δ *bgl2*Δ strain
444 was generated via single transformation of the nourseothricin resistance cassette to remove
445 both open reading frames from the genome. *R. toruloides* strains were grown from freezer
446 stocks on YPD (VWR) plates at 28°C for 2 d to 3 d prior to inoculation into liquid YPD to grow
447 strains at 28°C with constant shaking to exponential phase growth prior to starting experiments.
448 *N. crassa* strains used in this study are listed in Table S2. All strains were derived from
449 the wild type reference strain FGSC 2489 using standard genetic techniques and confirmed
450 using PCR (40, 41). *N. crassa* cells were grown from freezer stocks on Vogel's minimal medium
451 (42) + 2% sucrose + 1.5% agar (Bacto) slants for 2 d at 28°C in the dark and 4 d at 28°C in
452 constant light. *N. crassa* conidia were harvested from slants with sterile double distilled H_2O for
453 inoculation.
454 All *R. toruloides* experiments were done using yeast nitrogen base (YNB) with NH_4SO_4
455 without amino acids (MP Biomedicals) + 12 mM K_2HPO_4 (Fisher) + 38 mM KH_2PO_4 (Fisher) +
456 100 nM Fe(II)SO_4 pH 6.2 with the indicated carbon source at 1% w/v with the exception of
457 coumaric acid and ferulic acid, which were added at a concentration of 0.2%, and citrate which
458 was added at a concentration of 8.5 mM and a pH of 5.5. All *N. crassa* experiments were
459 performed using Vogel's minimal medium with 50mM NH_4Cl instead of 25mM NH_4NO_3 (VMM
460 NH_4Cl) and the indicated carbon source at 1% w/v. Carbon sources used are listed in Table S3.
461
462 *R. toruloides growth experiments*
463 Colonies of *R. toruloides* grown on solid YPD were inoculated into liquid YPD and grown
464 at 28°C with constant shaking until exponential phase. Cells were pelleted at 4000 x g and

465 washed three times with YNB lacking a carbon source. Washed cells were used to inoculate
466 750 µL of media in 48-well plates at an OD 600 of 0.1 for initial carbon screening experiments
467 (Figure S2) and 0.01 for all subsequent growth experiments. Growth assays were incubated at
468 28°C with constant shaking (3 mm orbit, 600 rpm for individual time points and 3 mm orbit 425
469 rpm for kinetic growth experiments). Kinetic growth experiments were performed using an
470 Agilent BioTek Epoch2 plate reader with OD 600 measurements taken every 20 or 30 minutes
471 depending on the experiment.
472
473 *N. crassa growth experiments*
474 For trehalose and xylose 2-deoxyglucose (TCI) flask growth experiments wild type *mat A*
475 (FGSC 2489) and Δ*clr2 mat A* conidia were inoculated at 10^6 conidia/mL into flasks with 100 mL
476 of filter sterilized VMM NH_4Cl with either 1% xylose or 1% trehalose. Filter sterilized 20% 2-
477 deoxyglucose dissolved in water was added to the 100mL media to achieve xylose + 2-
478 deoxyglucose concentrations of either 0%, 0.00195%, 0.00391%, or 0.0078125% and trehalose
479 + 2-deoxyglucose concentrations of either 0%, 0.04%, 0.0625%, or 0.125%. Cultures were
480 grown at 28ºC with constant light and shaking at 200rpm. After 24 hours biomass was
481 autoclaved, harvested, dried for 24 h at 60°C in a drying oven, and weighed.
482 For growth of *N. crassa* on TCA cycle intermediates and glucose, wild type mat A (FGSC
483 2489) and Δ*clr2 mat A* conidia inoculated at 10^6 conidia/mL in 24 deep-well plates with 3 mL of
484 filter sterilized VMM NH_4Cl with 1% succinate, 1% alpha-ketoglutarate, 1% malate, or 1%
485 glucose. Cultures were incubated at 28ºC with constant light and shaking (200 rpm). Biomass
486 from glucose cultures was harvested at 18 h post inoculation and biomass from TCA cycle
487 intermediate cultures was harvested 7 d post inoculation. Biomass was dried for 24 h in a drying
488 oven at 60ºC and weighed.
489
490 *Phylogenetic tree generation*

491 Protein sequences for CLR-1, CLR-2, and XLR-1, from *N. crassa* (OR74A) and ClrA,
492 ClrB, and XlnR from *Aspergillus nidulans* (A4) were obtained from Mycocosm (43). The protein
493 sequences were used to BLAST search the proteomes of *N. crassa*, *A. nidulans*,
494 *Saccharomyces cerevisiae* (S288C), and *R. toruloides* (IFO 0880). Identified homologs with an
495 e-value < 1E-10 were aligned via MUSCLE (44) in MEGA11 (45) and the phylogenetic tree was
496 generated using FastTree (46) using the maximum likelihood method based on the Jones-
497 Taylor-Thornton matrix-based model and visualized in MEGA11. The reliability of each split in
498 the tree was computed using the Shimodaira-Hasegawa test on three alternate topologies
499 around that split.
500
501 *Microscopy*
502 Fluorescent microscopy was performed using a Nikon Eclipse Ti2 inverted microscope
503 equipped with a prime BSI express CMOS camera using a CFI Plan Apochromat 100X oil
504 objective. *HTA1-mRuby2 CBR1-GFP* strains were grown to exponential phase in liquid YPD.
505 Cells were pelleted at 4000 x g and washed three times with YNB without a carbon source.
506 Cells were inoculated into a 48-well plate with YNB + 1% w/v of the indicated carbon source at
507 an OD 600 of 0.2 and incubated at 28ºC with shaking (3mm orbit, 600rpm) for 4 hours. Aliquots
508 of the cultures were placed onto agarose coated slides and imaged.
509
510 *RNA sequencing and transcript abundance*
511 Cells were grown to exponential phase in liquid YPD and washed three times in YNB
512 without a carbon source. Flasks containing 100 mL of YNB + 1% cellobiose, 1% succinate, or
513 no carbon source were inoculated at an OD 600 of 0.1. Cultures were incubated for 8 hours at
514 28ºC with shaking (200 rpm). Cells were harvested using centrifugation in 50 mL conical tubes
515 at 4000 x g. The supernatants were removed, and the cell pellets were flash frozen in liquid
516 nitrogen. Pellets were stored at -80ºC prior to RNA extraction.

517 RNA was extracted using the Quick-RNA Fungal/Bacterial Miniprep Kit according to the
518 manufacturer's instructions. RNA quality was checked via agarose gel electrophoresis and
519 BioAnalyzer analysis performed at the Transcriptional Regulation & Expression Facility at
520 Cornell University. RNA was submitted to the California Institute for Quantitative Biosciences at
521 UC Berkeley (QB3-Berkeley) for library preparation and sequencing. Libraries were prepared
522 using standard Illumina protocols and RNA was sequenced using an Illumina NextSeq 2000
523 150PE at a depth of approximately 20 million reads per sample.
524 The transcript abundance (transcripts per million, TPM) was quantified using Salmon v.
525 1.10.0 mapping to the *R. toruloides* IFO 0880 genome (v4) (11) using the –validateMappings
526 setting (47). Differential expression was determined using DESeq2 v. 1.44.0 (48). Genes were
527 denoted as differentially expressed if there was at least a 4-fold change in expression and the
528 average TPM across all samples was at least 5. RNAseq data used in this study were deposited
529 in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information
530 (NCBI) and are accessible through GEO series accession number GSE293943. Expression
531 data was visualized using the ComplexHeatmap R package (49).
532
533 *β-glucosidase enzyme activity assays*
534 *R. toruloides* wild type, *cbr1*Δ, and *bgl1*Δ *bgl2*Δ strains were struck out from freezer
535 stocks on 2% agar yeast extract peptone dextrose (YPD) plates from freezer stocks and
536 incubated for five days at 28ºC with constant light. Three independent colonies of each strain
537 were inoculated into flasks with 100 mL of YNB with 1% glucose and grown for 23 h at 28ºC in
538 constant light with shaking at 200 rpm. Cultures were transferred to 50mL conical tubes, and the
539 cells were pelleted at 4000 x g. Supernatants were discarded and pellets were resuspended in 5
540 mL YNB 1% glucose media. Each resuspended culture was then transferred into 3 independent
541 flasks with 100 mL YNB 1% glucose media at 0.004 OD/mL and cultures were grown for 8
542 generations at 28ºC, constant light, and shaking at 200 rpm. Cultures were transferred to 50mL

543 conical tubes, and the cells were pelleted at 4000 x g. Supernatants were discarded and pellets
544 were washed twice in 5mL YNB media without carbon. Cultures were transferred into YNB 1%
545 glucose, succinate, or cellobiose at 0.8 OD/mL and grown at 28ºC in constant light with shaking
546 at 200rpm for 20 h prior to measuring optical density. An aliquot from each culture was
547 centrifuged at 10,000 x g for 1 m and the culture supernatant was filtere sterilized with a 0.22µm
548 syringe filter and stored at -80ºC. Filtered supernatant aliquots were thawed on ice and β-
549 glucosidase activity was assessed by adding 50 uL of 100 mM sodium acetate (pH 5) and 25 uL
550 of 20 mM p-nitrophenyl-β-D-glucopyranoside (EMD Millipore Corp.) in a 96-well plate. The plate
551 was incubated on ice for five minutes and 25 uL of each supernatant aliquot was added, leaving
552 a set without supernatant added for a blank reading, and then incubated at 37ºC for 30 m.
553 Reactions were quenched by adding 100 uL of 200 mM Na_2CO_3 and 25 uL of each supernatant
554 aliquot was added to quenched blank wells. Optical densities were measured at 400 nm, and
555 blank readings were subtracted from their respective assay readings.
556
557 *Spent media growth assays*
558 The *R. toruloides* wild type strain was struck out from freezer stocks on a 2% agar YPD
559 plate and incubated at 28ºC for two days with constant light. Single colonies were inoculated
560 into 15 14 mL culture tubes with 5 mL YPD. The cultures were grown overnight at 28ºC with
561 constant light and shaking at 250rpm. The following day, five 5 mL cultures were combined into
562 sterile 50 mL conical tubes for each of three replicates. 25 mL cultures were spun down at 4000
563 x g and washed three times with YNB no carbon. Cells were inoculated at a density of 0.01
564 OD/mL into flasks with 100 mL of either YNB 1% glucose, 1% cellobiose, or 1% succinate.
565 Cultures were grown to an OD 600 of 1, 1.1, or 0.9, for glucose, cellobiose, and succinate,
566 respectively, prior to centrifugation in sterile 50 mL conicals at 4000 x g for 10 minutes.

567 The supernatants were sterilized with tube top vacuum 0.22 μm filters. Filter sterilized
568 supernatants were used the same day to grow wild type, *cbr1*Δ, and *bgl1*Δ *bgl2*Δ strains at 0.01
569 OD/mL in YNB with 1% cellobiose and YNB lacking a carbon source with spent, filtered
570 supernatant added to make up 6%, 12%, or 25% of the total 750 μL culture in 48-well plates.
571 OD 600 measurements of the 48-well plates were taken as indicated. β-glucosidase activity of
572 each supernatant was assessed according to β-glucosidase assay protocol above.
573
574 *Statistical significance tests*
575 All statistical analyses were performed in Rstudio (v2024.12.1; R v4.4.3). All experiments
576 were performed with a minimum of three biological replicates.
577
578 *Functional enrichment analysis*
579 Functional annotations for the *R. toruloides* IFO 0880 genome (v4) were downloaded
580 from Mycocosm
581 (https://mycocosm.jgi.doe.gov/Rhoto_IFO0880_4/Rhoto_IFO0880_4.home.html). Functional
582 category enrichment analyses were performed using the compareCluster function in the
583 clusterProfiler R package with function set to "enricher" (50). Differentially expressed genes
584 were compared for enrichment against all genes expressed at an average TPM of 5 or greater
585 across the experiment. The Benjamini-Hochberg procedure was used to correct for multiple
586 hypothesis testing. Data were visualized using the clusterProfiler package's dotplot function
587 (50).
588
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594

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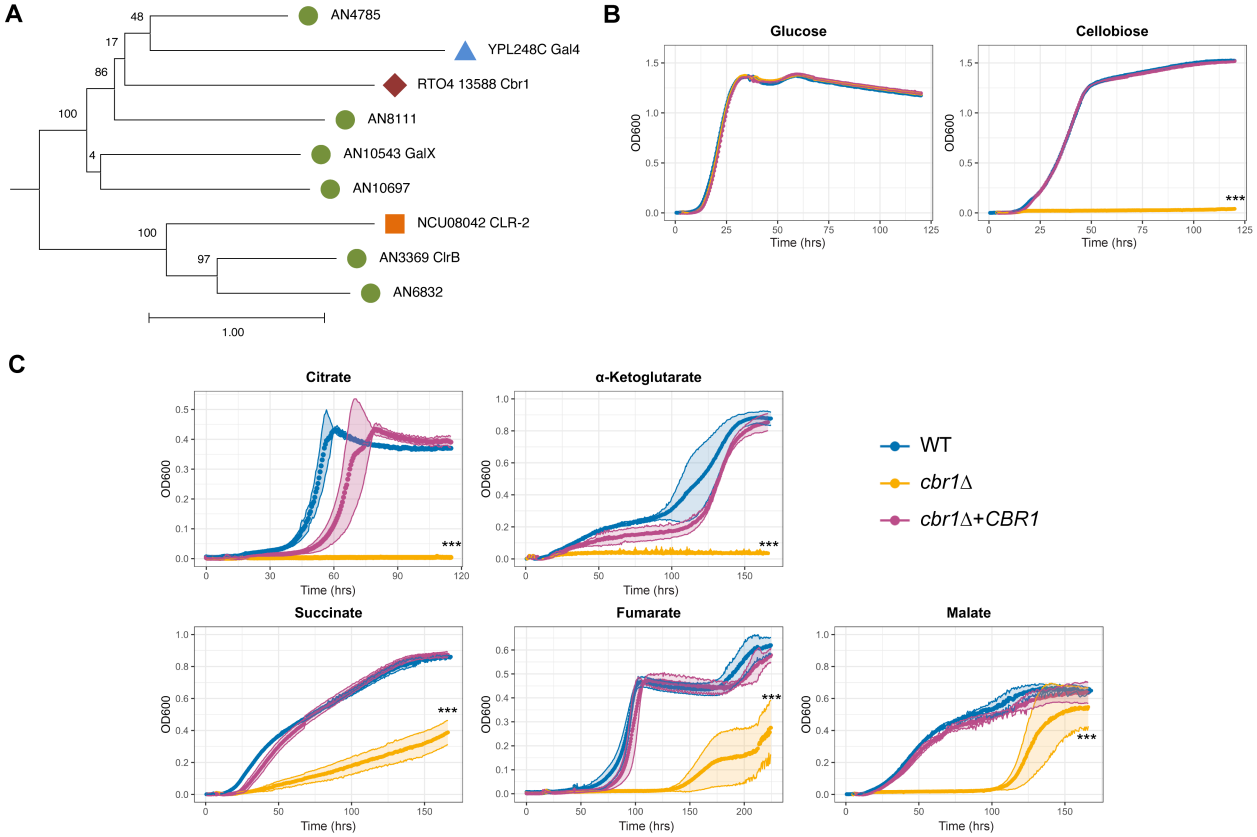
711

712 **FIGURES**

713



714

715 **Fig 1. *CBR1* regulates utilization of cellobiose and TCA cycle intermediates. (A)** Protein
716 sequences of *N. crassa* CLR-2 and *Aspergillus nidulans* ClrB homologs were used to build a
717 phylogenetic tree using the maximum likelihood method based on the Jones-Taylor-Thornton
718 matrix-based model using FastTree (46). The reliability of each split in the tree was computed
719 using the Shimodaira-Hasegawa test on three alternate topologies around that split. *R.*
720 *toruloides* proteins are indicated with maroon diamonds. *N. crassa* proteins are indicated with
721 orange squares. *A. nidulans* proteins are indicated with green circles. *Saccharomyces*
722 *cerevisiae* proteins are indicated with blue triangles. **(B-C)** Growth curves (OD 600) of wild type,
723 *cbr1*Δ, and *cbr1*Δ+*CBR1* complement strains grown in YNB with the indicated carbon source at
724 8.5 mM for citrate or 1% w/v for all other carbon sources. Dots are the average and colored bars

725 indicate standard deviation of three biological replicates. *** $p < 1E-10$ *cbr1*Δ vs WT and *cbr1*Δ

726 vs *cbr1*Δ+*CBR1* as determined by two-way ANOVA with a TukeyHSD post-hoc test.

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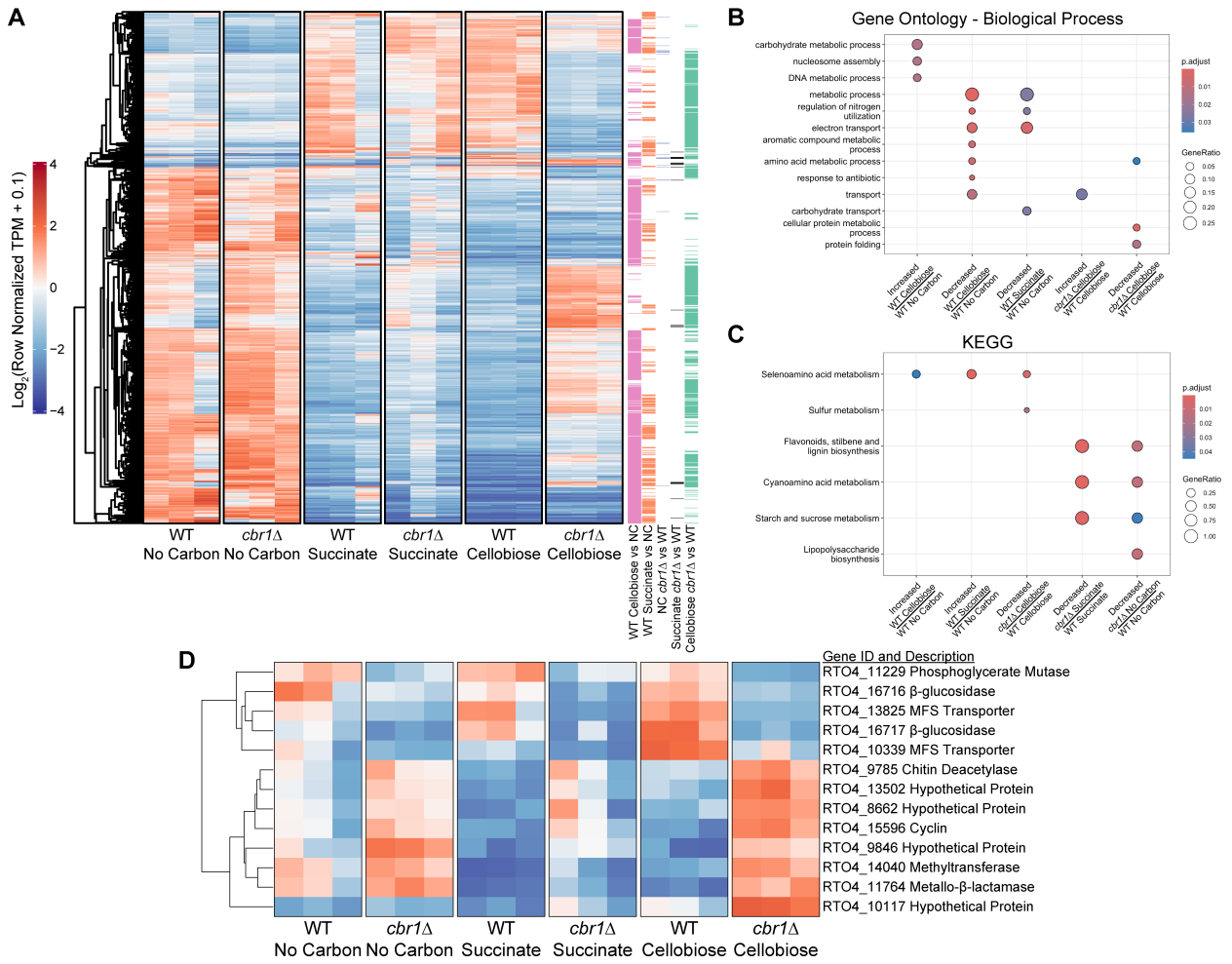


Fig 2. Cbr1 activates five genes in response to cellobiose and TCA cycle intermediates. **(A)** Heatmap of the expression of genes at least 4-fold differentially expressed between at least one of the following comparisons: wild type (WT) cells exposed to cellobiose as compared to carbon starvation (no carbon), wild type cells exposed to succinate as compared to carbon starvation, *cbr1*Δ cells as compared to wild type exposed to cellobiose, *cbr1*Δ cells as compared to wild type exposed to succinate, or *cbr1*Δ cells as compared to wild type exposed to carbon starvation. Genes were normalized by their average expression across all conditions, log transformed, and clustered using the Euclidean distance metric. Pink bars indicate genes that are at least 4-fold differentially expressed in wild type cells exposed to cellobiose as compared to carbon starvation (NC). Orange bars indicate genes that are at least 4-fold differentially

739 expressed in wild type cells exposed to succinate as compared to carbon starvation (NC). Blue
740 bars indicate genes that are at least 4-fold differentially expressed in *cbr1*Δ as compared to wild
741 type cells exposed to carbon starvation (NC). Black bars indicate genes that are at least 4-fold
742 differentially expressed in *cbr1*Δ as compared to wild type cells exposed to succinate. Green
743 bars indicate genes that are at least 4-fold differentially expressed in *cbr1*Δ as compared to wild
744 type cells exposed to cellobiose. **(B-C)** Genes significantly increased or decreased in
745 expression for each comparison were tested for gene ontology (GO) **(B)** and KEGG **(C)**
746 functional category enrichment. Comparisons were only displayed if they had at least one
747 statistically significantly enriched category for each analysis. Dot size corresponds to the
748 number of genes differentially expressed for a category divided by total genes in that category.
749 Color indicates p-value as determined by clusterProfiler (50) with a Benjamini-Hochberg multiple
750 comparisons correction. **(D)** Heatmap of the expression of genes differentially expressed by at
751 least 4-fold in *cbr1*Δ as compared to wild type cells exposed to cellobiose and exposed to
752 succinate. Genes were normalized by their average expression across all conditions, log
753 transformed, and clustered using the Euclidean distance metric. Gene IDs and annotations are
754 indicated to the right of the heatmap. The expression of *CBR1* was left out of the heatmaps,
755 GO, and KEGG enrichment analysis, since the change in expression of this gene was due to its
756 deletion in the *cbr1*Δ strain.
757

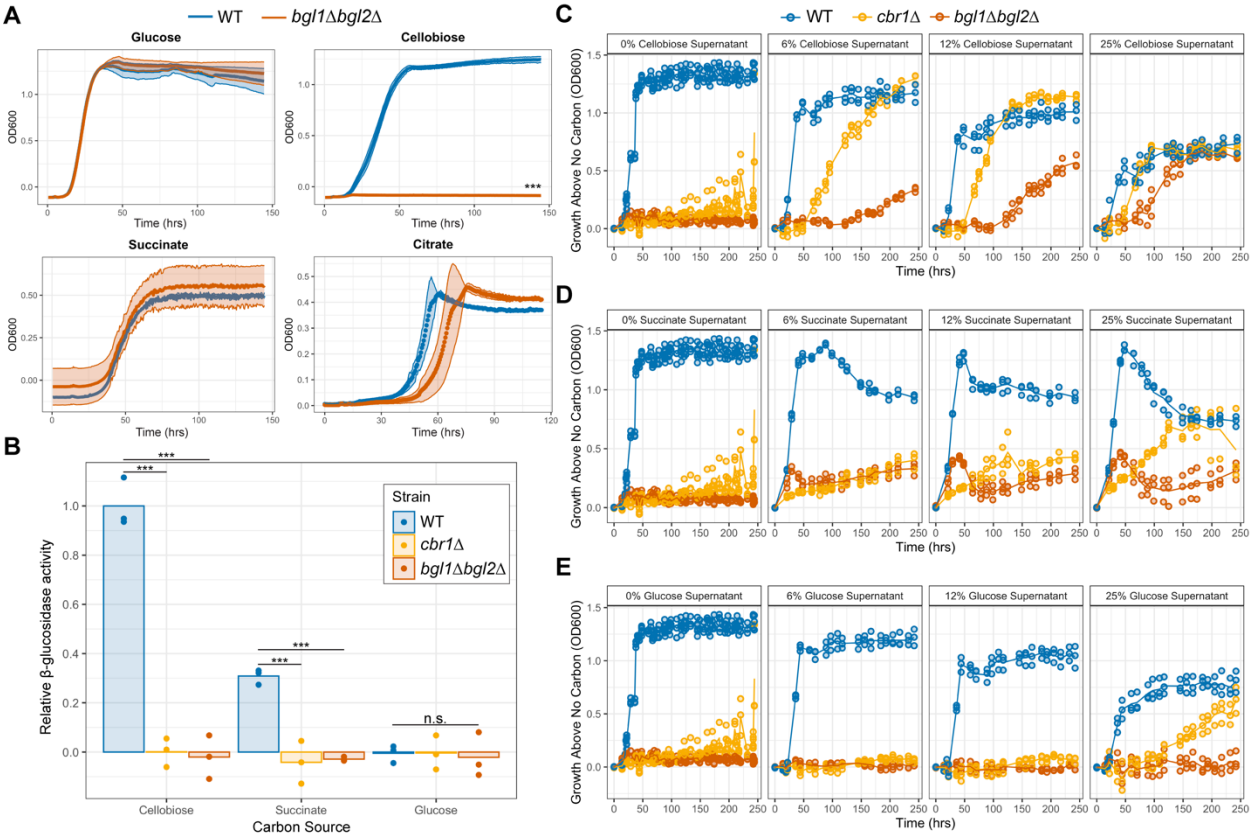
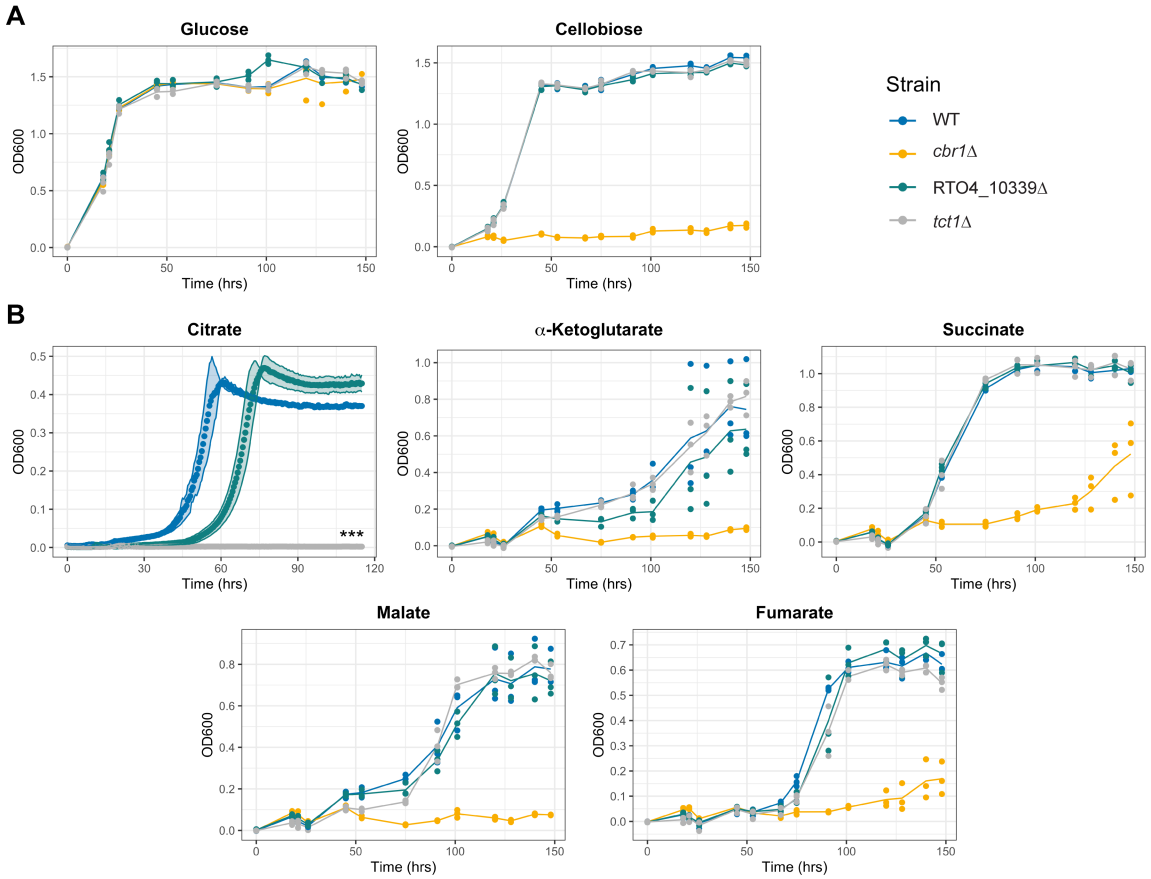


Fig 3. Cbr1-dependent expression of secreted β-glucosidase genes is necessary for cellobiose utilization. (A) Growth curves of wild type and *bgl1*Δ *bgl2*Δ cells grown in YNB with the indicated carbon source at 8.5 mM for citrate or 1% w/v for all other carbon sources. Dots are the average and colored bars indicate standard deviation of three biological replicates. *** $p < 1E-10$ *bgl1*Δ *bgl2*Δ vs wild type as determined by two-way ANOVA with a TukeyHSD post-hoc test. **(B)** β-glucosidase activity secreted into the supernatant of cultures of the indicated strain grown in YNB with the indicated carbon source at 1% w/v for 20 h relative to the β-glucosidase activity of wild type cells grown in cellobiose for 20 h. Bars are the averages of three biological replicates (dots). *** $p < 0.001$ as determined by two-way ANOVA with a TukeyHSD post-hoc test. **(C-E)** Growth (OD 600) of the indicated strains in YNB with 1% cellobiose supplemented with the indicated percentage of spent supernatant from wild type cells grown in YNB with 1% cellobiose **(C)**, 1% succinate **(D)**, or 1% glucose **(E)** for 6-7 generations. To normalize for the

771 growth of the indicated strain on the spent supernatant alone, the OD 600 of the indicated strain
772 grown on the indicated amount of spent supernatant alone was subtracted. Growth curves
773 without growth on spent supernatant alone subtracted, the growth curves on spent supernatant
774 alone, and the β-glucosidase activity of the spent supernatant are in Fig S6. Lines are the
775 averages of at least 3 biological replicates (dots).



776

777 **Fig 4. Cbr1-dependent expression of transporter genes is necessary for TCA cycle**

778 **intermediate utilization. (A)** Growth of the indicated strains in YNB with 1% glucose or 1%

779 cellobiose. Lines are the averages of three biological replicates (dots). **(B)** Growth of the

780 indicated strains in YNB with 8.5 mM citrate or 1% w/v of the indicated carbon source. For

781 growth on 8.5 mM citrate, dots are the average and colored bars are the standard deviation of

782 three biological replicates. For growth on all other carbon sources, lines are the average of three

783 biological replicates (dots). *** $p < 1E-10$ *tct1*Δ vs WT as determined by two-way ANOVA with a

784 TukeyHSD post-hoc test.

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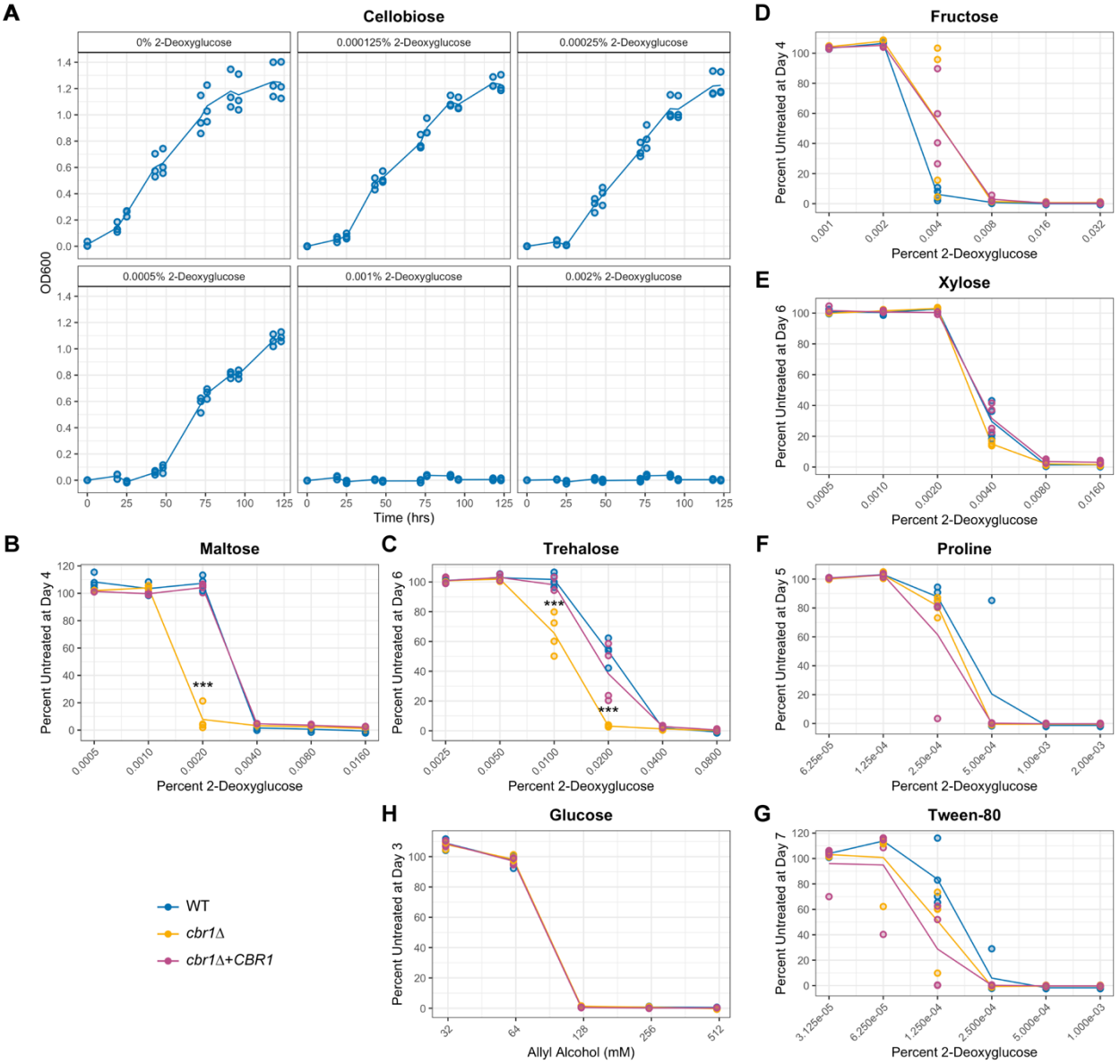

Fig 5. Cbr1 inhibits carbon catabolite repression during disaccharide utilization. (A) Growth (OD 600) of wild type in YNB + 1% cellobiose supplemented with the indicated concentration of 2-deoxyglucose. **(B)** Growth after 4 days of the indicated strain in YNB + 1% maltose supplemented with the indicated concentration of 2-deoxyglucose relative to growth after 4 days of the indicated strain in YNB + 1% maltose. **(C)** Growth after 6 days of the indicated strain in YNB + 1% trehalose supplemented with the indicated concentration of 2-deoxyglucose relative to growth after 6 days of the indicated strain in YNB + 1% trehalose. **(D)** Growth after 4 days of the indicated strain in YNB + 1% fructose supplemented with the

795 indicated concentration of 2-deoxyglucose relative to growth after 4 days of the indicated strain
796 in YNB + 1% fructose. **(E)** Growth after 6 days of the indicated strain in YNB + 1% xylose
797 supplemented with the indicated concentration of 2-deoxyglucose relative to growth after 6 days
798 of the indicated strain in YNB + 1% xylose. **(F)** Growth after 5 days of the indicated strain in
799 YNB + 1% proline supplemented with the indicated concentration of 2-deoxyglucose relative to
800 growth after 5 days of the indicated strain in YNB + 1% proline. **(G)** Growth after 7 days of the
801 indicated strain in YNB + 1% Tween-80 supplemented with the indicated concentration of 2-
802 deoxyglucose relative to growth after 7 days of the indicated strain in YNB + 1% Tween-80. **(H)**
803 Growth after 3 days of the indicated strain in YNB + 1% glucose supplemented with the
804 indicated concentration of allyl alcohol relative to growth after 3 days of the indicated strain in
805 YNB + 1% glucose. *** $p < 1E-9$ *cbr1*Δ vs WT and *cbr1*Δ vs *cbr1*Δ+*CBR1* as determined by two-
806 way ANOVA with a TukeyHSD post-hoc test.
807

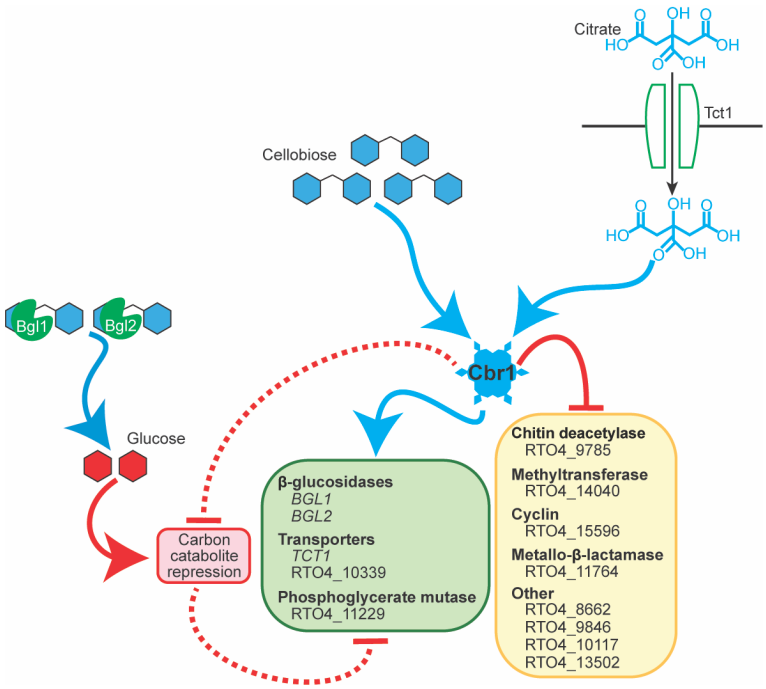

Fig 6. Model for Cbr1-mediated regulation of cellobiose utilization, TCA cycle intermediate utilization, and carbon catabolite repression. Cbr1 is activated by the presence of cellobiose and TCA cycle intermediates. Activated Cbr1 activates the expression of genes encoding β-glucosidases, transporters, and a phosphoglycerate mutase and represses the expression of genes encoding a chitin deacetylase, methyltransferase, cyclin, metallo-β-lactamase, and several hypothetical proteins. The Cbr1-regulated transporter *TCT1* is required for citrate utilization. The two β-glucosidase genes *BGL1* and *BGL2* encode secreted β-glucosidases that cleave cellobiose into two glucose molecules. These glucose molecules activate carbon catabolite repression, which likely represses the expression of genes necessary to utilize cellobiose and TCA cycle intermediates. Cbr1 plays a role in inhibiting carbon catabolite repression, specifically when a disaccharide is present as the nonpreferred carbon source. Solid lines indicate a genetic interaction for which direct genetic or biochemical data exists. Dotted lines indicate a genetic interaction for which indirect genetic data exists.