Development and applications of a CRISPR activation system for facile genetic overexpression in *Candida albicans*

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Abstract

For the fungal pathogen *Candida albicans*, genetic overexpression readily occurs via a diversity of genomic alterations, such as aneuploidy and gain-of-function mutations, with important consequences for host adaptation, virulence, and evolution of antifungal drug resistance. Given the important role of overexpression on *C. albicans* biology, it is critical to develop and harness tools that enable the analysis of genes expressed at high levels in the fungal cell. Here, we describe the development, optimization, and application of a novel, single-plasmid-based CRISPR activation (CRISPRa) platform for targeted genetic overexpression in *C. albicans*, which employs a guide RNA to target an activator complex to the promoter region of a gene of interest, thus driving transcriptional expression of that gene. Using this system, we demonstrate the ability of CRISPRa to drive high levels of gene expression in *C. albicans*, and we assess optimal guide RNA targeting for robust and constitutive overexpression. We further demonstrate the specificity of the system via RNA sequencing. We highlight the application of CRISPRa to overexpress genes involved in pathogenesis and drug resistance and contribute towards the identification of novel phenotypes. Together, this tool will facilitate a broad range of applications for the study of *C. albicans* genetic overexpression.

Keywords

Fungal genetics, CRISPR activation, Candida albicans, Gene regulation, Fungal pathogens

43 **Introduction**

44 Fungal pathogens are an increasingly important cause of human illness, with significant
45 impacts on morbidity and mortality (1). *Candida albicans* is an opportunistic fungal pathogen
46 that inhabits the microbiome of most healthy adults as a commensal organism (2, 3). However,
47 it is also responsible for invasive infections, particularly amongst immunocompromised
48 individuals, which are often fatal (4). Instances of *C. albicans* infections are becoming more
49 prevalent globally, in part due to an increasing population of susceptible elderly and
50 immunocompromised individuals (4). Illustratively, systemic *Candida* infections have been
51 identified in up to 15% of patients hospitalized for COVID-19, and patients with invasive
52 candidiasis spent longer in the hospital Intensive Care Unit (ICU) on average, and had more
53 severe symptoms than those COVID-19 patients without a *Candida* infection (5). The economic
54 cost of *Candida* infections is estimated to be ~1.4 billion dollars yearly in the United States
55 alone, hospitalizing more than 26,000 people (6). With limited development of novel antifungal
56 drugs (7) and increasing prevalence of antifungal drug-resistant strains and species (8–10),
57 *Candida* pathogens remain a serious threat to human health.

58 Given the importance of *C. albicans* and other fungal pathogens, it is imperative that
59 tools are developed and applied to manipulate these organisms and dissect genetic functions.
60 For *C. albicans*, along with countless other microbial species, CRISPR-based genetic
61 manipulation tools have revolutionized the ability of scientists to modify and study gene function
62 (11, 12). Such CRISPR platforms typically rely on a CRISPR-associated endonuclease protein,
63 often Cas9, targeted to a genomic region of interest via a single guide RNA (sgRNA), resulting
64 in targeted DNA double-strand breaks (DSBs), and subsequent repair via non-homologous end-
65 joining or homology-directed repair via a donor DNA repair template (13, 14). Since the initial
66 development of CRISPR tools for genetic editing in *C. albicans* (15), numerous systems have
67 been developed to modify this fungal genome with efficiency and specificity (11, 16–24), and to

68 build and screen genetic mutant libraries (24, 25). Together, these gene editing systems have
69 played an important role in providing novel biological insight into this fungal pathogen.
70 In addition to these canonical CRISPR-based genetic modification systems that
71 introduce mutations, insertions, or deletions into the genome, alternative CRISPR technologies
72 have been developed that modulate the expression of *C. albicans* genes (26, 27). These
73 CRISPR systems rely on the inactivation of the Cas9 endonuclease, to generate a nuclease-
74 dead (dCas9) protein, which can be fused to constructs from a transcriptional repressor or
75 activator that generate CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa)
76 systems, respectively (28, 29). These dCas9 fusions, when targeted to the promoter region of a
77 gene via the sgRNA, can repress or activate gene transcription from a gene of interest, and
78 have been widely applied in diverse organisms (14, 30–34). In *C. albicans*, these modified
79 CRISPR systems have enabled the modulation and study of important fungal genes, including
80 essential genes (26, 27, 35).
81 While a majority of CRISPR systems and other genetic manipulation platforms focus on
82 gene mutation, deletion, or downregulation, it is also critical to develop techniques that enable
83 the upregulation or overexpression of genes. Indeed, genetic overexpression in *C. albicans*
84 occurs readily via gene copy number amplification and aneuploidy, and these copy number
85 amplification events are frequently observed in genetically diverse clinical isolates (36–38). The
86 upregulation of gene expression plays a critical role in diverse aspects of fungal biology (39),
87 including the evolution of antifungal drug resistance (40–45), host colonization (46, 47), and
88 virulence (41, 48). Additionally, in other microbial systems, including bacteria, protozoa, and the
89 model yeast *Saccharomyces cerevisiae*, genetic overexpression libraries have proven to be
90 critical tools for identifying targets of drugs with uncharacterized mechanisms of action, as
91 mutants overexpressing the drug target are typically more resistant to the drug in question (49–
92 52). Overexpression screens can also help dissect molecular pathways (53–55). In *C. albicans*,
93 several techniques exist for genetic overexpression (39), including promoter replacement

94 strategies (55–58), the generation of ORFeome collection strains (59, 60), and CRISPR-based
95 methods (27). While these these tools have made important contributions to understanding
96 overexpression in *C. albicans*, there remains a need for simple and rapid methods to efficiently
97 target fungal genes of interest for overexpression, and to do so in diverse strain backgrounds.
98 Here, we introduce a CRISPR-based tool for genetic overexpression in *C. albicans*, to
99 bolster the existing functional genetic toolbox available to manipulate genes in this critical fungal
100 pathogen. Our CRISPRa tool exploits a *C. albicans*-optimized tripartite activator complex fused
101 to dCas9, which was previously developed for highly-efficient transcriptional regulation in *S.*
102 *cerevisiae* (61). This CRISPRa system is distinct from other CRISPR-based gene activation
103 systems for *C. albicans*, as it is an efficient single-plasmid system designed for rapid Golden
104 Gate cloning and facile fungal strain generation, which can be readily applied to diverse strain
105 backgrounds including clinical isolates. We test CRISPRa guide targeting principles based on
106 predicted transcriptional start sites, confirm on-target guide efficiency, and demonstrate the
107 ability of this CRISPRa system to drive high levels of expression of *C. albicans* genes. Using
108 this optimized CRISPRa technique, we validate its ability to drive overexpression of genes with
109 established roles in antifungal drug resistance and biofilm formation. Together, this work
110 introduces a novel CRISPRa system for genetic overexpression in *C. albicans*, with a wide
111 range of future applications.
112
113 **Results**
114 ***Development of a single-plasmid CRISPRa tool in C. albicans***
115 First, we designed a single-plasmid CRISPRa system for targeted gene activation,
116 optimized for *C. albicans*. We exploited an integrating plasmid backbone, which we previously
117 used for CRISPRi in *C. albicans*, containing a codon-optimized dCas9 and Golden Gate
118 assembly-compatible sgRNA cloning site (26). This plasmid can be linearized to integrate at the
119 *C. albicans NEUT5L* locus (a large intergenic region whose disruption does not affect fungal

120 fitness (62)), and contains a dual SapI cloning site for facile and highly-efficient Golden Gate
121 cloning of the sgRNA targeting sequence. This plasmid also contains a dominant nourseothricin
122 resistance (NATr) cassette, to enable strain generation in a diversity of fungal strains, including
123 clinical isolates.

124 To generate a version of dCas9 that could lead to strong constitutive activation of genes
125 of interest, we generated a plasmid with dCas9 fused to the tripartite activator complex, VP64-
126 p65-Rta (VPR) (**FIGURE 1a,b**). VPR consists of three previously-described transcriptional
127 activators: (1) VP64, a viral transcriptional activator domain composed of four tandem copies of
128 VP16 (Herpes Simplex Viral Protein 16), which has been used extensively in CRISPRa systems
129 (63); (2) p65, the NF-κB subunit responsible for the strong transcription activation in mammalian
130 cells (64); and (3) Rta, the Epstein-Barr virus R transactivator, with strong transcriptional
131 activation properties (65) (**FIGURE 1a,b**). Together the VPR activator complex fused to dCas9
132 and targetted to the promoter region of genes has previously been demonstrated to generate
133 strong gene activation in *S. cerevisiae* (61). Exploiting the ability of dCas9 to bind and be
134 targeted to genomic loci via sgRNAs enables us to design sgRNAs that will target dCas9 along
135 with these strong transcriptional activators to any gene promoter of interest.

136 This plasmid was generated by homology-based cloning and validated via Sanger
137 sequencing, and has been deposited to Addgene (**TABLE S1**, Addgene plasmid #182707). To
138 confirm if the expression of the dCas9-VPR construct had an impact on fungal fitness, we
139 compared the growth of a wild-type *C. albicans* strain with a strain transformed with the dCas9-
140 VPR CRISPRa plasmid (expressing a non-targeting sgRNA: a random sequence of nucleotides
141 that have no predicted complementarity to any region in the *C. albicans* genome). We found no
142 difference in growth between these strains (**FIGURE 1c**), suggesting no significant impact of the
143 CRISPRa system on *C. albicans* fitness.

144

145 ***Validation and sgRNA design principles for CRISPRa overexpression in C. albicans***

146 Next, we aimed to validate that the CRISPRa system could successfully overexpress
147 genes in *C. albicans*, and sought to determine the optimal sgRNA design strategy to maximize
148 genetic regulation. Our previous *C. albicans* CRISPRi system exploited sgRNAs ~ 50 - 150bp
149 upstream of the start codon to functionally repress genes of interest (26). However, CRISPRa
150 systems are known to have different optimal sgRNA targeting regions (66, 67), and our previous
151 design did not take into account the transcription start site (TSS), which is known to be an
152 important predictor of regulatory efficiency for CRISPRi/a systems (66, 68), but which had not
153 been robustly characterized across all *C. albicans* genes at the time. More recently, the TSS for
154 many *C. albicans* genes has been predicted (69). Thus, we sought to determine the optimal
155 design for CRISPRa sgRNAs based on the start codon and the TSS. We identified three *C.*
156 *albicans* genes with a predicted TSS that was particularly far upstream of the start codon
157 compared to all other genes in the genome (801-952 bp upstream): *KSP1*, *ALS3,* and *FGR28* .
158 We designed six sgRNAs for each gene, targeting upstream of the start codon (as previously
159 designed for CRISPRi (26)), as well as upstream of the TSS (based on predicted TSS (69)).
160 We generated CRISPRa plasmids based on these sgRNAs, used these to create *C.*
161 *albicans* strains, and monitored overexpression of each gene via quantitative reverse-
162 transcription PCR (RT-qPCR). This analysis validated our novel CRISPRa system and its ability
163 to overexpress *C. albicans* genes ~ 2 - 12-fold, based on different sgRNA designs and target
164 genes (**FIGURE 2**). We found that in some instances, such as for *KSP1*, the TSS was in fact a
165 better predictor of CRISPRa overexpression, as the most robust overexpression of this gene
166 was observed with sgRNAs targetted upstream of the TSS, while no overexpression was
167 observed based on targeting upstream of the start codon (**FIGURE 2a**). In other instances, such
168 as for *ALS3*, sgRNA targeting upstream of the start codon led to very high levels of
169 overexpression, while sgRNAs targeted to the TSS region led to moderate overexpression
170 (**FIGURE 2b**). For *FGR28*, modest overexpression (~2-fold) was achieved with sgRNAs
171 targeted to either the start codon or the TSS upstream regions (**FIGURE 2c**), together

172 suggesting that optimal sgRNA design can be predicted based on targeting the TSS and/or start
173 codon, but varies by gene. This data also suggests that our CRISPRa tool may have additional
174 applications in the functional assessment of key transcriptional regulatory regions in *C. albicans*.
175 These differences in sgRNA targeting may be based on differences in overall sgRNA efficiency
176 or may be attributed to inaccurate TSS predictions, or complex promoter dynamics and the
177 preferential use of different TSS regions in different conditions (70).
178
179 ***Use of CRISPRa to study antifungal drug resistance phenotypes***
180 To validate that the CRISPRa system could recapitulate phenotypes associated with *C.*
181 *albicans* gene overexpression, we targeted genes with known phenotypes associated with high
182 levels of expression. Initially, we focused on *CDR1*, a well-characterized *C. albicans* gene,
183 which encodes a well-characterized drug efflux pump (71), and whose overexpression is known
184 to play a role in drug resistance to azole antifungals in laboratory and clinical isolates (72–78).
185 To determine an optimal range in which to target our sgRNA in the *CDR1* promoter, we
186 designed three sgRNAs targeting loci approximately 90 bp, 200 bp, and 290 bp upstream of the
187 *CDR1* TSS. These sgRNAs were each cloned into our CRISPRa dCas9-VPR backbone and
188 transformed into *C. albicans*. Next, we quantified the expression of *CDR1* in the three different
189 fungal strains via RT-qPCR, compared with a control strain containing the CRISPRa plasmid
190 with a non-targeting sgRNA. We found that targeting the dCas9-VPR construct to the *CDR1*
191 promoter enhanced transcription by 2.9 - 5.6 fold (**FIGURE 3a**), indicating that this CRISPRa
192 system is indeed able to drive enhanced levels of gene expression from a target gene of
193 interest. For *CDR1*, we found the highest level of overexpression was obtained by targeting the
194 dCas9-VPR construct ~200 bp upstream of the TSS (**FIGURE 3a**), which helped inform the
195 design of sgRNAs for subsequent target genes.
196 In order to determine if *CDR1*-overexpressing strains had measurable changes in
197 antifungal drug resistance phenotypes, we performed minimum inhibitory concentration (MIC)

198 assays with these strains in the presence of the antifungal drug fluconazole. We found that
199 strains overexpressing *CDR1* exhibited increased resistance to fluconazole compared with the
200 control strain and that higher levels of overexpression corresponded with higher levels of
201 resistance (**FIGURE 3b**). Together, this confirms that our CRISPRa system can be exploited to
202 functionally overexpress target genes of interest, and recapitulate phenotypes associated with
203 high levels of gene expression. Further, targeting dCas9-VPR to different promoter loci leads to
204 variable levels of overexpression, which could be used to study correlations between expression
205 levels and associated phenotypes.
206 We next sought to determine the ability of our CRISPRa system to be employed to
207 characterize a mutant strain that, to our knowledge, has never been profiled with regard to
208 antifungal drug susceptibility. We selected the gene *YAK1* as a target for overexpression with
209 CRISPRa. *C. albicans YAK1* is a predicted serine-threonine protein kinase, inactivation of which
210 renders cells more sensitive to the antifungal amphotericin B (79). While *YAK1* overexpression
211 has been shown to promote filamentation in *C. albicans* (80), and there is emerging evidence
212 for a direct relationship between the two phenotypes (81), the role of overexpressing this factor
213 in antifungal drug resistance has not previously been established. We generated a CRISPRa
214 strain targeting *YAK1* for overexpression (**FIGURE 3c**) and profiled the ability of this strain to
215 grow in the presence of amphotericin B. We found that the *YAK1* CRISPRa strain was more
216 resistant to amphotericin B based on MIC testing on this strain compared to the control strain
217 (**FIGURE 3d**). This highlights our capacity to exploit this CRISPRa system to investigate novel
218 phenotypes in *C. albicans*.
219
220 ***Validation of CRISPRa overexpression targeting fungal pathogenesis traits***
221 Next, we aimed to validate the CRISPRa system as a means to assess *C. albicans*
222 genes involved in fungal pathogenesis traits. We focused on *ALS1*, a well-characterized *C.*
223 *albicans* adhesin gene, with important roles in adherence, biofilm formation, and virulence (25,

224 82–86). Previous work has demonstrated that overexpression of *ALS1* via promoter
225 replacement increases fungal adherence (83), therefore we chose to overexpress this gene
226 using our CRISPRa system. We designed and cloned two sgRNAs targeting approximately
227 130bp and 260bp upstream of the *ALS1* TSS, and generated *C. albicans ALS1* CRISPRa
228 strains with each of these constructs. We performed RT-qPCR to confirm the overexpression of
229 these strains, which enhanced the expression of *ALS1* by ~25 and ~6 fold, respectively
230 (**FIGURE 4a**).

231 To confirm phenotypes associated with overexpression of *ALS1*, we performed two
232 distinct biofilm growth assays that compared the *ALS1* CRISPRa strain to the CRISPRa control
233 strain. In the first assay, we allowed biofilms to establish over 24h in flat-bottom 96-well
234 polystyrene plates, removed planktonic cells, and quantified the metabolic activity of the biofilm
235 via XTT reduction, relative to the planktonic cell population. We found that the *ALS1* CRISPRa
236 strain formed significantly more robust biofilms, relative to the control strain ($P < 0.0005$;
237 **FIGURE 4b**). Interestingly, biofilm growth was not correlated with *ALS1* overexpression levels,
238 as the strain with ~25-fold *ALS1* overexpression formed less robust biofilms compared with the
239 strain expressing ~6-fold more *ALS1*. This suggests that very high levels of *ALS1* may
240 ultimately impair biofilm formation, or that the level of *ALS1* expression varies in the conditions
241 used for RT-qPCR analysis compared with biofilm growth, perhaps due to condition-specific
242 repositioning in the TSS.

243 Next, to mimic conditions associated with fungal biofilm formation in catheter-associated
244 urinary tract infections (CAUTIs), we used a urine-based assay and analyzed biofilm formation
245 of a *C. albicans* strain overexpressing *ALS1*, relative to its control strain, using crystal violet
246 staining. For this assay, we followed up on the *ALS1* CRISPRa strain with ~6-fold
247 overexpression of *ALS1*, which had more robust biofilm growth than the control strain (**FIGURE**
248 **4a,b**). The *C. albicans* strains were incubated for 48h in human urine supplemented with bovine
249 serum albumin (BSA) in fibrinogen-coated 96-well plates. Fibrinogen has been shown to be a

250 critical protein for pathogen binding in CAUTIs, by providing a scaffold for biofilm formation and
251 a nutritional source for pathogens (87–89). The *ALS1* CRISPRa overexpression strain showed
252 significantly greater biofilm formation compared to the corresponding wild-type strain ($P <$
253 0.0001; **FIGURE 4c**). This difference in biofilm formation suggests that *ALS1* promotes binding
254 to fibrinogen and plays a vital role in biofilm formation in urine.

255 To assess the specificity of this CRISPRa system and ensure no off-target effects from
256 the dCas9-sgRNA complex, we monitored global changes in transcription in the *ALS1* CRISPRa
257 strain via RNA-sequencing (RNA-seq). Since *ALS1* is a member of the large agglutinin-like
258 sequence (ALS) gene family (90, 91), it may be a likely candidate for any potential off-target
259 effects. We also considered *ALS1* as a strong candidate for this RNA-seq off-target profiling
260 since *ALS1* is not known to be a transcriptional regulator, and therefore its overexpression is
261 unlikely to lead to direct downstream transcriptional alterations that would obscure our analysis
262 of CRISPRa off-target effects. Therefore, we performed RNA-seq on the validated CRISPRa
263 strain overexpressing *ALS1* by ~25-fold as well as on a control strain containing the dCas9
264 cassette and a non-targeting sgRNA when grown in standard laboratory conditions. This
265 analysis confirmed the specific overexpression of *ALS1* (log2 fold change ~3.3 via RNA-seq
266 analysis, adjusted *P*-value 3.07E-142; **FIGURE 4d, TABLE S2**). Five additional transcripts were
267 identified as significantly modulated (adjusted *P*-value < 0.05, absolute log2 fold change > 1;
268 **TABLE S2**). However, their altered expression was much less significant than *ALS1* (**FIGURE**
269 **4d**), none of them having an adjusted P-value of less than 0.03, differing from the adjusted P-
270 value of 3e-142 for *ALS1.* We suspect these additional transcripts' altered expression is likely
271 unrelated to the CRISPRa system based on the fact that 4/5 genes were down-regulated
272 instead of up-regulated, and none of the genes are in proximity to the *ALS1* locus, nor do they
273 appear to share any sgRNA target similarities in their promoter regions. Together this suggests
274 that this CRISPRa system is likely specific with regards to gene targeting.
275

276 **Discussion**

277 Here, we present the development, validation, and application of a single-plasmid-based
278 CRISPRa platform for efficient genetic overexpression in *C. albicans*. We demonstrate the
279 ability of this system to overexpress genes ~2-20-fold, and recapitulate important biological
280 phenotypes associated with genetic overexpression, including biofilm formation and antifungal
281 drug resistance. In addition, we assess sgRNA design principles based on predicted TSS, and
282 validate the specificity of this system by monitoring off-target effects via RNA-seq. Together, this
283 work demonstrates an effective, efficient, and specific tool for genetic overexpression in *C.*
284 *albicans* to enhance the available techniques for genetic modulation in this important fungal
285 pathogen.

286 Studying genetic overexpression is an important strategy for functional genetic analysis,
287 as overexpressing genes can mimic natural biological states where genes are expressed at high
288 levels. In *C. albicans*, there are numerous scenarios in which the overexpression of genes
289 occurs, including mutations that modify the function of transcriptional regulators and promote
290 the expression of downstream genes (36, 92), as well as aneuploidies such as trisomies (38,
291 93–98), isochromosome formation (40, 94), and copy number variations (45, 93). These
292 overexpression events are frequently detected amongst *C. albicans* clinical isolates (93, 94, 98)
293 and can play an important role in drug resistance (40, 45, 94, 95), host adaptation (38, 99), and
294 other stress tolerance phenotypes (100, 101). For example, amplification of part or all of
295 chromosome 5, via trisomy or isochromosome formation has been tightly linked to the
296 development of resistance to azole antifungals in *C. albicans* via the increased copy number of
297 genes encoding the azole target, Erg11, and the transcriptional regulator of drug efflux, Tac1
298 (40). The prevalence and importance of these phenomena associated with increased gene
299 expression highlight the relevance of developing new tools to targetedly assess phenotypes
300 associated with genetic overexpression in *C. albicans*.

301 Harnessing the cell's natural transcriptional machinery to increase gene expression with
302 CRISPRa also leads to a unique set of obstacles. Off-target effects as a result of promiscuous
303 dCas9 binding in the cell presents the possibility of complications if the dCas9 is also targetting
304 other regions in the genome. While our RNA-seq results suggest that our CRISPRa system is
305 specific when paired with one sgRNA targeted to the *ALS1* gene, there is still the possibility of
306 off-target effects occurring in different CRISPRa constructs, especially as binding specificity with
307 CRISPR-Cas9 systems is highly dependent on the exact sgRNA sequence in use (102). Using
308 complementary genetic tools to confirm phenotypes observed from CRISPRa strains can help
309 mitigate this. One phenomenon shared among many different CRISPRa activation domains,
310 including VPR, is a reduced capacity to induce overexpression in genes that are already
311 expressed at a high levels (103). Incidentally, the gene that we achieved the lowest
312 overexpression with across all of our testing, *FGR28*, had the lowest basal expression (data not
313 shown) from our RT-qPCR experiments, opposing the idea that genes that are normally
314 expressed at low levels tend to be more easily overexpressed with CRISPRa systems.
315 However, efficiency of the sgRNA molecule and its exact position in the target region of the
316 gene of interest, as well as a host of promoter dynamics, can also influence CRISPR-Cas9
317 efficacy (69, 104, 105). This illustrates why multiple sgRNAs need to be designed when
318 targeting a gene with CRISPRa to maximize successful overexpression of the target gene.
319 While the rapid and inexpensive nature of CRISPRa strain construction easily justifies the need
320 to design several sgRNA molecules for any given gene, broader limitations as a result of the
321 specific "NGG" PAM recognition site by traditional *Streptococcus pyogenes* Cas9 proteins, like
322 the one used here, leads to the restraint that on rare occasions it may not be possible to
323 produce any highly-efficient sgRNA molecules in a suitable region of the promoter (11). Since
324 this PAM restriction is a feature shared among many CRISPR-Cas9 systems, it may therefore
325 be prudent to further expand the CRISPR toolbox in *C. albicans* by introducing novel Cas9
326 variants with differing PAM recognition sites into our system, or by constructing novel CRISPR

327 systems exploiting alternative Cas proteins with different and broader PAM recognition
328 capacities, offering the ability to target regions in the genome that cannot be manipulated with
329 current CRISPR-Cas9 tools in *C. albicans* (106, 107).
330 While large-scale CRISPRa libraries have not been generated in *C. albicans*, their
331 application in other organisms, namely mammalian cell lines, has enabled important discoveries
332 in cancer biology, stem cell reprogramming, cellular fitness, and drug resistance (30, 67, 108–
333 113). The development of CRISPRa systems in *C. albicans* could enable the development of
334 larger overexpression libraries for functional genomic screens, to complement existing fungal
335 gene deletion and depletion libraries (114–117) that have been subject to robust screening and
336 analysis under diverse conditions (118), to reveal critical new insight into fungal biology (119,
337 120), pathogenesis (115, 120–124), and mechanisms of drug and stress tolerance (125–128).
338 Currently, non-CRISPR-based overexpression libraries have been constructed and
339 screened in *C. albicans*, revealing novel insights into gene function related to hyphal
340 morphogenesis (60), cell cycle progression (129), drug tolerance (130), and biofilm formation
341 (131). Previous comparisons done between CRISPRa and ORF-based overexpression libraries
342 such as these in other species have highlighted the potential for these two complementary
343 strategies to both confirm the results from one another as well as unveil significant genetic hits
344 distinct from each other, even when libraries share similar gene targets (67). Compared with
345 other overexpression methods, the ability of the CRISPRa system to target any specified region
346 in the genome also confers the unique and powerful ability of allele-specific targeting which
347 could potentially be used to study heterozygosity in *C. albicans*, an aspect of its genomic
348 makeup that is critical to its pathogenicity (132). CRISPRa also offers the advantage of being
349 easily scalable into a pooled format whereby hundreds or thousands of unique plasmids and
350 resulting mutant strains can be constructed simultaneously, and mutant libraries in different
351 strain backgrounds can therefore be quickly constructed (133, 134). Non-CRISPR
352 overexpression libraries that have previously been assembled in *C. albicans* have the potential

353 weakness of missing important phenotypes during screening if the level of overexpression
354 achieved in each mutant strain is too low (46). The ability of our system to cause upwards of 25-
355 fold overexpression in gene targets is thus an important feature. Furthermore, corresponding
356 phenotypes in mutant strains may only be observable if the level of overexpression closely
357 mimics the level of upregulation of the gene induced by the cell in other relevant stress
358 conditions naturally (46). Future iterations of this CRISPRa system could also exploit an
359 inducible dCas9-VPR construct for tunability of gene expression. It is therefore imperative to
360 employ tools such as CRISPRa that possess the advantage of being inherently flexible and
361 easily tunable in order to study genetic overexpression thoroughly.

362 The application of similar CRISPRa systems to other fungal organisms would lend
363 further insight into fungal biology across this kingdom. Many CRISPR tools have been
364 developed in a diversity of fungal organisms (17, 22, 135–139), including numerous non-
365 *albicans Candida* species (11, 140–146), and other prominent pathogens such as *Cryptococcus*
366 *neoformans* (147–149) and *Aspergillus fumigatus* (150–153). These CRISPR platforms could be
367 adapted to similar CRISPRa tools in these organisms, with many possible applications for the
368 analysis of pathogen biology or drug target identification (49–52, 154).

369 Genetic overexpression systems also have many useful applications for industrially-
370 important fungi, as targeted genetic overexpression can be exploited for metabolic engineering
371 to enhance the production of commercially-important metabolites (136, 155–157). CRISPR
372 techniques have already been adapted to numerous industrially-relevant fungi (157–160), and
373 CRISPRa has recently been optimized for use in the filamentous fungi *Aspergillus nidulans* and
374 *Penicillium rubens* (161, 162). These CRISPRa systems were applied to activate
375 transcriptionally-silent biosynthetic gene clusters in these filamentous fungi, with important
376 applications for the discovery of novel bioactive molecules (161, 162). As many *Candida*
377 species play important industrial roles in manufacturing metabolites for food and pharmaceutical
378 industries, as well as remediating wastewater via hydrocarbon degradation (163–167), species-

379 specific adaptations to the CRISPRa tool presented here could produce optimized *Candida*
380 strains for valuable biomanufacturing and bioremediation applications.
381
382 **Materials and Methods**
383 ***Plasmid design and cloning***
384 The plasmid backbone used in this study was the *C. albicans*-optimized CRISPR-dCas9
385 plasmid (pRS143, Addgene #122377) used in our previous study (26), containing the *NEUT5L*
386 integration site, sgRNA cloning site (*SNR52* promoter, SapI cloning locus, and sgRNA tail), and
387 *dCAS9*. The dCas9-VPR fusion construct was generated via Gibson assembly. The VPR
388 tripartite complex was codon-optimized for *C. albicans* expression and synthesized as gBlocks
389 gene fragments from Integrated DNA Technologies (IDT). This gene fragment was cloned with
390 Gibson assembly into the *dCAS9* plasmid backbone. We have made the CRISPRa (dCas9-
391 VPR) plasmid available via Addgene (reference #182707). All plasmids are listed in **TABLE S1.**
392
393 ***sgRNA design and cloning***
394 The sgRNA CRISPR RNAs (crRNA; 20 nucleotide sequence complementary to the target DNA
395 genomic DNA) were designed based on efficiency and predicted specificity via the sgRNA
396 design tool Eukaryotic Pathogen CRISPR gRNA Design Tool (EuPaGDT;
397 http://grna.ctegd.uga.edu) (168). These sgRNA crRNA N20 sequences were cloned into the
398 dCas9-VPR CRISPRa plasmid at the sgRNA cloning locus (SapI cloning site, flanked by *SNR52*
399 promoter, and sgRNA tail) using Golden Gate cloning (169), as previously described (35, 170).
400 crRNA N20 sequences were obtained as two oligonucleotides from IDT in forward and reverse
401 complement orientation, each containing a SapI cloning site, and were reconstituted to 100μM
402 in a nuclease-free duplex buffer from IDT. Equal volumes of the two complementary
403 oligonucleotides were combined after being heated separately at 94°C, and then duplexed by
404 heating to 94°C for 2 min and cooling to room temperature. The duplexed fragment was then

405 cloned into the CRISPRa plasmid with the following Golden Gate cloning reaction: 10µl
406 miniprepped CRISPRa plasmid, 1µl duplexed oligonucleotide, 2µl 10X CutSmart buffer, 2µl
407 ATP, 1µl SapI, 1µl T4 DNA ligase, and 3µl nuclease-free water. This mixture was incubated in a
408 thermocycler under the following cycling conditions: (37°C, 2 min; 16°C, 5 min) for 99 cycles;
409 65°C, 15 min; 80°C, 15 min. After cycling, 1µl of additional SapI was added to each reaction
410 mixture, and the reaction was incubated at 37°C for 1h. All sgRNAs are listed in **TABLE S1.**
411
412 ***Media and growth conditions***
413 *Escherichia coli* DH5α cells were grown at 37°C in Lysogeny Broth (LB) and LB plates
414 supplemented with 100mg/mL ampicillin (AMP) and 250mg/mL nourseothricin (NAT) for plasmid
415 selection. *C. albicans* cells were grown at 30°C or 37°C in Yeast Peptone Dextrose (YPD) broth
416 and YPD plates supplemented with 250mg/mL nourseothricin (NAT) for plasmid selection.
417
418 ***Bacterial transformation***
419 High Efficiency 5-alpha Competent *E. coli* (NEB) cells were thawed on ice. 1µl of Golden Gate
420 cloning reagents were added to 50µl of competent cells and incubated on ice for 30 min, heat
421 shocked for 30s at 42°C, and incubated on ice for an additional 5 min. 950µl of SOC outgrowth
422 media was added to each cell culture and incubated at 30°C for 1.5h at 250 RPM. Transformed
423 cells were then plated on LB media containing AMP and NAT and grown at 30°C for 1 day.
424
425 ***C. albicans transformation***
426 Plasmids were transformed into *C. albicans* via a chemical transformation strategy, as
427 previously described (35, 170). Briefly, miniprepped CRISPRa plasmids were linearized via a
428 1.5X restriction digest mix using the PacI enzyme. A transformation master mix was prepared
429 as follows: 800µl of 50% polyethylene glycol (PEG), 100µl of 10X Tris-EDTA (TE) buffer
430 solution, 100µl of 1M lithium acetate (LiAc), 40µl of 10 mg/mL salmon sperm DNA, and 20µl of

431 1M dithiothreitol (DTT). The transformation mix was added to *C. albicans* cells and linearized
432 CRISPRa plasmid and left to incubate at 30°C for 1h, then heat-shocked at 42°C for 45 min.
433 Cells were washed with fresh YPD, then grown in YPD for 4h to allow for expression of the NAT
434 resistance construct. Transformed cells were then plated on YPD media containing NAT, and
435 grown at 30°C for 2 days. All *C. albicans* strains are listed in **TABLE S1.**
436
437 ***Growth curves***
438 Overnight cultures of *C. albicans* grown in YPD were diluted to an OD600 of 0.05 in fresh YPD
439 and added to a 96-well flat-bottomed plate with a total volume of 200µl per well. Growth was
440 measured at 30°C via optical density at 600nm at 15 min intervals over the course of 24h using
441 an Infinite 200 PRO microplate reader (Tecan), and plates were shaken orbitally for 900s at a
442 4mm amplitude in between growth measurements.
443
444 ***Minimum inhibitory concentration (MIC) assays***
445 Fluconazole MIC assays were performed in 96-well flat-bottomed plates. A suspension of
446 40µg/mL fluconazole was prepared in water, of which 100µl was added to the first column of
447 each plate containing 100µl of culture, to obtain a total volume of 200µl per well. The first
448 column was serially diluted 2-fold across the plate in water. The gradient of fluconazole,
449 therefore, ranged from 20µg/mL to 0µg/mL. Overnight cultures of *C. albicans* grown in YPD
450 were diluted to an OD600 of 0.1 in 2X RPMI 1640 with 40g/L of added D-glucose and mixed into
451 the plates in an equal volume such that the starting OD600 values of each strain were 0.05 and
452 the growth media was diluted to RPMI 1640 with 20g/L of added D-glucose. Plates were
453 incubated at 37°C at 900 RPM, and absorbance values at 600nm were read after 24h using an
454 Infinite 200 PRO microplate reader (Tecan). The amphotericin B MIC assays were also
455 performed in 96-well flat-bottomed plates similar to the protocol described above with a few
456 modifications. A starting concentration of 5µg/mL of amphotericin B was used to create a

457 gradient ranging from 2.5µg/mL to 0µg/mL. Strains were instead grown in YPD media using a
458 starting OD600 of 0.001. Plates were incubated at 37°C statically and absorbance values at
459 600nm were read after 72h using an Infinite 200 PRO microplate reader (Tecan).
460
461 ***Biofilm assays***
462 RPMI-based biofilm assays were performed as previously described (171), with minor
463 modifications. Overnight cultures of *C. albicans* grown in YPD were diluted to an OD600 of
464 0.001 in 5mL of RPMI 1640 with 20g/L of supplemented D-glucose. 100µl of RPMI 1640 was
465 mixed with 100µl of each diluted overnight culture into a 96-well flat-bottomed plate (12 wells
466 per strain). Plates were grown statically at 37°C for 24h to allow biofilms to form. 120µl of
467 planktonic cell supernatant was removed from each well and moved to a new 96-well flat-
468 bottomed plate. Density of the planktonic cells was measured at 600nm using an Infinite 200
469 PRO microplate reader (Tecan). Original plates from which planktonic cells were removed were
470 washed by adding 200µl 1X PBS to each well with an electronic multichannel pipette at a
471 moderate dispense speed. 1X PBS was then discarded, and this wash step was repeated.
472 Plates were then left to dry for 1 - 2h. Once biofilms were dry, 90µl of 1mg/mL XTT (prepared in
473 1X PBS and centrifuged to remove sediment prior to use) and 10µl of 0.32mg/mL PMS
474 (prepared in water) were added to each well. Plates were incubated statically at 30°C for 2h to
475 allow biofilms to reduce XTT, measured at 490nm using an Infinite 200 PRO microplate reader
476 (Tecan), and normalized to the growth of planktonic cells harvested previously.
477 For urine-based biofilm assays, biofilm formations were performed in 96-well flat-
478 bottomed plates. Plates were coated with 150µg/mL of fibrinogen and incubated overnight at
479 4°C. The *C. albicans* strains were cultured at 37°C with aeration in 5mL of YPD broth. The
480 inoculum was normalized to ~$1x10^6$ CFU/ml and then diluted (1:10) in human urine (female, pH
481 ~6.5). Urine was supplemented with 20mg/mL of BSA for carbon and nitrogen sources

482 mimicking the catheterized bladder environment. 200µl of the urine with inoculum was incubated
483 in each well of the 96-well plate at 37°C for 48h while static. Following the 48h incubation, the
484 cultures were removed and the wells were washed three times with 200µl 1X PBS to remove
485 unbound fungi. Plates were incubated with 200µl of 0.5% crystal violet for 15 min. Crystal violet
486 was removed, and plates were washed with water and dried. 200µl of 33% acetic acid was
487 added to the wells and 100µl was transferred to a new 96-well plate. Absorbance was measured
488 at 595nm using a SpectraMax ABS Plus microplate reader (Molecular Devices).
489
490 ***RNA extraction and real-time quantitative PCR***
491 To detect differences in gene expression, overnight cultures of *C. albicans* grown in YPD
492 were diluted to an OD600 of 0.05 in fresh YPD and grown to an OD600 of >0.2 at 30°C.
493 Cultures were pelleted and frozen at -80°C before RNA was extracted. RNA extractions were
494 performed using either a Presto Mini RNA Yeast Kit from FroggaBio (cat. RBYD050) or an
495 RNeasy Mini Kit from Qiagen (cat. 74104). To assess RNA integrity, an RNA ScreenTape assay
496 was performed on all samples using the TapeStation 4150 system following manufacturer's
497 instructions (Agilent Technologies). Only samples with RIN values of >5.0 were used for RT-
498 qPCR. Synthesis of cDNA from 1000ng (10µl) RNA was performed using a High Capacity cDNA
499 Reverse Transcription Kit from Applied BioSystems (cat. 4368814). Briefly, 20µl reaction
500 mixtures were prepared with 2µl 10X reverse transcription buffer, 0.8µl of 25X dNTPs at a
501 concentration of 100mM, 2µl of random primers, 1µl of MultiScribe Reverse Transcriptase at 50
502 U/µl, 4.2µl of nuclease-free water, and 10µl of RNA (1µg). Reverse transcription run conditions
503 were as follows: 10 min at 25°C, 120 min at 37°C, 5 min at 85°C. Real-time PCR assays were
504 conducted using a QuantStudio 7 Pro Real Time PCR system from Thermo Fisher Scientific Inc.
505 20µl reaction mixtures containing 10µl 2X SsoAdvanced Universal Inhibitor-Tolerant SYBR
506 supermix (Bio-Rad, cat: 172-5017), 0.8µl of PCR forward and reverse primer mix at 5µM (final
507 concentration of primer at 200 nM), 4.2µl of water and 5µl of diluted cDNA. The run conditions

508 were as follows: 3 min at 98°C polymerase activation step, followed by 40 cycles of a two-step
509 qPCR (10s of 98°C denaturation, 30s of 60°C combined annealing/extension). Primers are
510 listed in **TABLE S1**. Expression profiling calculations were performed according to the
511 comparative C_T method (172). Briefly, expression values for the experimental gene of interest
512 were compared to the housekeeping gene *ACT1* to obtain a ΔC_T value within each strain. The
513 ΔC_T values in the experimental CRISPRa strains were then compared to the non-targeting
514 CRISPRa control strain to obtain a $\Delta\Delta C_T$ value and finally a fold-difference in expression of the
515 experimental gene.
516
517 ***RNA sequencing***
518 RNA preparation and sequencing were performed as previously described, with minor
519 modifications (171). Strains were grown in YPD at 30°C overnight preceding RNA extraction.
520 RNA extraction, sample QC, library preparations, and sequencing reactions were conducted at
521 GENEWIZ, LLC./Azenta US, Inc. as follows: Total RNA was extracted using Qiagen RNeasy
522 Plus Universal kit following manufacturer's instructions (Qiagen). RNA samples were quantified
523 using Qubit 2.0 Fluorometer (ThermoFisher Scientific) and RNA integrity was checked with
524 4200 TapeStation (Agilent Technologies). ERCC RNA Spike-In Mix kit (cat. 4456740) from
525 ThermoFisher Scientific, was added to normalized total RNA prior to library preparation
526 following the manufacturer's protocol. The RNA sequencing library was prepared using the
527 NEBNext Ultra II RNA Library Prep Kit for Illumina using the manufacturer's instructions (New
528 England Biolabs). Briefly, mRNAs were initially enriched with Oligod(T) beads. Enriched mRNAs
529 were fragmented for 15 min at 94 °C. First-strand and second-strand cDNA were subsequently
530 synthesized. cDNA fragments were end-repaired and adenylated at 3'ends, and universal
531 adapters were ligated to cDNA fragments, followed by index addition and library enrichment by
532 PCR with limited cycles. The sequencing library was validated on the Agilent TapeStation
533 (Agilent Technologies), and quantified by using Qubit 2.0 Fluorometer (ThermoFisher Scientific)

534 as well as by quantitative PCR (KAPA Biosystems). The sequencing libraries were multiplexed
535 and clustered onto a flowcell. After clustering, the flowcell was loaded onto the Illumina HiSeq
536 4000 or equivalent instrument according to the manufacturer's instructions. The samples were
537 sequenced using a 2x150bp Paired End (PE) configuration. Image analysis and base calling
538 were conducted by the Illumina Control Software.

539 Raw sequence data (.bcl files) generated from the Illumina instrument was converted
540 into fastq files and de-multiplexed using Illumina bcl2fastq 2.20 software. One mis-match was
541 allowed for index sequence identification. Sequence reads were trimmed to remove possible
542 adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36, and trimmed
543 reads were mapped to the candida_albicans reference genome available on ENSEMBL using
544 the STAR aligner v.2.5.2b. The STAR aligner is a splice aligner that detects splice junctions and
545 incorporates them to help align the entire read sequences. BAM files were generated as a result
546 of this step. Unique gene hit counts were then calculated by using featureCounts from the
547 Subread package v.1.5.2. The hit counts were summarized and reported using the gene_id
548 feature in the annotation file. Only unique reads that fell within exon regions were counted. After
549 extraction of gene hit counts, the gene hit counts table was used for downstream differential
550 expression analysis. Using DESeq2, a comparison of gene expression between the control and
551 experimental groups of samples was performed. The Wald test was then used to generate p-
552 values and log2 fold changes. Genes with an adjusted p-value < 0.05 and absolute log2 fold
553 change > 1 were called as differentially expressed genes for each comparison. The data have
554 been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar *et al.* 2002) and are
555 accessible through GEO Series accession number GSE201904
556 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201904).
557
558 ***Data availability***

559 The *C. albicans* CRISPRa plasmid backbone was deposited to Addgene as plasmid number
560 #182707. RNA sequencing data was deposited in NCBI GEO repository (accession number
561 GSE201904; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201904).
562

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578
579

580 **Figure Legends**

581 **Figure 1. Design of a CRISPRa system for *C. albicans*. (a)** The single plasmid system
582 contains a catalytically inactive ("dead") dCas9 protein, paired with the VPR (VP64-P65-Rta)
583 complex of activator domains to cause upregulation at a target locus. The single-guide RNA
584 (sgRNA) region can be subjected to Golden Gate cloning with a custom N20 sequence in order
585 to target the dCas9-VPR complex to a desired region of DNA through complementary base-
586 pairing. The PacI enzyme is used to linearize the plasmid at the *NEUT5L* locus, which allows
587 the plasmid to be integrated into the cell's chromosome and remain in the cell following cell
588 division. Panel created with BioRender.com. **(b)** Once the sgRNA recognizes and binds to a
589 target sequence of DNA, the dCas9-VPR complex will cause the recruitment of transcription
590 initiation complex factors to the adjacent genetic material, thus increasing gene expression from
591 that region. Created with BioRender.com. **(c)** Wild-type *C. albicans* cells and cells containing a
592 non-targeting CRISPRa plasmid were grown in YPD at 30°C to compare fitness under standard
593 laboratory conditions. Growth was recorded via optical density at 600nm at 15 min intervals over
594 the course of 24h. Data points show the mean and standard deviation at each time point (n =
595 32).

596

597 **Figure 2. Determining optimal guide-design principles for CRISPRa overexpression in *C.***
598 ***albicans*.** CRISPRa strains were created using six different sgRNA molecules designed to
599 target different positions in the promoter region of each of **(a)** *KSP1*, **(b)** *ALS3*, and **(c)** *FGR28*
600 upstream of either the start codon or the predicted dominant transcriptional start site (TSS).
601 Fold-change in the expression of the target gene relative to the housekeeping gene *ACT1* in the
602 experimental strains and the non-targeting CRISPRa control strain was measured with RT-
603 qPCR and compared. Data points represent the mean fold-difference and standard deviation in
604 expression of the respective target gene in the CRISPRa strains compared to the non-targeting
605 CRISPRa control (n = 3).

606

607 **Figure 3. Using CRISPRa to validate antifungal drug resistance phenotypes. (a)** Three *C.*
608 *albicans* CRISPRa strains were created targeting the drug transporter gene *CDR1* for
609 overexpression. Fold-change in the expression of *CDR1* relative to the housekeeping gene
610 *ACT1* in the three experimental strains and the non-targeting CRISPRa control strain was
611 measured with RT-qPCR. All three experimental strains are represented by the position of their
612 sgRNA in the promoter region upstream of the proposed dominant *CDR1* transcriptional start
613 site. Data represent the mean fold-difference and standard deviation in expression of *CDR1* in
614 the CRISPRa strains compared to the non-targeting CRISPRa control (n = 3). **(b)** The fitness of
615 all three CRISPRa strains targeting *CDR1* for overexpression was measured in the presence of
616 the antifungal drug fluconazole with a minimum inhibitory concentration assay. The OD600
617 value of each strain in each concentration of fluconazole was measured to represent growth in
618 differing conditions, where OD600 values approaching 1.0 reflect strong fitness. **(c)** Fold-change
619 in the expression of *YAK1* relative to the housekeeping gene *ACT1* in the experimental strain
620 and the non-targeting CRISPRa control strain was measured with RT-qPCR. Data represent the
621 mean fold-difference and standard deviation in expression of *YAK1* in the CRISPRa strains
622 compared to the non-targeting CRISPRa control (n = 3). **(d)** The fitness of the CRISPRa strain
623 targeting *YAK1* for overexpression was measured in the presence of the antifungal drug
624 amphotericin B with a minimum inhibitory concentration assay. The OD600 value of each strain
625 in each concentration of amphotericin B was measured to represent growth in differing
626 conditions, where OD600 values approaching 1.0 reflect strong fitness.

627

628 **Figure 4. Validation of CRISPRa overexpression and target-specificity through targeting**
629 **fungal pathogenesis traits. (a)** Two *C. albicans* CRISPRa strains targeting different positions
630 in the promoter region of the adhesin protein gene *ALS1* were created. Fold-change in the
631 expression of *ALS1* relative to the housekeeping gene *ACT1* in both experimental strains and

632 the non-targeting CRISPRa control strain was measured with RT-qPCR. Experimental strains
633 are represented by the position of their sgRNA in the promoter region upstream of the proposed
634 dominant *ALS1* transcriptional start site. Data points represent the mean fold-difference and
635 standard deviation in expression of *ALS1* in the CRISPRa strains compared to the non-targeting
636 CRISPRa control (n = 3). **(b)** Two CRISPRa strains targeting *ALS1* and a non-targeting
637 CRISPRa control strain were grown in the biofilm-inducing media RPMI and left to grow
638 statically at 37°C for 24h to allow biofilms to form. The robustness of biofilms formed by all
639 strains was elucidated through an XTT reduction assay and measuring of the resulting OD490
640 values before normalizing final values to non-biofilm-forming planktonic cells. Data points
641 represent the mean and the standard error of the mean (n = 72). Differences between groups
642 were tested for significance using Dunnett's multiple comparison test. ****, p-value <0.0001. ***,
643 p-value <0.001. **(c)** A *C. albicans* CRISPRa strain overexpressing *ALS1* ~6-fold and a non-
644 targeting CRISPRa strain were grown in human urine supplemented with fibrinogen and BSA at
645 37°C for 48h to simulate a catheterized bladder environment. The robustness of biofilms formed
646 by both strains was measured by reading the OD595 value following a crystal violet assay. Data
647 points are combined from three independent experiments where n = 32 for the non-targeting
648 CRISPRa control and n = 48 for the CRISPRa *ALS1* overexpression strain. Differences
649 between groups were tested for significance using the Mann-Whitney U test. ****, p-value
650 <0.0001. **(d)** Global gene expression of a CRISPRa strain overexpressing *ALS1* ~25-fold, as
651 well as a non-targeting CRISPRa control strain, was measured using RNA-Seq. Differential
652 gene expression analysis showed that *ALS1* had a log2-fold change of ~3.3 in the *ALS1*
653 overexpression strain compared to the CRISPRa control strain, with an adjusted p-value of
654 3.07E-142, and showed no additional transcripts that had differential expression with an
655 adjusted p-value of less than 0.03.
656

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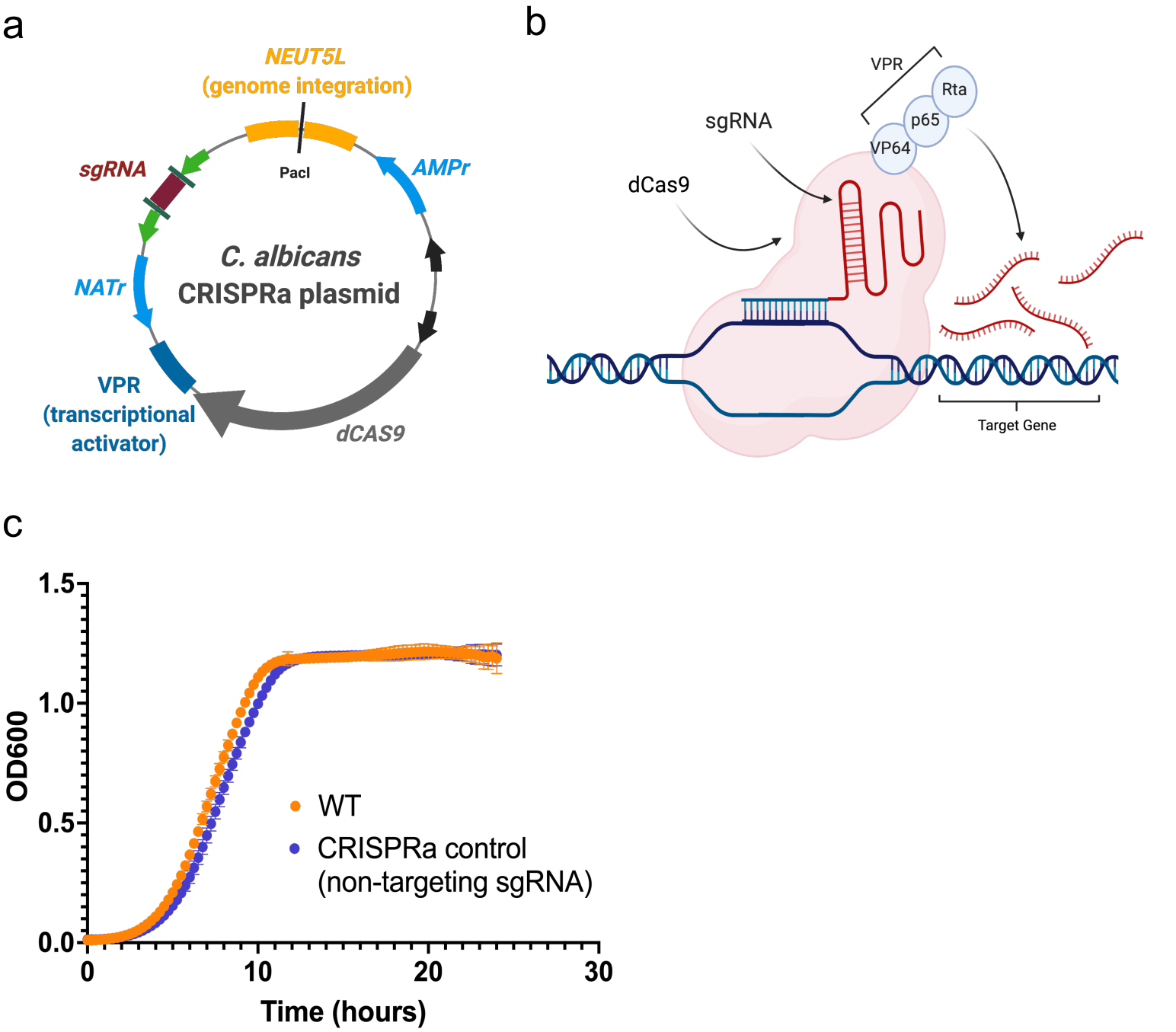

Figure 1. Design of a CRISPRa system for *C. albicans*. (a) The single plasmid system contains a catalytically inactive ("dead") dCas9 protein, paired with the VPR (VP64-P65-Rta) complex of activator domains to cause upregulation at a target locus. The single-guide RNA (sgRNA) region can be subjected to Golden Gate cloning with a custom N20 sequence in order to target the dCas9-VPR complex to a desired region of DNA through complementary base-pairing. The PacI enzyme is used to linearize the plasmid at the *NEUT5L* locus, which allows the plasmid to be integrated into the cell's chromosome and remain in the cell following cell division. Panel created with BioRender.com. **(b)** Once the sgRNA recognizes and binds to a target sequence of DNA, the dCas9-VPR complex will cause the recruitment of transcription initiation complex factors to the adjacent genetic material, thus increasing gene expression from that region. Created with BioRender.com. **(c)** Wild-type *C. albicans* cells and cells containing a non-targeting CRISPRa plasmid were grown in YPD at 30°C to compare fitness under standard laboratory conditions. Growth was recorded via optical density at 600nm at 15 min intervals over the course of 24h. Data points show the mean and standard deviation at each time point (n = 32).

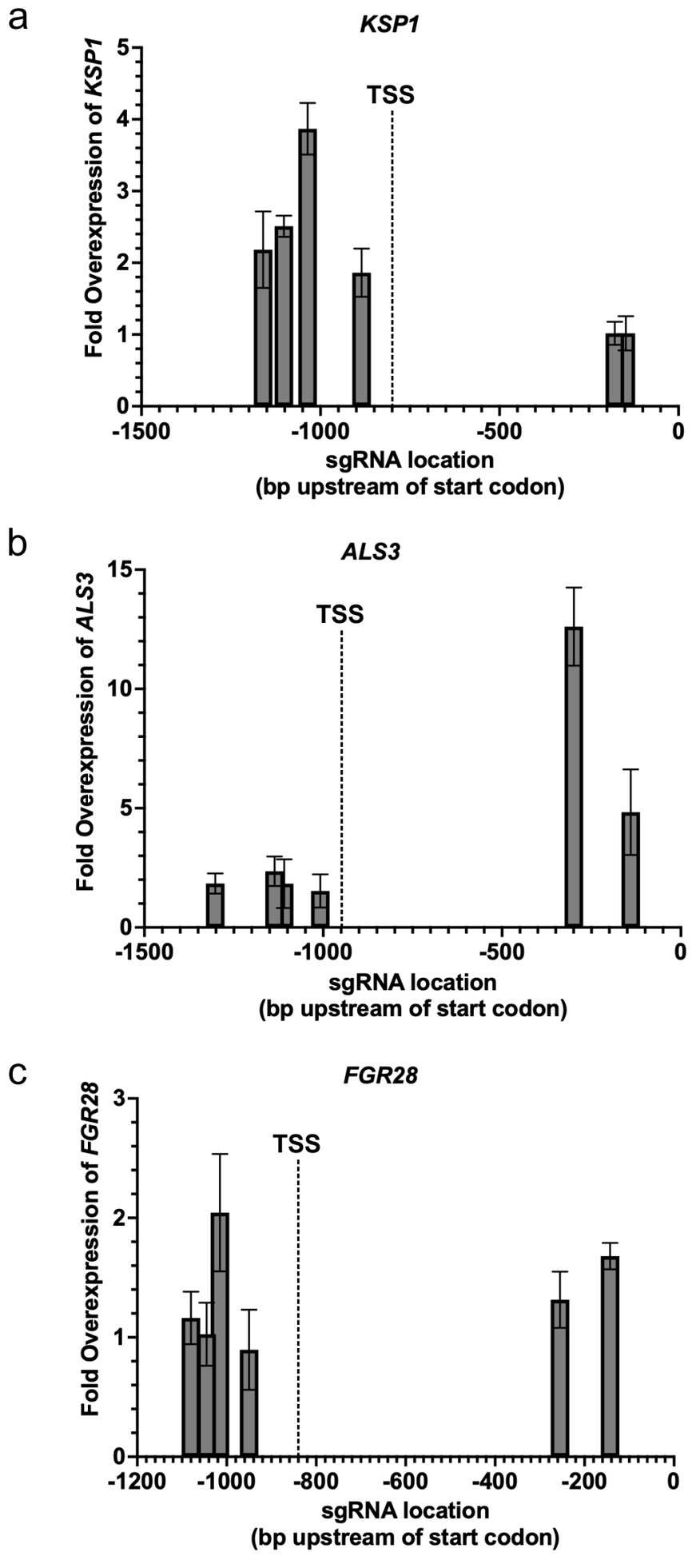

Figure 2. Determining optimal guide-design principles for CRISPRa overexpression in *C. albicans*. CRISPRa strains were created using six different sgRNA molecules designed to target different positions in the promoter region of each of **(a)** *KSP1*, **(b)** *ALS3*, and **(c)** *FGR28* upstream of either the start codon or the predicted dominant transcriptional start site (TSS). Fold-change in the expression of the target gene relative to the housekeeping gene *ACT1* in the experimental strains and the non-targeting CRISPRa control strain was measured with RT-qPCR and compared. Data points represent the mean fold-difference and standard deviation in expression of the respective target gene in the CRISPRa strains compared to the non-targeting CRISPRa control (n = 3).

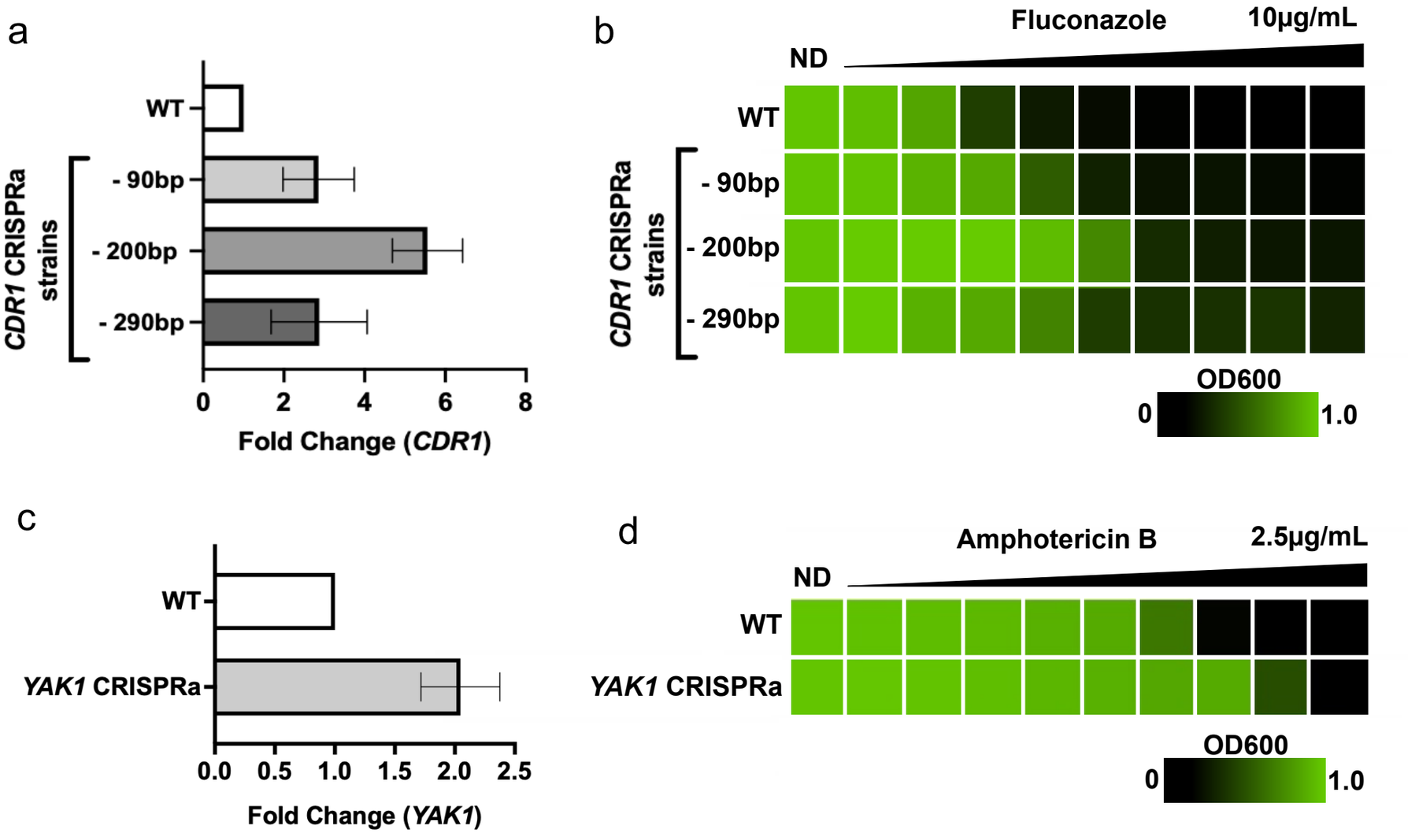

Figure 3. Using CRISPRa to validate antifungal drug resistance phenotypes. (a) Three *C. albicans* CRISPRa strains were created targeting the drug transporter gene *CDR1* for overexpression. Fold-change in the expression of *CDR1* relative to the housekeeping gene *ACT1* in the three experimental strains and the non-targeting CRISPRa control strain was measured with RT-qPCR. All three experimental strains are represented by the position of their sgRNA in the promoter region upstream of the proposed dominant *CDR1* transcriptional start site. Data represent the mean fold-difference and standard deviation in expression of *CDR1* in the CRISPRa strains compared to the non-targeting CRISPRa control (n = 3). **(b)** The fitness of all three CRISPRa strains targeting *CDR1* for overexpression was measured in the presence of the antifungal drug fluconazole with a minimum inhibitory concentration assay. The OD600 value of each strain in each concentration of fluconazole was measured to represent growth in differing conditions, where OD600 values approaching 1.0 reflect strong fitness. **(c)** Fold-change in the expression of *YAK1* relative to the housekeeping gene *ACT1* in the experimental strain and the non-targeting CRISPRa control strain was measured with RT-qPCR. Data represent the mean fold-difference and standard deviation in expression of *YAK1* in the CRISPRa strains compared to the non-targeting CRISPRa control (n = 3). **(d)** The fitness of the CRISPRa strain targeting *YAK1* for overexpression was measured in the presence of the antifungal drug amphotericin B with a minimum inhibitory concentration assay. The OD600 value of each strain in each concentration of amphotericin B was measured to represent growth in differing conditions, where OD600 values approaching 1.0 reflect strong fitness.

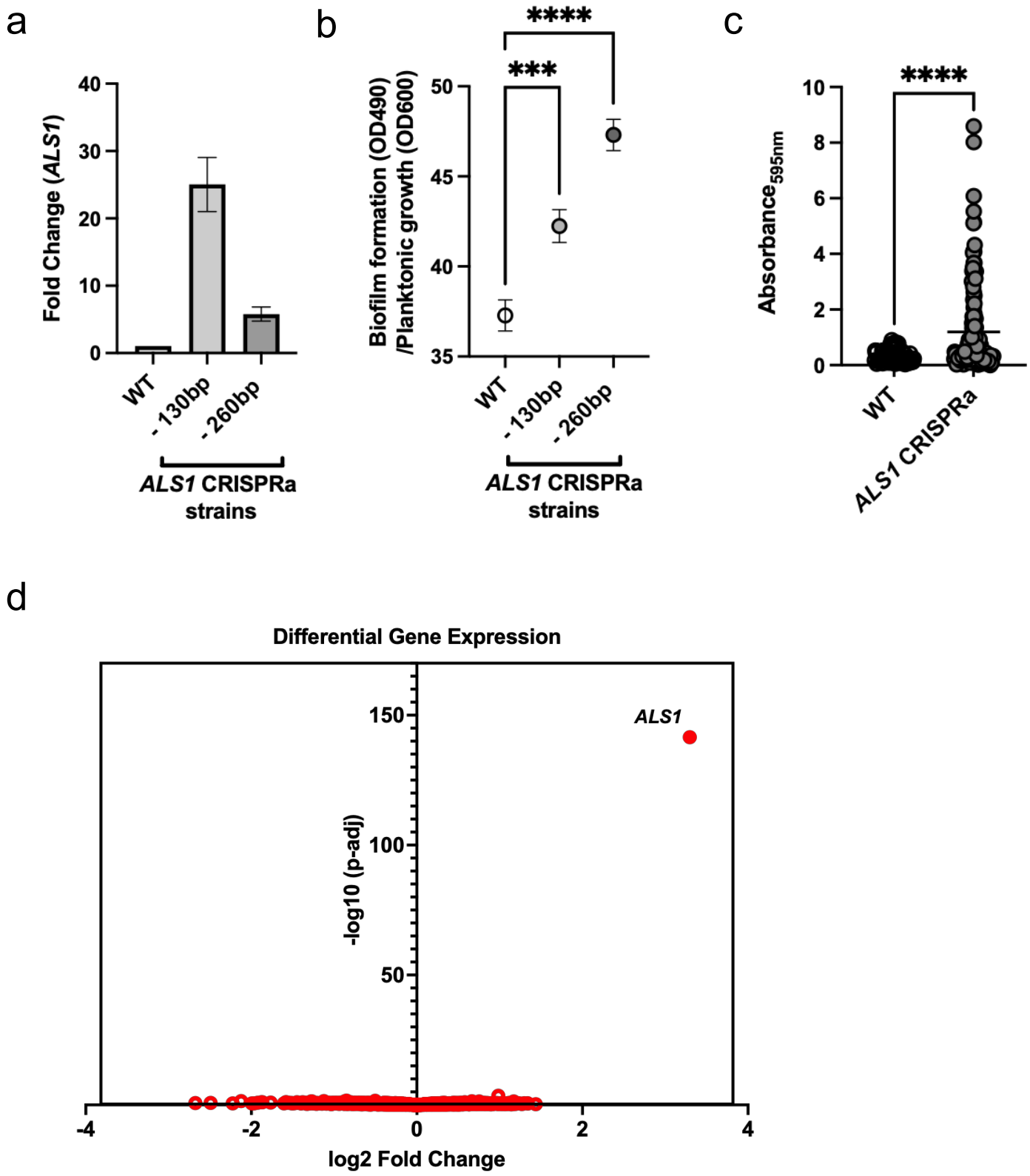

Figure 4. Validation of CRISPRa overexpression and target-specificity through targeting fungal pathogenesis traits. (a) Two *C. albicans* CRISPRa strains targeting different positions in the promoter region of the adhesin protein gene *ALS1* were created. Fold-change in the expression of *ALS1* relative to the housekeeping gene *ACT1* in both experimental strains and the non-targeting CRISPRa control strain was measured with RT-qPCR. Experimental strains are represented by the position of their sgRNA in the promoter region upstream of the proposed dominant *ALS1* transcriptional start site. Data points represent the mean fold-difference and standard deviation in expression of *ALS1* in the CRISPRa strains compared to the non-targeting CRISPRa control (n = 3). **(b)** Two CRISPRa strains targeting *ALS1* and a non-targeting CRISPRa control strain were grown in the biofilm-inducing media RPMI and left to grow statically at 37°C for 24h to allow biofilms to form. The robustness of biofilms formed by all strains was elucidated through an XTT reduction assay and measuring of the resulting OD490 values before normalizing final values to non-biofilm-forming planktonic cells. Data points represent the mean and the standard error of the mean (n = 72). Differences between groups were tested for significance using Dunnett's multiple comparison test. ****, p-value <0.0001. ***, p-value <0.001. **(c)** A *C. albicans* CRISPRa strain overexpressing *ALS1* ~6-fold and a non-targeting CRISPRa strain were grown in human urine supplemented with fibrinogen and BSA at 37°C for 48h to simulate a catheterized bladder environment. The robustness of biofilms formed by both strains was measured by reading the OD595 value following a crystal violet assay. Data points are combined from three independent experiments where n = 32 for the non-targeting CRISPRa control and n = 48 for the CRISPRa *ALS1* overexpression strain. Differences between groups were tested for significance using the Mann-Whitney U test. ****, p-value <0.0001. **(d)** Global gene expression of a CRISPRa strain overexpressing *ALS1* ~25-fold, as well as a non-targeting CRISPRa control strain, was measured using RNA-Seq. Differential gene expression analysis showed that *ALS1* had a log2-fold change of ~3.3 in the *ALS1* overexpression strain compared to the CRISPRa control strain, with an adjusted p-value of 3.07E-142, and showed no additional transcripts that had differential expression with an adjusted p-value of less than 0.03.