Restriction digest screening facilitates efficient detection of site-directed mutations introduced by CRISPR in *C. albicans UME6*

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Introduction of point mutations to a gene of interest is a powerful tool when determining protein function. CRISPR-mediated genome editing allows for more efficient transfer of a desired mutation into a wide range of model organisms. Traditionally, PCR amplification and DNA sequencing is used to determine if isolates contain the intended mutation. However, mutation efficiency is highly variable, potentially making sequencing costly and time consuming. To more efficiently screen for correct transformants, we have identified restriction enzymes sites that encode for two identical amino acids or one or two stop codons. We used CRISPR to introduce these restriction sites directly upstream of the *Candida albicans UME6* Zn^{2+}-binding domain, a known regulator of *C. albicans* filamentation. While repair templates coding for different restriction sites were not equally successful at introducing mutations, restriction digest screening enabled us to rapidly identify isolates with the intended mutation in a cost-efficient manner. In addition, mutated isolates have clear defects in filamentation and virulence compared to wild type *C. albicans*. Our data suggest restriction digestion screening efficiently identifies point mutations introduced by CRISPR and streamlines the process of identifying residues important for a phenotype of interest.

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2   by CRISPR in *C. albicans UME6*

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29   **Abstract:**

30   Introduction of point mutations to a gene of interest is a powerful tool when determining
31   protein function. CRISPR-mediated genome editing allows for more efficient transfer of a desired
32   mutation into a wide range of model organisms. Traditionally, PCR amplification and DNA
33   sequencing is used to determine if isolates contain the intended mutation. However, mutation
34   efficiency is highly variable, potentially making sequencing costly and time consuming. To more
35   efficiently screen for correct transformants, we have identified restriction enzymes sites that
36   encode for two identical amino acids or one or two stop codons. We used CRISPR to introduce
37   these restriction sites directly upstream of the *Candida albicans UME6* Zn^{2+}-binding domain, a
38   known regulator of *C. albicans* filamentation. While repair templates coding for different
39   restriction sites were not equally successful at introducing mutations, restriction digest
40   screening enabled us to rapidly identify isolates with the intended mutation in a cost-efficient
41   manner. In addition, mutated isolates have clear defects in filamentation and virulence
42   compared to wild type *C. albicans*. Our data suggest restriction digestion screening efficiently
43   identifies point mutations introduced by CRISPR and streamlines the process of identifying
44   residues important for a phenotype of interest.

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61   **Background:**

62   Site-directed mutagenesis may be used to introduce a mutation that leads to a change in protein
63   amino acid sequence (Shortle et al. 1981). Investigators can then assess the role of the altered
64   residue/s in particular phenotypes. Site-directed mutagenesis has been used to identify and
65   characterize enzyme active sites, assess the role of amino acid modification on protein function,
66   and characterize a wide variety of additional protein properties (Winter et al. 1982).

67   Alanine-scanning mutagenesis, where a particular amino acid/s is changed to alanine, is a
68   particularly popular application of this technique (Cunningham & Wells 1989; Weiss et al. 2000).
69   The methyl sidechain of Alanine is small and nonpolar. The benign nature of the alanine
70   sidechain often abolishes the functionality of the original residue while minimally disrupting
71   protein backbone structure. Additional protein variants can be made in analogous fashions. For
72   instance, in many cases, the carboxyl sidechain of a glutamate residue biochemically mimics a
73   phosphate. Thus, mutation of a threonine or serine to glutamate mimics constitutive
74   phosphorylation (Szewczuk et al. 2009; Thorsness & Koshland 1987). If an investigator
75   hypothesizes an aromatic or positively charged residue interacts with nucleic acid, a negatively
76   charged or small hydrophobic residue can be introduced to test if said residue is important for
77   nucleic acid binding (Bernstein & Keck 2005). Moreover, stop codons can be introduced to an
78   open reading frame to generate a C-terminal truncation and a stop codon introduced at the
79   beginning of the open reading frame will terminate translation before a functional protein is
80   made, generating a null protein phenotype. Furthermore, transcription initiation (Vo Ngoc et al.
81   2017) and splicing (Bortfeldt et al. 2008) are controlled in part by short nucleotide sequences
82   that can be readily interrogated by site-directed mutagenesis. As such, site-directed
83   mutagenesis is a powerful tool to elucidate not only protein but DNA and RNA sequence
84   function.

85   Historically, site-directed mutagenesis has been a multistep process. In yeast for instance, cells
86   are transformed with exogenous DNA containing a mutation and homologous recombination
87   incorporates the mutant sequence into the genome. Alternatively mutations can be subcloned
88   onto plasmids and transformed into yeast lacking the gene of interest (Cormack & Castano
89   2002). These techniques require cells perform homologous recombination at a relatively high
90   rate or maintain plasmids. In addition, detection of single nucleotide polymorphisms (SNPs)
91   between alleles in a diploid is challenging. Incorporation of restriction enzyme cut sites via PCR
92   has been used to detect SNPs using derived cleaved amplified polymorphic sequence analysis
93   (Hodgens et al. 2017; Neff et al. 1998).

94   One organism that has proven challenging to modify by site-directed mutagenesis is the human
95   fungal pathogen *Candida albicans*. Because *C. albicans* is diploid, introduction of a homozygous
96   point mutant has historically required multiple rounds of transformation and homologous
97   recombination (Jones et al. 2004; Muzzey et al. 2013). Furthermore, plasmids are not
98   maintained consistently in *C. albicans*. The development of the *Candida* CRISPR system however
99   has made introduction of mutations into the genome more efficient (Vyas et al. 2015; Vyas et al.
100   2018). During CRISPR-mediated genome editing, Cas9 nuclease bound to a guide RNA molecule
101   targets a specific genomic sequence to be modified using base pairing. Once bound to the
102   complementary sequence, Cas9 introduces a double-strand break. To introduce the desired

103 mutation and repair the double-strand break by homologous recombination, a double-stranded
104 DNA repair template possessing the altered sequence is cotransformed (reviewed in)
105 (Dominguez et al. 2016; Wang et al. 2016). Clones are sequenced to determine if the desired
106 mutation has been introduced, but efficiency of repair template incorporation can be a
107 substantial bottleneck. Furthermore, DNA sequencing is required to ensure the correct mutation
108 has been introduced into the genome.

109 We have developed a system to inexpensively screen potential point mutants using restriction
110 digestion. The system enables the investigator to screen for the introduction of two alanine, two
111 glutamate, two arginine, two glycine or one or two stop codons using colony PCR and restriction
112 digestion. As proof of concept, we introduced mutations into *UME6*, a transcription factor that
113 regulates *C. albicans* filamentation and virulence (Banerjee et al. 2008) and screened for repair
114 template incorporation by PCR and restriction digestion. Specifically, we introduced mutations
115 upstream of sequence encoding the Ume6 Zn^{2+} finger DNA-binding domain. We found the
116 frequency different repair templates were incorporated into the genome varied significantly. We
117 characterized the effects these mutations had on *C. albicans* filamentation. Strikingly,
118 introduction of a stop codon directly upstream of the *UME6* Zn^{2+} finger DNA binding domain
119 eliminated filamentation. In addition, we found mutation of two conserved positively charged
120 residues to glutamate directly upstream of the *UME6* Zn^{2+} finger DNA-binding domain resulted in
121 filamentation defects and a decrease in *C. albicans* virulence.

122 **Materials and Methods:**

123 **Cloning and Sequencing:** Primers used in this study are listed in **(Table 1)**. Ume6 guide RNA
124 primers (Ume6 Guide 2 fr and rv) were subcloned into pv1093 as previously described (Vyas et
125 al. 2015). Repair templates were made by PCR using indicated primer pairs. PCR products were
126 purified using a Zymo PCR Purification Kit. Lithium Acetate transformation was performed and
127 cells were plated on yeast extract-peptone-dextrose agar plates that contained 200 µg/ml
128 nourseothricin (Nat) (Gietz & Woods 2002). All growth media for this study was supplemented
129 with 27 mM uridine. After three days, Nat^r colonies were streaked for isolation on YPD medium
130 supplemented with 100 µg/ml Nat. Colony PCR was performed using primers Ume6 ch primer 2
131 fr and rv. 10 µl of colony PCR product was digested in accordance with the manufacturer's
132 recommended reaction conditions. Digestion and undigested PCR products were resolved on a
133 2% agarose gel, stained with ethidium bromide, and visualized. PCR products with digestion
134 patterns consistent with successful mutagenesis were sequenced by GeneWiz, and SNAPgene
135 was used to analyze the sequences.

136 **Growth and Filamentation Assays:** *C. albicans* strains were grown overnight at 25℃ in YPD and
137 diluted to OD 0.1. Fourfold serial dilutions were plated using a pin replicator on YPD and Spider
138 (Liu et al. 1994). Liquid filamentation was assessed by growing *C. albicans* variants for 24 hours
139 in Spider Media at 37℃.

140 ***Galleria mellonella* Infection:** Healthy (235 mg ± 45 mg) larvae were randomly assigned to
141 sterile PBS, wild type, or mutant groups. Log-phase cultures of *C. albicans* grown at 37℃ in YPD
142 were washed twice and suspended in sterile PBS. Prior to each injection, a Hamilton syringe was

143   sterilized via rinsing once with ethanol and then twice with sterile PBS. Larvae were swabbed

144   with 70% ethanol. 10 µl of either sterile PBS or 10^5 cfu of *C. albicans* suspended in sterile PBS

145   was injected per larva into the left rear proleg. Larvae were incubated at 37°C in a glass petri

146   dish, and survival was assessed daily by visual inspection and prodding with a sterile pipet tip.

147   Deceased larvae were removed from the dish daily.

148   **Results:**

149   One of the most challenging and costly aspects of site-directed mutagenesis is screening

150   transformants to identify those harboring the intended mutation. To reduce cost and increase

151   the speed by which transformants can be screened, we identified restriction enzyme recognition

152   sequences that, when introduced, convert two codons to codons encoding two alanines, two

153   arginines, two glutamates, two glycines, stop codons **(Table 2).** To test our strategy, we

154   cotransformed wild type *C.albicans* with a plasmid encoding Cas9 and a *UME6*-targeting guide

155   RNA and PCR-generated repair template DNA with *BseRI*, *PacI*, or *AcuI* recognition sequences.

156   Template-mediated repair replaced *UME6* residues 2259-2265 (encoding Lys754 and Lys755)

157   with either two negatively charged glutamate residues (repairEE; *BseRI* sequence), a TAA stop

158   codon (repairTAA; *PacI* sequence), or a TGA stop codon (repairTGA; *AcuI* sequence) **(Figure 1,**

159   **Table 2).**

160   Each transformation resulted in dozens of colonies. We used colony PCR to amplify *UME6* from

161   each transformant and digested the PCR products with the enzyme predicted to cut the repair

162   template. 2 of 48 transformants transformed with the repairEE template (designed to replace

163   Lys754/755 with glutamates) were successfully digested by BseRI. 2 of 8 transformants repaired

164   with the repairTAA template (designed to introduce the TAA stop codon) were successfully

165   digested with PacI, and 2 of 48 transformants repaired with repairTGA (designed to introduce

166   the TGA stop codon) were successfully digested with AcuI **(Figure 1 B)**. We sequenced

167   transformants with restriction digestion patterns consistent with successful mutagenesis. We

168   found successfully digested clones had correctly introduced homozygous point mutations in the

169   *UME6* gene **(Figure 1 D and E)**. In addition, we did not observe unintended mutations in *UME6*,

170   and *we* did not recover heterozygous mutants. We also sequenced PCR products that did not

171   digest; as predicted, those transformants retained wild type *UME6* sequence **(Figure 1 C)**.

172   Names and genotypes of strains used in further studies are listed **(Table 3)**. Our data establish

173   that restriction digestion is a rapid and accurate screening strategy for site-directed mutagenesis

174   in *C. albicans*.

175   Ume6 is a transcription factor important for *C. albicans* filamentation and the only domain

176   within Ume6 predicted by sequence homology is a Zn^{2+} finger DNA-binding domain (amino acids

177   760-810) (Banerjee et al. 2008). Ume6's C-terminal region (amino acids 811-844) is highly

178   conserved, but is not predicted to form a known structural domain (Inglis et al. 2012). We

179   hypothesized the Zn^{2+} finger and C-terminus of Ume6 are important for filamentation. We found

180   neither yeast harboring the premature stop codon (*ume6-stopTAA)* nor those expressing the

181   lysine-to-glutamate point mutants (*ume6-EE*) exhibited slow growth relative to wild type *C.*

182   *albicans* at a range of temperatures on rich media **(Figure 2 B)**. However, we did find wild type

183   yeast display a more pronounced crinkled colony morphology than either *ume6-EE* or *ume6-*
184   *stopTAA* yeast on rich media at 37°C **(Figure 2 B).**

185   Wild type, but not homozygous *ume6/ume6 C. albicans* undergo filamentation when cultured on
186   spider medium (Banerjee et al. 2008). We tested if the DNA-binding domain and conserved
187   upstream positively charged residues Lys754 and Lys755 are important for induction of *C.*
188   *albicans* filamentation. We analyzed filamentation of wild type, *ume6-EE*, and *ume6-stopTAA*
189   yeast on spider agar media. We found that, while wild type yeast filament robustly after 7 days,
190   *ume6-stopTAA* yeast failed to filament **(Figure 2 A)**. Furthermore, we found *ume6-EE* yeast
191   exhibit intermediate filamentation **(Figure 2 A)**. We observed similar filamentation patterns
192   when these strains were cultured in liquid spider media **(Figure 2C)**.

193   Next, we tested the effect of our point mutations on *C. albicans* virulence in a wax moth model.
194   *ume6/ume6* yeast exhibit lower virulence in a mouse model (Banerjee et al. 2008), and their
195   virulence has not been tested in the wax moth model system. Less than 30% of larvae injected
196   with wild type *C. albicans* survived after incubation for 200 hours. By comparison, the *ume6-*
197   *stopTAA* mutation significantly decreases virulence, with roughly 80% surviving after 200 hours
198   of incubation **(Figure 2 D)**. Consistent with our filamentation data, we found *ume6-EE* yeast
199   exhibited virulence intermediate to wild type and *UME6stop-TAA* yeast, with greater than 40%
200   of larvae surviving following 200 hours of incubation. Our data indicate the final 100 amino acids
201   of Ume6 that include both a Zn^{2+} finger domain and conserved C-terminal region are important
202   for *C. albicans* filamentation and virulence. Furthermore, our data suggest residues Lys754 and
203   Lys755 which lie N-terminal to the Zn^{2+} finger domain are also important for filamentation and
204   virulence as well.

205   **Discussion:**

206   We have identified restriction enzyme recognition sequences that, when translated, encode
207   consecutive identical amino acids or one or two stop codons. By replacing two codons with
208   these restriction sites, we were able to efficiently introduce and confirm the change of two
209   consecutive amino acids by PCR followed by restriction digestion. In the event that digestion and
210   sequencing are inconclusive, the incorporation of a restriction site also allows facile screening
211   for correct transformants by Southern Blot, a more time consuming, but potentially necessary
212   step to ensure additional copies of a DNA sequence are not present and your mutation of
213   interest has been introduced. We found not all repair templates are incorporated into the
214   genome with equal efficiency. This variability in successful recombination enhances the utility of
215   rapidly screening numerous colonies by digestion. Our technique enabled us to unambiguously,
216   inexpensively, and rapidly identify desired mutations in the *C. albicans* transcription factor
217   *UME6*. Although we did not observe heterozygotes, our restriction strategy should distinguish
218   homozygous from heterozygous mutations. Our data suggest that Ume6 Lys754 and Lys755 are
219   important for filamentation and virulence. In addition, our data suggests the Zn^{2+} finger DNA
220   binding domain and conserved C-terminal domain are also important for filamentation and
221   virulence.

222   We have identified restriction enzyme recognition sites that encode two consecutive alanines,
223   glutamates, arginines, and glycines and one or two stop codons. Classically, site-directed
224   mutagenesis introduces a single amino acid change, but screening for such changes using
225   restriction digestion is impractical. Introduction of restriction sites with four-base recognition
226   sites (e.g. GCGC which can be cleaved by HinP1I or HhaI) could be used to introduce a single
227   alanine; however, the additional base at the 5' or 3' end of the four-base recognition sequence
228   places upstream or downstream sequence constraints that limit its utility.

229   Introduction of stop codons can be used to truncate the C-terminus of a protein. However, stop
230   codon suppressor mutations could bypass these mutations. The introduction of an I-SceI
231   restriction site introduces two distinct stop codons (TAG and TAA) and is thus less likely to be
232   bypassed with suppressor mutations. We attempted to introduce I-SceI site to *UME6* using a
233   100-base pair repair template and screened over 50 transformants by restriction digestion.
234   Unfortunately, none of these transformants incorporated the repair template. This apparent
235   inefficiency could be due to slightly shortened regions of homology on the repair template in
236   comparison to the successful repair templates. A moderate increase in repair template size
237   might overcome this limitation. Alternatively, successive SpeI, PacI, or AcuI restriction sites in a
238   single repair template could be used to introduce sequential stop codons and reduce the
239   likelihood of suppression.

240   All codons are not used equally in genomes. For instance, in *C. albicans* GCN (N representing
241   any base) encodes alanine, but 43% of alanine are encoded by GCT and less than 5% by GCG
242   (Arnaud et al. 2005). Such codon bias must be appreciated when investigators decide which
243   restriction site to introduce. Rare codons may alter protein expression, confounding the
244   interpretation of phenotypes associated with protein variants (Quax et al. 2015). We have
245   identified three restriction sites that code for successive alanine residues **(Table 2)**. These
246   enzyme recognition sites are found at different frequencies throughout the genome and allow
247   for the introduction of any alanine codon. This cadre of enzymes provides the investigator
248   flexibility when designing a mutagenesis experiment.

249   Site-directed mutagenesis restriction digest screening could be exceptionally useful for
250   investigation of protein localization. Proteins use short conserved amino acid sequences to
251   localize to a variety of intracellular compartments including the endoplasmic reticulum,
252   mitochondria, peroxisome, and nucleus (Nakai 2000). These sequence motifs are often charged
253   or hydrophobic. Disruption of such sequence motifs is critical when dissecting the function of
254   these proteins *in vivo*. Site-directed mutagenesis could replace these amino acids with others
255   possessing different biochemical characteristics. Our screening system would enable efficient
256   interrogation of the roles signal motifs play in protein localization.

257   We piloted our restriction digestion-based strategy to screen for point mutations in *UME6*.
258   Ume6 is a transcriptional regulator of filamentation, and deletion of *UME6* causes defects in
259   filamentation and decreased virulence in mouse models (Banerjee et al. 2008). However, little
260   was known about the function of structural elements within Ume6. Ume6 consists of a
261   predicted Zn^{2+} finger DNA binding domain, conserved C-terminal domain, and a poorly

262   conserved N-terminal domain of unknown function **(Figure 1A)**. Our data show the Zn^{2+} finger
263   domain and/or the C-terminal domain plays a role in filamentation, as introduction of a stop
264   codon upstream of these domains led to decreased filamentation and virulence **(Figure 2A-C)**.
265   One potential interpretation is   altering conserved lysine residues directly N-terminal to the
266   Zn^{2+} finger domain causes defects in filamentation and virulence. This suggests Lys754 and/or
267   Lys755 play a previously unappreciated role in filamentation program activation. Alternatively,
268   changing Lys754 and/or Lys755 could lead to destabilization and/or lower expression of
269   functional protein. The Glu codons that were introduced code for 21% of Glu residues in
270   *Candida* so it is unlikely that codon usage is limiting. While Ume6's N-terminal domain is not as
271   highly conserved as its Zn^{2+} finger or C-terminal domains, it does contain a number of highly
272   conserved residues. Further site-directed mutagenesis of the Ume6 N-terminal domain will help
273   elucidate the role of Ume6 in *C. albicans'* filamentation program.

274   Site-directed mutagenesis coupled with restriction digest screening has the potential to be
275   broadly useful in a number of ways. First, the development of CRISPR-mediated genome editing
276   systems has significantly expanded the breadth of organisms that can be readily genetically
277   modified (Reardon 2016). These advances are likely to revolutionize knockout library generation
278   in a variety of organisms. For organisms that require a repair template to introduce mutations
279   like *C. albicans* (Vyas et al. 2015), introduction of stop codons that can be screened by restriction
280   digestion could be an important component of efficient and accurate library preparation and
281   verification. Second, undergraduate research projects are a critical component of education, but
282   identifying projects that both excite students and can be performed during the confines of an
283   undergraduate curriculum is challenging. This can be especially difficult for students interested
284   in molecular biology, where cloning and reagent development are often times required before
285   one can begin to test a hypothesis experimentally. Introducing mutations using CRISPR and
286   screening for correct mutations using restriction digestion is a quick method whereby
287   undergraduate students can learn and apply multiple molecular biology techniques. The
288   relatively high success rate and moderate cost of these techniques allow students to move
289   projects forward while asking and answering important biological questions. Furthermore, the
290   low cost and speed of these procedures makes these exercises suitable for undergraduate
291   teaching laboratories.

292   **Conclusions:**

293   We have identified restriction enzymes cut sites that code for successive Alanine, Glutamate,
294   Arginine, Glycine, or stop codons. The incorporation of these sites allows the efficient
295   identification of correct point mutations using restriction digestion. Using CRISPR we introduced
296   these sites to *C. albicans UME6*, changing Lys754 and Lys755 to either two Glutamate residues
297   or a stop codon. We found Lys754 and Lys755 are important for filamentation and virulence.
298   Site-directed mutagenesis coupled with restriction digest screening is a quick cost effective
299   method of screening potential point mutants.

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399

400

Figure 1

Introduction of restriction sites enable efficient screening for correct point mutants.

A. Cartoon of Ume6 domains and key residues for this study. Red line indicates CRISPR cut site. **B.** Restriction digestion of colony PCR of *UME6* from representative *ume6TAAStop* and *ume6-EE C. albicans*. Lane **1.** Ladder, **2.** *ume6TAAStop* undigested, **3.** *ume6TAAStop* digested with Pac1 **4**. *ume6-EE* undigested **5.** *ume6-EE* digested with BseRI **C-E.** Sequence analysis of site of *ume6* mutagenesis of representative failed mutant (wild type sequence) (**C**), *ume6-EE* (**D**), and *ume6TAAStop* (**E**) *C. albicans* amino acid sequence is presented above DNA sequence. Red E designates site of lysine-to-glutamate point mutations. Red octagon designates site of introduced stop codon.

A

1 ······ 760 ······ 810 ······ 844

| N-terminal Domain | K755 K754 | Zinc Finger Domain | C-Terminal Domain |

B

1   2   3   4   5

1000 bp
500 bp
400 bp
300 bp
200 bp
100 bp

C

M V K K K H

A T G G T G A A G A A A A A C A T

D

M V E E K H

A T G G T G G A G G A G A A A C A T

E

M V 🛑

A T G G T T T A A T T A A A A A C A T

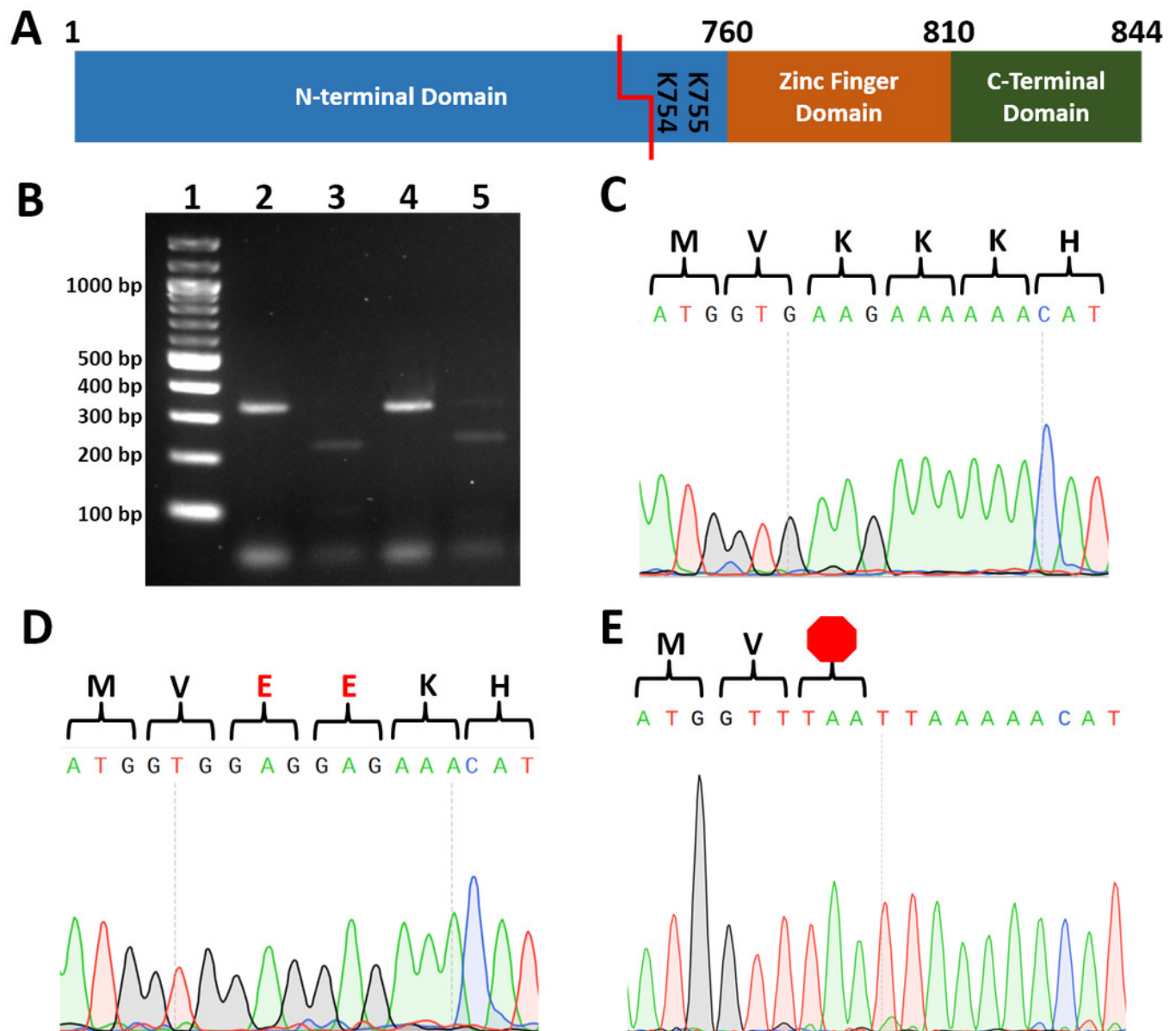
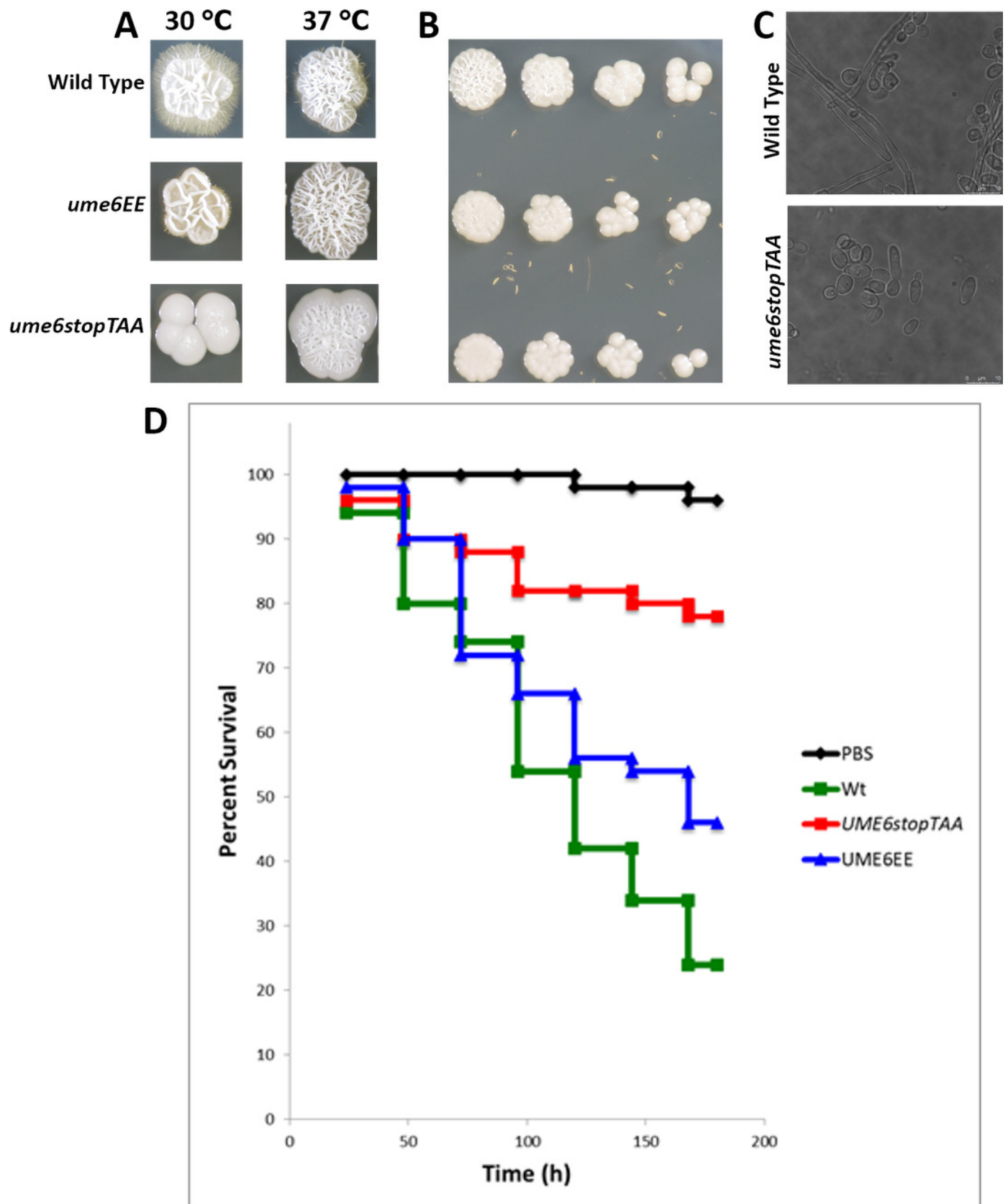



Figure 2

Assessment of UME6 mutations on filamentation and virulence.

A. Wild type, *ume6-EE*, and *ume6-stopTAA C. albicans* were cultured on Spider media at 30 and 37°C for 7 days and assessed for filamentation. **B.** Fourfold serial dilutions of wild type, *ume6-EE*, and *ume6-stopTAA C. albicans* were incubated on YPD media at 37 ℃ for 3 days. **C.** Wild Type and *ume6-stopTAA* incubated in liquid Spider media were assessed for filamentation after 24 hours. **D.** 10^5 cfu of wild type, *ume6-EE*, and *UME6-stopTAA C. albicans* were injected into wax moth larvae, which were subsequently incubated at 37°C. Survival of larvae was tracked over time. Wt was more virulent than ume6-EE (P = 0.036) and more virulent than *ume6-stopTAA* as well (P < 0.001). Additionally *ume6-EE* was more virulent than *ume6-stopTAA* (P = 0.003). Results represent total survival of 5 independent experiments with 10 larvae per treatment. Survival curves were created using the Kaplan-Meier method and statistical analyses were performed using the log rank test for multiple comparisons (IBM SPSS Statistics).

Oligos used in this study.

Restriction Sites Sequences are bolded and capitalized in repair templates. Nucleotides added for cloning into the CRISPR plasmids are bolded and italicized in the guide primer sequences.

Oligo Name	Primer Sequence	Restriction Site
Ume6 Guide 2 fr	***ATTTG***tacttctacttctaatccaa***G***	XXXXXX
Ume6 Guide 2 rv	***AAAAC***ttggattagaagtagaagta***C***	XXXXXX
Ume6 ch primer 2 fr	ggtcatgatcatgatgatgaaaat	XXXXXX
Ume6 ch primer 2 rv	ctccacaaattggtgtgacttc	XXXXXX
Ume6 rp 2_Glu fr	atggcactaacaccaatactgattctacttctacttctaatccaatggtg**GAGGAG**aaac	BseRI
Ume6 rp 2_Glu rv	catcctttttagatctaggtaataatcttcttcttgtatgttt**CTCCTC**caccattgga	BseRI
Ume6 rp 2_TAA fr	aatggcactaacaccaatactgattctacttctacttctaatccaatggt**TTAATTAA**aa	Pac1
Ume2 rp 2_TAA rv	tcctttttagatctaggtaataatcttcttcttgtatgttt**TTAATTAA**accattggat	Pac1
Ume6 rp 2_TGA fr	atggcactaacaccaatactgattctacttctacttctaatccaatggt**CTGAAG**aaaca	Acu1
Ume6 rp 2_TGA rv	acatcctttttagatctaggtaataatcttcttcttgtatgttt**CTTCAG**accattgga	Acu1

Restriction sites that encode consecutive amino acids or stop codons.

Nucleotide code is as follows: **W** = A or T, **M** = A or C, **K** = G or T, **R** = A or G, **N** = A T C or G, /=cleavage site. Numbers in parentheses correspond to how far upstream a cut site will occur from the recognition sequence.

Translation Product	Codon	Restriction Enzyme Recognition Site Sequence
2xAlanine	GCN	**BbvI** GCAGCN (8/12), **Fnu4HI** GC/NGCN, **TseI** G/CWGCN
2xArginine	CGN	**Hpy99I** CGWCGN/
2xGlutamate	GAR	**BseRI** GAGGAG (10/8)
2xGlycine	GGC+GGA	**EciI** GGCGGA (11/9)
1x Stop Codon	TAG	**SpeI** A/C*TAG*T
1x Stop Codon	TAA	**PacI** T*TAA*T/TAA
1x Stop Codon	TGA	**AcuI** C*TGA*AG(16/14)
2x Stop Codon	TAG+TAA	**I-SceI** *TAG*GGA*TAA*CAGGGTAAT(-9/-13)

Strains used in this study.

Name	Genotype	How strain is referenced in text
SC5314	*UME6:UME6*	Wild Type
DAB898	*ume6-LYS754E LYS755E:ume6-LYS754E LYS755E*	*ume6-EE*
DAB894	*ume6-LYS754stop:ume6-LYS754stop*	*ume6-stopTAA*