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1 **Secreted Acb1 Contributes to the Yeast-to-Hypha Transition in *Cryptococcus***

2 ***neoformans***

3 Running title: Acb1 regulates *Cryptococcus* morphotype transition

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9 **ABSTRACT**

10 Adaptation to stress by eukaryotic pathogens is often accompanied by transition in cellular
11 morphology. The human fungal pathogen *Cryptococcus neoformans* is known to switch between
12 the yeast and the filamentous form in response to amoebic predation or during mating. Like the
13 classic dimorphic fungal pathogens, morphotype is associated with cryptococcal ability to infect
14 various hosts. Many cryptococcal factors and environmental stimuli are known to induce the
15 yeast-to-hypha transition, including pheromones (small peptides) and nutrient limitation. We
16 recently discovered that secreted matricellular proteins could also act as intercellular signals to
17 promote the yeast-to-hypha transition. Here we showed that the secreted acyl-CoA binding
18 protein, Acb1, plays an important role in enhancing this morphotype transition. Acb1 does not
19 possess a signal peptide. Its extracellular secretion and consequently its function in filamentation
20 are dependent on the GRASP unconventional secretion pathway. Surprisingly, the recruitment of
21 Acb1 to the secretory vesicles intracellularly is independent of Grasp. In addition to Acb1, Grasp
22 possibly controls the secretion of other cargos, because the *grasp*Δ mutant, but not the *acb1*Δ
23 mutant, is defective in capsule production and macrophage phagocytosis. Nonetheless, Acb1 is
24 likely the major or the sole effector of Grasp in terms of filamentation. Furthermore, we found
25 that Acb1's key Y80 residue for acyl-binding is critical for its proper subcellular localization,
26 secretion, and cryptococcal morphogenesis.

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27 **INTRODUCTION**

28 Adaptation to changing environment by eukaryotic microbes is often accompanied by transition
29 in cellular morphology. The human fungal pathogen, *Cryptococcus neoformans*, causes
30 devastating cryptococcal meningitis that claims the lives of hundreds of thousands of people
31 each year (1). Late diagnosis, limited options of antifungals, and the lack of vaccines to prevent
32 cryptococcosis all contribute to the high mortality rate of this disease (2). *Cryptococcus* typically
33 grows as yeasts, but it can switch from yeasts to filaments (hyphae or pseudohyphae) in response
34 to predation (e.g. amoeba) or during sexual reproduction (3-7). Like many other fungal
35 pathogens, cryptococcal morphotype shapes its interaction with various hosts (8). As we
36 demonstrated recently, the hyphal form is associated with virulence attenuation in mouse models
37 of cryptococcosis because hyphal morphotype elicits strong and protective host immune-
38 responses (9, 10). On the other hand, the hyphal morphotype assists the fungus to resist
39 predation from soil amoeba (8), increases its ability to explore the environment (11), and is
40 linked to its unisexual and bisexual reproduction (3, 12-14). Thus it is important to understand
41 the factors that promote cryptococcal hyphal growth.

42 Many environmental stimuli and few cryptococcal factors have been identified to
43 promote hyphal growth in *Cryptococcus* (12, 15-18). Pheromone is the most prominent
44 cryptococcal molecule that stimulates mating and filamentation. We recently discovered that the
45 matricellular and hypha-specific protein Cfl1, when released from the cell wall, can also act as
46 an intercellular communication signal to stimulate yeast-to-hypha transition (19, 20). Here, we
47 decided to investigate the potential role of the secreted protein Acb1 in filamentation and sexual
48 reproduction in *Cryptococcus*.

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49 The acyl-CoA binding protein, Acbp, was first identified in mammals because its
50 processed peptide inhibited diazepam binding to the GABA receptor, which gave rise to its name
51 diazepam binding inhibitor (DBI) (21). DBI's homolog in *Dictyostelium discoideum*, called the
52 peptide signal spore differentiation factor 2 (SDF2), activates sporulation within the fruiting
53 body (22). Similarly, SDF2 is processed from *Dictyostelium* AcbA (23, 24). In the absence of
54 AcbA, *D. discoideum* fruiting bodies generate about 10% as many viable spores as the wild type.
55 Interestingly, co-incubation of the *acbA*Δ mutant with the wild-type cells restored the level of
56 sporulation to that of the wild type (23). It was proposed that secreted AcbA from wild type was
57 sufficient to complement the sporulation defect of the *acbA*Δ mutant in *Dictyostelium*.

58 Acbps are later found to be widely distributed in the eukaryotic domain and they play
59 important roles in a wide range of biological processes (25-27). In higher eukaryotes, there are
60 multiple copies of Acbp-encoding genes in one genome and these proteins vary in size and in
61 their subcellular localization (28-34). Nonetheless, all Acbps are conserved in the acyl-CoA-
62 binding domain (34). Not surprisingly, Acb1 in *Saccharomyces cerevisiae* helps transport newly
63 synthesized acyl-CoA esters from the fatty acid synthase to acyl-CoA consuming processes (35).
64 Acb1 plays an important role in fatty acid elongation, membrane assembly, and protein
65 trafficking in *S. cerevisiae* (36, 37).

66 Despite the predicted cytosolic-localization of AcbA due to its absence of a classical
67 secretion signal or any transmembrane domain (38), AcbA was found to be in puncta or vesicles
68 in the cortical region in *D. discoideum* (39). The vast majority of AcbA proteins are intracellular,
69 with less than 5% being secreted extracellularly (39). However, extracellular secretion and post-
70 secretion processing are critical for its signaling function in *Dictyostelium* in promoting spore

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71 generation (23, 39). The extracellular secretion of AcbA requires an unconventional pathway that
72 is dependent on Golgi-Associated Protein Grasp in *D. discoideum* (38).

73 In the present study, we set out to investigate the role of secreted Acb1 in yeast-to-hypha
74 transition and sporulation in *C. neoformans.* We found that Acb1 contributes to cryptococcal
75 yeast-to-hypha transition. Interestingly, the secretion of Acb1 is dependent on its acyl-CoA
76 binding ability and the Grasp protein in *Cryptococcus*. Accordingly, the mutation of Acb1's
77 acyl-CoA binding domain or the deletion of the *GRASP* gene impairs cryptococcal hyphal
78 growth.

79

80 **MATERIALS AND METHODS**

81 **Media, strains, and *in vitro* phenotypic assay.** The YPD medium (2% Bacto peptone, 1% yeast
82 extract, and 2% glucose) was used for routine culture. For phenotypic assays, the YNB medium
83 (6.7 grams/liter of yeast nitrogen base w/o amino acids and ammonium) without glucose was
84 used as the base medium to test the utilization of a specific nutrient source supplemented to the
85 final concentration of 2% as indicated in the texts and figures. For the phenotypic assays, wild-
86 type and mutant cells were suspended at the same cell density. Cell suspensions with serial
87 dilutions were spotted onto the relevant medium and incubated for two to three days before
88 photographs were taken. For the filamentation assay, we used V8 juice agar (50 ml V8 juice, 0.5
89 g KH_2PO_4, in 1 liter, pH 5 or 7 adjusted with KOH), YNB, or YPD medium. V8 juice medium is
90 a commonly used mating medium in laboratory research (40).YNB is a minimal medium and
91 YPD is a nutrient-rich medium. All mutant strains were generated in the reference strain XL280

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92 (serotype D) and H99 (serotype A) backgrounds. Both XL280 and H99 have publically available
93 genomes and congenic pairs (41, 42). Strains used in this study are listed in Table S1.

94 For bisexual mating, parental strains (α and **a**) with equal number of cells were co-
95 cultured together on the YNB, the V8 juice, or the YPD medium in the dark at 22°C. Mating was
96 examined microscopically for the formation of mating hyphae and spores. For unisexual mating
97 (self-filamentation without a partner of an opposite mating type), individual isolates at the same
98 cell density were dropped onto the YNB or the V8 juice medium alone. Self-filamentation and
99 sporulation were examined microscopically as described previously (14). XL280 is a hyper-
100 filamentous strain and it filaments robustly on V8 media, which renders the reduction in
101 filamentation of the *acb1*Δ mutant less obvious. Reduction in filamentation of the *acb1*Δ mutant
102 is much more pronounced on the less optimal YNB media. In comparison, H99 strains filament
103 poorly during bisexual mating on all these media and the reduction in filamentation of the *acb1*Δ
104 mutant is evident irrespective of the medium used.

105 **Confrontation assay.** The confrontation assay was used to test whether secreted products
106 from the wild type could restore the defect in bisexual mating of the *acb1*Δ mutant. The
107 procedure was performed as we described previously (19). Briefly, the co-cultured α and **a** cells
108 at 1:1 ratio of either the wild type or the mutant strains were spotted onto the relevant medium
109 (YNB medium or YPD medium) as donor strains. After the donor strains were incubated for 3
110 days, the recipient α-**a** co-cultures (wild-type or mutant strains) were spotted onto the medium in
111 a close proximity to the donor cells (distance <5 mm). After additional 48 hours of incubation,
112 the colony morphology and the formation of hyphae in the recipients were photographed.

113 **Gene deletion and complement.** The knockout and the complementation constructs
114 were generated as previously described (14, 43). To disrupt the *ACB1* or the *GRASP* gene, we

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115 amplified the 1 kb 5' and 3' flanking sequences of the coding region using the genomic DNA
116 isolated from the strain XL280α or H99α as template and the NEO or NAT dominant drug
117 marker amplified from the plasmid pAI1 or pJAF1 respectively. The knockout constructs with 5'
118 and 3' flanking sequences bordering the selective marker gene were generated by overlap PCR
119 as we described previously (43). The knockout constructs were introduced into strains XL280α,
120 XL280**a**, H99α, and KN99**a** by biolistic transformation as described previously (44). The
121 resulting transformants were screened for gene replacement *via* homologous recombination
122 events by PCR. The genetic linkage between the phenotype and the gene deletion was confirmed
123 by analyzing the segregation pattern of the meiotic progeny generated from a bisexual cross
124 between the mutant and a wild-type mating partner (43). For complementation, the wild-type
125 genes with 1-1.5 kb upstream of their ORF were amplified by PCR, digested with proper
126 restrictive digestion enzymes, and introduced into the pXL1-mCherry plasmid (9). The resulting
127 plasmid, pXL1-*ACB1*-mCherry, was confirmed by enzyme digestion and gel electrophoresis.
128 The plasmids were then linearized and transformed into the relevant *Cryptococcus* strains
129 through biolistic transformation or electroporation as we described previously (43). Primers and
130 plasmids used for this study are listed in Table S2.

131 **Target site-directed mutagenesis.** To mutate the acyl-CoA binding site, the key residue
132 Y80 of Acb1 was mutated to A (Y80A) using the site directed mutagenesis kit (Quickchange II,
133 Aglilent Technologies) according to the manufacturer's instructions. The fragment with the
134 mutated allele of *ACB1* and the 1 kb sequences upstream of the *ACB1* ORF was ligated into the
135 plasmid PXL1-mCherry (9). The resulting plasmid PXL1-Acb1(Y80A)-mCherry was linearized
136 and transformed into XL280 or H99 as described earlier.

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137 **Microscopic examination.** To examine the sub-cellular localization of Acb1::mCherry
138 or Acb1(Y80A)::mCherry, the relevant strains were cultured on the YPD or the YNB agar
139 medium at 30°C for 24 hrs. Images were acquired and processed with a Zeiss M2 imaging
140 system with the AxioCam MRm camera and the software Zen 11 (Carl Zeiss Microscopy).

141 **RNA extraction and qPCR**. RNA extraction and qPCR were performed as described
142 previously (9). Briefly, strains with opposite mating types were co-cultured on the YNB agar
143 medium for the indicated durations. Cells were harvested, washed with cold water, immediately
144 frozen in liquid nitrogen, and then lyophilized. Cells were broken into fine powder with glass
145 beads and total RNA was extracted with the PureLink® RNA Mini Kit (life technology)
146 according to the manufacture's instruction. First strand cDNA was synthesized with Superscript
147 III cDNA synthesis kit (Invitrogen) according to the manufacture's instruction. The house-
148 keeping gene *TEF1* was used as the endogenous control. The relative transcript levels were
149 determined using the comparative ΔΔCt method as described previously (9). Three biological
150 replicates were performed for each sample and their values were used to calculate the mean
151 value and standard error.

152 **Protein extraction and Western blot.** Strains carrying the Acb1-mCherry or the
153 Acb1(Y80A)-mCherry in the wild-type or the *grasp*Δ mutant background were cultured in YNB
154 liquid medium with proteinase inhibitors PMSF and TACK (Roche Inc.) for 48 hours. The
155 culture supernatant was separated from the cell pellet by centrifugation. The supernatant was
156 concentrated with Amicon Ultra-15 Centrifugal Filter (EMD Millipore) and denatured with the
157 SDS-containing loading buffer before electrophoresis in a SDS-gel. The cell pellet was washed
158 twice with cold PBS and then lyophilized. The dried cells were disrupted by cell disruptor (Next
159 Advance) with glass beads. The total proteins were extracted with the lysis buffer (in mM: 25

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160 Hepes, pH 7.5, 300 NaCl, 2 EDTA; plus proteinase inhibitor cocktail) and then denatured with
161 the SDS-containing loading buffer before electrophoresis in a SDS-gel. The Western blotting
162 process was executed as previously described (19, 45). Briefly, the samples were separated on
163 SDS/12% PAGE gel and transferred to a PVDF membrane (Millipore) for 1 h at 30 V in TE70
164 ECL semi-dry transfer unit (GE Healthcare). The blots were incubated with the anti-mCherry
165 primary antibody (1/2,000 dilution), washed, and then incubated with a rabbit anti-mouse
166 secondary antibody (1/10,000 dilution) (Clonetech Inc). Signal detection was performed using
167 the ECL system according to the instruction provided by the manufacture (Pierce).

168 **Phagocytosis assay.** Phagocytosis assay was performed as we previously described (8).
169 Briefly, macrophage cell line J774A.1 (ATCC® TIB-67™) was cultured in Dulbecco's Modified
170 Eagle's Medium (DMEM, catalog no. 30-2002) with 10% Fetal bovine serum (FBS). Three
171 hundred microliters of culture with 2.5 x 10^5 freshly grown J774A.1 cells were seeded into each
172 well of the 24 well microtiter plate. The macrophage cells were cultured at 37ºC with 5% CO_2
173 overnight and then replaced with fresh medium. *Cryptococcus* cells were inoculated to each well
174 to achieve MOI =3. After 30s of rock mixing, the co-cultures were incubated at 37ºC with 5%
175 CO_2 for an additional three hours. The co-cultures were then washed three times with warm
176 phosphate buffered saline (PBS; 500 µL/well) to remove medium and non-adherent cells. Then
177 the PBS+0.1% Tween-20 was added to the culture and incubated for 10 min at 37°C to lyse
178 macrophage cells. The supernatant was then harvested, serially diluted, and spread onto YNB
179 agar plates. The cryptococcal colony forming units (CFUs) were counted after 2 days of
180 incubation at 30°C.

181

182 **RESULTS**

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183 **The deletion of the *ACB1* gene reduced hyphal growth.** *ACB1* is one of the abundantly
184 expressed genes based on our RNA-seq data (46). As secreted and processed Acb1 is involved in
185 sexual reproduction as a signal molecule in other species (23, 39), we decided to test whether
186 Acb1 is also important for *Cryptococcus* sexual reproduction. We first examined the transcript
187 level of *ACB1* during bisexual mating. We found that the expression of *ACB1* was modestly
188 increased during mating (Fig. 1C), suggesting a possible role of Acb1 in this biological process.

189 To examine the role of Acb1 in sexual reproduction and hypha formation in
190 *Cryptococcus*, the *ACB1* gene (CNM01420) was deleted in the hyper-filamentous serotype D
191 strain XL280α and its congenic strain XL280**a** (3, 47). No *ACB1* transcript could be detected in
192 the *acb1*Δ mutants (Fig. 1C), as expected. Under mating-inducing condition on V8 juice agar
193 media, the **a**-α mating pair of the *acb1*Δ mutant showed dramatically reduced filamentation
194 relative to the wild type, as reflected in the less white and fluffy mutant colony (Fig. 1A).
195 Introduction of a wild-type allele of *ACB1* ectopically into the *acb1*Δ mutant restored the defect
196 (Fig. 1A), supporting the role of Acb1 in filamentation. Under mating-suppressing condition on
197 YPD agar medium, the colony derived from the wild-type **a**-α mating pair was wrinkled with
198 some degree of filamentation at the colony edge (Fig. S1A). By contrast, the colony derived from
199 the *acb1*Δ **a**-α mating pair was smooth and almost barren at the edge (Fig. S1A). During the
200 unisexual mating with only α cells, the wild-type XL280α cultured on the YNB medium
201 produced a wrinkled colony with hyphae at the colony edge (Fig. S1B). The *acb1*Δ mutant
202 generated a smooth colony with very few hyphae at the colony edge (Fig. S1B). Introduction of
203 the wild-type *ACB1* gene ectopically into the *acb1*Δ mutant restored self-filamentation to the
204 wild-type level. These observations indicate that Acb1 also enhances hyphal growth during self-
205 filamentation.

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206 To test whether the role of Acb1 in filamentation is conserved in other *Cryptococcus* sub-
207 species, we deleted the *ACB1* gene (CNAG_06140) in the serotype A reference strain H99α and
208 the mating type **a** strain KN99**a**. Similarly, the *acb1*Δ **a**-α mating pair in H99 background also
209 showed drastic reduction in filamentation when cultured on V8 juice agar medium, and robust
210 filamentation could be restored by the introduction of a wild-type copy of *ACB1* (Fig. 1B).
211 Taken together, the results indicate that Acb1 has a conserved role in enhancing filamentation
212 during bisexual mating in *Cryptococcus*. Because sporulation is proceeded by yeast-to-hypha
213 transition in *Cryptococcus* (48), and Acb1 in *Dictyostelium* is known to trigger sporulation
214 within fruiting bodies (23), we decided to test if Acb1 is required for sporulation in *Cryptococcus*.
215 We examined the spore production by the **a-**α bisexual mating on V8 juice medium of the wild-
216 type XL280 and the corresponding *acb1*Δ mutants. Although filamentation was reduced in the
217 *acb1*Δ mutants, both mating pairs produced 4 chains of basidiospores (Fig. 2A-B) and we did not
218 observe any apparent defect or drastic reduction in spore production.

219 **The deletion of *ACB1* reduced the transcript level of the pheromone gene *MFα* and**
220 **the hypha-specific gene *CFL1*.** *Cryptococcus* undergoes yeast-to-hypha transition during both
221 unisexual and bisexual mating. The pheromone signaling pathway initiates the process under
222 mating-inducing conditions (14) and the activation of the filamentation pathway eventually leads
223 to hyphal growth (9, 14). To understand how the loss of *ACB1* affects filamentation, we decided
224 to measure the impact of the *ACB1* deletion on the transcript level of *MF*α, *CFL1*, and *PUM1*
225 during bisexual mating. The pheromone MFα is the initial signaling factor initiating mating (49).
226 The secreted protein Cfl1 is a specific marker for filamentation (19). Pum1 is a genetic linker
227 between filamentation and sporulation (48). The basal level of all the three transcripts at time
228 point 0 was similar between the wild type and the *acb1*Δ mutant (Fig. 1C). The transcript levels

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229 of *MFα* and *CFL1* were both induced in the wild type as well as in the *acb1*Δ mutant during
230 mating (Fig. 1C). However, the degree of induction for both *MFα* and *CFL1* was lower in the
231 *acb1*Δ mutant compared to that in the wild type (Fig. 1C). This is consistent with the reduced
232 filamentation observed in the *acb1*Δ mutant. Interestingly, the transcript level of *PUM1,* a gene
233 that connects filamentation with sporulation in *Cryptococcus* (48), was comparable between the
234 wild type and the *acb1*Δ mutant at all three time points examined (Fig. 1C). Given that the
235 deletion of *PUM1* causes the formation of barren basidial heads without spores (48), the finding
236 that the *PUM1* expression is unaltered in the *acb1*Δ mutant is consistent with the observation that
237 the *acb1*Δ mutant displays no specific defects in sporulation (Fig. 2B). Taken together, the
238 observations indicate that secreted Acb1 contributes to cryptococcal morphotype transition at
239 least partly through its effect on the pheromone signaling and the filamentation pathway.

240 **Secreted products from the wild type, but not the *acb1*Δ mutant, could enhance**
241 **hyphal formation in the nearby *acb1*Δ recipient strain.** Extracellular Acb1 secreted from
242 wild-type cells acts as a signal and can compensate for the loss of *ACB1* in the nearby mutant
243 cells in *Dictyostelium* in terms of sporulation (23). As Acb1 is important for filamentation in
244 *Cryptococcus* and it is highly expressed, we hypothesize that secreted Acb1 from the wild type
245 may also act as a signal in promoting filamentation in *Cryptococcus acb1*Δ mutant. To test this
246 hypothesis, we performed confrontation assays, in which the donor and the recipient were placed
247 in close proximity but not physically touching each other. The wild-type recipient filamented
248 well regardless whether the donor was the wild type or the *acb1*Δ mutant (Fig. 2C-D, Fig. S2).
249 However, more *acb1*Δ recipient colonies formed filaments when the donor was the wild type
250 rather than the *acb1*Δ mutant. Despite increased frequency of the number of *acb1*Δ recipient
251 colonies to form hyphae, the hyphae formed by the mutant were rudimentary at the time point

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252 examined (Fig. S2). Nonetheless, the evidence suggests that products secreted from the wild-type
253 donor, but not from the *acb1*Δ mutant donor, enhanced the frequency of filamentation of the
254 nearby *acb1*Δ recipient cells.

255 **Acb1 promotes the utilization of alternative carbon source.** As an acyl-CoA binding
256 protein, Acb1 regulates growth in different media and conditions, as demonstrated in *S.*
257 *cerevisiae* (35-37, 50, 51). To our surprise, we did not observe any apparent growth defect of the
258 *Cryptococcus acb1*Δ mutants in either the rich YPD medium or the minimum YNB medium (Fig.
259 3 and Fig. S3-4). The mutants were also no different from the corresponding wild-type strains in
260 their tolerance to SDS and antifungal drugs such as capsofungin, polymyxin B, and fluconazole
261 (not shown). In yeast and mammalian cells, the *acb1*Δ mutant showed severe defect in long
262 chain fatty acid metabolism (35, 52). However, in *Cryptococcus*, the *acb1*Δ mutant grew well on
263 lipids as the sole carbon source, just like the wild type (Fig. S5).

264 More surprisingly, the *acb1*Δ mutant in either the XL280 or the H99 background grew
265 equally well as the corresponding wild-type strains on media with different carbon sources
266 (glucose, galactose, glycerol, NaAc, or ethanol) or different nitrogen sources ($(NH_4)_2SO_4$,
267 $NaNO_3$, glycine, aspartic acid, or thiamine) (Fig. 3 and Fig. S3-4). This is again different from
268 what is observed for the *acb1*Δ mutant in *S. cerevisiae*, which showed growth defects in different
269 carbon sources (35, 37, 50).

270 The presence of the preferred carbon source (usually glucose) represses the utilization of
271 other carbon sources (53, 54). This is called catabolite repression. Catabolite repression can be
272 observed with the addition of glucosamine, a glucose mimic (55, 56). The idea is that the
273 presence of glucosamine (GlcN) suppresses the catabolism of other carbon sources and thus
274 inhibits growth even when other carbon sources are available. Indeed, we found that the addition

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275 of glucosamine inhibited the wild type in utilizing NaAc, glycerol, and ethanol (Fig. 3 and Fig.
276 S4). The *acb1*Δ mutant showed more severe growth deficiency than the wild type in using NaAc
277 or ethanol in the presence of glucosamine (Fig. 3 and Fig. S4). This suggests that Acb1 in the
278 wild type might be involved in relaxing catabolite repression, which could be useful for an
279 organism found in soil and decaying vegetation where complex carbon sources other than
280 glucose are more likely to be present.

281 **The Y80 residue in the acyl-CoA binding domain is critical for Acb1's function and**
282 **its subcellular localization.** The acyl-CoA binding domain is highly conserved among Acb
283 proteins (Fig. 4A), suggesting the importance of this domain to the function of Acb1. In this
284 region, Y80 is shown to be a conserved and important residue for binding acyl-CoA, as a
285 mutation of this residue can decrease the proteins' acyl-binding ability by 1000 fold (28, 57-59).

286 To examine if the acyl-CoA binding domain is critical for *Cryptococcus* Acb1's function
287 in filamentation, we made Y80A mutated allele of Acb1 through site-directed mutagenesis. The
288 Acb1 (Y80A) mutated allele, when introduced into the *acb1*Δ mutant, could not restore the
289 mutant's filamentation defect, in contrast to the wild-type allele (Fig. 4B). This result suggests
290 that the acyl-CoA binding ability is critical for Acb1's function in filamentation.

291 Acb1, despite its predicted cytosolic location, is known to be recruited to the secretory
292 pathway in other organisms (28, 39). Under wide-field epi-fluorescence microscope, the
293 mCherry-labeled Acb1 in *Cryptococcus* was located in intracellular puncta (Fig. 4C) that are
294 consistent with secretory vesicles. In *Dictyostelium*, Acbp is also localized to intracellular puncta
295 (39). The Acb1(Y80A)-mCherry, however, showed a diffused cytoplasmic localization (Fig. 4C).
296 The cytosolic localization of Acb1(Y80A) in *Cryptococcus* is consistent with previous

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297 observations in *S. cerevisiae* and *D. discoideum*, where decreased or abolished ability of Acbp to
298 bind to acyl-CoA is associated with increased cytosolic localization (28, 39). Thus previously
299 published literature and our observation collectively indicate that the recruitment of Acb1 to the
300 secretory pathway requires its acyl-CoA-binding capability. It is tempting to speculate that its
301 binding partner might help bring this otherwise cytosolic protein to the secretory pathway.

302 In *Dictyostelium*, the minor proportion of AcbA proteins were secreted extracellularly
303 (39). This is also true in *Cryptococcus* as we found most of the Acb1 proteins in the total cell
304 lysate and some in the culture supernatant (Fig. 5B). Because Acb1(Y80A) is localized to the
305 cytosol, we speculate that extracellular secretion of this mutated protein would be abolished.
306 Indeed, we could not detected any Acb1(Y80A) in the culture supernatant although we could
307 easily detected the protein from the cell lysate. This suggests that the Acb1(Y80A) mutated
308 protein was not released to the environment. Thus, the alteration of this key residue affects
309 Acb1's function as well as its subcellular localization (Fig. 5B).

310 **Acb1's extracellular secretion, but not its recruitment to the secretory pathway, is**
311 **dependent on Grasp.** As mentioned earlier, Acb1 lacks a signal peptide and is not a typical
312 secretory protein that uses the conventional or the general secretion pathway. In *S. cerevisiae* and
313 *D. discoideum*, Acb1's secretion was shown to be dependent on GRASPs (Golgi reassembly
314 stacking proteins (38, 60-62), which were originally identified as factors required for the
315 stacking of Golgi cisternae and the tethering of vesicles destined to fuse with Golgi (63, 64). In
316 addition to Acb1, the secretion of other factors such as integrain and CFTR also depends on the
317 Grasp-mediated unconventional pathway in other organisms (38, 65-68). The mammalian
318 genomes encode two orthologues of *GRASP* genes (66) whereas only one *GRASP* gene is found
319 in *C. neoformans* and yeast previously (61, 69). It is important to note that in both fungal species,

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320 Grasp was shown to be not involved into Golgi stacking but important for molecular secretion
321 (61, 69).

322 To test if Grasp in *Cryptococcus* is involved in the Acb1's recruitment to the secretory
323 pathway and/or its extracellular secretion, we deleted the *GRASP* gene. We then examined the
324 localization and extracellular secretion of Acb1-mCherry in the *grasp*Δ mutant background.
325 Interestingly, we found that Acb1-mCherry was localized to vesicles in the *grasp*Δ mutant, as
326 observed in the wild type (Fig. 5A). This suggests that recruitment of Acb1 to the secretory
327 pathway is independent of Grasp.

328 Next, we tested whether the absence of Grasp affects the extracellular secretion of Acb1
329 in *C. neoformans* using the P_{ACB1}-*ACB1-mCherry* as the reporter. We detected a strong signal of
330 Acb1-mCherry from the cell lysate of the *grasp*Δ mutant (Fig. 5B). This is consistent with our
331 microscopic observation of Acb1-mCherry in intracellular vesicles in the wild type as well as in
332 the *grasp*Δ mutant. However, no Acb1-mCherry was detected in supernatant derived from the
333 *grasp*Δ mutant (Fig. 5B), in contrast to the supernatant derived from the wild-type background.
334 This suggests that extracellular release of Acb1-mCherry is abolished in the *grasp*Δ mutant.
335 Taken together, the recruitment of Acb1 to the secretory pathway is independent of Grasp, but its
336 extracellular secretion requires Grasp.

337 **The *grasp*Δ mutant recapitulated the *acb1*Δ mutant phenotype in terms of**
338 **filamentation.** The evidence presented earlier indicates that Acb1 proteins are predominantly
339 localized intracellularly, with some being secreted extracellularly. As Acb1 is important for
340 filamentation and that secreted products from the wild-type donor, but not the *acb1*Δ mutant
341 donor, can enhance the filamentation in the nearby *acb1*Δ cells, we hypothesize that the released
342 extracellular Acb1 is important for filamentation. As Grasp is required for Acb1's extracellular

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343 secretion, but not Acb1's production or its intracellular localization, we decided to test our
344 hypothesis using the *grasp*Δ mutant. The *grasp*Δ mutant in the H99 background showed reduced
345 filamentation during bisexual mating on V8 medium, as observed for the *acb1*Δ mutant (Fig. 6A).
346 Similarly, the *grasp*Δ mutant in the XL280 background yielded a colony with smoother colony
347 morphology and was less robust in hyphal production during bisexual mating on YNB medium,
348 resembling the *acb1*Δ mutant (Fig. 6B). Consistently, the *grasp*Δ **a**-α mixed culture gave rise to a
349 smooth colony on YPD medium, in contrast to the wrinkled colony generated by the wild-type **a**-
350 α mixed culture (Fig. 6C). Thus, the *grasp*Δ mutant displayed the same phenotypes as the *acb1*Δ
351 mutant in terms of colony morphology and filamentation. This suggests that Grasp regulates
352 cryptococcal morphogenesis mainly through the extracellular secreted Acb1.

353 **The deletion of *GRASP*, but not *ACB1*, affects the production of capsule and**
354 **phagocytosis by macrophages.** We showed in the above paragraph that the *grasp*Δ mutant
355 recapitulates the same phenotypes as the *acb1*Δ mutant in terms of colony morphology and
356 filamentation. It was shown previously that the *grasp*Δ mutant in *Cryptococcus* is defective in
357 the production of capsule and in macrophage phagocytosis (69). To test if such defect in the
358 *grasp*Δ mutant is caused by its defect in Acb1's extracellular secretion, we first examined the
359 production of capsule and melanin in the *acb1*Δ mutant. To our surprise, the deletion of *ACB1*
360 had no apparent impact on capsule production and melanization. By contrast, the deletion of
361 *GRASP* greatly reduced capsule size (Fig. 7A), consistently with the previous report (69). There
362 might be a slight reduction in melanization of the *grasp*Δ mutant (Fig. 7B). Next, we tested the
363 *acb1*Δ mutant in both XL280 and H99 backgrounds in phagocytosis by murine macrophage
364 J774A.1 cells. Although there might be a slight reduction in phagocytosis of the *acb1*Δ mutants
365 compared to the corresponding wild-type strains (Fig. 7C), the differences were not statistically

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366 significant. By contrast, phagocytosis of the *grasp*Δ mutants was significantly lower compared to
367 the wild-type cells in both XL280 and H99 backgrounds (Fig. 7C), as being demonstrated
368 previously for the *grasp*Δ mutant in the H99 background (69). The results indicate that Acb1 is
369 not the only effector of Grasp in *Cryptococcus*, but it is likely the major or the sole effector of
370 Grasp in terms of filamentation.

371

372 **DISCUSSION**

373 We previously demonstrated that the secreted matricellular protein Cfl1, a downstream target of
374 global regulator Znf2 (9, 14), plays important roles in cellular and colony morphogenesis in the
375 environmental fungal pathogen *C. neoformans* (19, 70). In this study, we investigated the role of
376 the secretory protein Acb1 in cryptococcal yeast-to-hypha morphological transition and
377 sporulation given the importance of its ortholog in sporulation in *Dictyostelium* and *Pichia*
378 *pastoris* (23, 62). In contrast to Acbp in *Dictyostelium* (23), we found that Acb1 in *Cryptococcus*
379 is not critical for sporulation *per se*, but secreted Acb1 is important for hyphal growth that
380 precedes sporulation during both unisexual and bisexual reproduction. The function of Acb1 in
381 cellular and colony morphology is conserved in both serotype A and serotype D, two subspecies
382 of the *C. neoformans* species complex (71). However, in contrast to *CFL1*, *ACB1* is unlikely to
383 be controlled by Znf2 at the transcript level, despite the modest increase of *ACB1* transcripts
384 during bisexual mating. First, *ACB1* is not among the differentially expressed genes in the
385 *ZNF2*^{oe} strain or the *znf2*Δ strain compared to the wild-type control (9, 14). Second, Znf2
386 controlled genes are typically expressed at low levels during yeast growth and are highly induced
387 during filamentous growth (9, 19). This is not the case for *ACB1*. The transcript level of *ACB1*
388 gene is high even during yeast growth in YPD. In fact, *ACB1* ranks ~ top 5% among all

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389 cryptococcal expressed genes based on our recent RNA-seq data analyses (46). However,
390 whether Znf2 directly or indirectly affects Acb1's activity at other regulatory levels (e.g.
391 translation, protein localization, secretion, or modification) is yet to be investigated.

392 The cryptococcal genome carries two genes that encode proteins with an acyl-CoA
393 binding domain. One is CNAG_06140 that we named Acb1 in this study, which is predicted to
394 encode a protein of a little over 100 amino acids (Fig. 4A). The other is CNAG_01191, which is
395 predicted to encode a long-chain fatty acid transporter of 458 amino-acid long. Given that AcbP
396 in *Dictyostelium* and Acb1 in *Saccharomyces* are composed of 84 and 87 amino acids
397 respectively (23, 26, 35, 72), we considered CNAG_06140/Acb1 in *Cryptococcus* a more likely
398 ortholog of Acb proteins. Furthermore, the transcript level of *ACB1* is about 10 folds higher than
399 that of CNAG_01191 based on our RNA-seq data (46). Thus, we focused on *ACB1* in this study.
400 However, it is likely that the lack of defects of the *Cryptococcus acb1*Δ mutant in utilizing
401 various carbon sources could be due to functional redundancy in fatty acid metabolism of Acb1
402 and the protein encoded by CNAG_01191.

403 The observation that secreted products from the wild type, but not the *acb1*Δ mutant, can
404 partially restore the filamentation defect of the nearby *acb1*Δ mutant suggests that secreted Acb1
405 proteins can act intercellularly. Given that filamentation of the *acb1*Δ mutant is not as robust as
406 the wild type, even when it is confronted by the wild-type donor, Acb1 likely functions in a
407 paracrine fashion. We hypothesize that it is the extracellular Acb1 proteins and not the
408 intracellular Acb1 proteins that are critical for filamentation. This hypothesis is consistent with
409 its predicted paracrinal signaling function. This hypothesis is also corroborated by the result that
410 the *grasp*Δ mutant, which is defective in secreting Acb1 to the environment but not defective in
411 recruiting Acb1 to the secretory pathway, displays a similar drastic reduction in filamentation as

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412 the *acb1*Δ mutant. The hypothesis is further supported by the observation that mis-localization
413 of Acb1(Y80) to the cytosol and consequently the lack of protein secretion render the protein
414 non-functional.

415 One interesting aspect of Acb1 is its unconventional recruitment to the secretory pathway.
416 Although Grasp is critical for its extracellular secretion, Grasp is not involved in recruiting Acb1
417 to the secretory vesicles intracellularly. Since Y80 mutation is known to disrupt its acyl-binding
418 property (58, 59, 73) and Acb1(Y80A) showed diffused localization in the cytosol, it is tempting
419 to speculate that Acb1's binding partner, be it a lipid or a protein, might help recruit Acb1 to the
420 vesicles through the interaction with its acyl-binding domain. How Grasp recognizes Acb1 after
421 Acb1 is recruited to secretory vesicles and how it assists Acb1 to be secreted extracellularly is
422 unknown. It is clear that Grasp is involved in the secretion of Acb1 and additional factors in
423 *Cryptococcus* based on this and a previous study (69). Consistent with this idea, the secretion of
424 integrin in *Drosophila* and CFTR in mammalian cell also depends on the Grasp-mediated
425 unconventional secretion (67, 68, 74). Given that many proteins that are found to be secreted
426 extracellularly are atypical proteins that possess no signal peptide in fungi (75-79), it would be
427 important to continue the investigation into these atypical proteins and the corresponding
428 unconventional secretory pathways.

429 Consistent with the idea that Grasp is responsible for the secretion of factors in addition
430 to Acb1, the *grasp*Δ mutant showed drastically decreased capsule production [here and previous
431 study (69)], while the *acb1*Δ mutant showed normal capsule production. Similarly, in contrast to
432 the severe defect in phagocytosis of the *grasp*Δ mutant, the *acb1*Δ behaves similarly to the wild
433 type in the phagocytosis assay. Thus Grasp likely affects the secretion of various molecules (e.g.
434 capsule and possibly some cell wall proteins) that contribute to the phenotypic defects of the

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435 *grasp*Δ mutant in various assays. Nonetheless, in terms of Grasp's effect on hyphal growth,

436 Acb1 appears to be the major if not the sole factor.

437

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448

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683

684 **FIGURE LEGENDS**

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685 **FIG 1**. The deletion of *ACB1* impairs filamentation during bisexual mating in *Cryptococcus*. (A)
686 The **a**-α mating pairs of the WT XL280 strains, the *acb1*Δ mutants, and the *ACB1* complement
687 strains were cultured on YNB medium for 48 hrs. (B) The **a**-α mating pairs of the WT H99
688 strains, the *acb1*Δ mutants, and the *ACB1* complement strains were cultured on YNB medium for
689 9 days. (C) Q-RT PCR measurement of the transcript levels of *MFα*, *CFL1*, *PUM1*, and *ACB1*.
690 The transcript level of each gene in the wild type at time point 0 was set as 1 for comparison (0
691 in the Log_2 value). RNA samples were extracted from bisexual matings of the WT XL280 and
692 the *acb1*Δ mutants on YNB medium at the time point of 0 hour, 24 hours, and 48 hours.

693

694 **FIG 2**. Secreted products from the wild type, but not the *acb1*Δ mutant, enhanced filamentation
695 in the neighboring cells. (A) Colony images of the **a**-α bisexual mating of the WT XL280 and the
696 corresponding *acb1*Δ mutant on V8 pH7 medium. (B) Images of the colony edge (top panel) and
697 basidiospores (lower panel) generated by the bisexual mating of WT strains or the *acb1*Δ
698 mutants on V8 medium. (C) Confrontation assay using WT and mutant mating pairs as donor or
699 recipient. (D) Quantification of the frequency of recipient colonies that formed filaments at 48
700 hrs post the inoculation. 80% of the *acb1*Δ mutant recipient colonies formed hyphae when the
701 donor was WT. In contrast, 30% of the *acb1*Δ mutant recipient colonies formed hyphae when
702 confronted with the *acb1*Δ mutant donor.

703

704 **FIG 3**. Growth of the *acb1*Δ mutant and the corresponding wild type strain on different carbon
705 sources in the presence of glucosamine.

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707 **FIG 4**. Mutation of the key residue Y80 affects Acb1's function and subcellular localization. (A)
708 Acb1 is a highly conserved protein among different species. Multiple alignment of proteins from
709 the following species: Hs, *Homo sapiens*; Mus, Mouse Species; Cn, *Cryptococcus neoformans*;
710 Sc, *Saccharomyces cerevisiae*; Pp, *Pichia Pastoris*; Dd, *Dictyostelium discoideum*; Af,
711 *Aspergillus fumigatus*; An, *Aspergillus nidulans*. (B) The mutated Acb1(Y80A) could not restore
712 the filamentation defect of the *acb1*Δ mutant. The *acb1*Δ mutant, the *acb1*Δ mutant transformed
713 with the wild type allele [$ACB1^c$], and the *acb1*Δ mutant transformed with the Y80A allele
714 [$ACB1(Y80A)^c$] were cultured on YNB medium for 48 hours. (C) The subcellular localization of
715 Acb1-mCherry and Acb1(Y80A)-mCherry. The fluorescent image from non-transformed wild
716 type cells were used as the negative control.

717

718 **FIG 5**. The subcellular localization of Acb1 is independent of Grasp, but the secretion of Acb1
719 requires Grasp. (A) Intracellular localization of Acb1-mCherry in the wild-type or the *grasp*Δ
720 mutant background. (B) The western blotting analysis of the supernatant from different strains.
721 (1, Acb1-mCherry in the *acb1*Δ mutant; 2, Acb1-mCherry in the *grasp*Δ mutant; 3, Acb1
722 (Y80A)-mCherry in the *acb1*Δ mutant; 4, negative control without any mCherry, XL280; 5,
723 Acb1-mCherry in WT XL280).

724

725 **FIG 6**. The *grasp*Δ mutant recapitulates the phenotype of the *acb1*Δ mutant in terms of colony
726 morphology and filamentation. (A) The **a**-α co-culture of the wild type, the *acb1*Δ mutant, or the
727 *grasp*Δ mutant in the H99 background on V8 juice medium (pH5). (B) The **a**-α co-culture of the
728 wild type, the *acb1*Δ mutant, or the *grasp*Δ mutant in the XL280 background on YNB medium.

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729 (C) The **a**-α co-culture of the wild type, the *acb1*Δ mutant, or the *grasp*Δ mutant in the XL280
730 background on YPD medium.
731
732 **FIG 7**. The *grasp*Δ mutant showed slightly deficiency in multiple classic *cryptococcal* virulence
733 traits. (A) The WT, the *grasp*Δ mutant, and the *acb1*Δ mutant after cultured on RPMI medium
734 for 2 days. The capsule (halo surrounding the yeast cells) was visualized by Indian ink negative
735 staining. (B) The WT, the *grasp*Δ mutant, and the *acb1*Δ mutant were cultured on L-DOPA
736 medium for 3 days to test melanin formation. The dark brown pigment indicates melanin. (C)
737 The macrophage cells were inoculated with the WT, the *grasp*Δ mutant, and the *acb1*Δ mutant
738 on RPMI medium and were co-cultured for 3 hours. The number of phagocytosed *Cryptococcus*
739 cells were measured by CFU counting and graphed.

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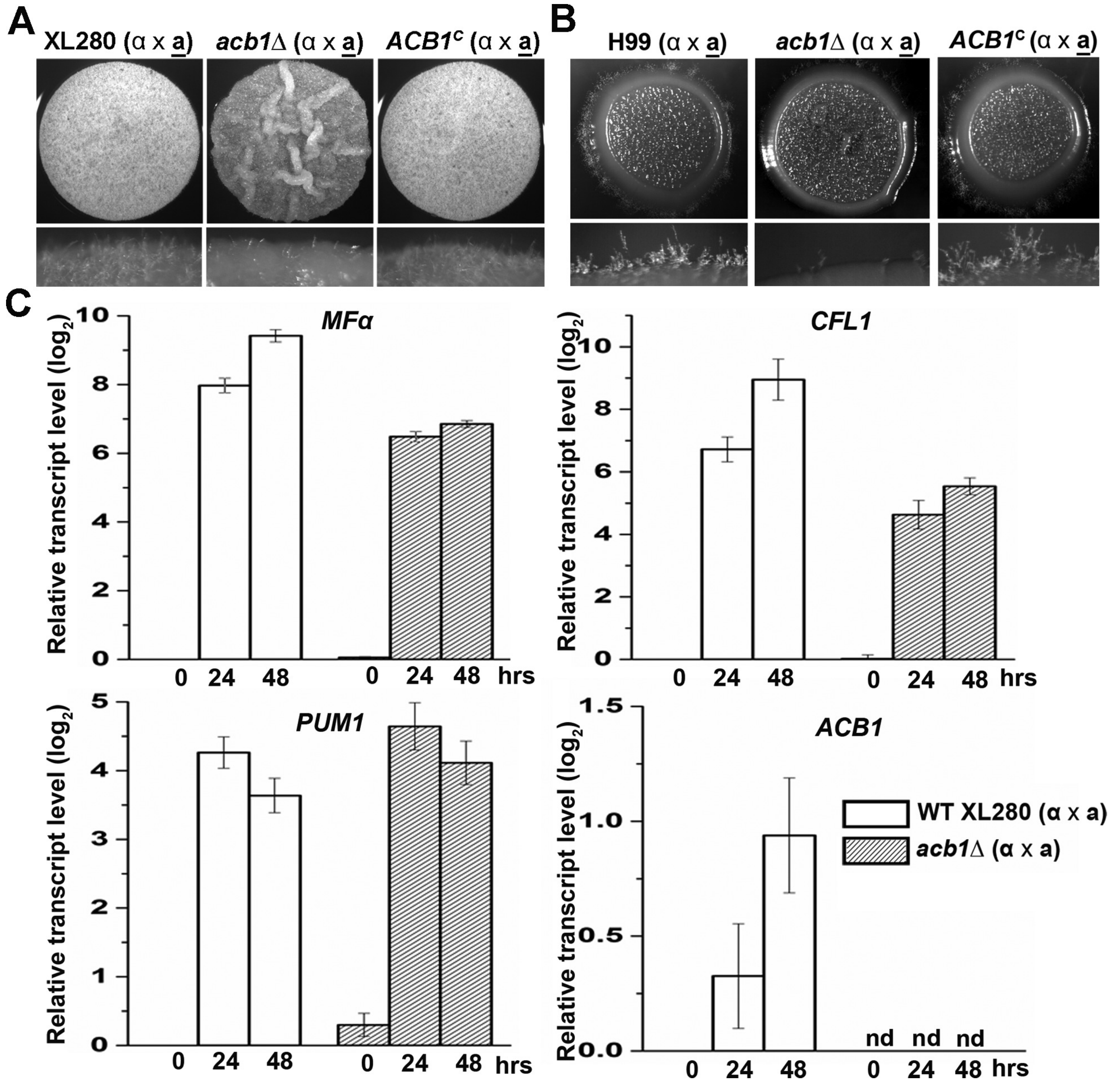

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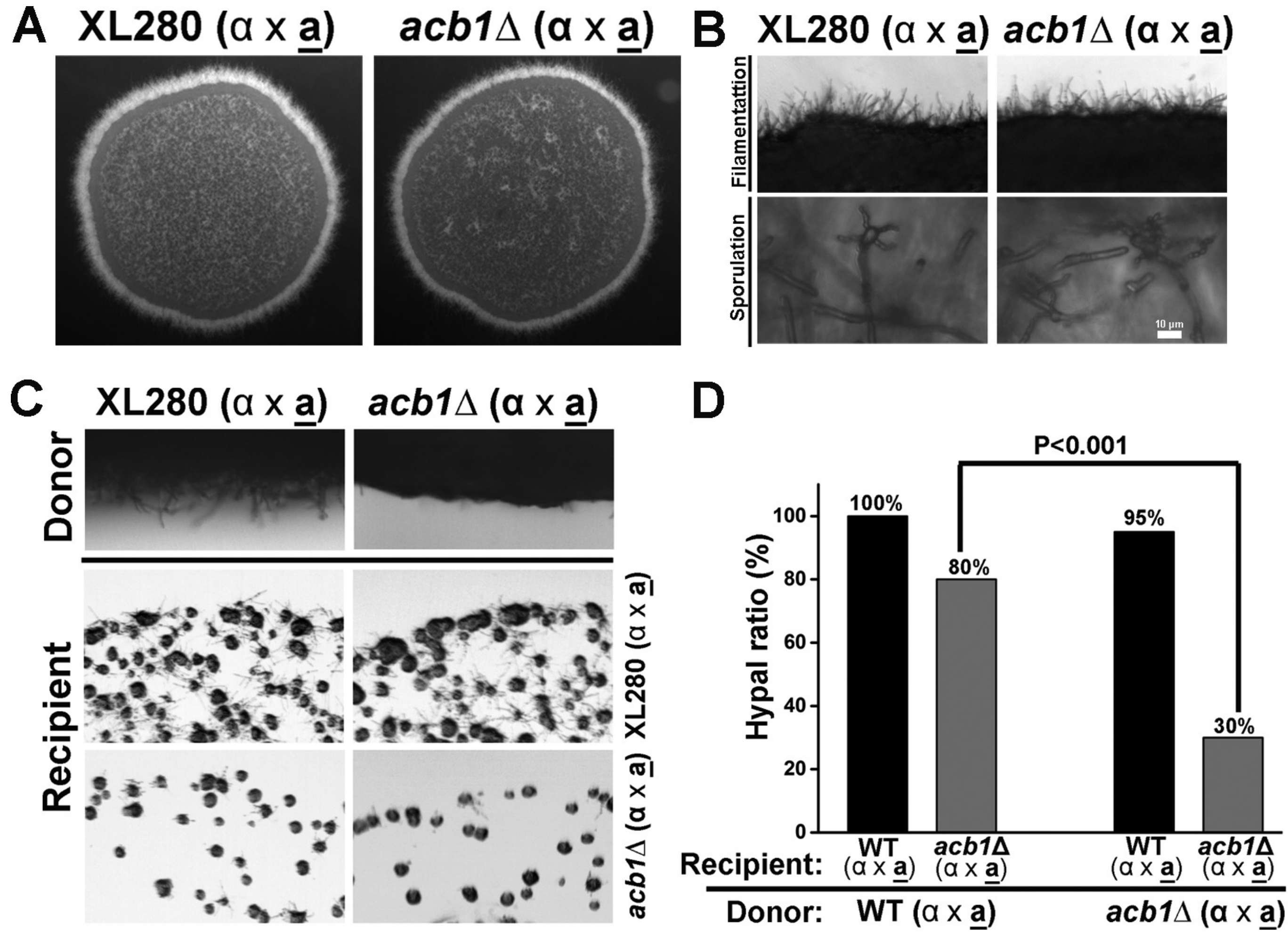

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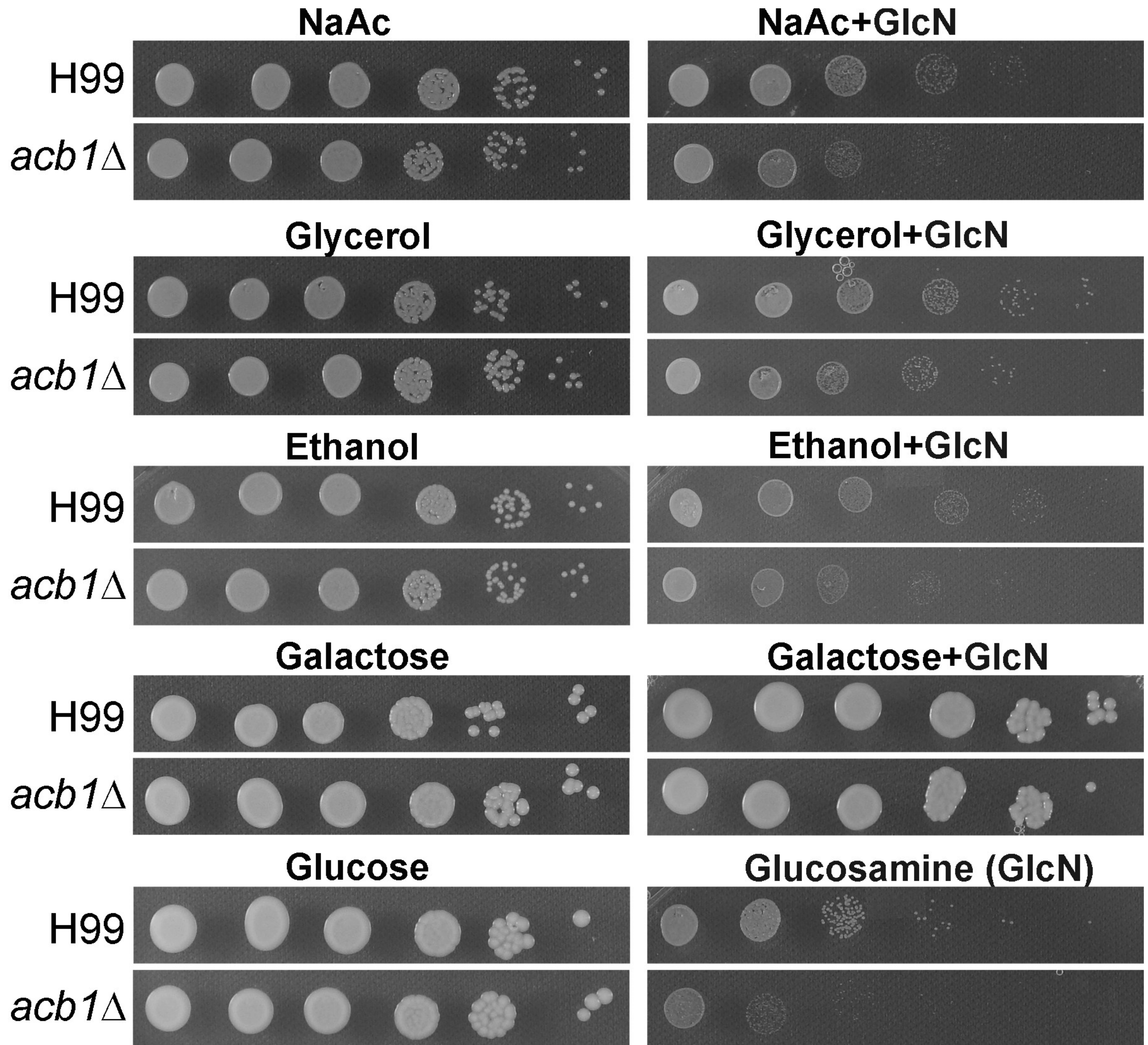

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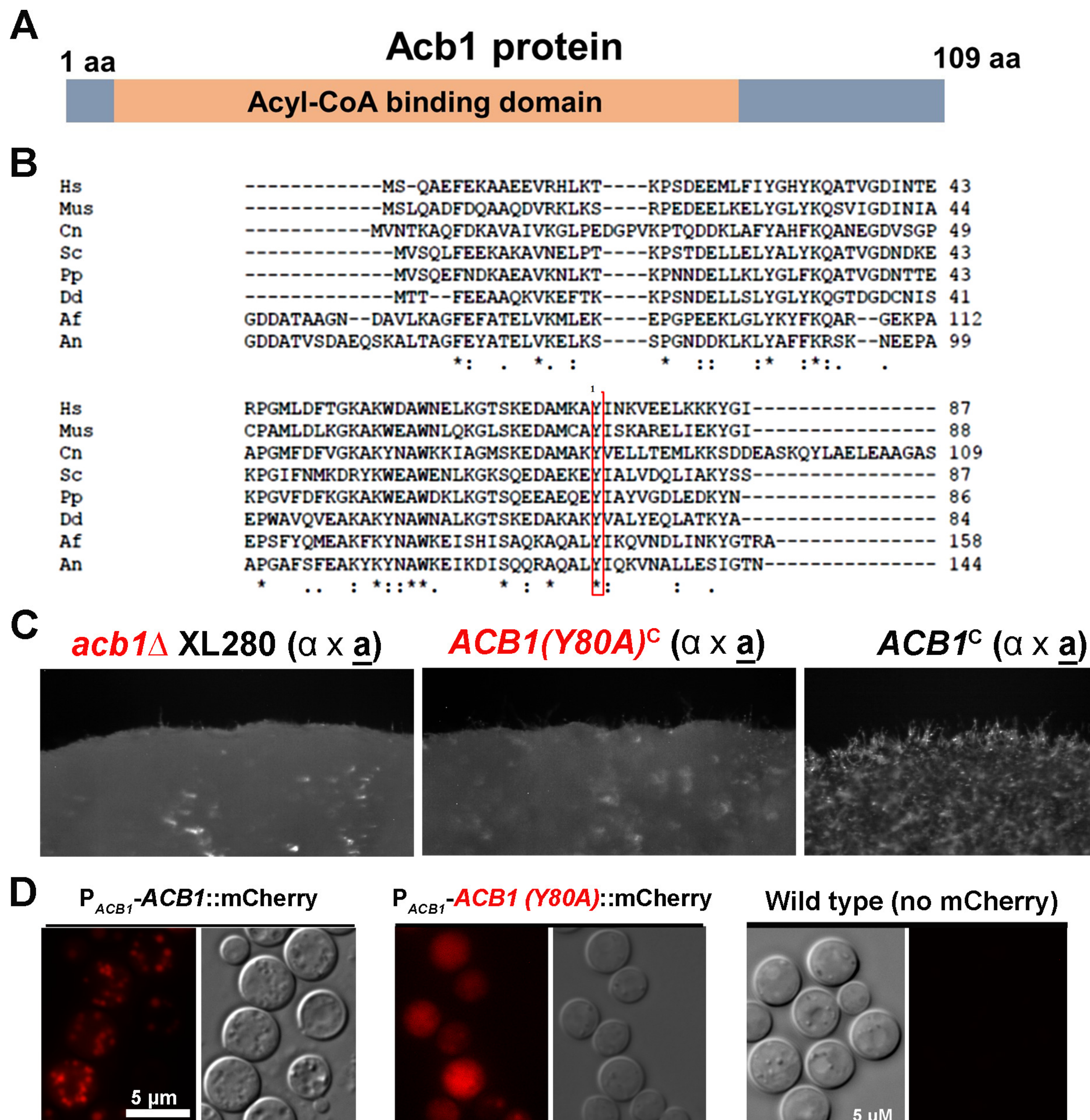

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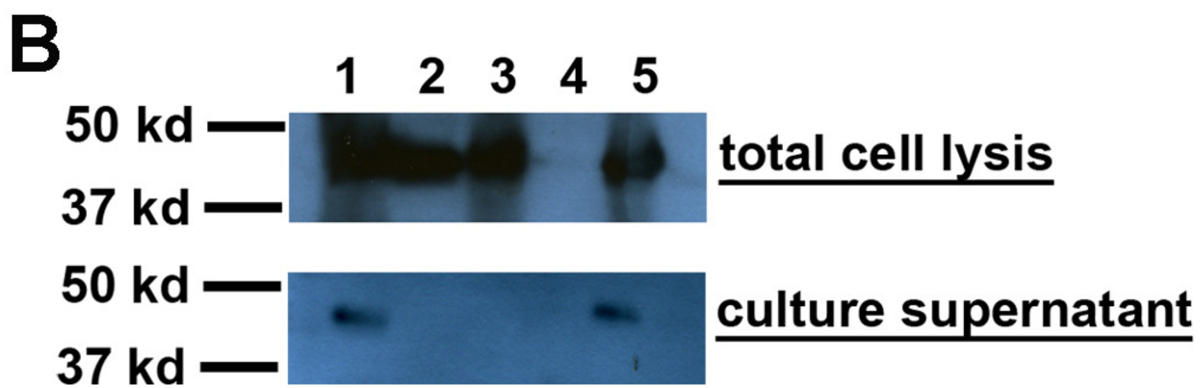
A

P_{GPD1}-Acb1-mCherry

WT

*grasp*Δ

10 µm



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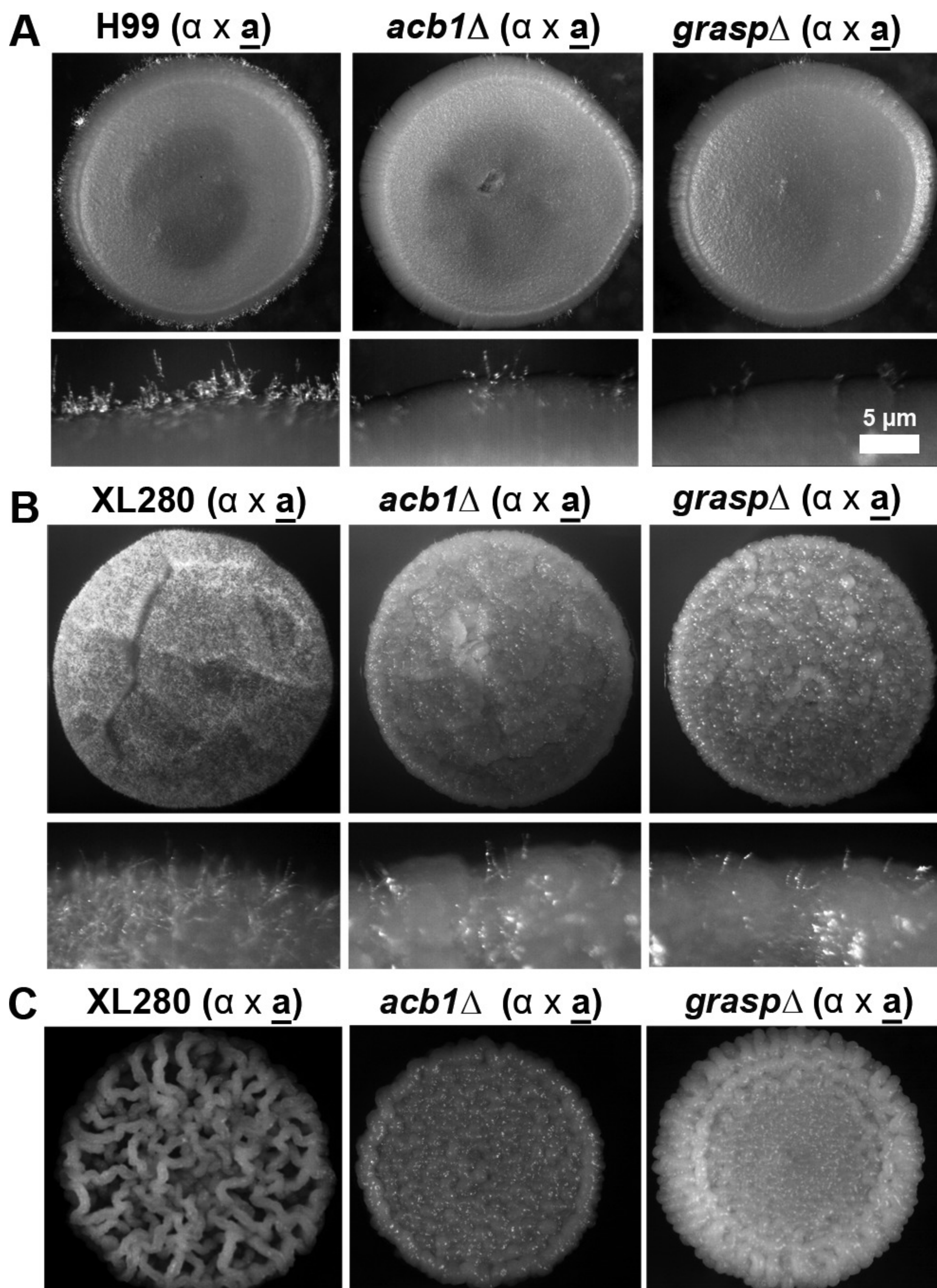

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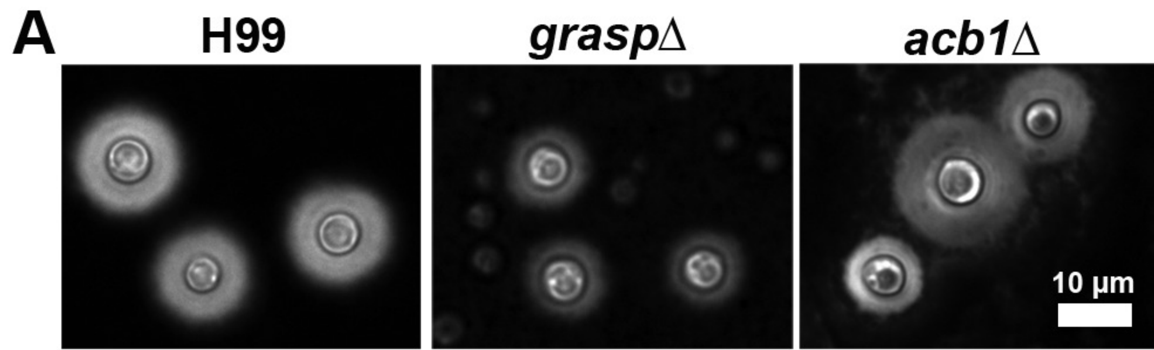

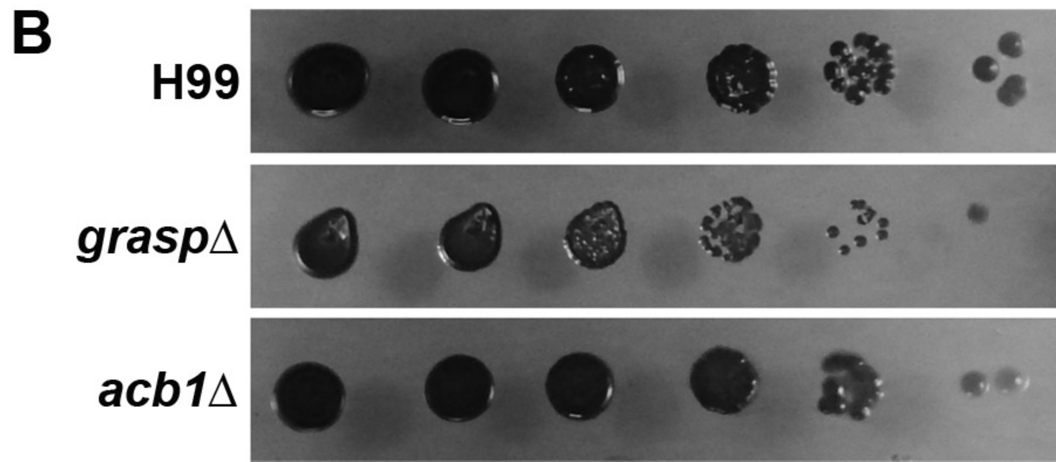

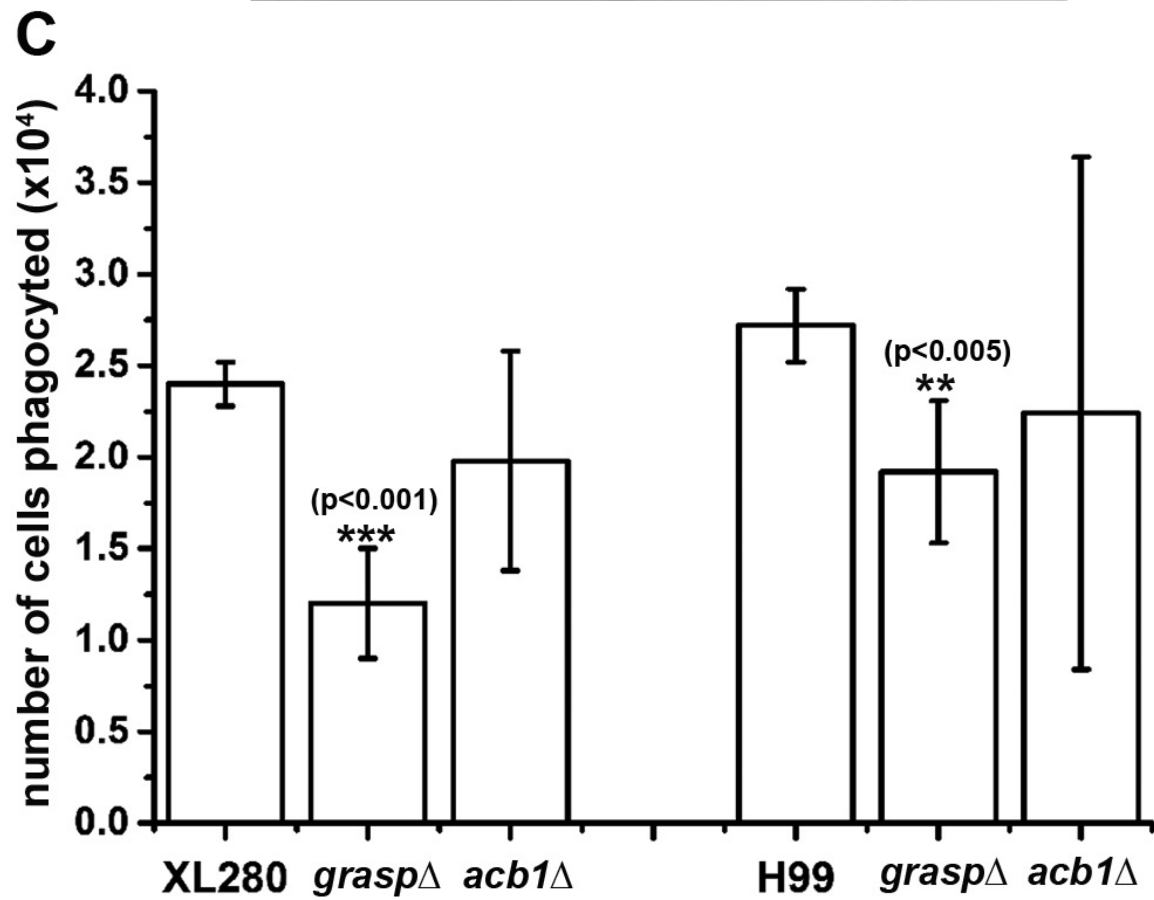

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