

1 **Redistribution of a glucuronoxylomannan epitope towards the capsule surface**
2 **coincides with Titanisation in the human fungal pathogen *Cryptococcus***
3 ***neoformans***

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43 **Abstract**

44 Disseminated infections with the fungal species *Cryptococcus neoformans* or, less
45 frequently, *C. gattii*, are a leading cause of mortality in immunocompromised
46 individuals. Central to the virulence of both species is an elaborate polysaccharide
47 capsule that consists predominantly of glucuronoxylomannan (GXM). Due to its
48 abundance, GXM is an ideal target for host antibodies, and several monoclonal
49 antibodies (mAbs) have previously been derived using purified GXM or whole
50 capsular preparations as antigen. In addition to their application in the diagnosis of
51 cryptococcosis, anti-GXM mAbs are invaluable tools for studying capsule structure.
52 In this study, we report the production and characterisation of a novel anti-GXM
53 mAb, Crp127, that unexpectedly reveals a role for GXM remodelling during the
54 process of fungal Titanisation. We show that Crp127 recognises a GXM epitope in
55 an *O*-acetylation dependent, but xylosylation-independent, manner. The epitope is
56 differentially expressed by the four main serotypes of *Cryptococcus neoformans* and
57 *gattii*, is heterogeneously expressed within clonal populations of *C. gattii* serotype B
58 strains and is typically confined to the central region of the enlarged capsule.
59 Uniquely, however, this epitope redistributes to the capsular surface in Titan cells, a
60 recently recognised subset of giant fungal cells that are produced in the host lung
61 and are critical for successful infection. Crp127 therefore highlights hitherto
62 unexpected features of cryptococcal morphological change and may hold significant
63 therapeutic potential in differentially identifying cryptococcal strains and subtypes.

64

65 **Importance**

66 *Cryptococcus neoformans* and *Cryptococcus gattii* are the etiological agents of
67 cryptococcosis, an invasive fungal infection responsible for approximately 200,000
68 deaths each year and 15% of AIDS-related deaths annually. Whilst the main

69 virulence factor for both species is a highly variable polysaccharide capsule,
70 formation of Titan cells also underlies the pathogenesis of *C. neoformans*. Previous
71 studies have shown that capsule composition differs between yeast and Titan cells,
72 however no clear distinctions in the expression or localisation of specific capsular
73 epitopes have been made. In this study, we characterise a novel monoclonal
74 antibody (mAb) specific to a capsular epitope that is differentially distributed
75 throughout the capsules produced by yeast and Titan cells. Whilst this epitope is
76 found within the midzone of yeast capsules, the presentation of this epitope on the
77 surface of Titan cell capsules may represent a way in which these cell types are
78 perceived differently by the immune system.

79

80 **Introduction**

81 As the two main etiological agents of cryptococcosis, *Cryptococcus neoformans* and
82 *Cryptococcus gattii* are major contributors to the global health burden imposed by
83 invasive fungal infections (1). Whilst *C. neoformans* typically manifests as meningitis
84 in immunocompromised individuals, *C. gattii* infections are not associated with
85 specific immune defects and have been responsible for fatal outbreaks of pneumonia
86 (2–4). Central to the virulence of both species is an elaborate polysaccharide
87 capsule, without which *Cryptococcus* is rendered avirulent (5, 6). The composition of
88 this capsule is highly variable and differs between yeast cells and Titan cells, the
89 latter representing a subset of giant cells (>10 µm in cell body diameter) formed by
90 *C. neoformans* within the host lung (7–9). Titan cells contribute to pathogenesis by
91 resisting phagocytosis, enhancing dissemination of yeast to the central nervous
92 system and altering host immune status (7, 9–13).

93

94 The cryptococcal capsule consists of ~90% glucuronoxylomannan (GXM), ~10%
95 glucuronoxylomannogalactan (GXMGal) and <1% mannoproteins (MPs) (14). GXM
96 is a megadalton polysaccharide containing a backbone of α -(1,3)-mannan that is
97 decorated with β -(1,2)-glucuronic acid, β -(1,2)-xylose and β -(1,4)-xylose substituents
98 (15). The backbone mannan can also be O-acetylated, although the position at
99 which this modification is added remains unclear for most strains (14–16). Seven
100 repeat motifs – called structure reporter groups (SRGs) – contribute to structural
101 variation in GXM (15). All SRGs contain a β -(1,2)-glucuronic acid on their first
102 mannose residue, however the number of β -(1,2)- and β -(1,4)-xylose substituents
103 varies (15). The extent and position of O-acetyl groups in each SRG remain unclear,
104 however xylose and O-acetyl groups attached to the same mannose residue appear
105 to be mutually exclusive (17). SRG usage differs between the four main serotypes of
106 *Cryptococcus*, with each strain designated a serotype based on the reactivity of its
107 capsular material with antibody preparations (18). *C. neoformans* serotypes A and D
108 tend to biosynthesise GXM containing SRGs with fewer xylose substituents than
109 those from *C. gattii* serotypes B and C (15, 19).

110

111 Whilst capsule structure differs between serotypes of *Cryptococcus*, a flexible
112 biosynthetic pathway enables rapid remodelling of the capsule under different
113 environmental conditions (20). *In vitro*, changes in O-acetylation have been
114 associated with cell ageing in *C. neoformans* (21), reaffirming earlier reports that
115 capsules produced within clonal populations are far from homogeneous (19, 22). *In*
116 *vivo*, changes in capsule size and structure coincide with the infection of different
117 organs and likely enhance fitness through the evasion of host immunity (23–25). In
118 light of these observations, it is perhaps unsurprising that capsules produced by

119 Titan cells are structurally distinct from those produced by typical yeast cells (7, 11,
120 26). As the increased chitin content of cell walls produced by Titan cells is
121 associated with activation of a detrimental T_H2 immune response during
122 cryptococcosis (27), it is possible that hitherto unidentified structural differences in
123 Titan cell capsules also contribute to the modulation of host immunity by this *C.*
124 *neoformans* morphotype.

125

126 Alterations in capsule structure are likely to affect how *Cryptococcus* is perceived by
127 host immune molecules, with antibodies particularly sensitive to small changes in
128 molecular structures. Following exposure to cryptococci, immunoglobulin M (IgM)
129 antibodies are the most abundant isotype of antibody produced in response to GXM
130 (28). As a repetitive capsular polysaccharide, GXM is a T-independent type 2
131 antigen and antibodies generated against it utilise a restricted set of variable region
132 gene segments (29). Using monoclonal antibodies (mAbs) in conjunction with
133 mutants harbouring specific defects in GXM modification (17, 30, 31), it has been
134 determined that O-acetylation and, to a lesser extent, xylosylation of GXM are
135 important for epitope recognition by anti-GXM antibodies (16, 30). Whilst there is no
136 consensus surrounding the effect of GXM O-acetylation on virulence (17, 32), its
137 influence on antibody binding suggests that changes in GXM O-acetylation could be
138 a strategy deployed by cryptococci to avoid recognition by immune effectors.
139 Additionally, despite the immunomodulatory roles for GXM O-acetylation that have
140 been identified (30, 33), receptors that bind O-acetylated GXM remain elusive (34).
141 Due to the enigmatic nature of this modification within the primary virulence factor of
142 cryptococci, further investigation of GXM O-acetylation will help unravel the

143 complexities of cryptococcal capsule structure with the ultimate aim of understanding
144 the strategies deployed by this fatal fungal pathogen to evade host immunity.

145

146 In the present study, we report the generation of Crp127, a murine IgM mAb, using a
147 cocktail of heat-killed *C. neoformans* H99 (serotype A), heat-killed *C. gattii* R265
148 (serotype B) and their lysates as an immunogen. Characterisation of Crp127
149 demonstrated that it is an O-acetyl-dependent anti-GXM mAb specific to an epitope
150 expressed by the four *Cryptococcus* serotypes in a serotype-specific manner. Having
151 subsequently found that this epitope is heterogeneously expressed within serotype B
152 populations and is spatially confined to distinct regions of the enlarged capsule
153 across all strains tested, we then turned our attention to its expression by Titan cells.
154 Intriguingly, we noticed that the spatial distribution of this epitope differs within the
155 capsules produced by the three *C. neoformans* morphotypes found within Titanising
156 populations. Further analysis revealed that, under conditions permissive for
157 Titanisation, cell enlargement coincides with the gradual redistribution of this epitope
158 to the capsule surface.

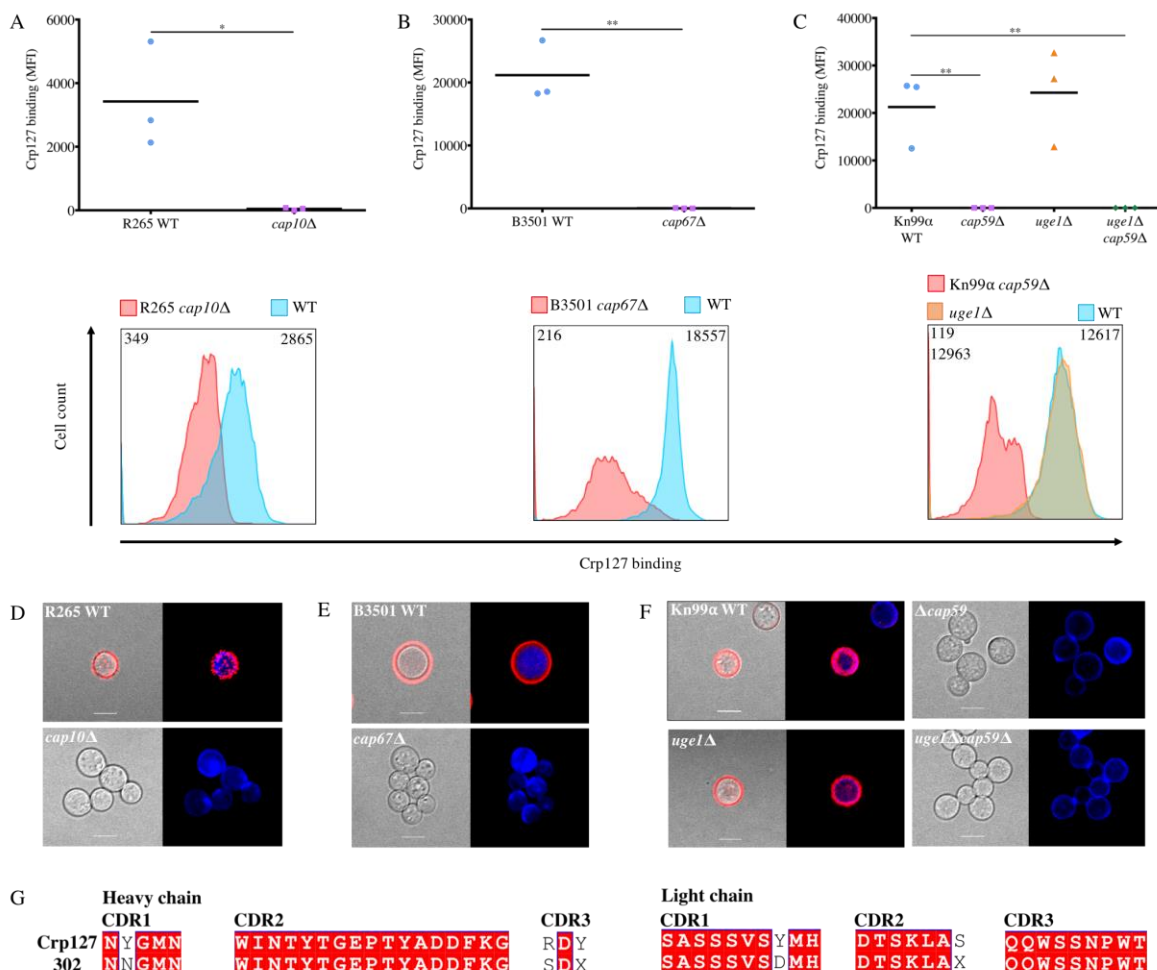
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160 **Results**

161 **Crp127 recognises a capsular epitope located in GXM**

162 During hybridoma screening, Crp127 was identified as staining the outer zone of live
163 cryptococci. We first assessed whether Crp127 recognises a capsular component by
164 performing flow cytometric analysis of three GXM-deficient mutants (R265 *cap10* Δ ,
165 Kn99 α *cap59* Δ and B3501 *cap67* Δ), a GXMGal-deficient mutant (Kn99 α *uge1* Δ) and
166 a mutant lacking both GXM and GXMGal (Kn99 α *cap59* Δ *uge1* Δ), using an Alexa-
167 488-conjugated anti-IgM secondary antibody to label Crp127. Unlike their

168 corresponding wild-type strains, the GXM-deficient mutants were not recognised by
 169 Crp127 (fig. 1A-C; *cap10*Δ *P* < 0.05; *cap67*Δ *P* < 0.01; *cap59*Δ *P* < 0.01;
 170 *cap59*Δ*uge1*Δ *P* < 0.01, Student's t-test). In contrast, the GXMGal-deficient *uge1*Δ
 171 mutant was bound at levels similar to the wild-type strain (fig. 1C; *P* > 0.05).
 172 Confocal microscopy corroborated these observations, with no observable binding of
 173 Crp127 to GXM-deficient mutants but clear binding of Crp127 to the GXMGal-
 174 deficient mutant (fig. 1D-F). Taken together, these experiments demonstrated that
 175 the epitope recognised by Crp127 – hereon referred to as the Crp127 epitope – is a
 176 component of GXM.



177

178 **Fig. 1. Crp127 is an anti-GXM mAb.** The ability of Crp127 to bind to GXM- and
 179 GXMGal-deficient mutants of *C. gattii* and *C. neoformans* was quantified via flow

180 cytometry. Scatter plots (top row) and representative histograms (bottom row) are
181 presented for **A)** R265 cap10 Δ , **B)** B3501 cap67 Δ , **C)** Kn99 α cap59 Δ , Kn99 α uge1 Δ ,
182 Kn99 α cap59 Δ uge1 Δ and their corresponding wild-type strains. For scatter plots,
183 corrected median fluorescence intensity (MFI) values were calculated by subtracting
184 the MFI value of isotype control cells from the MFI value of the Crp127-treated cells,
185 with data points representing MFI values calculated from three biological replicates
186 performed as independent experiments. A Student's t-test was used to test for
187 statistically significant differences between R265 cap10 Δ and B3501 cap67 Δ and
188 their corresponding wild-type strains, whilst one-way ANOVA followed by Dunnett's
189 multiple comparison test was used to test for statistically significant differences
190 between Kn99 α cap59 Δ , Kn99 α uge1 Δ cap59 Δ , Kn99 α uge1 Δ and the wild-type
191 strain KN99 α (n=3) (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$). Histograms show a
192 representative distribution of Crp127 binding for one or all of the strains in the above
193 scatter plot, with the colour-coded key provided for reference. Numerical values in
194 the top left and right of each histogram correspond to the MFI value calculated from
195 the strain labelled directly above. **D)** R265 cap10 Δ , **E)** B3501 cap67 Δ , **F)** Kn99 α
196 cap59 Δ , uge1 Δ , uge1 Δ cap59 Δ and their wild-type strains were labelled for chitin
197 using calcofluor-white (CFW; blue) and Crp127 (far-red; goat Alexa-647-conjugated
198 anti-mouse IgM μ -chain) and maximum-intensity projections generated from confocal
199 microscopy z-stacks. Presented are representative images merged for transmitted
200 light and Crp127 (left panels) and Crp127 and chitin (right panels). Scale bars
201 represent 5 μ m. **G)** Amino acid sequences of the CDRs from mAbs Crp127 and 302
202 were aligned. Residues highlighted in red are identical. X denotes amino acid
203 residues that are undetermined.

204

205 In order to draw comparisons with previously described anti-GXM mAbs, we next
206 sequenced both the heavy-chain variable (V_H) and light-chain variable (V_L) regions of
207 Crp127 (fig. S1). Antibody sequence analysis revealed that the Crp127 V_H region
208 uses the V_{H9} (VGAM family)- J_{H2} combination with a D-segment consisting of four
209 amino acids, whilst the V_L region uses a combination of $V_{k4/5}$ - J_k1 . This gene usage
210 profile is remarkably similar to that of anti-GXM IgG1 mAb 302, which differs only in
211 respect to the length of its D-segment (35). In line with this, alignment of Crp127 and
212 302 protein sequences revealed 86.6% and 98.1% sequence identity between V_H
213 and V_L regions, respectively (fig. S1A-B). When the complementarity determining
214 regions (CDRs) of mAbs Crp127 and 302 were aligned, we found identical V_H CDR2
215 and V_L CDR3 regions whilst the remaining CDRs differed at one or two positions (fig.
216 1G). Notably, however, the sequence available for mAb 302 is incomplete and may
217 contribute additional variation (fig. 1G). We also compared the CDRs of Crp127 with
218 four other well-characterised anti-GXM IgM mAbs – 2D10, 12A1, 13F1 and 21D2 –
219 with less similarities identified between Crp127 and these mAbs (fig. S1C-D).

220

221 **GXM O-acetylation is required for Crp127 epitope recognition**

222 Considering the importance of O-acetylation and xylosylation to the antigenic
223 signature of GXM (30), we proceeded to investigate the effect of these modifications
224 on Crp127 epitope recognition. We firstly tested the ability of Crp127 to recognise
225 two xylose-deficient mutants (JEC155 $uxs1\Delta$ (serotype D) and Kn99 α $uxs1\Delta$
226 (serotype A)). No significant differences were found between either $uxs1\Delta$ mutant
227 and their corresponding wild-type strains (fig. 2A; JEC155 $uxs1\Delta$ $P > 0.05$; Kn99 α
228 $uxs1\Delta$ $P > 0.05$), indicating that xylosylation does not impact Crp127 binding. In
229 contrast, however, antibody binding was completely abrogated in the O-acetyl-

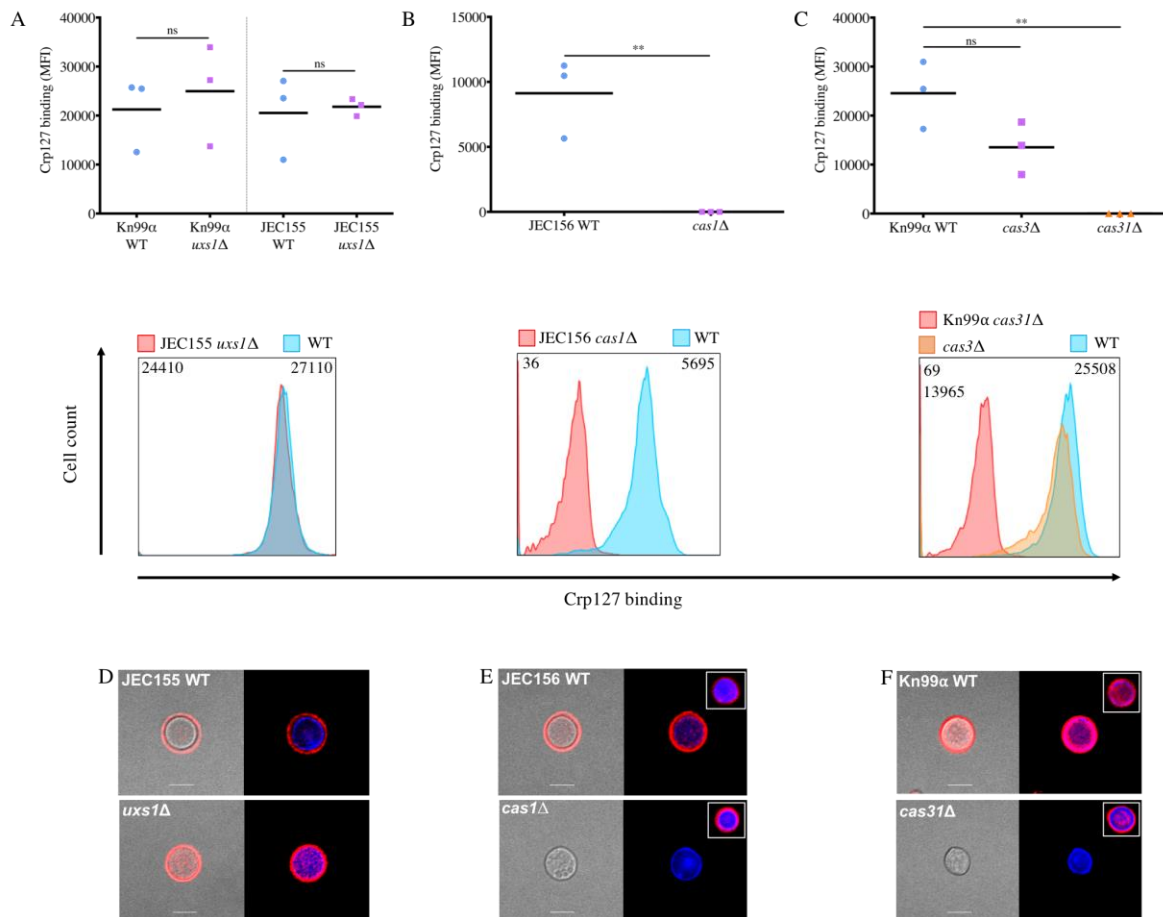
230 deficient *cas1*Δ mutant (fig. 2B; $P < 0.01$), indicating that O-acetylation of GXM is an
231 essential prerequisite for Crp127 epitope recognition.

232

233 Having made this observation, we proceeded to test two further mutants in genes
234 implicated in GXM O-acetylation. *Kn99*α *cas3*Δ exhibits a ~70% reduction in GXM O-
235 acetylation, whereas *Kn99*α *cas31*Δ exhibits subtle differences in sugar composition
236 of GXM but no reduction in GXM O-acetylation (36). Binding of Crp127 to the *cas3*Δ
237 mutant was slightly reduced but statistically to the wild-type strain (fig. 2C; $P > 0.05$).

238 This may reflect reduced density of O-acetylation in GXM produced by this mutant
239 Surprisingly, however, Crp127 completely failed to recognise the *cas31*Δ mutant
240 despite this strain retaining an O-acetylation profile similar to the wild-type (36) (fig.
241 2C; $P < 0.01$). To be certain that the O-acetyl-defective mutants tested still produced
242 capsule, we confirmed the binding of O-acetyl-independent anti-GXM mAb F12D2 to
243 each strain (fig. 2E-F; insets). Thus, *CAS1* and *CAS31* contribute to the formation of
244 | an O-acetylation dependent Crp127 epitope.

245



246

247 **Fig. 2. Crp127 requires O-acetylation, but not xylosylation, of GXM for epitope**

248 **recognition.** The ability of Crp127 to recognise mutants with specific defects in GXM

249 modification was quantified via flow cytometry. Scatter plots (top row) and

250 representative histograms (bottom row) are presented for **A)** Kn99α *uxs1Δ*, JEC155

251 *uxs1Δ* and corresponding wild-type strains; **B)** JEC156 *cas1Δ* and JEC155 wild-type

252 and **C)** Kn99α *cas3Δ*, Kn99α *cas31Δ* and Kn99α wild-type. For scatter plots,

253 corrected MFI values were calculated by subtracting the MFI value of isotype control

254 cells from the MFI value of the Crp127-treated cells, with data points representing

255 MFI values calculated from three biological replicates performed as independent

256 experiments. A Student's *t*-test was used to test for statistically significant differences

257 between Kn99α *uxs1Δ*, JEC155 *uxs1Δ* and JEC156 *cas1Δ* and their corresponding

258 wild-type strain whilst Dunnett's multiple comparison test was used to test for

259 *statistically significant differences between Kn99 α Δ cas3, Kn99 α Δ cas31 mutants*
260 *and the Kn99 α wild-type strain (n=3) (ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$).*
261 *Histograms show a representative distribution of Crp127 binding for one or all of the*
262 *strains in the above scatter plot, with a colour-coded key provided for reference.*
263 *Numerical values in the top left and right of each histogram correspond to the MFI*
264 *value calculated from the strain labelled directly above. **D)** JEC155 *uxs1* Δ , **E)***
265 *JEC156 *cas1* Δ , **F)** Kn99 α *cas31* Δ and corresponding wild-type strains were labelled*
266 *for chitin (blue; CFW) and Crp127 (far-red; goat Alexa-647-conjugated goat anti-*
267 *mouse IgM μ -chain) and maximum-intensity projections generated via confocal*
268 *microscopy. Presented are representative images merged for transmitted light and*
269 *Crp127 (left panels) and Crp127 and chitin (right panels). Insets in the top right of*
270 *images show representative cells labelled for chitin (blue) and O-acetyl-independent*
271 *mAb F12D2 (far-red; Alexa-647-conjugated F(ab')₂ goat anti-Mouse IgG (H+L)) used*
272 *as a control. Scale bars represent 5 μ m.*

273

274 Lastly, we confirmed that GXM O-acetylation is essential for Crp127 recognition by
275 testing the binding of Crp127 to *Cryptococcus* cells that had been chemically de-O-
276 acetylated via alkaline hydrolysis. For both strains tested (H99 and B3501), Crp127
277 failed to bind chemically de-O-acetylated cells, just as it failed to recognise the *cas1* Δ
278 mutant (fig. S2A-C; H99 $P < 0.01$ and B3501 $P < 0.01$). However, binding of O-
279 acetylation independent antibodies F12D2 and 18B7 remained unaltered (fig. S2C-
280 D; $P > 0.05$). Taken together, our results demonstrate an essential role for O-
281 acetylation in recognition of GXM by Crp127.

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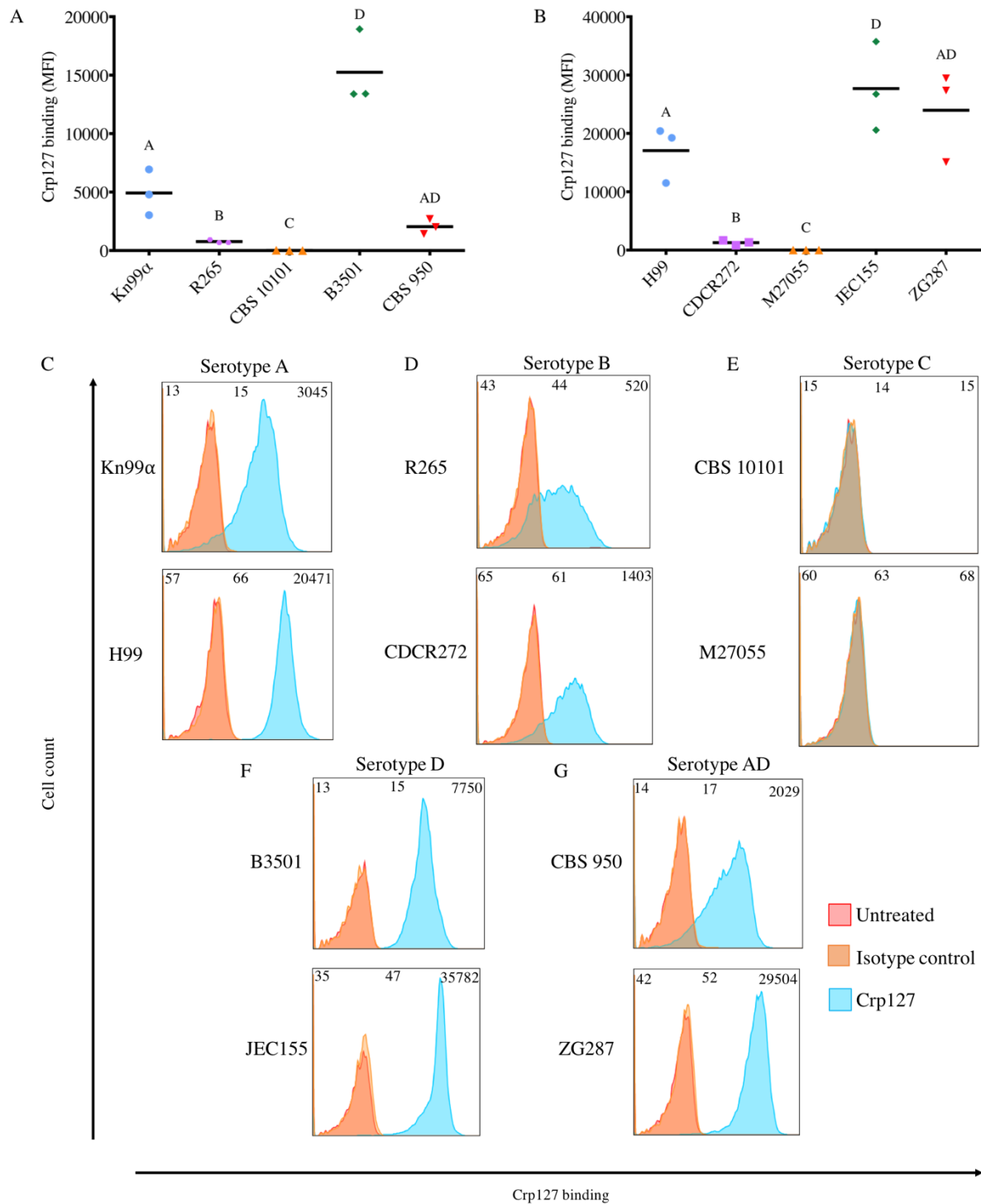
283 ***Cryptococcus* serotypes differ in their level of Crp127 epitope recognition**

284 Differences in the O-acetylation state of GXM contributes towards serotype
285 classification and is a source of structural variation within the capsule of cells from a
286 clonal population (21, 30). Therefore, with Crp127 recognising an O-acetyl-
287 dependent epitope, we next checked for differences in Crp127 staining between the
288 five recognised serotypes of *Cryptococcus neoformans* and *gattii*, testing two
289 independent strains of each serotype. Flow cytometry analysis demonstrated that
290 Crp127 consistently bound most effectively to serotype D strains (B3501 and
291 JEC155) (fig. 3A-B), with all cells within these populations exhibiting high-level
292 accessibility of the Crp127 epitope (fig. 3F). We detected slightly lower binding to
293 serotype A strains (fig. 3A-B), with high-level homogeneous staining also seen in the
294 case of H99, but a proportion of unstained cells from strain Kn99 α (fig. 3C).
295 Interesting, the two AD hybrid strains tested (CBS 950 and ZG287) were notably
296 different in regard to Crp127 binding (fig. 3A-B), with CBS 950 exhibiting low-level
297 heterogeneous staining and ZG287 showing high-level homogeneous staining (fig.
298 3G).

299

300 The two remaining cryptococcal serotypes, B and C, together represent *C. gattii*.
301 Serotype B strains R265 and CDCR272 demonstrated significantly lower epitope
302 recognition than *C. neoformans* serotypes (fig. 3A-B) and considerable heterogeneity
303 within the population (fig. 3D). Interestingly, however, serotype C strains were
304 completely unrecognised by Crp127, with neither strain CBS 10101 or M27055
305 showing detectable staining (fig. 3A, B and E). From this, we conclude that there are
306 serotype-specific differences in the availability of the Crp127 epitope, with epitope
307 accessibility being related to serotype in a pattern of D > A >> B >>> C.

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309

310 **Fig. 3. Recognition levels of the Crp127 epitope are associated with serotype.**

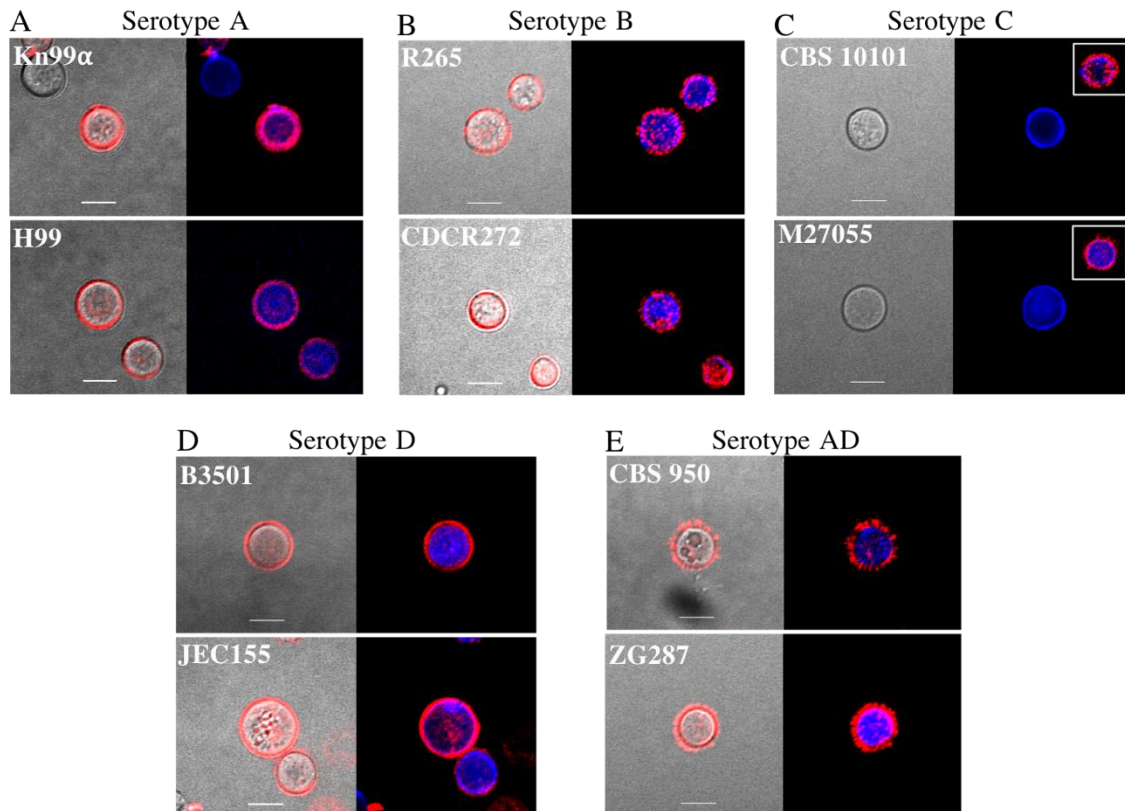
311 *The ability of Crp127 to bind to two different strains from each Cryptococcus*
 312 *serotypes A, B, C, D and AD was quantified using flow cytometry. A-B) Scatter plots*
 313 *show corrected MFI values for each strain, which were calculated by subtracting the*
 314 *MFI value of isotype control cells from the MFI value of the corresponding Crp127-*

315 *treated cells. Data points represent MFI values calculated from three biological*
316 *replicates performed as independent experiments (n=3). Histograms show a*
317 *representative distribution of Crp127 binding for C) serotype A strains Kn99 α and*
318 *H99, D) serotype B strains R265 and CDCR272, E) serotype C strains CBS 10101*
319 *and M27055, F) serotype D strains B3501 and JEC155 and G) serotype AD hybrid*
320 *strains CBS 950 and ZG287. Fluorescence intensity values for untreated, isotype*
321 *control and Crp127-treated cells are presented in red, blue and orange, respectively,*
322 *with corresponding MFI values displayed in the top left, centre and right of each*
323 *panel.*

324

325 **Crp127 exhibits serotype-specific binding patterns that are not associated with**
326 **opsonic efficacy**

327 Having identified differential levels of the Crp127 epitope between serotypes using
328 flow cytometry, we next examined their patterns of binding by immunofluorescence
329 microscopy. Indirect immunofluorescence revealed an annular binding pattern for all
330 four strains representing serotypes A and D (fig. 4A and D). In line with their
331 differences in flow cytometry, the two AD hybrid strains tested showed different
332 patterns of binding, with CBS 950 showing punctate binding and ZG287 showing a
333 mix of annular and punctate staining. Both *C. gattii* serotype B strains exhibited
334 punctate binding (fig. 4B and E) whilst, in agreement with flow cytometry, no Crp127
335 binding was detected when imaging serotype C strains CBS 10101 or M27055.
336 However, O-acetyl-independent mAb F12D2 bound well to these strains, suggesting
337 that the lack of Crp127 binding reflects changes in GXM O-acetylation rather than
338 loss of capsular material (fig. 4C; inset).



339
340
341

Fig. 4. The immunofluorescence-binding pattern of Crp127 correlates with serotype. Two *Cryptococcus* strains from **A)** serotype A (Kn99 and H99), **B)** serotype B (R265 and CDCR272), **C)** serotype C (CBS 10101 and M27055), **D)** serotype D (B3501 and JEC155) and **E)** serotype AD (CBS 950 and ZG287) were labelled for chitin (blue; CFW) and Crp127 (far-red; goat Alexa-647-conjugated anti-mouse IgM μ -chain) and maximum-intensity projections generated via confocal microscopy. Representative images are shown for each strain. Images are merged for transmitted light and Crp127 (left panels) and Crp127 and chitin (right panels). Insets in the top right of images show representative cells labelled for chitin (blue) and O-acetyl-independent mAb F12D2 (far-red; Alexa-647-conjugated F(ab')₂ goat anti-Mouse IgG (H+L)). Scale bars represent 5 μ m.

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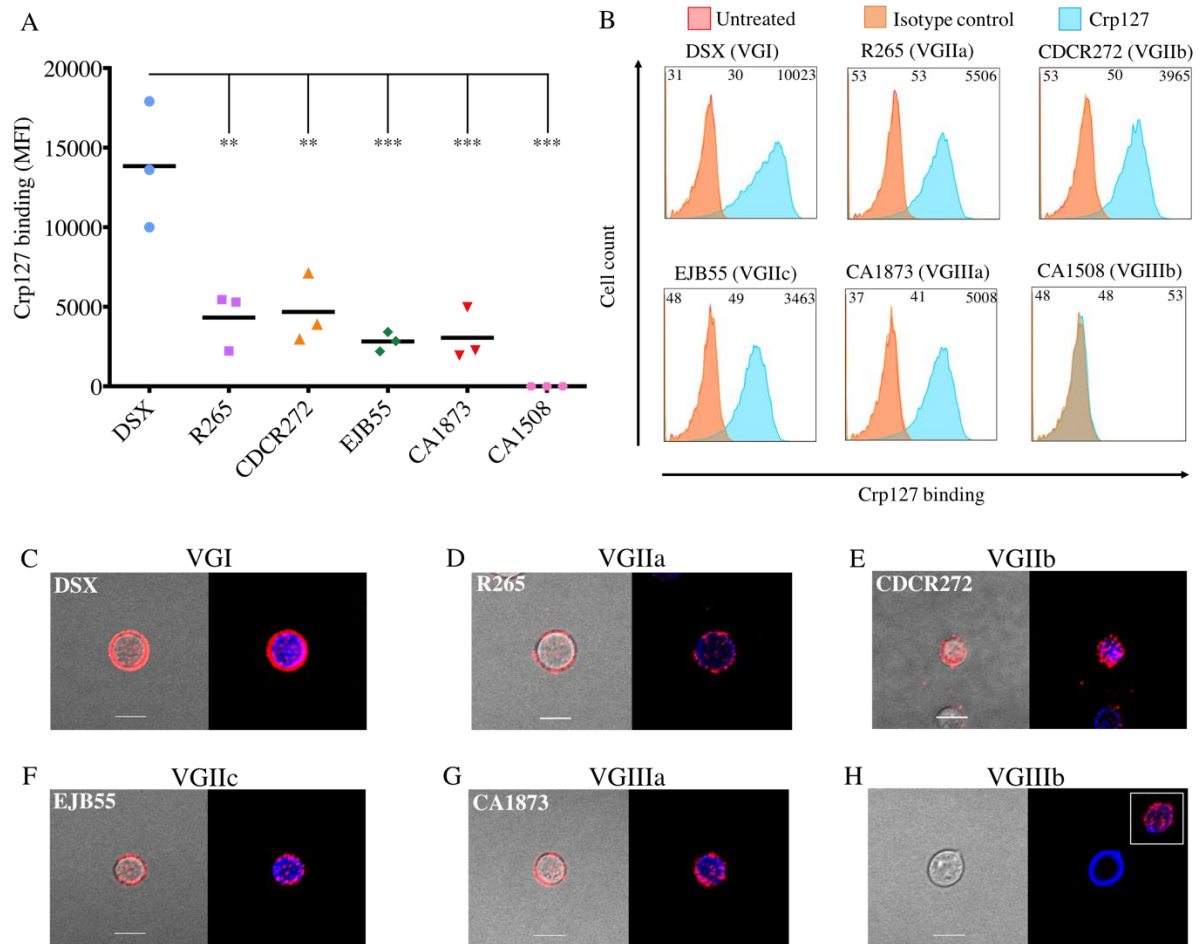
354 As annular and punctate binding patterns have been associated with opsonic and
355 non-opsonic anti-GXM IgM mAbs, respectively, we tested the ability of Crp127 to
356 opsonise cells from strains Kn99 α (annular) and R265 (punctate). Unlike positive
357 control treatments mAb 18B7 and pooled human serum, Crp127 did not enhance
358 phagocytosis of either strain by J774 macrophage-like cells in the presence or
359 absence of serum (fig. S3). In summary, annular binding patterns are associated
360 with the high-level binding of Crp127 to *C. neoformans* serotypes A and D strains.
361 On the other hand, punctate binding is associated with low-level binding of Crp127 to
362 serotype B strains. However, under the conditions tested in this study, neither
363 binding pattern is clearly associated with opsonic efficacy.

364

365 **Crp127 epitope recognition reflects serotype differences within *C. gattii***

366 Our data above indicate that Crp127 binding accurately reflects known serotyping of
367 cryptococcal strains. However, recent genomic data indicate that *C. gattii* may in fact
368 be composed of several cryptic species (37). We therefore extended our analysis of
369 this species group by investigating a further four *C. gattii* strains, representing
370 molecular subtypes VGI-VGIII. Similar levels of Crp127 epitope recognition was seen
371 for serotype B strains R265 (VGIIa), CDCR272 (VGIIb), EJB55 (VGIIc) and CA1873
372 (VGIIIa) (fig. 5A-B; $P > 0.05$), however significantly higher recognition was seen for
373 the serotype B strain DSX (VGI) (fig. 5A-B; $P < 0.01$). Indirect immunofluorescence
374 corroborated these findings, with punctate binding seen for the four strains
375 presenting the epitope at low levels (fig. 5D-H) and annular binding seen for strain
376 DSX (fig. 5C). We also tested strain CA1508 (VGIIIb), a *C. gattii* strain that, to our
377 knowledge, has not previously been serotyped. Both flow cytometry and indirect
378 immunofluorescence showed that Crp127 did not recognise this strain (fig. 5A and

379 H), implying that it is a serotype C strain. In combination with the data presented in
 380 figure 3, our finding that four out of five serotype B strains were bound similarly by
 381 Crp127 suggests that availability of this epitope is fairly well conserved within this
 382 serotype.



383

384 **Fig. 5. Recognition of the Crp127 epitope is largely consistent within *C. gattii***
 385 **serotypes.** The ability of Crp127 to bind to six strains from *C. gattii* that encompass
 386 molecular types VGI-VGIIIb was quantified via flow cytometry. **A)** Scatter plots show
 387 corrected MFI values for each strain, which were calculated by subtracting the MFI
 388 value of isotype control cells from the MFI value of the corresponding Crp127-treated
 389 cells. Data points represent MFI values calculated from three biological replicates
 390 performed as independent experiments. Tukey's multiple comparisons test was used
 391 to test the statistical significance of differences between the six strains ($n=3$) ($ns P >$

392 0.05; ** $P < 0.01$; *** $P < 0.001$). **B)** Histograms show a representative distribution of
393 Crp127 binding for strains DSX (VGI), R265 (VGIIa), CDCR272 (VGIIb), EJB55
394 (VGIIc), CA1873 (VGIIIa) and CA1508 (VGIIIb). Fluorescence intensity values for
395 untreated, isotype control and Crp127-treated cells are presented in red, blue and
396 orange, respectively, with corresponding MFI values displayed in the top left, centre
397 and right of each panel. *C. gattii* strains **C)** DSX, **E)** R265, **F)** CDCR272, **G)** CA1873
398 and **H)** CA1508 were labelled for chitin (blue; CFW) and Crp127 (far-red; goat Alexa-
399 647-conjugated anti-mouse IgM μ -chain) and maximum-intensity projections
400 generated via confocal microscopy. Presented are representative images merged for
401 transmitted light and Crp127 (left panels) and Crp127 and chitin (right panels). Scale
402 bars represent 5 μ m. Insets in the top right of images show representative cells
403 labelled for chitin (blue) and O-acetyl-independent mAb F12D2 (far-red; Alexa-647-
404 conjugated F(ab')₂ goat anti-Mouse IgG (H+L)). Scale bars represent 5 μ m.

405

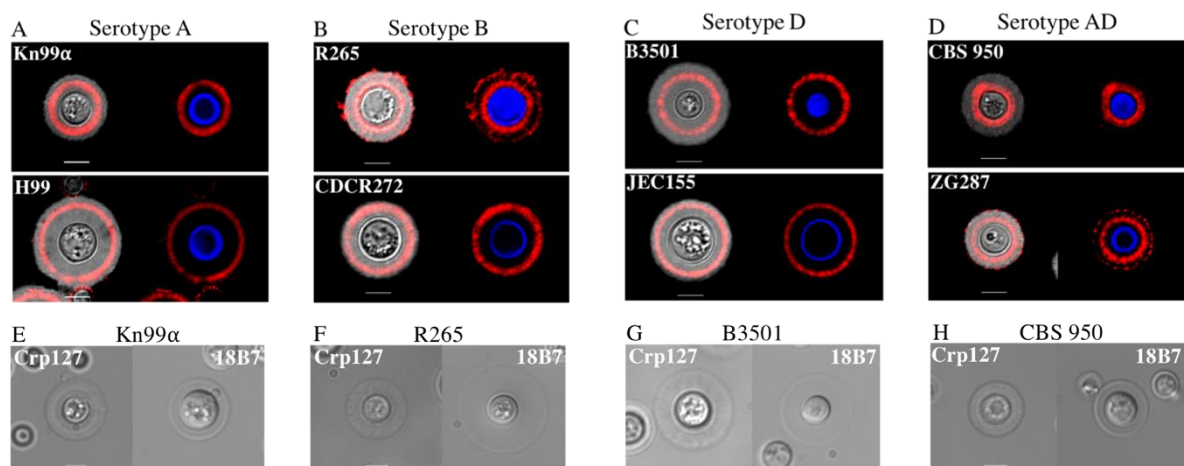
406 **The Crp127 epitope localises to spatially confined zones of the enlarged** 407 **capsule and binding elicits capsular swelling reactions**

408 Having investigated the binding of Crp127 to cells with a small capsule, we next
409 wished to investigate cells that had been grown in capsule-inducing conditions, given
410 that capsule enlargement occurs shortly after infection of the host. Interestingly, in all
411 of the strains tested we saw that the Crp127 epitope was spatially confined to distinct
412 capsular regions (fig. 6). For serotype A and D strains, antibody binding was
413 detected in the central zone of the capsule (fig. 6A and D). Serotype B strains
414 differed, with regions adjacent to the cell wall and on the capsule surface bound by
415 Crp127 in the case of strain R265 but only the single region proximal to the surface
416 bound in the case of CDCR272 (fig. 6B). Serotype AD strain ZG287 exhibited a

417 similar pattern of binding to R265, with Crp127 binding to both an inner and outer
418 region of the capsule, however strain CBS 950 was bound in a region adjacent to the
419 cell wall (fig. 6D).

420

421 The binding of mAbs to capsular GXM alters the refractive index of the enlarged
422 capsule, resulting in capsular swelling reactions that can be visualised using DIC
423 microscopy (38). In testing the ability of Crp127 to produce a capsular swelling
424 reaction with strains Kn99 α , R265, B3501 and CBS 950, we observed no discernible
425 differences in the reaction pattern produced between strains, with a highly refractive
426 outer rim and a textured inner capsule characteristic for each strain (fig. 6E-H; left
427 panels). Notably, however, Crp127 reaction patterns differed from those elicited by
428 18B7, which also exhibited a highly refractive outer rim but lacked texture throughout
429 the capsule (fig. 6E-H; right panels). Taken together, our studies of Crp127 binding
430 to capsule-induced cells demonstrate that the Crp127 epitope is localised to specific
431 capsular regions and that Crp127 binding produces capsular swelling reactions that
432 are independent of serotype.



433

434 **Fig. 6. The Crp127 epitope is spatially confined to distinct capsular regions**
435 **and binding elicits capsular swelling reactions distinct from those of 18B7.**

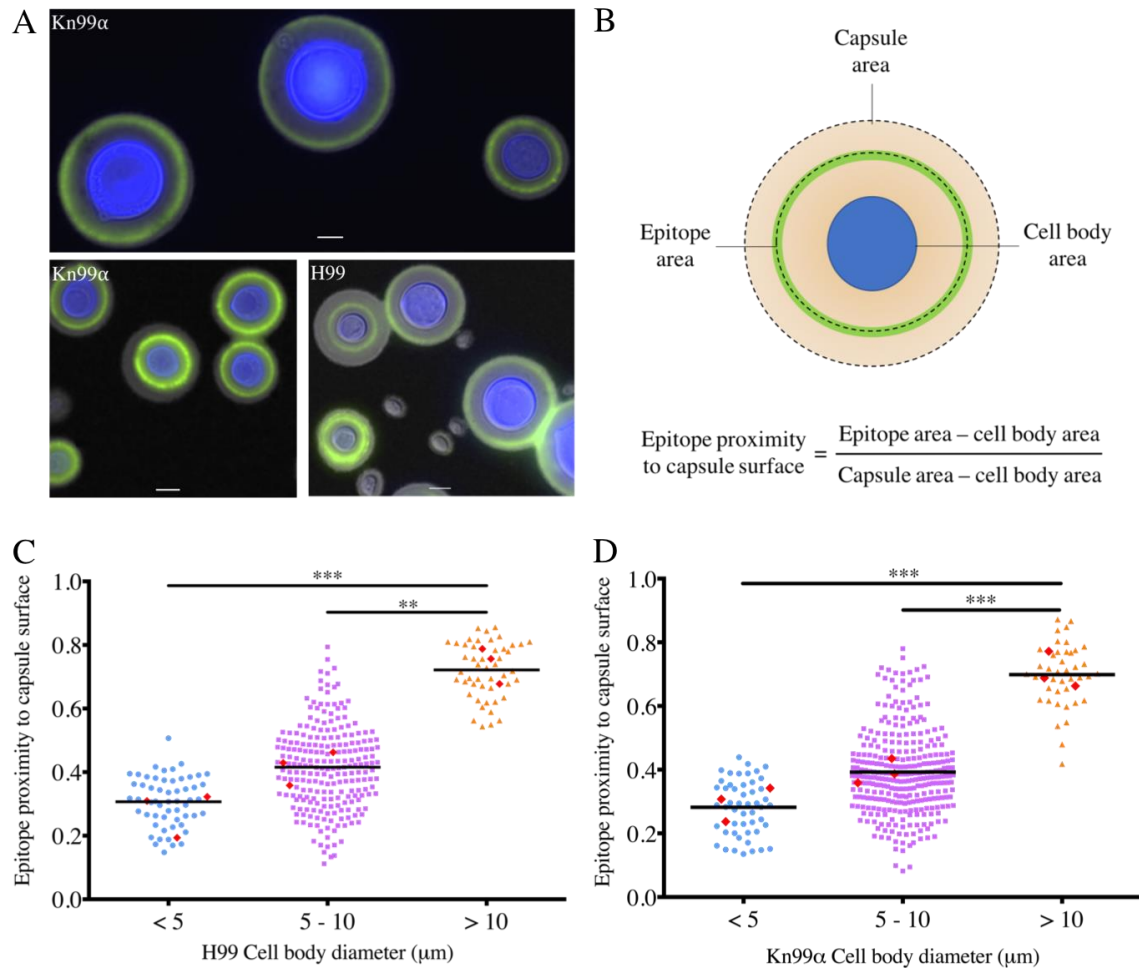
436 *Cryptococcus* cells were grown in capsule-inducing conditions and imaged to
437 determine the location of the Crp127 epitope within the enlarged capsule and
438 characterise the capsular reaction patterns elicited by this antibody. *Cryptococcus*
439 strains from **A)** serotype A (*Kn99 α* and H99), **B)** serotype B (*R265* and *CDCR272*,
440 **C)** serotype D (*B3501* and *JEC155*) and **D)** serotype AD (*CBS 950* and *ZG287*) were
441 labelled for chitin (blue; CFW) and Crp127 (far-red; goat Alexa-647-conjugated anti-
442 mouse IgM μ -chain), suspended in Indian ink to visualise the capsule and imaged
443 using confocal microscopy. Representative images of a single focal plane are shown
444 for each strain. Presented are images merged for transmitted light and Crp127 (left
445 panels) and Crp127 and chitin (right panels). Capsule-induced cells of strains **E)**
446 *Kn99 α* , **F)** *R265*, **G)** *B3501* and **H)** *CBS 950* were also treated with either Crp127
447 (left panels) or 18B7 (right panels) and imaged using DIC microscopy to observe
448 capsular reaction patterns. Scale bars represent 5 μ m.

449

450 **Spatial distribution of the Crp127 epitope differs within the capsules produced** 451 **by Titanide, yeast-like and Titan cells**

452 Following infection of the host lung, a proportion of *C. neoformans* cells differentiate
453 into Titan cells, a very large morphotype that facilitates pathogenesis and is
454 associated with poor clinical outcomes (8, 12). When grown under Titanising
455 conditions *in vitro*, *C. neoformans* forms a heterogeneous population of small oval-
456 shaped Titanide cells (<5 μ m cell body diameter), yeast-like cells (~5 μ m) and large
457 Titan cells (>10 μ m) (9). As differences in capsule structure – but not mAb binding
458 pattern – are known to exist between yeast and Titan cells (26, 39), we tested
459 whether Crp127 could distinguish the morphological subtypes found in Titanising
460 populations from strains H99 and *Kn99 α* . Indeed, when imaging cells grown in

461 Titanising conditions *in vitro*, we noticed differences in the spatial distribution of the
462 Crp127 epitope within the capsules produced by cells of different sizes (fig. 7A).
463 Specifically, Crp127 bound to a capsular region adjacent to the cell wall in smaller
464 Titanide cells, within the central zone of the capsule in yeast-like cells and close to
465 the capsule surface of Titan cells. In order to quantify how cell size affects capsular
466 distribution of the Crp127 epitope, we determined the ratio between the area of
467 capsule encompassed by the Crp127 epitope and the area of the whole capsule;
468 using this metric, a ratio approaching 1 is indicative of the epitope being found in
469 close proximity to the capsule surface (fig. 7B). Across three biological repeats (with
470 a mean number of 111 and 133 cells measured for strains H99 and Kn99 α ,
471 respectively), mean (\pm standard error of the mean) ratios of 0.28 ± 0.04 and $0.30 \pm$
472 0.03 were calculated for Titanide cells ($<5 \mu\text{m}$ in diameter) for strains H99 and
473 Kn99 α , respectively, consistent with our initial observations that the Crp127 bound
474 near to the cell wall of these small cells (fig. 7C-D). For yeast-like cells ($5\text{-}10 \mu\text{m}$ in
475 diameter), mean ratios were 0.42 ± 0.03 and 0.39 ± 0.02 for strains H99 and Kn99 α ,
476 respectively (fig. 7C-D), indicating the Crp127 epitope is predominantly located in the
477 central zone of the capsule in yeast-like cells, as we had previously observed (fig.
478 6A). Finally, the mean ratios for Titan cells ($>10 \mu\text{m}$ in diameter) were 0.72 ± 0.03
479 and 0.71 ± 0.03 for strains H99 and Kn99 α respectively, making them significantly
480 higher than those calculated for both Titanide (fig. 7C-D; H99 $P < 0.001$; Kn99 α $P <$
481 0.001) and yeast-like cells (fig. 7C-D; H99 $P < 0.01$; Kn99 α $P < 0.001$). In summary,
482 our results demonstrate that Crp127 binds closer to the capsule surface of Titan cells
483 than Titanide and yeast-like cells.



484

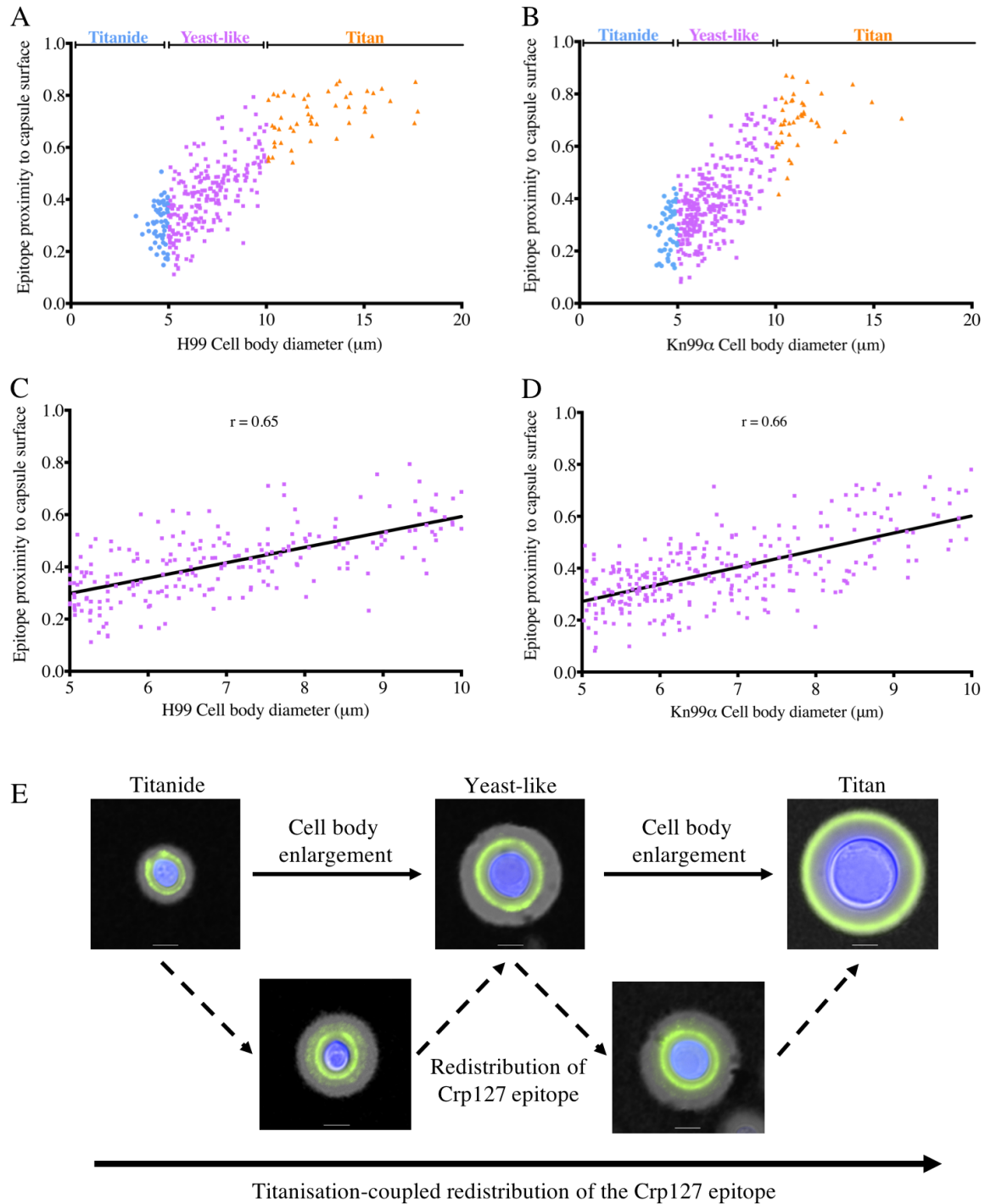
485 **Fig. 7. Spatial distribution of the Crp127 epitope differs within the capsule of**
486 **the three cell subtypes found in Titanising populations. Cultures of *C.***
487 **neoformans strains H99 and Kn99 α that derived solely from Titan cells were**
488 **investigated for differences in the capsular distribution of the Crp127 epitope. **A)****
489 **Representative images of cells from strains H99 and Kn99 α grown under conditions**
490 **permissive for Titanisation. Scale bars represent 5 μm . **B)** A schematic**
491 **representation of how Crp127 epitope proximity to the capsule surface was**
492 **quantified through the analysis of micrographs using ImageJ software. The proximity**
493 **of the Crp127 epitope to the capsule surface of **C)** H99 and **D)** Kn99 α cells with cell**
494 **body diameter <5 μm (blue circles), 5-10 μm (purple squares) and >10 μm (orange**
495 **triangles) was quantified. Data points represent all individual cells for which the**

496 *location of the Crp127 epitope was quantified, whilst the horizontal bar represents*
497 *the mean. Red diamonds represent mean values calculated from three biological*
498 *repeats. Tukey's multiple comparisons test was used to test for statistically*
499 *significant differences between the three groups (n=3) (** P < 0.01; *** P < 0.001).*

500

501 **Migration of the Crp127 epitope towards the surface of the capsule coincides**
502 **with cell enlargement**

503 To investigate the effect of small changes in cell size on Crp127 epitope distribution,
504 we plotted cell body diameter against epitope proximity to the capsule surface for all
505 cells measured (fig. 8A-B). Having done so, we noticed a correlation between the
506 cell body diameter and epitope proximity to the capsule surface of yeast-like cells. In
507 agreement with this, when plotting only cells with a cell body diameter between 5-10
508 μm , we found a positive correlation between cell body diameter and epitope
509 proximity to the capsule surface in both strains tested (fig. 7F-G; H99 $r = 0.65$;
510 Kn99 α $r = 0.66$). Unlike cell body diameter, capsule diameter did not correlate with
511 epitope proximity to the capsule surface, indicating that changes in capsule size do
512 not explain changes in the proximity of the Crp127 to the capsule surface (fig. S4).



513

514 **Fig. 8. Cell size positively correlates with proximity of the Crp127 epitope to**

515 **the capsule surface and suggest a model of Titanisation-coupled epitope**

516 **redistribution.** Cell body diameter was plotted against the epitope proximity to the

517 capsule surface for all cells measured from strains **A) H99** and **B) Kn99α**. Data

518 points represent individual cells with cell body diameter $< 5 \mu\text{m}$ (blue circles), 5-10

519 μm (purple squares) and $>10 \mu\text{m}$ (orange triangles). Linear regression analysis of
520 cells with cell body diameter between 5-10 μm was used to assess the correlation
521 between cell size and Crp127 epitope proximity to the capsule surface for strains **C)**
522 *H99* and **D)** *Kn99 α* . **E)** Positive correlation between the cell body diameter and
523 proximity of epitope to the capsule surface suggests a model by which Titanisation is
524 coupled to redistribution of the Crp127 epitope towards the capsule surface.
525 Representative images of Titanide, yeast-like and Titan cells exhibiting a typical
526 annular immunofluorescence binding pattern – in addition to Titanide and yeast-like
527 cells exhibiting a second more faint ring of antibody binding pattern outside of this –
528 are presented to propose a model by which epitope is added closer to the capsule
529 surface as cells enlarge. Images are merged for transmitted light, and Crp127
530 (green) and chitin (blue). Scale bars represent 5 μm .

531

532 Our results suggest that the Crp127 epitope moves gradually to the capsule surface
533 as cells enlarge and age, raising the question of how this may occur. Throughout our
534 imaging experiments, the binding of Crp127 to the majority of Titanide and yeast-like
535 cells (in addition to all Titan cells) produced an annular immunofluorescence binding
536 pattern (fig. 8E). However, we also noticed that some Titanide and yeast-like cells
537 produced a second more faint ring of Crp127 epitope outside of this typical annular
538 ring (fig. 8E). This may represent the addition of Crp127 epitope closer to the
539 capsule surface, partially explaining how redistribution of this epitope coincides with
540 cell enlargement.

541

542 **Discussion**

543 In this study, we demonstrated that a capsular epitope recognised by Crp127 – an
544 anti-GXM mAb produced in our laboratory – contributes to serotype-specific
545 differences in capsule structure. This epitope traverses the capsule as cells enlarge
546 under conditions permissive for Titanisation, resulting in its differential distribution
547 throughout the capsule of the three *C. neoformans* morphotypes found within
548 Titanising populations. Detailing the accessibility and localisation of this epitope adds
549 to the existing body of literature surrounding the variability of the cryptococcal
550 capsule between strains and reveals yet another way in which Titan cell capsules
551 are structurally distinct from those produced by yeast cells (21–23, 32, 40).

552

553 Based on our examination of a panel of mutants harbouring capsule defects, we
554 propose that Crp127 is an anti-GXM mAb whose binding depends on GXM O-
555 acetylation, but not xylosylation. When comparing sequences of the CDRs from
556 Crp127 with four previously characterised anti-GXM IgM mAbs – namely 2D10,
557 12A1, 13F1 and 21D2 – we found that Crp127 CDRs were significantly different,
558 particularly with regard to the light-chain variable (V_L) CDRs. These differences
559 reflect differential gene usage and are likely to manifest as differences in epitope
560 specificity (41, 42). In contrast, when we aligned the heavy-chain variable (V_H) and
561 V_L sequences from Crp127 with those from anti-GXM IgG1 mAb 302, we noticed that
562 the sequences were extremely similar as a result of identical variable region gene
563 segment usage by these two mAbs. Identical gene segment usage is not entirely
564 surprising given the restricted set of antibody gene segments utilised by antibodies
565 specific to capsular polysaccharides (29), however the two mAbs were produced in
566 response to GXM derived from different serotypes of *Cryptococcus*. Whereas mAb
567 302 was generated following the immunisation of a mouse with serotype D GXM

568 (ATCC 24064) (43), we generated Crp127 through the immunisation of a mouse with
569 a cocktail containing both serotype A (H99) and serotype B (R265) GXM. Whichever
570 serotype of GXM activated the B cell from which Crp127 derives, the sequence
571 similarities between mAbs Crp127 and 302 demonstrate that nearly identical
572 antibodies can be elicited during infection by at least two different serotypes of
573 *Cryptococcus*.

574

575 Crp127 binding shows strong serotype dependence, with serotype D strains being
576 recognised most strongly, followed by serotype A strains. *C. gattii* serotype B strains
577 show lower, heterogeneous Crp127 epitope recognition and a punctate
578 immunofluorescence binding pattern, whilst serotype C strains entirely fail to bind the
579 antibody. Interestingly, the predominant SRG found in GXM produced by serotype D,
580 A, B and C contains 1, 2, 3 and 4 xylose substituents, respectively (15, 44). Together
581 with the previous observation that β -(1,2)-xylose and O-acetyl groups are not added
582 to the same backbone mannose residue (17, 36), this differential SRG usage may
583 explain the variable Crp127 epitope recognition in one of two ways. For example, the
584 additional xylose substituents present in the predominant SRG found in serotype B
585 and C GXM may prevent addition of O-acetyl groups in such a way that the Crp127
586 epitope is not formed. Alternatively, the extra xylose substituents found in these
587 SRGs may sterically hinder binding of Crp127 to its epitope. Studies that further
588 elucidate the roles of specific proteins in GXM biosynthesis – together with advances
589 in techniques that enable chemical synthesis of GXM oligosaccharides – will
590 enhance our understanding of molecular how epitope recognition is achieved by anti-
591 GXM mAbs like Crp127. Intriguingly, a recent transcriptomics study identified CAS31
592 as being absent from the genome of strain CBS 10101, a serotype C isolate that we

593 subsequently found was not recognised by Crp127. Whilst we cannot rule out the
594 possibility that other factors contribute to the inability of Crp127 to recognise
595 serotype C strains, it is tempting to speculate that the loss of *CAS31* function in this
596 lineage may explain its lack of reactivity with Crp127 (32, 34).

597

598 Perhaps our most striking observation regarding the Crp127 epitope was its
599 differential distribution throughout the capsules produced by Titanide, yeast-like and
600 Titan cells. No such differences have been consistently observed for other anti-GXM
601 mAbs E1, 2D10, 13F1 or 18B7 (7, 26), making this a unique feature of Crp127. The
602 positive correlation between cell size and Crp127 epitope proximity to the capsule
603 surface is suggestive of a scenario whereby the epitope in question is initially
604 produced in a capsular region adjacent to the cell wall in small Titanide cells, before
605 redistributing first to the midzone of yeast-like cells and eventually to the capsule
606 surface of Titan cells. This finding raises the intriguing question of how formation and
607 removal of the Crp127 epitope is so tightly spatially controlled within the capsule?
608 One possibility is that the epitope could be formed at the cell surface and then move
609 outwards as the capsular material elongates. Therefore, we speculate that since the
610 epitope moves outwards at a faster rate than the capsule expands, and since the
611 amount of epitope that initially surrounds a smaller Titanide or yeast-like cell would
612 not be sufficient to form the perimeter of capsule encasing a much larger Titan cell,
613 we instead favour a model in which the epitope is enzymatically removed and added
614 to different regions of the capsule during growth. For instance, it is possible that
615 GXM decorated with *O*-acetyl groups is added closer to the capsule surface in larger
616 cells or that such regions are “unmasked” in a different capsular region as the
617 capsule is reshaped during Titanisation (26). To summarise, our findings

618 demonstrate that the differential distribution of specific epitopes within the
619 cryptococcal capsule is yet another way in which Titan cells can be distinguished
620 from canonical yeast cells, prompting further investigation into how redistribution of
621 such epitopes occurs and what impact this has on the outcome of infection.

622

623 **Materials and methods**

624 **Reagents, strains and mAbs**

625 All reagents were purchased from Sigma-Aldrich unless stated otherwise. The
626 *Cryptococcus* strains used in this study are described in table S1. The anti-GXM
627 mAbs used in this study are described in table S2.

628

629 **Growth of cryptococci**

630 *Cryptococcus* strains were preserved at -80°C in MicroBank™ tubes (Thermo Fisher
631 Scientific) prior to being stored on yeast extract peptone dextrose (YPD) agar plates
632 at 4°C for a maximum of 30 days. Unless stated otherwise, strains were cultured on
633 a rotary wheel at 20 rpm for 24 h at 25°C in round-bottom culture tubes containing 3
634 mL YPD broth. To induce capsule growth, *Cryptococcus* cells were grown in round-
635 bottom culture tubes containing 3 mL Dulbecco's Modified Eagle's Media (DMEM)
636 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin
637 and 10% foetal bovine serum (FBS) for 72 h in an incubator at 37°C and 200 rpm.

638

639 **Hybridoma production and mAb purification**

640 Cultures of *C. neoformans* H99 and *C. gattii* R265 were microfuged (4000 x g for 5
641 min) and washed three times in 1 mL Dulbecco's phosphate-buffered saline (PBS).
642 Washed cultures were then heat killed for 60 minutes at 65°C. Following heat killing

643 20 μ L was plated onto YPD to confirm there were no viable cells. Heat-killed H99
644 and R265 cells were then either lysed (see below) or mixed 1:1 and stored at -20°C
645 prior to inoculation. Fungal cells were lysed using Precellys tubes (UK05 03961-1-
646 004) using programme 6400-2x10-005. Following lysis, lysis beads were microfuged
647 ($3000 \times g$ for 1 min) and supernatant collected. H99 and R265 lysates were mixed
648 1:1 and stored at -20°C .

649

650 BALB/c mice were hyper-immunised with heat-killed H99 and R265 in addition to
651 their lysates. Hybridomas were generated by a method that has previously been
652 described (45). NS0 immortal fusion partner cells were fused with splenocytes
653 mediated by polyethylene glycol (StemCell Technologies). All animal work was
654 conducted in accordance with Home Office guidelines and following local ethical
655 approval granted under animal licence 30/2788. Supernatants from clones were
656 screened for reactivity with H99 and R265 cells using 96-well plates, with FITC-
657 conjugated anti-mouse IgG and anti-mouse IgM antibodies used to identify positive
658 clones via epifluorescence microscopy. Positive clone 127 was cultured in RPMI
659 1640 with IgG-depleted FBS and supernatant collected in a MiniPerm bioreactor
660 (Sarstedt). MAb Crp127 was purified from supernatant using affinity chromatography
661 and ProSep Thiosorb (Millipore).

662

663 **Hybridoma sequencing and antibody sequence analysis**

664 Sequencing of hybridomas was carried out by Absolute Antibody Ltd (UK).
665 Sequencing was performed by whole transcriptome shotgun sequencing (RNA-Seq).
666 In brief, hybridomas were cultured in Iscove's Modified Dulbecco's Media (IMDM)
667 supplemented with 10% FBS in an incubator at 37°C and with 5% CO_2 . Total RNA

668 was extracted from cells and a barcoded cDNA library generated through RT-PCR
669 using a random hexamer. Sequencing was performed using an Illumina HiSeq
670 sequencer. Contigs were assembled and annotated for viable antibody sequences
671 (i.e those not containing stop codons) to confirm the species and isotype of mAb
672 Crp127 as murine and IgM, respectively.

673

674 Variable region gene usage was determined using VBASE2 software (46) and CDRs
675 were predicted using the Kabat numbering system (47). Heavy-chain variable (V_H)
676 and light-chain variable (V_L) sequences of mAb Crp127 were aligned with antibody
677 sequences that have previously been described (35, 48). Amino acid sequences
678 were aligned using Clustal Omega software (49) and annotated using ESript
679 software (50).

680

681 **Immunolabelling**

682 *Cryptococcus* cells were immunostained for flow cytometry and microscopy
683 experiments. 1 mL of fungal culture was transferred to a 1.5 mL microcentrifuge
684 tube, microfuged (15,000 x g for 1 min) and washed 3x in PBS. Cell density was
685 determined using a haemocytometer and adjusted to 10^7 cells/mL in a final volume
686 of 200 μ L. 20 μ g/mL Crp127, F12D2, 18B7 or mouse anti-human IgG (IgM isotype
687 control) were added and samples mixed on a rotary wheel at 20 rpm for 1 h at room
688 temperature. Untreated cells for use in flow cytometry were left untreated. After
689 primary antibody treatment, samples were microfuged (15,000 x g for 1 min) and
690 washed 3x in PBS to remove unbound primary antibody. 2 μ g/mL Alexa-488-
691 conjugated goat anti-mouse IgM (heavy chain) (Thermo Fisher Scientific), Alexa-
692 647-conjugated goat anti-mouse IgM μ -chain (Abcam) or Alexa-647-conjugated

693 F(ab')₂-Goat anti-Mouse IgG (H+L) (Thermo Fisher Scientific) were added to
694 antibody-treated samples and samples mixed on a rotary wheel at 20 rpm for 1 h at
695 room temperature. Secondary antibody was also added to isotype control samples
696 for flow cytometry. For microscopy experiments, 5 µg/mL calcofluor-white (CFW)
697 was also added at this stage to label chitin. Following incubation with secondary
698 antibody, samples were again microfuged (15,000 x g for 1 min) and washed 3x to
699 remove unbound secondary antibody and CFW.

700

701 **Flow cytometry**

702 Flow cytometry experiments were performed with an Attune NxT Flow Cytometer
703 equipped with an Attune Autosampler (Thermo Fisher Scientific). Untreated, isotype
704 control and either Crp127 or 18B7 samples were prepared for each strain or
705 conditions tested. Following immunostaining, samples were diluted to 5×10^6
706 cells/mL and 200 µL of *Cryptococcus* put into individual wells of a plastic round-
707 bottom 96-well plate ready for insertion into the Attune Autosampler. Sample was
708 collected from each well at a rate of 100 µL/min until 10,000 events were recorded.
709 The 488 nm laser was used to detect primary antibody bound by Alexa-488-
710 conjugated secondary antibodies, with the same voltage used to power the laser
711 within each experiment. Flow cytometry data was then analysed using FlowJo (v10)
712 software. Debris was excluded by using the FSC-A vs. SSC-A gating strategy,
713 followed by exclusion of doublets using the FSC-A vs. FSC-H gating strategy (fig.
714 S5). Where GXM-deficient mutants were analysed, samples were only gated to
715 exclude debris due to the inseparable large aggregates formed by these mutants as
716 a result of budding defects. After gating, histograms of fluorescence intensity were
717 plotted and the median fluorescence intensity (MFI) determined. Corrected MFI

718 values were calculated by subtracting the MFI value of the mAb-treated sample by
719 the corresponding isotype control sample in the case of Crp127 or untreated sample
720 where 18B7 was used. Across all experiments, MFI values returned from isotype
721 control cells were extremely similar to those returned from untreated cells.

722

723 **Confocal microscopy**

724 Following the final washes of the immunostaining procedure, 2 μ L of stained
725 cryptococcal cells were spotted onto a glass slide and placed under a square glass
726 coverslip. Where visualisation of the capsule was necessary, 2 μ L Indian ink was
727 also added to the glass slide. Imaging was performed on a Nikon A1R laser
728 scanning confocal microscope using a 100x object lens and oil immersion. Alongside
729 transmitted light, 639 nm and 405 nm lasers were used to detect Alexa-647-
730 conjugated secondary antibodies and CFW, respectively. For cells with small
731 capsules, Z-stacks spanning 8 μ m were generated using steps of 0.27 μ m. For
732 capsule-induced cells, Z-stacks were taken across 20 μ m using steps of 0.66 μ m.
733 Generation of maximum intensity projections (MIPs) and other image processing
734 was performed using NIS-Elements and ImageJ software.

735

736 **Chemical de-O-acetylation of capsular GXM**

737 Where chemical de-O-acetylation of the capsule was required, cells were grown in
738 YPD broth that had been adjusted pH 11 with NaOH and sterilised with a 0.22 μ m
739 filter. Round-bottom culture tubes containing 3 mL of pH 11 YPD broth was then
740 placed on a rotary wheel turning at 20 rpm for 24 h at 25°C. This method was
741 adapted from that used in a previous study (21).

742

743 **Phagocytosis assays**

744 Phagocytosis assays were performed using the murine macrophage-like J774A.1
745 cell line (mouse BALB/cN; ATCC[®] TIB-67[™]). Cells were cultured in DMEM
746 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin
747 and 10% FBS, before 1×10^5 cells were seeded onto round glass coverslips that had
748 been placed into wells of a flat-bottom 24-well plate and incubated for 24 h at 37°C
749 and 5% CO₂. Cells of strain R265 and Kn99 α were opsonised with 18B7 or Crp127
750 as described for the first incubation of the immunostaining procedure. In the same
751 way, cells were opsonised with 10% AB-human serum alone or in combination with
752 Crp127. To achieve a multiplicity of infection (MOI) of 10, 10^6 R265 or Kn99 α cells
753 were then resuspended in serum-free DMEM and added to each well of J774A.1
754 cells. Following the infection, each well was gently washed 3x with 1 mL of warmed
755 PBS to remove extracellular yeast. The contents of each well were then fixed with
756 4% paraformaldehyde prior to being washed a further 3x. Cover slips were then
757 extracted from their well, any residual PBS removed by briefly submersing in sterile
758 dH₂O and mounted onto glass slides with Prolong Gold Antifade Mountant (Thermo
759 Fisher Scientific). The total number of internalised yeast cells per 100 J774A.1 cells
760 (phagocytic index) was determined by microscopic examination using a Nikon
761 TE2000-U microscope with a 60x objective lens and oil immersion.

762

763 **Capsular swelling reactions**

764 Capsule-induced cells were treated with 50 μ g/mL Crp127 or 18B7 as described for
765 the immunostaining procedure. 2 μ L of *Cryptococcus* cells were then dropped onto a
766 glass slide and placed under a square glass coverslip. Imaging was performed on
767 the differential interference contrast (DIC) channel of a Nikon TE2000-U microscope

768 using a 60x objective lens with oil immersion. Image processing was performed
769 using NIS-Elements and ImageJ software.

770

771 **Titan cell experiments**

772 Titan cells that exhibit all the properties of *in vivo* Titan cells were induced *in vitro*
773 using a previously described protocol (9). *C. neoformans* H99 and Kn99 α cells were
774 cultured in glass conical flasks containing 10 mL yeast nitrogen base (YNB) + 2%
775 glucose at 30°C and 200 rpm for 24 h. Cells were adjusted to an OD₆₀₀ reading of
776 0.001 before being transferred into 10% heat-inactivated foetal calf serum (HI-FCS)
777 at a final volume of 3 mL in a plastic six-well plate and grown for 72 h at 37°C and
778 5% CO₂. To begin a culture derived solely from Titan cells, cells were passed
779 through an 11 μ m filter, trapping only larger cells on the filter paper. This filter paper
780 was then washed in PBS to re-suspend Titan cells. Between 10³ and 10⁴ Titan cells
781 were then transferred into 3 mL HI-FCS in a plastic six-well plate and cultured for a
782 further 72 h at 37°C and 5% CO₂. Titanising populations were prepared for imaging
783 according to the method described for immunostaining. Imaging was performed on a
784 Nikon TE2000-U microscope using a 60x objective lens with oil immersion.

785

786 To quantify the proximity of the Crp127 epitope to the capsule surface was
787 quantified, ImageJ software was used to draw regions of interest (ROIs) around the
788 cell body, the immunofluorescence binding pattern of the Crp127 and the capsule
789 surface (as determined by Indian ink staining). For each cell measured, the area of
790 these three ROIs was determined before the area of the cell body was subtracted
791 from the areas calculated for both the Crp127 epitope ROI and the capsule surface
792 ROI. Finally, the area of the Crp127 epitope ROI was divided by the capsule surface

793 ROI as a means of quantifying the proximity of the Crp127 epitope to the capsule
794 surface. A mean number of 111 and 133 cells were measured per biological replicate
795 for strain H99 and Kn99 α , respectively. Image processing was performed using NIS-
796 Elements software.

797

798 **Experimental design and statistical analysis**

799 For each experiment described, three biological repeats were performed as
800 independent experiments that were carried out on different days. All datasets were
801 analysed using GraphPad Prism 7 software.

802

803 **Acknowledgements**

804 **Provision of antibodies, strains and assistance**

805 We gratefully acknowledge our colleagues Tamara Doering (Washington University),
806 Guilhem Janbon (Institut Pasteur), Arturo Casadevall (Johns Hopkins) and Thomas
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811

812 **Author contributions**

813 Experiments were designed and conducted by MP, XZ and EB. The Crp127 antibody
814 was raised and initially characterised by SAJ and MG. ERB and RCM helped design
815 and oversee this project. Data figures and text were prepared by MP and then edited
816 and revised by all the other authors.

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818 **Competing interests**

819 | The authors declare no competing interests with this work.

820

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829

830 **Data and materials availability:** All data needed to evaluate the conclusions drawn
831 in this paper are present in the paper and/or the supplementary materials. Additional
832 data related to this paper may be requested from the authors. The Crp127 antibody
833 described here is available via Ximbio.com.

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835 **References**

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