Saccharomyces spores are born prepolarized to outgrow away from spore-spore connections and penetrate the ascus wall

Running Title: Upon germination budding yeast spores grow away from each other

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1. Abstract

How non-spore haploid *Saccharomyces* cells choose sites of budding and polarize towards pheromone signals in order to mate has been a subject of intense study. Unlike non-spore haploids, sibling spores produced via meiosis and sporulation by a diploid cell are physically interconnected and encased in a sac derived from the old cell wall of the diploid, called the ascus. Non-spore haploids bud adjacent to previous sites of budding, relying on stable cortical landmarks laid down during prior divisions, but since spore membranes are made de novo it was assumed that, as is known for fission yeast, *Saccharomyces* spores break symmetry and polarize at random locations. Here we show that this assumption is

26   incorrect: *Saccharomyces cerevisiae* spores are born prepolarized to outgrow, prior to

27   budding or mating, away from interspore bridges. Consequently, when spores bud within

28   an intact ascus, their buds locally penetrate the ascus wall, and when they mate, the

29   resulting zygotes adopt a unique morphology reflective of re-polarization towards

30   pheromone, which we dub the derrière. Long-lived cortical foci containing the septin Cdc10

31   mark polarity sites, but the canonical bud site selection program is dispensable for spore

32   polarity, thus the origin and molecular composition of these landmarks remain unknown.

33   These findings demand further investigation of previously overlooked mechanisms of

34   polarity establishment and local cell wall digestion, and highlight how a key step in the

35   *Saccharomyces* life cycle has been historically neglected.

2. Introduction

37   Most steps in the *Saccharomyces cerevisiae* life cycle have been described in great detail at

38   the cellular and molecular levels. For example, in the YeastBook series of reviews published

39   by the journal *GENETICS,* forty three articles to date "span the breadth of *Saccharomyces*

40   biology" (https://www.genetics.org/content/yeastbook). Here and in other sources can be

41   found numerous mechanistic insights into the ways that budding yeast cells choose bud

42   sites by positioning polarity factors according to mating-type-specific cortical landmarks;

43   how a potent extrinsic signal, mating pheromone, can override these landmarks; and how

44   cells use hydrolytic enzymes to remodel their cell walls upon budding and cell fusion. The

45   unique morphology yeast cells adopt as they grow chemotropically toward a pheromone

46   source even garnered an enduring and endearing name, the shmoo, based on its

47   resemblance to a comic strip character from the mid 1900s. Indeed, early studies of the

48   signal transduction cascades underlying the pheromone response and the ways that yeast

49   cells repolarize to track pheromone gradients were foundational in our general

50   understanding of eukaryotic signal transduction and polarity determination.

51   Accordingly, we find it remarkable how little is known about a step of the *Saccharomyces*

52   life cycle for which polarity, chemotropism, and cell wall remodeling are crucial.

53   Germination is the process by which spores awaken from a nearly dormant state, break out

54   of a rigid, specialized cell wall, and grow in a single direction by producing new membrane

55   and cell wall. Only a single review dedicated to germination has been published, over a

56   decade ago (Geijer et al., 2010).

57   Since most natural isolates of *S. cerevisiae* are diploid, most spores are haploid, and

58   germinating spores are capable of mating immediately (without a prior budding event) if a

59   suitable mating partner is sufficiently close by (Joseph-Strauss et al., 2007). The

60   assumption from the literature is that mating between spores follows the same rules as

61   what has been worked out for non-spore haploids, but this has not been rigorously tested.

62   Alternatively, and often regardless of the proximity of a mating partner (McClure et al.,

63   2018), following outgrowth haploid spores can enter S phase and bud from the tip of the

64   outgrowth. The most recent comprehensive review of cell polarity in yeast states that "in

65   yeasts, germinating spores lack obvious positional cues and appear to break symmetry to

66   initiate polar growth" (Chiou et al., 2017). This statement is clearly true for the fission

67   yeast *Schizosaccharomyces pombe*, where an elegant study demonstrated that polarity

68   factors, including active Cdc42, wander randomly around the cortex of growing spores until

69   the outer spore wall breaks, whereupon polarity is stabilized at the site of wall rupture

70   (Bonazzi et al., 2014). In *S. cerevisiae*, on the other hand, a single polarity site is already

71   apparent in dormant spores, prior to germination; the only known marker of this site is the

72   septin protein Cdc10 (Joseph-Strauss et al., 2007). Polarization of the actin cytoskeleton, a

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73 prerequisite for outgrowth, occurs only after germination begins (Kono et al., 2005). In

74 non-spore cells, septins are recruited to the site of future budding by active Cdc42 and

75 Cdc42-interacting proteins (Iwase et al., 2006); it is not known how Cdc10 is deposited

76 during sporulation at a single site on each spore. During sporulation the spore cortex is

77 synthesized de novo, precluding any influence on bud site selection by persistent cortical

78 landmarks produced by previous budding events.

79 Yeast sporulation takes place within a single (usually diploid) cell and generates (usually

80 four) spores encased within the original cell wall of the sporulating cell, called the ascus

81 (Neiman, 2011). Whereas in *S. pombe* the ascus wall is globally digested by cell wall

82 hydrolases immediately upon the successful completion of meiosis (Dekker et al., 2007;

83 Encinar del Dedo et al., 2009; H. Guo & King, 2013), in *Saccharomyces* the ascus wall

84 remains intact throughout sporulation. In the absence of external factors, such as those

85 found in insect guts (A. E. Coluccio et al., 2008), the *Saccharomyces* ascus wall breaks

86 down slowly during germination, often persisting long enough for spores to mate and bud

87 following germination. *Saccharomyces* spores (but not spores of *S. pombe*) are also

88 interconnected by cell wall junctions called interspore bridges that persist following ascus

89 wall digestion (A. Coluccio & Neiman, 2004). Outgrowth by germinating spores first

90 requires local break down of a rigid outer spore wall that confers stress resistance via layers

91 of chitosan and polymerized dityrosine (in *Saccharomyces* (Briza et al., 1990)) or a

92 proteinaceous coat (in *S. pombe* (Fukunishi et al., 2014)). Subsequent budding or mating

93 requires local breakdown of the newly-emerged cell wall at the site of budding or fusion,

94 respectively. If *Saccharomyces* spores bud when the ascus wall is still intact, those buds can

95 locally penetrate the ascus wall, as visualized by electron microscopy many decades ago

96 (Hashimoto et al., 1958; Rij, 1978; Sando et al., 1980). One of us (M.M.) noticed this

97 phenomenon using light microscopy in the context of a more recent study, and verified that

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98   the protrusions were buds by visualizing bud necks marked with fluorescently tagged septin

99   proteins (McMurray & Thorner, 2008). The mechanisms by which germinating

100   *Saccharomyces* spores digest cell walls during outgrowth, budding and mating have not

101   been investigated.

102   Here we describe how the sites where spores bud and fuse upon mating, and the

103   localization of Cdc10 with relation to the position of other haploid spores produced by the

104   same diploid mother, demonstrate that each *S. cerevisiae* spore is born pre-polarized to

105   direct outgrowth away from its "sibling" spores. Spore buds are thereby positioned to

106   penetrate the ascus wall.

3. Materials and Methods

3.1. Yeast strains and media

Stable cortical foci of the septin Cdc10 at the ascus periphery

110   All strains used in the new experiments described here are of the S288C strain background,

111   specifically derived from the "designer deletion" strains BY4741 (*MATa his3Δ1 leu2Δ0*

112   *met15Δ0 ura3Δ0*), BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), and BY4743 (the diploid

113   formed by mating BY4741 and BY4742). Unless specified otherwise in the figure legend, all

114   experiments were done with a diploid strain made by mating FY2742 (*MATα his3Δ1*

115   *leu2Δ0 lys2Δ0 ura3Δ0 TAO1 MKT1 RME1*) and FY2839 (*MATa his3Δ1 leu2Δ0 lys2Δ0*

116   *ura3Δ0 TAO1 MKT1 RME1*), two haploid strains that carry at three loci (*TAO1*, *MKT1*, and

117   *RME1*) dominant alleles from the efficiently-sporulating SK-1 strain background

118   (Kloimwieder & Winston, 2011). JTY3985 carries *CDC10-GFP* integrated at the *CDC10*

119   locus using the *URA3* selectable marker and is otherwise isogenic to BY4741 (Johnson et

120   al., 2015). MMY0341 (*gas1Δ::kanMX /gas1Δ::kanMX*) MMY0286 (*acf2Δ::kanMX,*

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121 *acf2Δ::kanMX*), MMY0291 (*rsr1Δ::kanMX, rsr1Δ::kanMX*) were retrieved from the

122 homozygous diploid deletion collection derived from BY4743 (Giaever et al., 2002).

123 Previously published Gas1-GFP localization data reproduced here used a diploid strain of

124 the SK-1 background in which both genomic copies of *GAS1* were deleted and Gas1-GFP

125 was expressed from a high-copy plasmid (Rolli et al., 2011).

126 Haploid strains were mated together using sterile toothpicks by mixing approximately equal

127 amounts on the surface of YPD agar (per liter: 10 g yeast extract, 20 g peptone, 20 g

128 dextrose, 2% agar) in a petri dish. After overnight incubation at 30˚C, cells from the

129 mixture were streaked with a toothpick to agar media that was either selective for the

130 diploid, or non-selective (YPD). In the latter case, diploid clones were identified as

131 individual colonies containing cells that were able to sporulate.

132 To induce sporulation, diploid cells were cultured in 5 mL liquid YPD (per liter: 10 g yeast

133 extract, 20 g peptone, 20 g dextrose) in glass culture tubes rotated in a roller drum

134 overnight to near-saturation. For analysis of *rsr1Δ/rsr1Δ* cells in "old" rich medium, the

135 culture time in YPD was extended for 6 additional days. A 200-μL aliquot of these cells was

136 washed with 5 mL sterile water and resuspended in 2.5 mL sporulation medium (1%

137 potassium acetate, 0.05% glucose, 20 mg/L leucine, 40 mg/L uracil) in a new tube to an

138 optical density at 600 nm of approximately 0.5. These cell suspensions were rotated at

139 22˚C for at least 4 days, after which time the percentage of cells that had formed mature

140 asci did not noticeably increase. To induce germination, aliquots of cells from sporulation

141 cultures were pelleted and resuspended in 1 mL YPD in 1.7-mL microcentrifuge tubes and

142 rotated at 22˚C or 30˚C for 4-6 hours (or as indicated in figure legends). To digest the

143 ascus wall, a 50-μL aliquot of cells from sporulation culture was pelleted and resuspended

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144   in 1 mg/mL Zymolyase-20T (#320921, MP Biomedicals) dissolved in water and incubated

145   at 30˚C for 10 minutes.

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3.2. Fluorescence labeling

148   FM™ 4-64 dye (N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl)

149   Hexatrienyl) Pyridinium Dibromide, from Molecular Probes, Inc. # T3166) was dissolved in

150   dimethyl sulfoxide to make a 1.6 μM stock solution. An aliquot of cells from a sporulation

151   culture that had been in sporulation medium for 18 hours was pelleted and resuspended in

152   50 μL ice-cold YPD to which 1 μL of the dye stock was then added. After 20 minutes on ice

153   in the dark, the cells were pelleted again and washed twice by resuspension in 1 mL ice-

154   cold water each time. Finally, the cells were suspended in 50 μL water and visualized by

155   microscopy using the Texas red LED filter cube.

156   Calcofluor white M2R was dissolved in water at a concentration of 10 mg/mL. 1 μL was

157   added to a 100-μL aliquot of cells that had been in sporulation medium for 4 days, after

158   which the cells were washed three times with water by pelleting and resuspension. After

159   the third wash, the cells were resuspended in 1 mL YPD and incubated at 30˚C for 6 hours,

160   then pelleted and resuspended in 50 μL before imaging.

3.3. Microscopy and imaging

162   Aliquots of cells from sporulation cultures were imaged directly on agarose pads made with

163   1% agarose in water or, in the case of cells expressing Cdc10-GFP, before and after a 3.5-

164   hour interval of incubation at 30˚C on an agarose pad made with 1% agarose in

165   sporulation medium. Germinating cells were pelleted and resuspended in water before

166   applying to agarose pads. All images were captured on an EVOSfl all-in-one microscope

167   (ThermoFisher Scientific, Waltham, MA) with an Olympus 60× Plan-Apo oil objective

168   (numeric aperture 1.42). Filter cubes were as follows: GFP (AMEP4651, excitation 470/22

169   nm, emission 510/42 nm), Texas red (AMEP4655, excitation 585 nm, emission 624 nm),

170   RFP (AMEP4652, excitation 531/40 nm, emission 593/40 nm), and DAPI (AMEP4650,

171   excitation 357/44 nm, emission 447/60 nm). Images were cropped and adjusted (always

172   the same way for each image of the same type from the same experiment), and inverted in

173   Photoshop (Adobe, San Jose, CA).

174   4. Results

175   4.1. Stable cortical foci of the septin Cdc10 at the ascus periphery

176   That single *S. cerevisiae* spores are born pre-polarized was known from the discrete

177   localization of the septin Cdc10 to a single site on the spore cortex from which cell wall

178   outgrowth occurs (Joseph-Strauss et al., 2007). However, it was not known how the site of

179   spore pre-polarization relates to the spatial relationship between the spores, the interspore

180   bridges, and the ascus wall. If spore buds commonly penetrate the ascus wall, then spores

181   should be polarized to outgrow and bud away from the interspore bridges at the center of

182   the ascus. To test this prediction, we visualized Cdc10-GFP in spores within intact, mature

183   asci. As is true of diverse fluorescently-tagged proteins, strong signal was observed in the

184   areas between the spores (Figure 1A), presumably reflecting Cdc10-GFP molecules that

185   were outside the prospore membranes as they closed and thus remained in the ascal

186   cytoplasm, which becomes concentrated between the spores as the ascus wall compresses

187   tightly to surround the spores during the final stages of ascus maturation. Cdc10-GFP signal

188   is not found at these locations in spores that are isolated from asci (Joseph-Strauss et al.,

189   2007). Apart from this signal, single Cdc10-GFP puncta were found in many (but not all)

190   spores (Figure 1A); the failure to observe a punctum in every spore may reflect the fact that

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191   only one allele of *CDC10* in the diploid strain expresses the GFP fusion. While it is known

192   that all spores from such *CDC10-GFP/CDC10* heterozygous diploids inherit some pre-

193   existing Cdc10-GFP protein, regardless of their haploid genotype, those that fail to inherit

194   the *CDC10-GFP* allele have fainter signal (Joseph-Strauss et al., 2007). When they were

195   visible, Cdc10-GFP puncta were found in locations consistent with a mode of outgrowth

196   upon germination that precedes spore budding through an intact ascus wall (Figure 1A and

197   data not shown).

198   Spores can survive for long periods in nutrient-free conditions and then, once nutrients are

199   provided, germinate efficiently. We see no obvious effect on the site of budding during

200   germination of the length of time between sporulation and germination (unpublished

201   observation); indeed, the images in Figure 1A were taken of asci that had been in

202   sporulation medium for over 7 days, with the vast majority of asci in these cultures having

203   visibly completed maturation by day 4. If Cdc10 foci mark the location of spore pre-

204   polarization, then these cortical foci must not diffuse to any great extent over time.

205   Alternatively, they may be able to diffuse on regions of the cortex far from the interspore

206   bridges, but may be prevented from diffusing into the cortical areas near interspore bridges.

207   To distinguish between these possibilities, we visualized Cdc10-GFP foci before and after an

208   interval of 3.5 hrs. We could detect no change in location during this time (Figure 1B).

209   These data suggest that diffusion of the cortical Cdc10-GFP foci is highly restricted,

210   potentially allowing spores to maintain cortical polarity for long periods of time.
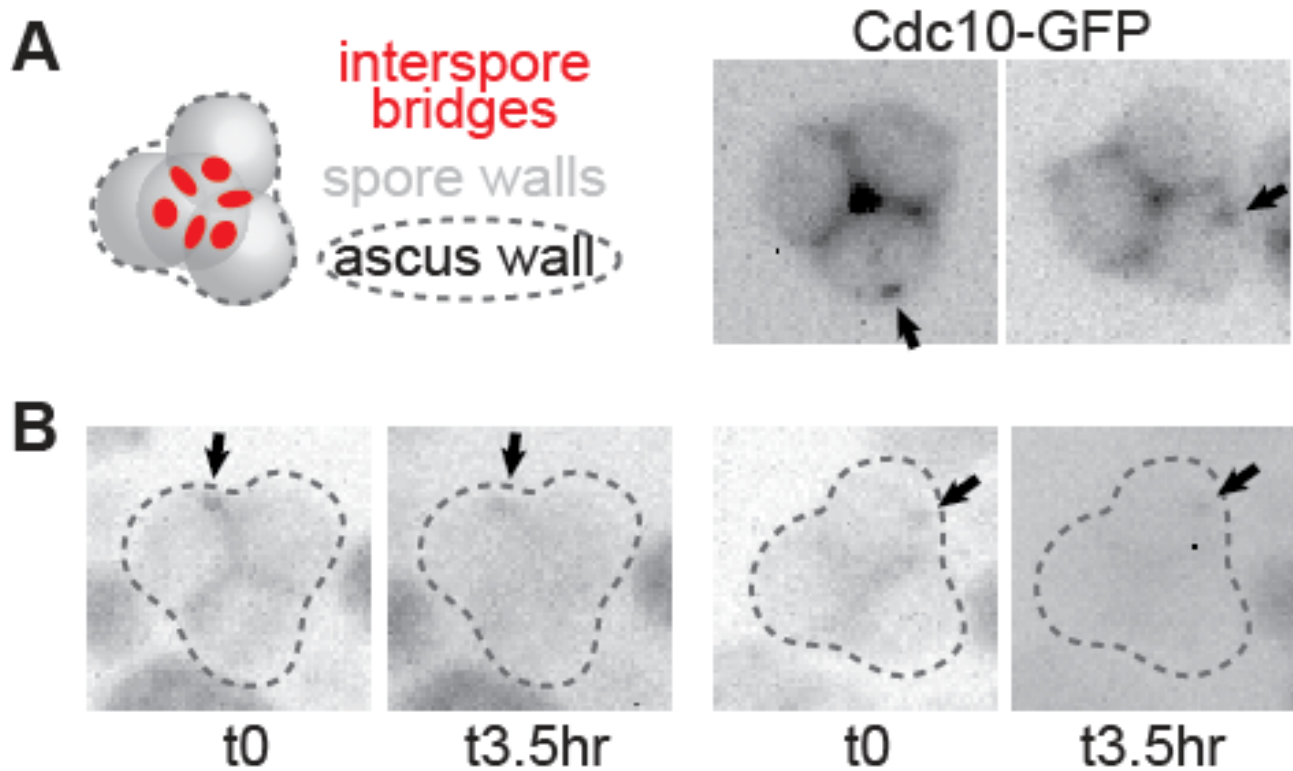
Figure 1. Stable cortical foci of the septin Cdc10 persist on the periphery of asci. (A) At left is an illustration of a typical pyramidal four-spored ascus in which the three spores at the "base" of the pyramid are in the same focal plane. Red circles indicate interspore bridges. At right, Cdc10-GFP fluorescence in intact asci from sporulation culture. Arrows indicate cortical foci. (B) As in (A), but the same asci were visualized before and after a 3.5-hr interval on solid sporulation medium. Cells are of diploid strain made by mating haploid strains JTY3985 and FY2742.

## 4.2. Ascus wall penetration upon spore budding points to prepolarization of spores away from interspore bridges

If spores are pre-polarized to outgrow upon germination towards the periphery of the ascus, then when germinating spores bud while they are still within the ascus, they should locally penetrate the ascus wall, such that the buds protrude from the ascus surface. If, instead, spore pre-polarization is random, then spore budding should frequently result in budding in between the spores, within the ascus, without penetrating the ascus wall. We

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226 exploited the weak red autofluorescence of the post-germination spore wall (Joseph-Strauss

227 et al., 2007) to identify trapped buds (non-fluorescent cell wall outgrowth) within the

228 crowded environment of an intact ascus full of germinating spores. Among hundreds of

229 such asci we have only seen a single likely case of a bud "trapped" within the ascus (Figure

230 2A).

231 As an independent way to visualize bud penetration of the ascus wall, we performed a

232 pulse-chase with calcofluor white, which fluorescently labels cell wall chitin (Cabib &

233 Bowers, 1975). The ascus wall in mature asci was labeled with calcofluor, and then excess

234 dye was washed away prior to the induction of germination by addition of rich (YPD)

235 medium (Figure 2B). New cell wall synthesized in the absence of calcofluor white is non-

236 fluorescent. Spore buds emerged from holes in the ascus wall, which otherwise remained

237 intact (Figure 2B). Thus budding by germinating spores is directed away from the

238 interspore bridges that connect spores to each other, and towards the ascus wall that

239 surrounds them. Budding away from interspore bridges was also apparent for

240 interconnected spores in which the ascus wall was enzymatically removed (Figure 2C).

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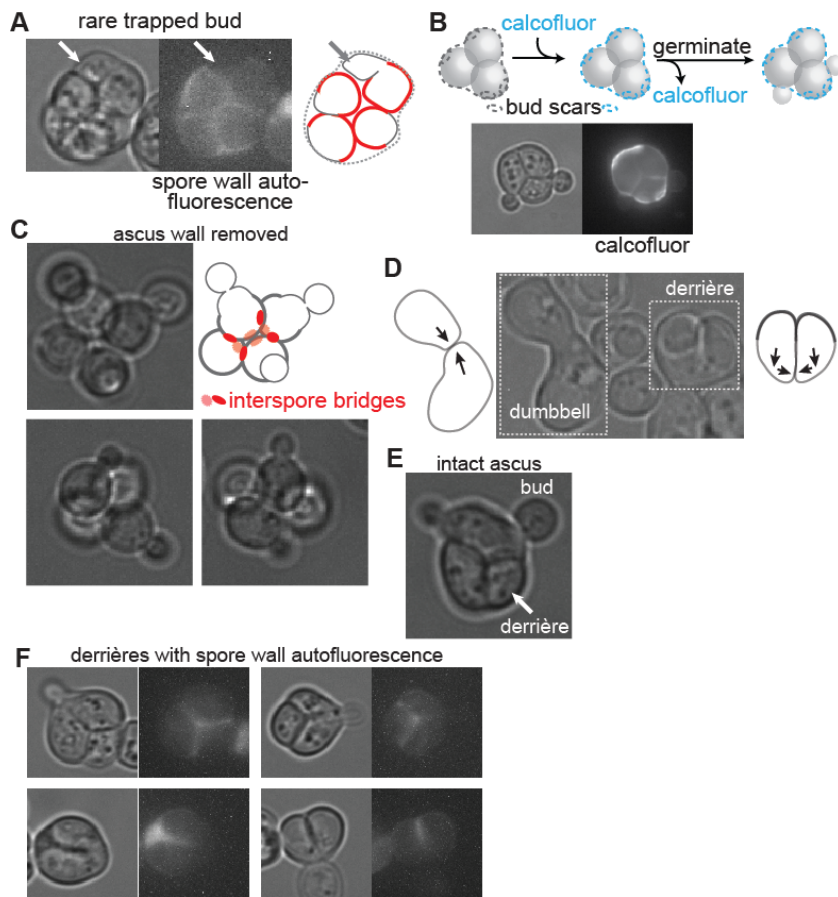
Figure 2 *Saccharomyces* spores outgrow away from interspore bridges upon germination.

(A) Germinating ascus as viewed by transmitted light and with autofluorescence of the spore wall visualized with an RFP filter. (B) According to the illustration, asci were exposed to the chitin-binding dye calcofluor white and then, after washing away free dye, allowed to germinate. Pre-existing bud scars on the ascus wall were deposited during diploid budding events prior to sporulation. Calcofluor fluorescence and transmitted light are shown. (C) Tetrads for which the ascus wall was removed by exposure to Zymolyase prior to germination. In the illustration red circles are interspore bridges, and the spore wall is thicker than the new, vegetative cell wall. (D) Image taken several hours after asci were allowed to germinate, showing dumbbell-shaped zygote and derrière-shaped zygote, with illustrations of presumptive directions of outgrowth prior to fusion. (E) Germinating ascus showing two buds penetrating the ascus wall and the other two spores fused into a

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255   derrière. (F) As in (A), after 7.75 hours of germination, showing localization of the

256   autofluorescent spore wall with regard to the shape of the derrière.

257 ## 4.3. Unique zygote morphology provides independent evidence of spore

258 prepolarization

259 We noticed independent evidence of spore pre-polarization in the morphology of zygotes

260 produced by spores that mated within the ascus. Figure 2D shows an image of cells from a

261 population of germinating asci in which a zygote formed by non-spore haploid cells (or

262 possibly a spore with a non-spore cell) is adjacent to a zygote formed by mating between

263 sibling spores. The dumbbell morphology of the zygote formed by non-spore mating is

264 consistent with the morphology established in the literature for mating between wild-type

265 haploids (Sena et al., 1973), where prior to cell fusion each partner grows directly towards

266 the other, following a pheromone gradient. The zygote formed from intra-ascus mating

267 between spores is distinctly different, as if both mating partners initially grew in

268 approximately the same direction, roughly perpendicular to a line directly connecting them,

269 and then redirected growth towards each other prior to fusion (Figure 2D). Similar to the

270 use of the term "shmoo" to refer to the unique morphology adopted by non-spore cells just

271 prior to mating, we sought a new term to refer to the unique morphology of zygotes

272 produced by mating between sibling spores. Inspired by the inescapable resemblance of one

273 side of the resulting shape to the shape of the human buttocks, we propose the term

274 "derrière". Derrières were also seen within intact asci after germination (Figure 2E).

275 The rigid outer spore wall is likely a barrier to outgrowth, which requires cell wall

276 breakdown at the site of fusion. Cell wall expansion upon germination is thus restricted to

277 the site of outer spore wall breakdown, and the residual spore wall changes little upon

278 germination and thereafter. If spores break down the outer spore wall at sites

279 approximately opposite from the interspore bridges, then two adjacent spores within an

280 ascus should grow along vectors that do not converge and instead are parallel or, more

281 likely, divergent (Figure 2D). If redirected growth towards a pheromone source is only

282 possible for vegetative cell wall, where polarized exocytosis targets cell wall synthesis

283 enzymes to specific sites, then some amount of cell wall outgrowth is presumably required

284 before that cell wall outgrowth can be redirected towards the pheromone source. This

285 model predicts derriére-shaped zygotes with spore wall in the twin bulges. To test this

286 model, we visualized spore wall autofluorescence within derrières. As expected, spore wall

287 fluorescence was restricted to the twin bulges of derrières (Figure 2F). Buds then emerged

288 from the site of fusion (Figure 2D,F).

289 ## 4.4. The old plasma membrane of the sporulating diploid cell disappears prior

290 to germination

291 In addition to local digestion via budding, the ascus wall breaks down globally during

292 germination, albeit on a slower time scale. In *S. pombe*, two hydrolytic enzymes, the α-1,3-

293 glucanase Agn2 and the endo-β-1,3-glucanase Eng2, reside in the ascal cytoplasm because

294 they lack signal sequences to drive secretion (Dekker et al., 2007; Encinar del Dedo et al.,

295 2009). It has been speculated that after completion of spore wall synthesis, the old plasma

296 membrane of the diploid fission yeast cell "may disintegrate through an unknown

297 mechanism" (Dekker et al., 2007) allowing the two enzymes access to their substrates.

298 If in *Saccharomyces* spore buds frequently penetrate the ascus wall, we wondered if in

299 doing so they also penetrate the old plasma membrane of the sporulating cell, or if, as is

300 thought for fission yeast, that membrane has already been destroyed. Late in sporulation,

301 vacuole lysis releases hydrolytic enzymes that destroy, among other things, nuclei that were

302 not protected by prospore membrane engulfment (Eastwood et al., 2012). The functional

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303   integrity of old plasma membrane becomes compromised at the same time, suggesting that

304   this membrane may also be destroyed by vacuole lysis (Eastwood & Meneghini, 2015). A

305   direct examination of plasma membrane persistence during sporulation has not, to our

306   knowledge, been reported. To label the plasma membrane in cells undergoing sporulation,

307   we exposed an asynchronously sporulating culture to FM4-64, a lipophilic dye (Vida & Emr,

308   1995). As can be seen in Figure 3, very early in sporulation, when spore walls had not yet

309   been made, only the plasma membrane was labeled. Slightly later, when spore walls had

310   been made but the ascus wall had not yet compressed around the spores, the old plasma

311   membrane was labeled and, in some cases, the spore membranes were also labeled. In

312   mature asci, in which the ascus wall had compressed tightly around the spores, only spore

313   membrane labeling was visible. We interpret these results as an intact plasma membrane

314   "shielding" the spore membranes from labeling early in sporulation, then losing structural

315   integrity later in sporulation, and finally disappearing altogether in mature asci. If our

316   interpretation is correct, when spores germinate and bud within an intact ascus, they only

317   penetrate an old cell wall, and not an old membrane, in order to exit the ascus.

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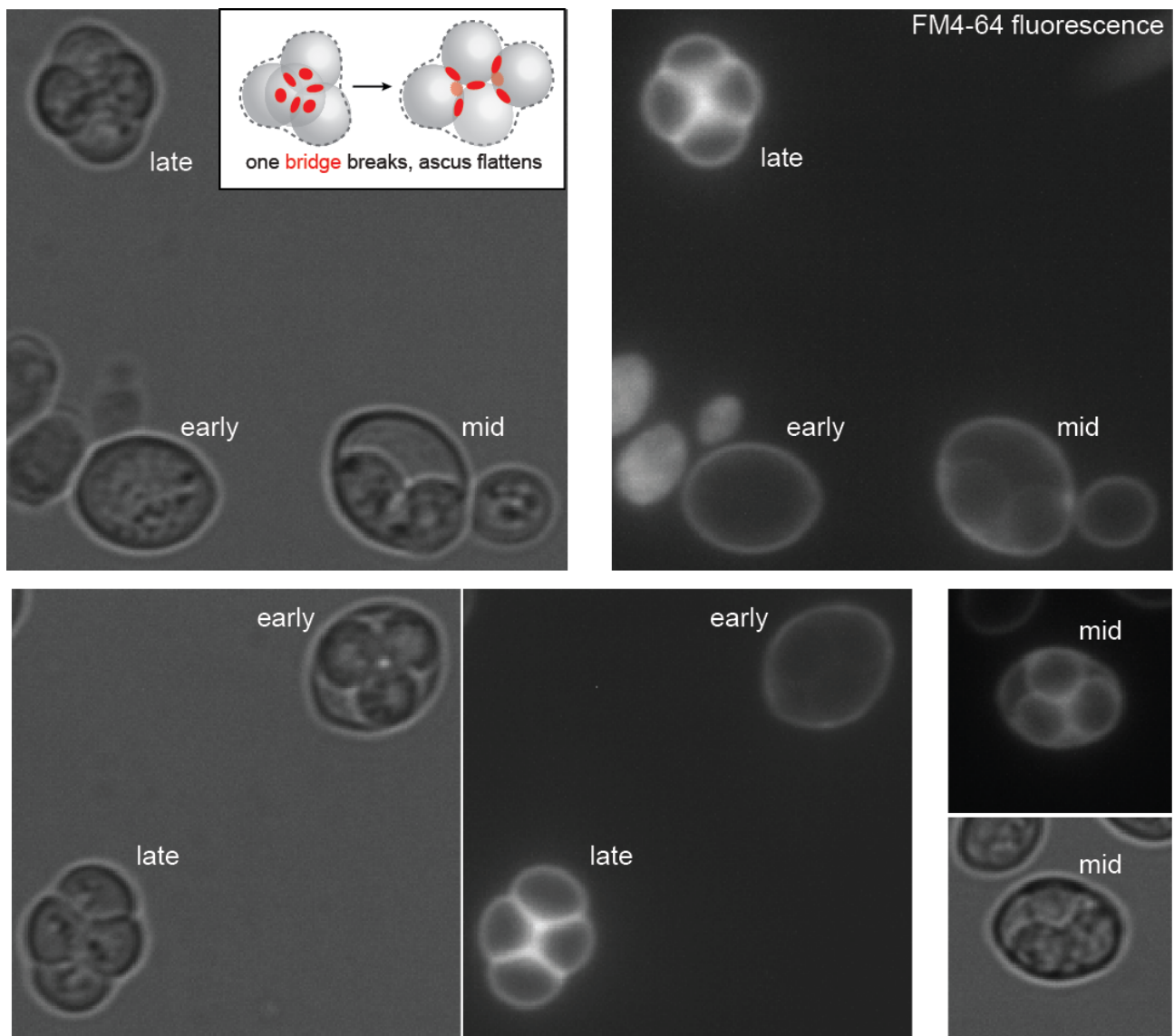
Figure 1 Gradual loss of the old plasma membrane during sporulation. An asynchronously
sporulating culture was exposed briefly to the lipophilic dye FM™4-64. The stage of
sporulation is labeled and was estimated based on spore and ascus wall appearance by
transmitted light. Inset, an illustration of the arrangement of spores in a tetrahedral ascus
with all six interspore bridges (red circles) intact, and in a rhomboid ascus in which one of
the bridges has broken (dashed red circle) as an ascus flattens under a coverslip.

### 4.6. Global ascus wall digestion does not require the putative cell wall hydrolase Acf2

In the *S. cerevisiae* genome, the *ACF2* gene (alias *ENG2*) encodes a homolog of *S. pombe* Eng2, one of the enzymes responsible for global ascus wall digestion (Encinar del Dedo et al., 2009). Like Eng2, Acf2 lacks a predicted signal sequence. In *S. pombe*, the absence of either Agn2 or Eng2 is sufficient to almost completely prevent global ascus wall digestion, resulting in mature spores trapped within an ascus wall (Dekker et al., 2007; Encinar del Dedo et al., 2009). If in *S. cerevisiae* Acf2 digests the ascus wall during germination, we predicted that it should either be translated near the end of sporulation and then secreted into the ascal cytoplasm upon germination, or both translated and secreted immediately upon germination. No translation data are available for germination, but existing ribosome profiling data from synchronously sporulating cells (Brar et al., 2012) demonstrate that Acf2 translation spikes at the last stage of sporulation, just before mature spores are produced (Figure 4A). If gradual Acf2-mediated digestion gradually thins the ascus wall during germination, we predicted that in asci lacking Acf2, the ascus wall might remain too thick and inhibit penetration by spore buds. However, we saw no discernible difference in the frequency or morphology of instances in which the buds of *acf2Δ* spores penetrated the ascus wall upon germination (Figure 4B). In *S. cerevisiae*, either Acf2 performs a different function than Egn2, or loss of a single digestive enzyme is not enough to toughen the ascus wall to an extent that is impenetrable by buds.

4.5. Local ascus wall digestion does not require the putative cell wall hydrolase Gas1

To identify candidate enzymes that may mediate local digestion of the ascus wall during germination, we searched the literature for published localization patterns of cell wall

349 hydrolytic enzymes, with the logic that a protein responsible for local wall digestion should

350 localize at the site of digestion. Translation of the β-1,3-glucanosyltransferase Gas1

351 translation increases at the end of sporulation (Figure 4A), and while this pattern was not

352 specifically noted by the authors, examination of a GFP-tagged allele of Gas1 in spores

353 germinating within an intact ascus revealed that Gas1-GFP clearly localizes to a broad

354 region of the spore cortex opposite from interspore bridges (Rolli et al., 2011) (Figure 4C).

355 To ask if Gas1 is required for the ability of buds to penetrate the ascus wall, we monitored

356 germination by *gas1Δ/gas1Δ* mutant diploid cells. Consistent with the phenotype reported

357 in the literature for non-spore haploid *gas1Δ* cells (Watanabe et al., 2009), the buds

358 produced by *gas1Δ* spores were oddly-shaped, but they had no problems penetrating the

359 ascus wall (Figure 4D). Thus deletion of *GAS1* is insufficient to prevent local ascus wall

360 digestion by germinating spores.

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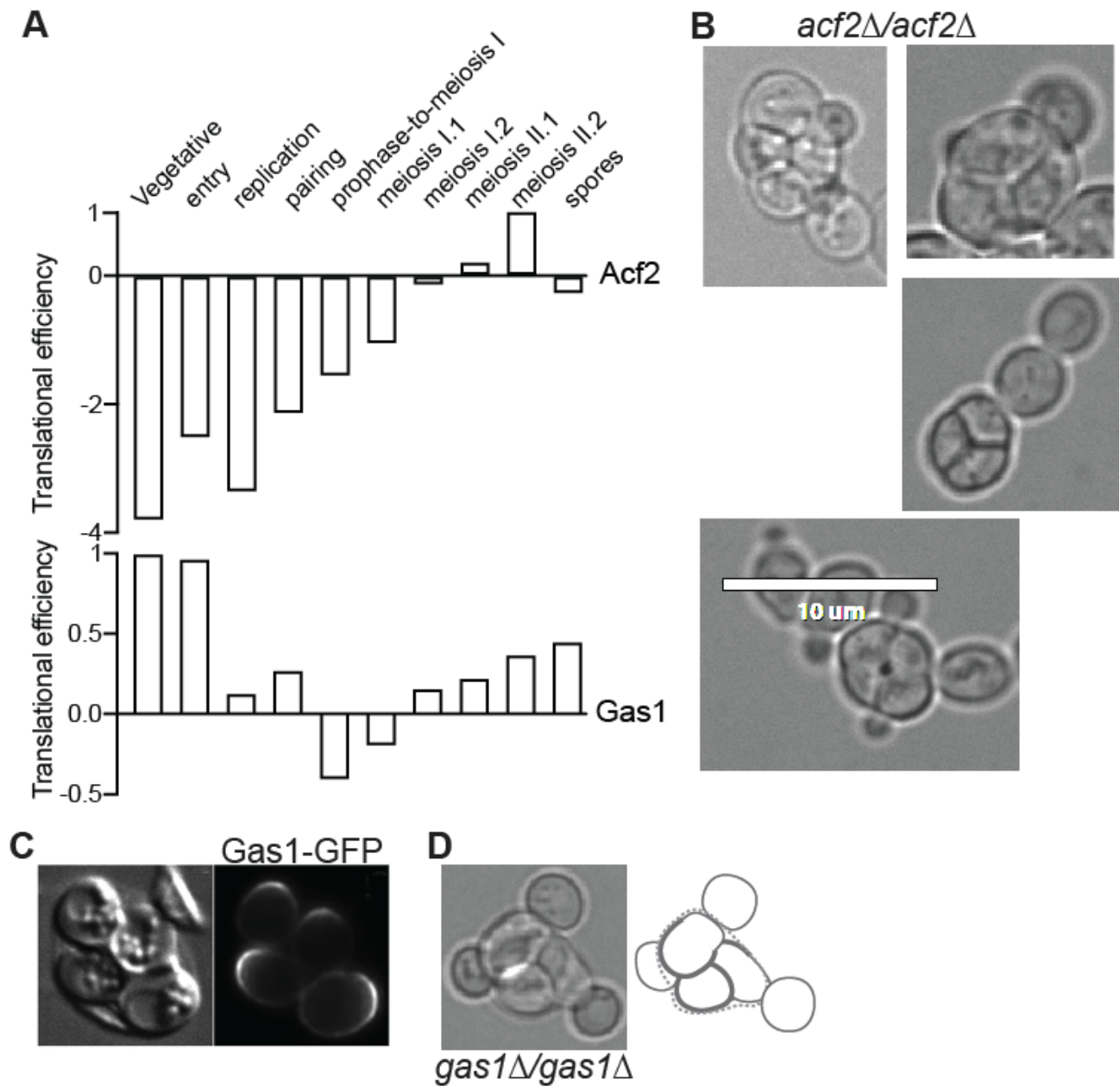
Figure 2 Penetration of the ascus wall by spore budding does not require Gas1 or Acf2. (A)

Translational efficiencies (a measure of ribosome occupancy per mRNA) for Acf2 and Gas1

at various timepoints during sporulation. Data are from (Brar et al., 2012). (B)

Germinating asci of *acf2Δ/acf2Δ* strain MMY0286 showing penetration of the ascus wall by

spore budding. Scale bar, 10 μm. (C) Localization of Gas1-GFP to sites of spore outgrowth

upon germination. Reproduced from (Rolli et al., 2011). (D) A germinating ascus of
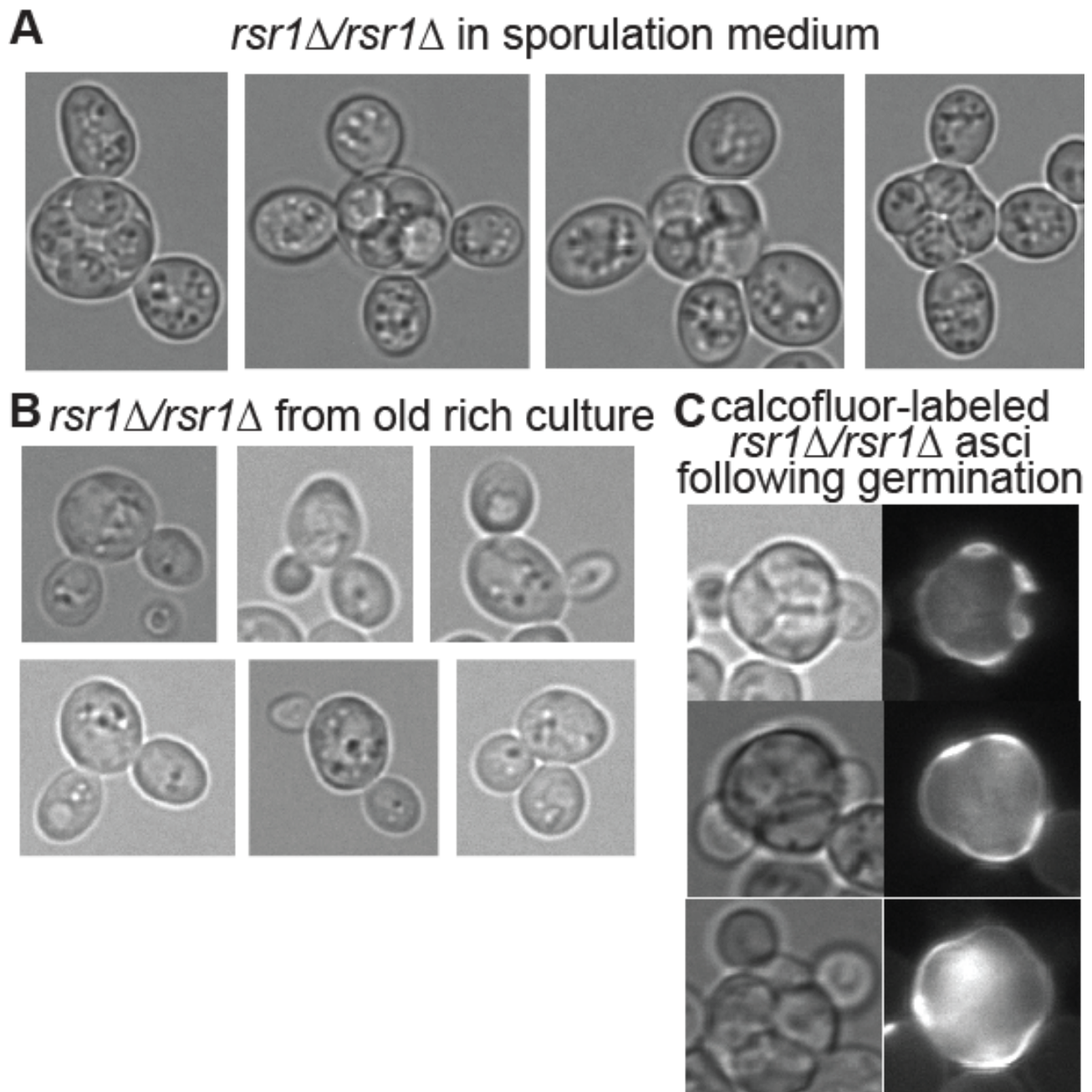
gas1Δ/gas1Δ strain MMY0341.

19

4.6. The canonical bud site selection pathway does not drive polarity in spores

Spores polarize approximately opposite from the cluster of interspore bridges that connect them to the other spores within in ascus. Similarly, diploid non-spore cells usually polarize opposite from the previous bud site (Chiou et al., 2017). We noticed that two genes required for bipolar bud site selection by non-spore cells, *RSR1* (Bender & Pringle, 1989) and *SPH1* (Roemer et al., 1998), are transcriptionally induced early in germination (Joseph-Strauss et al., 2007). We wondered if the same pathway that controls bipolar budding in non-spore diploids also controls spore polarization. We sporulated *rsr1Δ/rsr1Δ* diploids and monitored budding upon germination.

Analysis of budding was complicated by the fact that many of the mutant cells had multiple buds even prior to germination (Figure 5A). Unlike mitotic DNA replication, premeiotic DNA replication is usually uncoupled from bud emergence. The few singly-budded asci found in nominally wild-type strain backgrounds presumably arise from diploid cells that were in a small window of early S phase when sporulation began and proceeded directly into meiosis upon completion of DNA replication (Croes et al., 1976). In the *rsr1Δ/rsr1Δ* mutants, however, either buds formed during multiple prior cell cycles failed to separate from the mother, or multiple buds formed simultaneously upon premeiotic S phase entry. We favor the latter interpretation, considering that simultaneous multiple budding is a known phenotype of *rsr1Δ* haploid cells expressing a synthetic fusion of the polarity scaffold protein Bem1 to the v-SNARE Snc2, and that this phenotype is exacerbated when cells are cultured in minimal, as opposed to rich, medium (Howell et al., 2009). The starvation conditions induced by sporulation medium may bypass the need for the Bem1-Snc2 fusion in driving multiple budding events. Indeed, we found that starving *rsr1Δ/rsr1Δ*

20

393   diploid cells by prolonged culture (1 week) in rich medium, which does not induce

394   sporulation, was sufficient to induce a bi-budded phenotype (Figure 5B).

395   To unambiguously identify buds produced during germination, we applied the calcofluor

396   pulse-chase method. The vast majority of *rsr1Δ/rsr1Δ* asci that were not budded prior to

397   germination showed buds penetrating the ascus upon germination (Figure 5C and data not

398   shown). We conclude from these results that spore polarity is not determined by the

399   canonical pathway that drives polarity during budding by non-spore cells.

A *rsr1Δ/rsr1Δ* in sporulation medium

B *rsr1Δ/rsr1Δ* from old rich culture

C calcofluor-labeled *rsr1Δ/rsr1Δ* asci following germination

400

401 **Figure 3 Cells lacking the polarity factor Rsr1 form multiple buds upon starvation but spore**

402 **buds are able to penetrate the ascus wall.** *rsr1Δ/rsr1Δ* strain MMY0291 was cultured in (A)

403 sporulation medium, (B) rich medium for 1 week, or (C) sporulation medium, followed by

404 pulse labeling with calcofluor white, and then rich medium to induce germination.

405 Fluorescence images show calcofluor fluorescence.

22

5. Discussion

The evidence for prepolarization of *S. cerevisiae* spores has been hiding in plain sight for decades. For every published image we could find in which the orientation of a germinating spore relative to its sibling spores is discernible, the direction of outgrowth, budding and/or mating is consistent with prepolarization away from the interspore bridges. In *S. pombe* spores, on the other hand, lack of prepolarization has been clearly demonstrated (Bonazzi et al., 2014), and an assumption that *S. cerevisiae* is the same is understandable, given the many similarities with regard to mechanisms of sporulation. Several key differences are worth noting, however. Fission yeast spores lack interspore bridges, and ascus wall breakdown is a programmed event taking place immediately following successful completion of meiosis (H. Guo & King, 2013). Hence *S. pombe* spores are designed for dispersal, consistent with a mostly haploid lifestyle in this species, whereas *Saccharomyces* species appear to have evolved to prioritize return to diploidy following meiosis. From this perspective, why not prepolarize a spore directly towards its meiotic siblings, at least two-thirds of which will be of compatible mating type? A somewhat trivial explanation may be that interspore bridges represent a physical barrier to outgrowth, analogous to the difficulties that non-spore yeast cells encounter when mutations drive them to re-bud through a chitin-rich bud scar (Tong et al., 2007). We propose another model. In a crowded four-spored ascus, navigating gradients of multiple pheromones in order to identify a compatible partner is non-trivial (Rappaport & Barkai, 2012). If germinating spores grew into the center of the ascus, the situation would become even more complex, especially if spores budded and then switched mating types. Hence, prepolarization away from the center may facilitate pheromone sensing by simplifying the gradients of signals spores must navigate to find a mate.

430 For other septin functions, e.g., cytokinesis, Cdc10 acts together with other septin proteins

431 as a stable hetero-oligomeric protein complex that act as diffusion barriers and scaffolds for

432 the recruitment and cortical retention of other proteins (Oh & Bi, 2011). Polymerization of

433 hetero-oligomeric septin complexes into filaments is required for cytokinesis (McMurray et

434 al., 2011); no single septin is sufficient. Hence while we have not yet asked if other septins

435 co-localize with Cdc10 to cortical foci in spores, we speculate that they do. What specific

436 function, if any, Cdc10 might perform at these foci is an intriguing question for future

437 inquiry. In non-spore cells, septins encircle active Cdc42 at both the site of bud formation

438 (Okada et al., 2013) and the shmoo (Kelley et al., 2015), but the cortical puncta in spores

439 are not obviously ring-shaped, and it is not known if Cdc42 (and/or another Rho-family

440 GTPase) is also there.

441 How does Cdc10 arrive at this location? Cdc10 and other septins localize around the

442 spindle pole bodies at the end of meiosis metaphase II, where the prospore membrane first

443 appears, and then localize as bars and horseshoes associated with the prospore membrane

444 as it grows (Pablo-Hernando et al., 2008). The nuclear envelope and prospore membrane

445 are connected via the spindle pole body until the meiosis-specific components of the

446 "meiotic outer plaque", Mpc54 and Mpc70, are destroyed just after meiosis II (Knop &

447 Strasser, 2000). The exocyst complex, which targets exocytic vesicle docking and fusion, is

448 found (along with septins) at the meiotic outer plaque (Mathieson et al., 2010), at the

449 shmoo tip (Kelley et al., 2015), and at the site of future budding by non-spore cells (W.

450 Guo et al., 2001). Meiotic anaphase II pushes the spindle pole bodies toward the periphery

451 of the ascus. If septins and/or the exocyst persist on the spore membrane at the former site

452 of contact with the meiotic outer plaque, these "landmarks" will be near the periphery of

453 the ascus, with the sites of prospore membrane fusion (and interspore bridges) in the

454 center. Provided cortical diffusion of the landmarks is limited in mature spores, these sites

24

455 would correspond to the Cdc10 localization patterns and sites of outgrowth that we observe

456 upon germination. Future work will be required to test this model.

457 Ascus wall penetration by buds has been documented previously (Hashimoto et al., 1958;

458 Rij, 1978; Sando et al., 1980) but the implications for spore polarity were not considered.

459 The mechanism of local ascus digestion upon spore budding also remains unknown.

460 Budding itself requires cell wall digestion at a single site on an unbudded cell. Thus when a

461 spore buds inside an ascus, two walls are locally digested: the wall of the spore and the

462 ascus wall. Another step in the yeast life cycle also requires local digestion of two walls: cell

463 fusion upon mating. Here it is thought that targeted secretion of enzymes followed by cell-

464 contact-limited diffusion restricts digestion to a narrow pore (Huberman & Murray, 2014).

465 We imagine that a similar mechanism, perhaps involving the same enzymes, mediates ascus

466 wall penetration during budding. Our results show that, despite localizing to the right place

467 at the right time, Gas1 is dispensable for this process; other enzymes may act redundantly.

468 Intra-ascus mating events do not locally digest the ascus wall because repolarization

469 towards the mating partner directs the digestive enzymes elsewhere. On the other hand,

470 during inter-ascus mating events *Saccharomyces* spores penetrate four walls in order to

471 fuse. Crowding promotes inter-ascus mating (Murphy & Zeyl, 2010), which emphasizes the

472 importance of considering where germination occurs in nature and thus how evolution

473 acted upon it. Here we know very little. Sporulation is most frequent at the colony

474 periphery (Purnapatre & Honigberg, 2002), but to what extent wild yeast grow in such

475 colonies is unknown.

476 Indeed, while studies of polarity determination and mating by isolated non-spore

477 *Saccharomyces* cells in the laboratory setting have provided numerous insights into basic

478 biology, considering what we now know about the *Saccharomyces* life cycle outside the lab

479 (Tsai et al., 2008), budding and mating by non-spore haploids may represent a kind of

480 backup plan for circumstances in which a spore is physically separated from, or chooses to

481 ignore (McClure et al., 2018), its meiotic siblings. Even the spore-based proposed rationale

482 for axial budding by haploids – to place two cells of one mating type adjacent to two cells

483 of opposite mating type following mating-type switching by an isolated spore (Gimeno &

484 Fink, 1992) – can now be viewed in an additional light: axial budding would help ensure

485 that if a spore is still within an intact ascus when it buds a second time, the second bud will

486 also penetrate the ascus wall. Our work thus highlights outstanding questions and lays a

487 foundation for future studies.

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