Dormancy-to-death transition in yeast spores occurs due to gradual loss of gene-expressing ability

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Short summary (256 characters with space)

This study identifies systems-level quantities that decay during dormancy in *Saccharomyces cerevisiae* spores and thereby reveals the meaning of ageing for dormant yeast spores and shows that they die when their gene-expressing ability is irreversibly lost.

Highlights

- For a given glucose concentration, a dormant yeast spore has a well-defined probability of germinating ("germination ability").
- A spore's germination ability positively correlates with its RNAP I-III levels and the gene-expression (GFP) level it can realize when the expression is forced without nutrients.
- Ageing during dormancy means gradual decreases in germination ability, RNAP levels, and GFP-level realizable when expression is forced.
- Spores die after sufficiently losing gene-expressing ability and drugs that inhibit gene expression during dormancy shorten spores' lifespans (e.g., from months to a day).

ABSTRACT

Dormancy is colloquially considered as extending lifespan by being still. Starved yeasts form dormant spores that wake-up (germinate) when nutrients reappear but cannot germinate (die) after some time. What sets their lifespans and how they age are open questions because what processes occur - and by how much - within each dormant spore remains unclear. With single-cell-level measurements, we discovered how dormant yeast spores age and die: spores have a quantifiable gene-expressing ability during dormancy that decreases over days to months until it vanishes, causing death. Specifically, each spore has a different probability of germinating that decreases because its ability to - without nutrients - express genes decreases, as revealed by a synthetic circuit that forces GFP expression during dormancy. Decreasing amounts of molecules required for gene expression - including RNA polymerases - decreases gene-expressing ability which then decreases chances of germinating. Spores gradually lose these molecules because they are produced too slowly compared to their degradations, causing gene-expressing ability to eventually vanish and, thus, death. Our work provides a systems-level view of dormancy-to-death transition.

(175 words)

INTRODUCTION

When starved of nutrients, microbes can become dormant by forming spores (Rittershaus *et al,* 2013; Neiman *et al,* 2011; Stragier & Losick, 1996; Errington, 2003; Lennon & Jones, 2011; Nachman *et al,* 2007; Chu *et al,* 1998; Eldar *et al,* 2009; Suel *et al,* 2006; Suel *et al,* 2007). Dormancy is a state in which gene expression, metabolism, and all other cellular processes are thought to have nearly or completely ceased. But exactly to what extent each process of life has halted inside dormant spores remain ambiguous as attaching firm numbers to them has been challenging due to the barely detectable levels of activities inside dormant spores. Entering dormancy is a multi-step process that does not necessarily end with the spore formation. In fact, after sporulation, spores of some species are not dormant right away. For example, *Bacillus subtilis* spores - a model for prokaryotic spores - undergoes a week-long "ageing" process during which they are metabolically active, and they actively degrade or produce key transcripts to tailor their dormancy to the environmental temperature during this process (Segev *et al,* 2012). The spores of budding yeast, *Saccharomyces cerevisiae* - a model for eukaryotic spores - takes between a few hours to a few days to fully enter dormancy (Brengues *et al,* 2002; Thacker *et al,* 2011). During this time, the not-yet-dormant yeast spores express various genes - such as those involved in completing the spore wall - before gradually turning off gene expressions, at the end of which the spores are considered to be dormant (Brengues *et al,* 2002; Thacker *et al,* 2011). After entering dormancy, both bacterial and yeast spores are thought to have greatly reduced metabolism and vanishingly low, if any, genome-wide expression levels (Rittershaus *et al,* 2013; Segev *et al,* 2012; Brengues *et al,* 2002). Supporting this view for yeast spores are the recent discoveries of proteins and messenger RNPs (e.g., mRNAs bound to translational machineries) becoming inactive by aggregating into macromolecular structures inside dormant yeast spores and other fungal spores (Laporte *et al,* 2008; Petrovska *et al,* 2014). The resulting solid-like, glassy cytoplasm - being packed with these aggregates - greatly hinders proteins' movements and enzymatic activities (Ablett *et al,* 1999; Cowan *et al,* 2003; Dijksterhuis *et al,* 2007; Parry *et al,* 2014; Joyner *et al,* 2016; Munder *et al,* 2016). Taken together, previous findings support the widely accepted view that dormant yeast spores have nearly ceased all their activities (Rittershaus *et al,* 2013). But technical and conceptual difficulties lie in the phrase, "nearly ceased". Technically, the nearly ceased activities inside dormant yeast spores might, for instance, occur too slowly so that many measurement techniques would lack the required sensitivity to detect those activities, especially in individual dormant spores rather than in lysates of many spores. On the conceptual side: given that dormant yeast spores already appear still and that, as time passes without nutrients, they eventually die - meaning that nutrients can no longer revive

them - an intriguing and underexplored challenge is explaining how yeast spores age over time to transition from being dormant (i.e., alive) to dead (i.e., what sets a spore's lifespan?). Addressing this question would deepen our understanding of dormancy - which remains either poorly understood or misunderstood for many species as the emerging discoveries show (Laporte *et al,* 2008; Petrovska *et al,* 2014) - and the meaning of cellular death. We sought to address this question in the context of yeast spores.

To address our question, we first reasoned that a dormant yeast spore's lifespan is likely encoded by amounts of its stored intracellular factors (e.g., specific proteins and RNAs) which are required for restarting replicative life. Their depletions below some values may cause spores to die. For dormant spores of some species, researchers have identified several intracellular factors that affect their revival (Segev *et al,* 2012; Dworkin & Shah, 2010; Mutlu *et al,* 2018; Donnini *et al,* 1988; Geijer *et al,* 2012; Herman & Rine, 1997; Sturm & Dworkin, 2015; Sinai *et al,* 2015). For example, alanine is more likely to revive *B. subtilis* spores that have more alanine dehydrogenase stored in them than spores that stored less of it (Mutlu *et al,* 2018). But it is unclear how the known and as-yet-unknown intracellular factors that affect revivability - perhaps the numbers of stored ribosomes and RNA polymerases - collectively affect a dormant yeast or bacterial spore's lifespan, which can be months or decades (Nagtzaam & Bollen, 1994) and thus vastly longer than typical biomolecules' lifetimes. A brute-force way to address this question is identifying all intracellular factors that affect spores' revival, then measuring how the amounts of all those factors may change over time within a dormant spore, and then deducing when the spore dies, thereby determining all combinations of intracellular factors and their amounts that are necessary for sustaining dormancy. But this approach remains elusive because, for one, it requires using a current snapshot of numbers (i.e., amounts of intracellular factors now) to predict a capacity for achieving a complex future behavior - whether the dormant spore still has an ability to wake-up if nutrients were to reappear. Addressing our question requires circumventing this difficulty.

As we will show, we found a single, readily measurable, systems-level metric - ability for a dormant yeast spore to express genes - that reveals how a dormant yeast spore eventually dies. In the process, we propose here a view of dormancy as a precisely measurable, continuous quantity that a yeast spore loses over time as it approaches death. Our study begins by giving various glucose concentrations to dormant yeast spores. From this, we discovered that not all yeast spores wake-up (germinate) to re-enter a replicative life and that those that do not germinate are primed - they undergo accelerated germinations if they encounter more glucose hours-to-

138 days later. These phenomena, which were previously unnoticed, led us to investigate why only a
139 fraction of genetically identical, dormant yeast spores in a population germinate despite having
140 ample glucose. To investigate this, we used a synthetic circuit to induce, in dormant yeast spores,
141 expression of a gene (*GFP*) that plays no role in germination. We found that one can induce GFP
142 expression in dormant yeast spores that are in water without any nutrients (no extracellular amino
143 acids and glucose) and that, surprisingly, the inducible GFP level in a dormant spore tells us the
144 probability of that spore germinating for a given concentration of glucose. Strikingly, we found that
145 dormant yeast spores, when induced, can express genes at levels that are as high as those of
146 vegetative yeasts. To our knowledge, we provide here one of the first evidences and
147 quantifications of active transcription in intact, dormant yeast spores at a single-cell resolution.
148 We found that the inducible GFP level in a dormant yeast spore is strongly and positively
149 correlated with the amounts of all three RNA polymerases - RNAP I-III - that the spore has but is
150 virtually uncorrelated with the amount of ribosomal RNA, which is a proxy for the amount of
151 ribosomes in spores. Having identified these links, our paper proceeds to show how key quantities
152 - gene-expression ability, amount of RNA polymerases, and ability to germinate - decrease over
153 time during dormancy, up until death. This led us to quantitatively determine when dormancy ends
154 (death occurs) for a yeast spore. Putting the results together, our paper ends by formulating a
155 new conceptual model that explains how yeast spores gradually lose dormancy and die: key
156 molecules that are required for gene expression, including RNA polymerases, are gradually lost
157 during dormancy because spores cannot produce them faster than they degrade - we measured
158 firm numbers (tens of days) for these extremely slow processes. These molecules eventually
159 reach below some threshold amounts after which the spore has permanently lost the ability to
160 express genes, meaning that even ample glucose cannot restore the gene-expressing ability.
161 Since, after receiving glucose, spores must express genes in order to build a new cell that will
162 bud off the spore (i.e., germinate), the permanent loss of gene expressing ability at the final
163 moments of dormancy marks the spore's death.
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166 **RESULTS**
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168 **Not all genetically identical spores germinate despite encountering ample glucose**
169 We began by re-examining the conventional test for determining whether a yeast spore is dormant
170 or dead. The test involves giving ample glucose to yeast spores and then observing whether they
171 germinate or not (Brengues *et al,* 2002; Joseph-Strauss *et al,* 2007) (Fig. 1A).

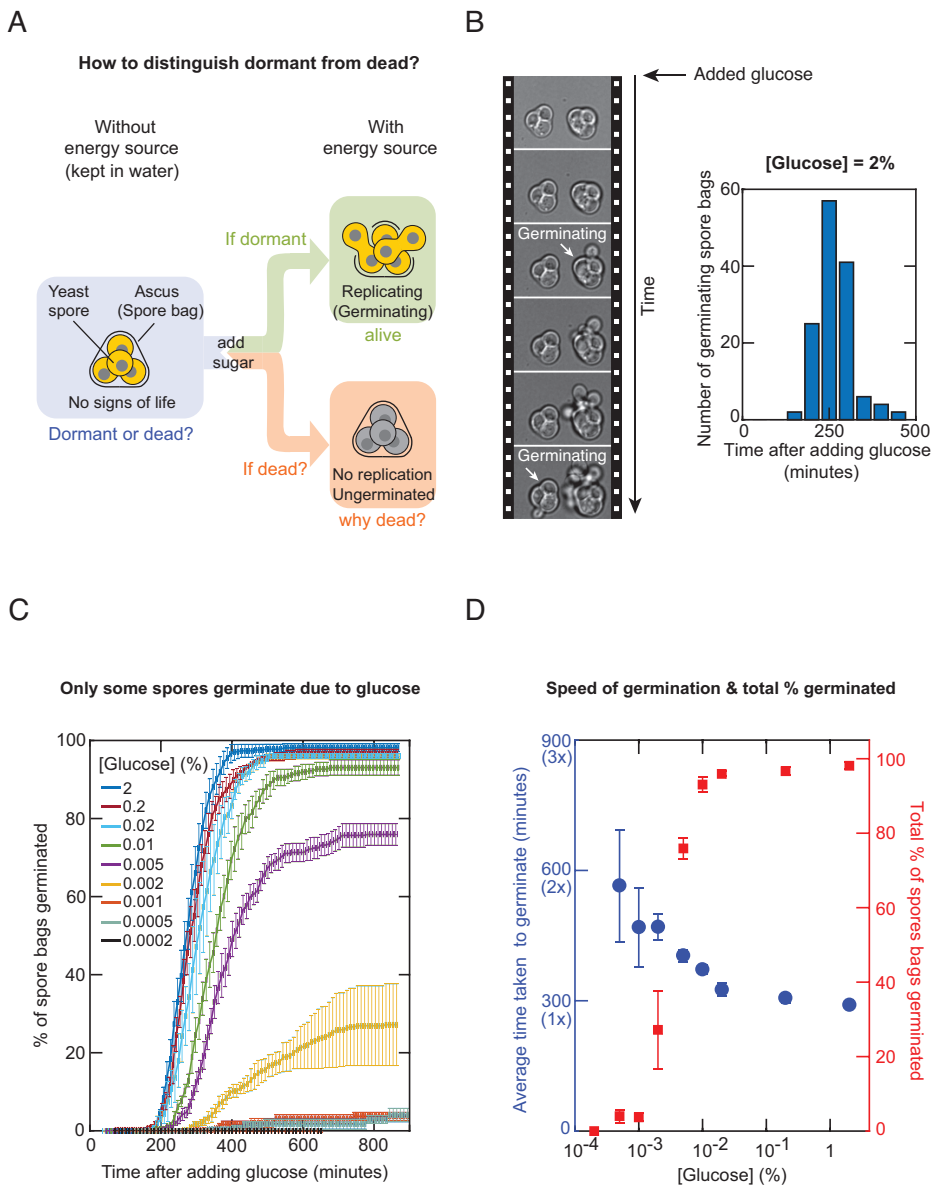

Figure 1 - Glucose germinates only a fraction of yeast spores.
(A) Conventional test to determine whether a spore bag (i.e., ascus) containing four haploid spores, in the absence of any external nutrients (blue box), is dormant or dead. Green box: outcome if dormant. Red box: outcome if dead. **(B)** Left: Filmstrip of a time-lapse movie in which a 2%-glucose was added at the beginning of the movie (10 minutes between frames). A spore bag is counted as having germinated at the moment that at least one budding cell emerges from the spore bag (white arrows). Right: Time taken by each "wild-type" spore bag to germinate in the time-lapse movie. *n* = 137 spore bags from a representative time-lapse movie. (**C)** Percentage of wild-type spore bags that germinated as a function of time after adding glucose to the minimal medium. Different colors represent different glucose concentrations (from 0.0002% to 2%). *n* = 3; error bars are s.e.m. **(D)** Average time taken to germinate (blue circles) and the total percentage of spore bags that germinated (red squares), both 16 hours (960 minutes) after receiving glucose (i.e., the plateau values for each color in Fig. 1C). Both are functions of glucose concentration. *n* = 3; error bars are s.e.m.

If the spore germinates, then it is considered to have been dormant whereas if it does not, then it is considered to have been dead. This test, however, does not reveal why a spore that does not germinate is dead in the first place and when it died. For instance, the test cannot distinguish

175 between a spore that died *while* it was being formed versus a spore that was alive (i.e., dormant)
176 after forming but died *during* its dormancy (and why and when the death occurred). Motivated by
177 this deficiency, we re-examined the test by asking whether all yeast spores can indeed germinate
178 after receiving ample glucose (Fig. 1A). Starving a laboratory-standard ("wild type") homozygous
179 diploid yeasts caused them to form genetically identical, haploid spores. Specifically, each diploid
180 cell formed a single "spore bag" (i.e., ascus) that contained four genetically identical haploid
181 spores (Fig. 1A). We incubated a population of these spore bags in a "minimal medium" - this has
182 all the essential amino acids but no glucose - and, as a modification to the conventional test,
183 supplemented the minimal medium with a less-than-saturating concentration of glucose (less than
184 2%) instead of the usual, 2%-glucose. We used a wide range of glucose concentrations that
185 spanned a 10,000-fold range, from 0.0002% to 2%. For each glucose concentration, we used a
186 wide-field microscope to track individual spore bags and count how many of them germinated -
187 that is, how many spore bags contained at least one spore that germinated (i.e., replicated) - as
188 a function of time after we added glucose (Fig. 1B and Appendix Fig S1). We focused on spore
189 bags instead of individual spores that are within each spore bag because we sought to assess
190 whether a diploid cell successfully formed at least one spore that germinates. With a saturating
191 glucose concentration (2%), nearly every spore bag in the population germinated (Fig. 1C). But
192 with lower glucose concentrations (i.e., less than ~0.01%), a noticeable percentage of spore bags
193 in the population - about 10% or more - did not germinate regardless of how many hours we
194 waited after adding the glucose (Fig. 1C). The percentage of spore bags that germinated changed
195 in a sharp, step-like (sigmoidal) manner as a function of the glucose concentration (Fig. 1D - red
196 points), with the change in step located at a glucose concentration of ~0.003% (i.e., at this
197 concentration, ~50% of spore bags germinate). In contrast, the average time taken to germinate
198 only weakly depended on the glucose concentration, increasing by at most 2-fold despite a
199 10,000-fold decrease in the glucose concentration from 2% to 0.0002% (Fig. 1D – blue points).
200 This indicates that glucose weakly affects the speed of germination. Importantly, the germinations
201 did not stop because the spores ran out of glucose for any of the glucose concentrations because
202 when we measured how much glucose was left in the minimal medium after all the germinations
203 stopped (i.e., ~600 minutes after adding glucose for all glucose concentrations), we always found
204 a large fraction of the original glucose remaining in the medium: the glucose concentration hardly
205 decreased even for the lowest starting glucose concentrations that we used (e.g., 0.002% and
206 0.001%) (Appendix Fig S2). Moreover, we observed that vegetative, diploid cells of the wild-type
207 strain - the same strain as the spores - could replicate multiple times even with the lowest glucose
208 concentration (i.e., 0.0002%) (Appendix Fig. S3). Hence, even the lowest glucose concentration

was ample enough for a vegetative cell to divide multiple times. These results establish that yeast spores do not necessarily germinate even when there is ample glucose.

Spores that do not germinate after encountering ample glucose are not necessarily dead

To explain why some spores do not germinate even with ample glucose, we considered two possibilities. One was that the spore bags that did not germinate (i.e., "un-germinated spore bags") died while trying to germinate. The other possibility was that the un-germinated spore bags were still able to germinate and thus were still alive (i.e., dormant). We distinguished these two possibilities by repeating the above experiments but now by adding glucose in two steps (Fig. 2A). First, we gave the spores a relatively low concentration of glucose. We then waited, typically ~16 hours (~1000 minutes), after which no more germinations occurred. Then, we added more glucose to increase the total glucose concentration and then observed if this led to any more germinations. We found that some of the spore bags that did not germinate the first time were germinating after receiving more glucose (Fig. 2B and Appendix Figs S4-S5). Yet, some of the spore bags still did not germinate after receiving the second batch of glucose if the resulting, final concentration was not the saturation value, 2% (Fig. 2C - top panel). For example, when the final concentration was 0.002%, nearly 60% of the spore bags in the population remained un-germinated. Intriguingly, this 60% is close to the percentage that would have remained as un-germinated if we had given the 0.002%-glucose all at once instead of in two steps (compare Fig. 2C with Fig. 1C). This suggests that each spore bag may be pre-programmed to germinate for certain glucose concentrations. Accordingly, nearly every spore bag eventually germinated if the second batch of glucose increased the total glucose concentration to 2% (Fig. 2C - bottom panel). These results establish that spores that do not germinate after encountering ample glucose are not necessarily dead.

Spores that do not germinate after encountering low glucose concentrations are primed so that they accelerate their germinations later

To better understand why only some spore bags germinated for a given glucose concentration, we examined whether the un-germinated spore bags had any measurable response to the glucose that they first encountered.

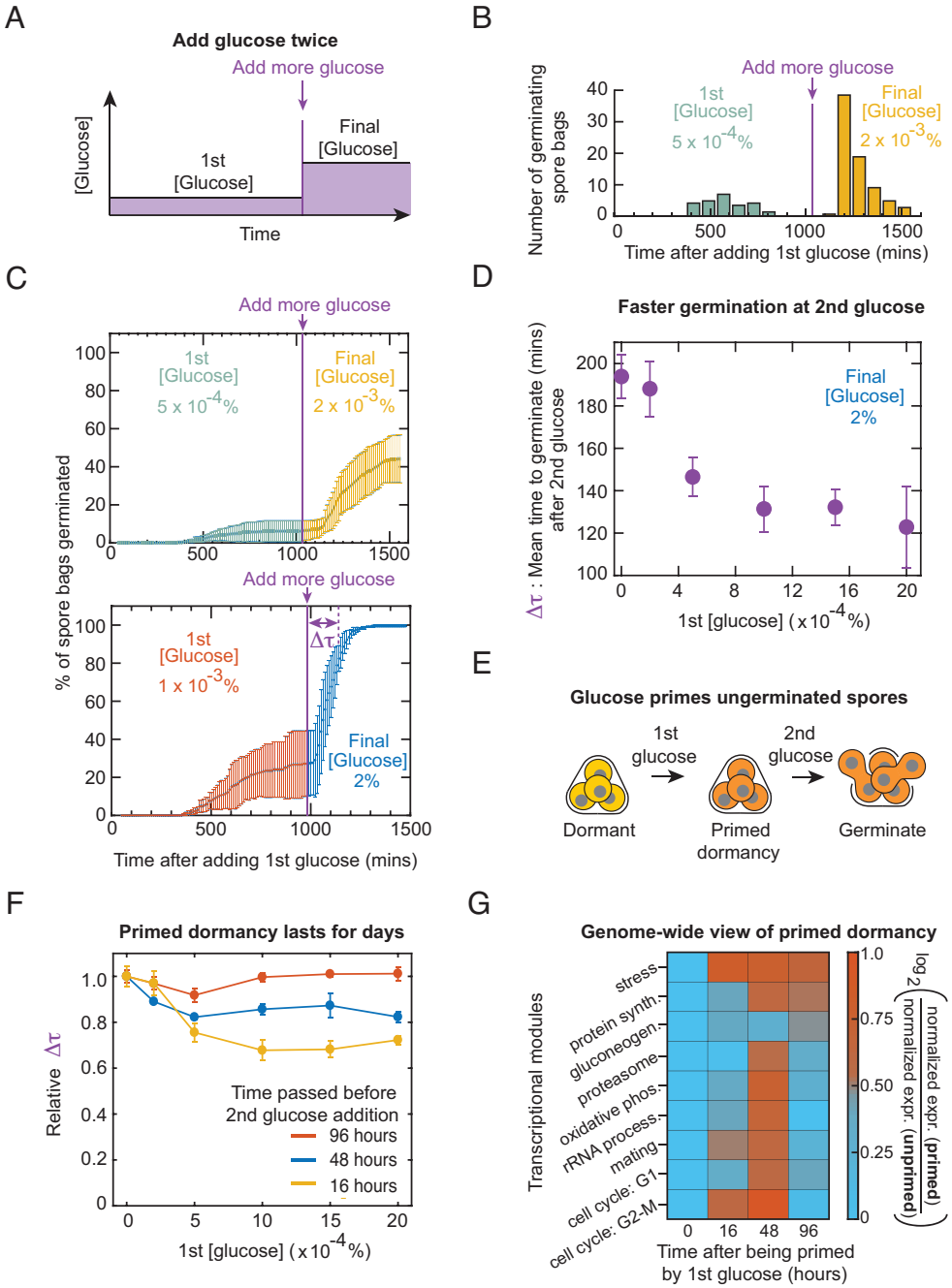

Figure 2 - Un-germinated spores, primed by a low glucose concentration, germinate faster upon encountering more glucose a few days later.
(A) Wild-type spores are first incubated in a low glucose concentration ("1st [glucose]") before we add more glucose later to increase the glucose concentration to "final [glucose]". **(B)** For experiment in (A), number of spore bags germinated within each binned time. First glucose concentration is 0.0005% (from 0 to 16 hours) (green bars) and the final concentration is 0.002% (from 16 to 32 hours) (orange bars). *n* = 143 spore bags (representative data). **(C)** For the experiment in (A), percentage of spore bags that germinated as a function of time since the first glucose addition (1st and final concentrations as indicated). More glucose added 1000 minutes after the first glucose (purple vertical line). *n* = 3; error bars are s.e.m. **(D)** For the experiment in (A), average time taken for a spore bag to germinate - denoted $\Delta\tau$ in bottom panel of (C) - after stepping up the glucose concentration. We varied the first glucose concentration, but the final concentration was always 2%. *n* = 3; error bars are s.e.m. **(E)** Spore bags that do not germinate after encountering the first glucose are "primed" to germinate faster upon encountering more glucose. **(F)** For the experiment in (A), average time ($\Delta\tau$) taken for a spore bag to germinate after stepping up the glucose concentration (final glucose concentration is 2%), plotted as a function of the first, low glucose concentration. Different colors represent different times at which we added more glucose after the first addition: 16 hours (yellow), 48 hours (blue) and 96 hours (red). "Relative $\Delta\tau$" is the average time $\Delta\tau$ divided by the $\Delta\tau$ of spore bags that were incubated in minimal media without any glucose (0%) for the same duration of time (after which they received a 2%-glucose). *n* = 3; error bars are s.e.m.

(G) Heat map showing transcriptome-wide changes in un-germinated spores at 0, 16, 48, and 96 hours after being primed by a 0.002%-glucose (via RNA-Seq). Appendix Table S1 lists the genes for each transcriptional module (each row in the heat map). Also see Appendix Fig S8. For each transcriptional module, we first divided the expression level of each gene in that module by its expression level at 0-hours - this yields "normalized expression level" for that gene for both primed and unprimed spores (unprimed spores were in minimal media without glucose for 0, 16, 48, and 96 hours). We averaged these values over all genes in a transcriptional module to obtain "normalized expression (primed)" and "normalized expression (unprimed)" for each transcriptional module. Colors represent ratio of these two values, averaged over three biological replicates (n = 3).

When we added glucose in two steps so that the final concentration was 2% (Fig. 2A), we found that the spore bags took less time to germinate, in response to the second batch of glucose, than they would have if they had received the entire 2%-glucose all at once (Fig. 2D). Specifically, if a spore bag was in a minimal medium without any glucose for 16 hours and then encountered a 2%-glucose, it needed an average of ~200 minutes to germinate. But this time decreased by about half (i.e., to ~120 minutes) if a spore bag was first in a minimal medium with a low glucose concentration - ranging from 0.0002% to 0.002% - for 16 hours and then received more glucose so that the final concentration was 2% (Fig. 2D). Thus, encountering a very low amount of glucose "primes" some spores so that, upon encountering a saturating level of glucose at a later time, they would germinate faster - up to two times faster on average - compared to spores that did not previously encounter any glucose (Fig. 2E). Furthermore, when we primed the spores with a very low glucose concentration and then waited between 16 hours to 4 days before increasing the glucose concentration to 2%, the spores were still primed - they germinated faster compared to spores that were kept in minimal medium without any glucose for the same amount of time. They were primed for up to two days after we added the first glucose but no longer primed four days after we added the first glucose (Fig. 2F and Appendix Fig S6). Thus, primed dormancy lasts for and decays over days.

Transcriptome-wide view of primed dormancy

Before turning to the question of what causes only some spore bags to germinate for a given glucose concentration, we sought to uncover gene expressions that underlie the primed dormancy. We first primed the spores by incubating them with a low glucose concentration (0.002%) for either 16 hours, 1 day, 2 days, or 4 days. We then used zymolyase, as is the standard (Coluccio *et al,* 2004), to isolate the un-germinated spores from the surrounding vegetative cells (Appendix Fig S7) and then analyzed their transcriptomes with RNA-seq. As a control, we also analyzed the transcriptome of un-primed spores, which were incubated in minimal media without glucose for the same amounts of time as the primed spores. Following an insightful previous study (Joseph-Strauss *et al,* 2007) that analyzed the yeast spores' transcriptome over several hours

271 after they received a 2%-glucose, we grouped multiple genes together into a set, called a
272 "transcriptional module" (Joseph-Strauss *et al,* 2007; Ihmels *et al,* 2002), if those genes
273 participate in the same process (e.g., protein synthesis) (Appendix Table S1). For both the primed
274 and un-primed spores, we averaged the expression levels of all genes in a given module to obtain
275 one expression value for that module. For six of nine transcriptional modules, the primed spores
276 had higher expression values than the un-primed spores after 16 hours and 48 hours of
277 incubations. But both types of spores had nearly the same expression value after four days of
278 incubation (Fig. 2G - last six rows and Appendix Fig S8). This trend mirrors the trend followed by
279 the average time taken by primed spores to germinate (i.e., accelerated germinations up to 48
280 hours after being primed but no accelerated germinations after four days) (Fig. 2F). Two
281 transcriptional modules showed this trend in a particularly pronounced manner. One of them is
282 the module for mating - haploid cells mate after germinating (Fig. 2G - seventh row). The other is
283 the module for transitioning from cell cycle's G2-phase to mitosis - a crucial final step of
284 germination (Fig. 2G - last row and Appendix Fig S8). Intuitively, these results make sense since
285 one expects that getting ready for mitosis and mating would accelerate germinations. Together,
286 these results establish that very low glucose concentrations can trigger transcriptome-wide
287 changes in un-germinated spores to prime them, so that they can germinate faster when they
288 encounter more glucose hours-to-days later.
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291 **Hypothesis on why not every yeast spore germinates with low glucose concentrations**
292 Although we now understand how un-germinated spores respond after receiving glucose, we
293 have not addressed our original question: what determines, in the first place, which spore bags
294 germinate? We hypothesized that each diploid, vegetative yeast forms a spore bag with a distinct
295 "internal state". Many intracellular factors may define an internal state, including amounts of ATPs
296 or ribosomes that are stored inside the spores or combinations of these and other molecules that
297 are stored inside spores. We then hypothesized that, for each glucose concentration, only some
298 of the spore bags have the "right" internal states that enable germination. A difficulty in testing
299 this hypothesis is that in our experiments thus far, we gave glucose to spores *and then* observed
300 their subsequent actions, including for discovering primed dormancy. But in such experiments,
301 due to all measurements occurring *after* the spores receive glucose, we cannot infer the spores'
302 internal states that existed *before* they encountered glucose since glucose likely changes the
303 internal states. Therefore, we sought to manipulate the internal states without giving glucose. In
304 particular, we reasoned that depleting any internal resources (e.g., ATPs or amino acids) that are

305 stored inside spores *before* adding glucose would either decrease - or alter in more complex ways
306 - the percentage of spore bags that germinate for each glucose concentration.
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309 **Synthetic circuit to induce *GFP* in dormant yeast spores without any nutrients**
310 To test our hypothesis, we built a synthetic gene-circuit in vegetative diploid yeasts so that
311 doxycycline - a small inducer molecule that easily diffuses through the yeast's cell wall - would
312 cause the cells to produce the Green Fluorescent Protein (GFP). In vegetative yeasts, this
313 synthetic circuit functions in such a way that increasing the doxycycline concentration increases
314 the GFP expression. We formed spores out of these engineered diploid cells (Fig. 3A). We
315 reasoned that if doxycycline can induce GFP expression in these spores without any nutrients
316 (i.e., in water), then we might deplete the stored resources inside the spores and thereby alter the
317 percentage of spore bags that germinate for a given glucose concentration. But it was unclear
318 whether it was even possible to induce GFP expression or any arbitrary gene in dormant yeast
319 spores without nutrients. For one, if gene induction were possible, then it is unclear why,
320 apparently, almost all gene expressions are suppressed in dormant yeast spores (Rittershaus *et*
321 *al,* 2013) since being able to induce GFP expression would mean that there must be active RNA
322 polymerases and ribosomes, and chromosomal regions that are accessible to them. Moreover,
323 recent studies established that starved vegetative yeasts, dormant yeast spores, and other
324 dormant fungal spores have a solid-like, glassy cytoplasm that is packed with macroscopic
325 aggregates of proteins and mRNPs (mRNAs bound by proteins) that inhibit gene-expression
326 machineries and movement of molecules inside the cytoplasm (Laporte *et al,* 2008; Petrovska *et*
327 *al,* 2014). By aggregating, proteins needed for metabolism and gene expression would be
328 inactivated as well (Ablett *et al,* 1999; Cowan *et al,* 2003; Dijksterhuis *et al,* 2007; Parry *et al,*
329 2014; Joyner *et al,* 2016; Munder *et al,* 2016). Indeed, to our knowledge, there has not yet been
330 a direct, single-cell-level observation of gene expression occurring in yeast spores during
331 dormancy. A previous, bulk-level (population-level) study (Brengues *et al,* 2002) has shown, from
332 lysates of many yeast spores, that two genes - *PGK1* (involved in gluconeogenesis) and *SPS100*
333 (involved in forming spore walls during spore formation) - are expressed but that they turn off a
334 few days after sporulation (i.e., while the spores enter dormancy). But it remains unclear whether
335 their expressions completely cease or remain at low, non-zero levels during dormancy. This is
336 because these bulk-level measurements were based on finding ribosomes bound to mRNAs after
337 lysing populations of yeast spores, meaning that the measurements cannot clearly distinguish
338 between zero and very low expression levels.

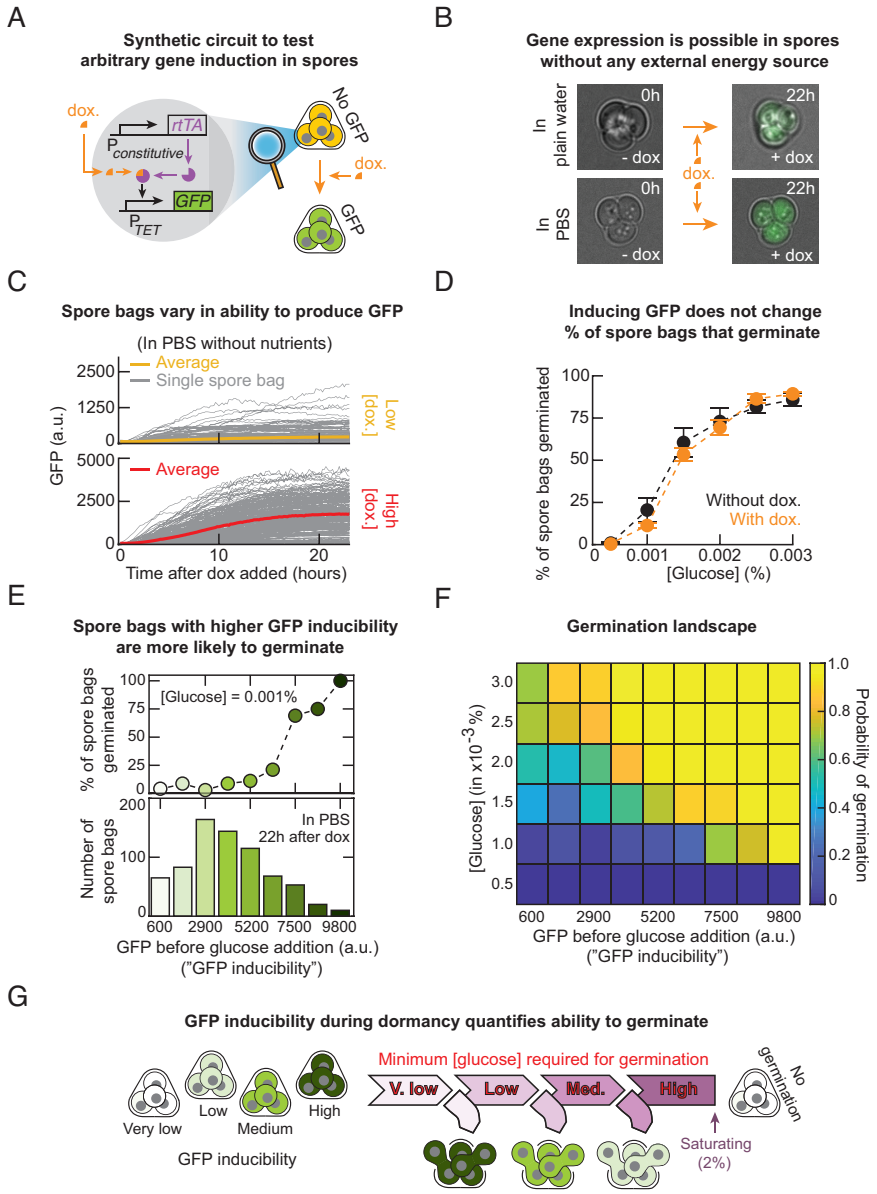

Figure 3 - Inducing GFP expression during dormancy with synthetic circuit shows that spores that can realize higher GFP level (higher GFP inducibility) are more likely to germinate (higher germination ability) for any glucose concentration.
(A) Synthetic gene-circuit that constitutively expresses a transcription factor, rtTA (with ADH1-promoter) and an inducible promoter (TET-promoter) controlling *GFP* expression. Increasing doxycycline increases GFP expression. **(B)** Engineered spore bags (shown in (A)) transcribe and translate GFP in water (without any nutrients) with 25 μg/ml of doxycycline (top row) and in a saline solution (PBS) with 50 μg/ml of doxycycline (bottom row). Snapshots of GFP expression shown 22 hours after adding doxycycline**. (C)** GFP levels of individual spore bags (grey curves) over time (measured every 10 minutes with a wide-field epifluorescence microscope) after incubation in PBS with 10 μg/ml of doxycycline (top panel: n = 104 spore bags) and 100 μg/ml of doxycycline (bottom panel: n = 150 spore bags). **(D)** Engineered spore bags (shown in (A)) were first incubated for 22 hours in either PBS without any doxycycline or with 100 μg/ml of doxycycline. Next, we transferred them to minimal medium with various glucose concentrations. Plot shows the total percentage of the engineered spore bags that germinated (measured 20 hours after incubating with glucose) for spores pre-incubated in PBS without doxycycline (black points) and in PBS with 100 μg/ml of doxycycline (orange points). n = 3; error bars are s.e.m. **(E)** Top: Percentage of spore bags with the same GFP level ("GFP inducibility") from (D) that germinated after receiving a 0.001%-glucose. Percentages are averaged over all spore bags with the same binned GFP level (in the histogram below). Representative data shown (n = 145 spore bags in a population). **(F)** Germination landscape: colors represent the probability that a spore bag with a particular steady-state GFP level ("GFP inducibility") germinates for each glucose concentration (i.e., data in top panel of (E) represents a single row of this heat map). To measure each pixel, as in the experiment described in (D), we incubated spore bags in PBS with 100 μg/ml of doxycycline for 22 hours before adding glucose ([glucose] as indicated along the rows). Columns indicate GFP inducibility of a spore bag, measured at 22 hours after adding the doxycycline. Each pixel is an average over 3 replicate populations (n = 3). **(G)** Given a spore bag, its GFP inducibility is a read-out of the probability to

germinate ("germination ability") for each glucose concentration and the minimal glucose concentration required to guarantee germination. Spore bags with lesser gene-expressing abilities without nutrients require more glucose to germinate.

Moreover, the ribosomes bound to mRNAs may be from the macroscopic aggregates that formed before the spores entered dormancy (Laporte *et al,* 2008; Petrovska *et al,* 2014), which may disable translation of those ribosome-bound mRNAs (Ablett *et al,* 1999; Cowan *et al,* 2003; Dijksterhuis *et al,* 2007; Parry *et al,* 2014; Joyner *et al,* 2016; Munder *et al,* 2016). Adding to the ambiguity is the fact that a bulk-level study revealed a depletion of a minute fraction of radioactive uracil and methionine from an extracellular medium by a dense population of yeast spores (Brengues *et al,* 2002). This finding indicates that transcription (proxied by the depleted uracil) and translation (proxied by the depleted methionine) may be occurring in dormant yeast spores, though these are indirect measurements since they did not directly visualize gene-expression dynamics inside individual, intact spores. Time-lapse imaging of gene expression in individual yeast spores - as we will show with our synthetic circuit - would help in resolving these ambiguities and subtleties of gene regulations in dormant yeast spores.

Before testing the hypothesis posed in the previous section, we investigated whether GFP induction in dormant spores is even possible by incubating the engineered spores with doxycycline in either water or saline solution (PBS). Surprisingly, we discovered that doxycycline fully induced GFP expression in these spores (Fig. 3B and Appendix Figs S9-S10). Varying the doxycycline concentration tuned the spores' GFP levels over the same, wide range of values as in vegetative cells that also have the synthetic circuit (Appendix Fig. S9-S11). We found that both the rate of GFP production and the final (steady state) level of GFP widely varied among spore bags of the same population (Fig. 3C and Appendix Fig S12). The most striking feature was that all spore bags expressed GFP very slowly – GFP levels plateaued at steady-state values after ~20 hours of doxycycline whereas the vegetative yeasts needed ~8 hours of doxycycline for the GFP level to plateau (Appendix Fig S11). But a more puzzling discovery was that the spores' GFP levels stabilized at steady-state values in the first place. This is puzzling because the spores were not dividing and hence their GFP - a highly stable protein - could not be diluted away by cell divisions. In replicating (vegetative) yeasts, highly stable proteins such as GFP reach steady-state levels because their production rate matches the cell-division rate. After the spores' GFP levels reached steady-state values, we incubated the spores in PBS without doxycycline for two days during which their GFP levels remained virtually unchanged (Appendix Fig S13). This means that GFP levels reached steady-state values in dormant spores because the spores stopped

371 producing GFP after a day, despite the saturating concentration of doxycycline (100 μg/ml) still
372 being present in the medium after a day, with barely detectable degradations (Appendix Fig S14).
373 Together, these results provide a direct proof, with intact spores, that one can fully activate - to
374 the same level as in vegetative yeasts - transcription and translation of an arbitrary gene such as
375 *GFP* in dormant yeast spores without any nutrients. These results also establish that gene
376 expression in dormant yeast spores can exhibit starkly different dynamics when compared to
377 vegetative yeasts, such as vastly slower timescales and gene regulations.
378
379
380 **Inducing GFP expression in dormant yeast spores does not alter percentages of spore**
381 **bags that germinate**
382 Returning to our hypothesis that depleting yeast spores' internal resources through GFP induction
383 hinders germination, we incubated spores in PBS with a saturating concentration of doxycycline
384 (100 μg/ml) for 24 hours, after which the GFP levels plateaued at steady-state values (Fig. 3C
385 and Appendix Fig S13). We then washed away the doxycycline and then incubated the spores in
386 a minimal medium with a fixed concentration of glucose. Then, with time-lapse microscopy, we
387 tracked individual spore bags to determine how many spore bags germinated. For comparison,
388 we used the same method to determine how many spore bags without GFP induction germinated
389 - these spores were incubated for 24 hours in PBS without doxycycline and then in a minimal
390 medium with the same concentration of glucose as the GFP-induced spores (Fig. 3D and
391 Appendix Fig S15). For every glucose concentration that we examined, we found that inducing
392 GFP expression did not appreciably alter the percentage of spore bags that germinated and that
393 it also did not appreciably alter the average times taken by the spores to germinate (Appendix Fig
394 S16). Thus, our hypothesis is incorrect: expressing *GFP* does not alter the spores' ability to
395 germinate. While it may now appear that we are "back to square one" - since we still have not yet
396 uncovered what causes only some of the spore bags to germinate for a given glucose
397 concentration - we discovered the answer, as we explain in the next section, when we examined
398 the GFP levels of individual spore bags rather than the average GFP level of the entire spore
399 population as we have just done.
400
401
402 **GFP level that a spore can realize (GFP inducibility) encodes the probability of germinating**
403 **(germination ability) for each glucose concentration**
404 In the previous experiment, we measured the steady-state GFP levels just before the spores

encountered glucose. This led to the finding that spore bags that produced more GFP - spore bags with higher "GFP inducibility" - were more likely to germinate (Fig. 3E and Appendix Figs S17-S18). For example, after encountering a 0.001%-glucose, nearly 100% of the spore bags with the highest, steady-state GFP levels (highest GFP inducibility) germinated whereas, in the same population, only ~10% of the spore bags with half the GFP inducibility germinated. In fact, for every glucose concentration, we could precisely determine the probability of germinating for a spore bag once we knew its GFP inducibility (Appendix Fig S18). We thereby established a quantitative relationship between the inducible GFP level and the ability to germinate. Importantly, this means that just because a spore bag can express any amount of GFP without nutrients does not mean that it will germinate after receiving a certain amount of glucose. There is no binary relationship between the GFP expression and germination. The quantitative relationship between the GFP inducibility and the probability of germinating ("germination ability") for a given glucose concentration establishes that the stochastic variability (Padovan-Merhar & Raj, 2013; Balazsi *et al,* 2011) in the induced GFP expression among dormant spores is meaningful and predictive, despite GFP not having any functional role in the complex, multi-step process that is germination.

Germination landscape visually represents germination ability as a function of glucose concentration and GFP inducibility

To visualize our results, we plotted a “germination landscape” - a heat map whose color represents a probability that a spore bag with a given GFP inducibility germinates (i.e., germination ability) for each glucose concentration (Fig. 3F and Appendix Fig S19). In the germination landscape, yellow represents a near-certain germination (i.e., germination probability of nearly 1), green represents a germination probability of ~0.5, and dark blue represents a germination probability of nearly zero. The germination landscape shows a "coastline" of nearly-yellow pixels moving up towards higher rows (i.e., towards higher glucose concentrations) as one moves from right to left (i.e., as the GFP inducibility decreases), meaning that more glucose is required to guarantee a germination for a spore bag with a lesser GFP inducibility. The blue-green pixels are almost immediately below the coastline of yellow pixels, indicating that the probability of germinating, for a fixed value of GFP inducibility, is a sharp step-like function of the glucose concentration. We confirmed this by quantitatively extracting (by log-regression), from the germination landscape, the minimum glucose-concentration required for a spore with a given value of GFP inducibility to have a 99%-chance of germinating (Appendix Fig S20). We call this concentration, given the sharpness of the nearly step-like probability function, the "minimum

439 glucose concentration required for germination". We determined that as a spore bag's GFP
440 inducibility decreases, the minimum glucose concentration required for germination increases
441 (Fig 3G and Appendix Fig S20). Importantly, since inducing GFP expression does not alter the
442 total percentage of spore bags that germinate for any glucose concentration (Fig. 3D), inducing
443 GFP expression in a spore bag does not change - neither increase nor decrease - the probability
444 of germinating. Hence, GFP inducibility merely indicates which spore bags are more likely to
445 germinate for a given glucose concentration but does not cause or hinder germination. Hence, for
446 the same glucose concentration, spores with higher GFP inducibility have higher germination
447 ability than spores with lesser GFP inducibility. Moreover, spores with a higher GFP inducibility
448 require less glucose to guarantee that they germinate (i.e., have 100% chance of germinating)
449 than spores with lesser GFP inducibility (Fig. 3G and Appendix Fig S20C).
450
451
452 **Transcribing within a day during dormancy is crucial for spores to remain alive**
453 GFP has no role in germination. So, it is puzzling why GFP inducibility so precisely predicts the
454 germination ability for each glucose concentration. To make sense of this, we reasoned that the
455 GFP inducibility represents the dormant spore's ability to express genes in general, not just *GFP*,
456 when they are forced to do so without any nutrients (e.g., inducers such as doxycycline force an
457 activation of GFP expression). This is because there is nothing special about GFP.
458 Notwithstanding the conventional view that gene expression has nearly ceased in dormant yeast
459 spores, we then reasoned that GFP inducibility is a proxy for gene expressions that are actually
460 occurring in dormant spores and which are required for germination. According to this reasoning,
461 inhibiting global (genome-wide) transcription and translation during dormancy would decrease the
462 percentages of spore bags that germinate (i.e., decrease germination ability) for each glucose
463 concentration. To test this idea, we incubated spores in PBS for one day with one of three drugs
464 - thiolutin, cycloheximide, and antimycin A (Fig. 4A). Each drug inhibits some key part of gene
465 expression. Thiolutin globally inhibits transcription (Grigull *et al,* 2004; Parker *et al,* 1991; Guan
466 *et al,* 2006; Lauinger *et al,* 2017; Pelechano & Perez-Ortin; 2008). Cycloheximide globally inhibits
467 translation (Belle *et al,* 2006; Buchanan *et al,* 2016). Antimycin A inhibits oxidative
468 phosphorylation and, in turn, synthesis of ATP - a molecule that is required for expressing *GFP*
469 and other genes (Ocampo *et al,* 2012). After the day-long drug treatment, we washed away the
470 drug and then added a 2%-glucose with minimal medium to check how many spores could still
471 germinate (i.e., were still alive) (Fig. 4A).

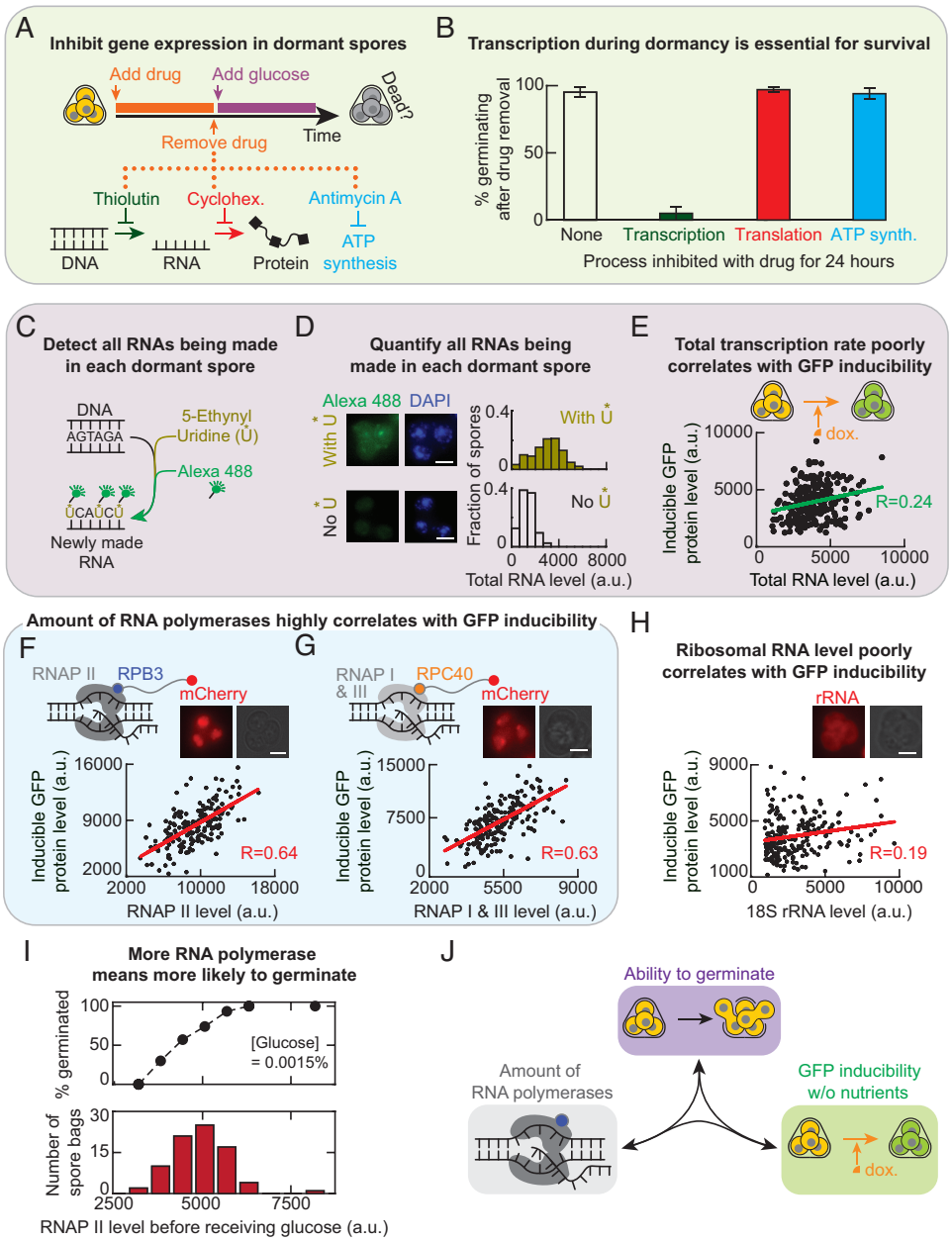

Figure 4 - More RNA polymerases I-III means higher germination ability and GFP inducibility
(A) Protocol for (B). See "Protocol for Fig. 4B" in Methods. Thiolutin inhibits transcription. Cycloheximide inhibits translation. Antimycin A inhibits ATP synthesis by inhibiting oxidative phosphorylation. **(B)** For experiment in (A), percentage of spore bags that germinated. *n*=3; error bar are s.e.m. **(C)** Method to detect all RNAs being made in yeast spores with a single-cell resolution. Spores were incubated for 24 hours in PBS with 1 mM of 5-Ethynyl Uridine (5-EU; denoted U*) that incorporates into freshly made RNAs. We fix the spores afterwards and let fluorophore (Alexa 488) enter the spores and bind U* (see Methods). "Total RNA level" of a spore bag is the total fluorescence from all the 5-EU labeled RNAs. **(D)** Microscope images show a spore bag (from strain "TS3") after either incorporating 5-EU as described in (C) (top two images) or, as a control, following the protocol in (C) but without the 5-EU (bottom two images) (also see Appendix Fig S23A). Scale bar: 2 μm. Top histogram: total RNA level per spore bag with 5-EU (i.e., Alexa 488 fluorescence; *n* = 103 spore bags). Bottom histogram: fluorescence per spore bag in control population (i.e., without 5-EU; *n* = 95 spore bags). Also see Appendix Fig. S22A. **(E)** Each dot is from a single spore bag ("TS3" strain) with GFP-inducing circuit (Fig. 3A). For each spore bag, we measured its GFP protein level ("GFP inducibility") and total RNA level (5-EU fluorescence) after incubating the spores for 24 hours in PBS with both 100-μg/ml doxycycline and 1 mM of 5-EU (see (C)). *n* = 245 spore bags. Alexa 594 fluorophore attached to 5-EU (see Methods). Green line: linear regression with R=0.24 and Pearson p-value = 0.00018. **(F)** See "Protocol for Fig. 4F" in Methods. GFP inducibility per spore bag (each dot, "TS8" strain) measured as in (E) but now with live time-lapse without the 5-EU. "RNAP II level" is the mCherry fluorescence per spore bag due to the mCherry protein fused to Rpb3, a subunit of RNA polymerase II. *n* = 182 spore bags; Red line: linear regression with R=0.64 and Pearson p-value = 3.02 x 10^{-22}. Scale bar = 2 μm. **(G)** Same protocol as in (F) but with "TS9" spores. "RNAP I & III level" is the mCherry fluorescence

per spore bag due to the mCherry protein fused to Rpc40, a subunit of both RNAP I and RNAP III. *n* = 185 spore bags; Red line: linear regression with R=0.63 and Pearson p-value = 6.6 x 10^{-22}. Scale bar = 2 μm. **(H)** Same protocol as in (F) but with "TT14" spores fixed after 24 hours of incubation in PBS with 100-μg/ml doxycycline. 18s rRNA level is from CAL Fluor Red 610 fluorescence emitted by single-molecule FISH probes bound to 18S rRNAs (see Methods). *n* = 213 spore bags; Red line: linear regression with R = 0.19 and Pearson p-value = 0.005. **(I)** Bottom: RNAP II levels of spore bags ("TS8" strain) in a population, measured as in (F). Top: As a function of the RNAP II level (binned in the histogram), percentage of spore bags that germinated after receiving a 0.0015%-glucose, averaged over all spore bags with the same binned RNAP II level. *n* = 80 spore bags. **(J)** Triangular relationship. Any pair of the following three are positively correlated: Germination ability for each glucose concentration (purple), GFP inducibility (green), and amounts of RNA polymerases I-III.

By convention, being a dead spore means that it cannot germinate even after receiving a 2%-glucose. This definition of death is sensible in light of the germination landscape (Fig. 3F): since spores are more likely to germinate with a higher glucose concentration than with a lower glucose concentration, a dead spore is one that has no chance of germinating with the saturating concentration of glucose and, thus, no chance of germinating for lesser glucose concentration either. Hence measuring the percentage of spore bags that do not germinate after receiving a 2%-glucose tells us the percentage of spore bags that contain only dead spores.

We verified that all three drugs indeed inhibited GFP expression in yeast spores (Appendix Fig. S21). Nearly 100% of the spores were still alive after we inhibited either ATP synthesis with antimycin A or global translation with cycloheximide for 24 hours (Fig. 4B). In contrast, inhibiting global transcription with thiolutin for 24 hours killed almost all spores: less than 5% of the spore bags were still able to germinate with a 2%-glucose after the thiolutin treatment (Fig. 4B). These results establish that transcription - and potentially RNA metabolism in general - is the major process that keeps dormant spores alive.

Method devised for detecting RNA synthesis in dormant yeast spores at single-cell level

To further explore how transcribing during dormancy is connected to germination ability - and thus to survival - for a given glucose concentration, we first developed a method to measure all the RNA that is actively being made in dormant yeast spores with a single-cell resolution. We modified an existing method for mammalian cells in which the cells uptake 5-Ethynyl Uridine (5-EU) and incorporates it into RNA that is being synthesized, after which the cells are fixed and fluorophores (e.g., Alexa 488) enter the fixed cells to bind the 5-EU-labeled RNAs (Jao & Salic, 2008) (Fig. 4C). Since 5-EU would only be found in RNAs that are synthesized while the extracellular 5-EU was present, this method lets us determine the total amount of RNAs that are made during a specified time window (i.e., only while the 5-EU was present). The method therefore distinguishes

500 these freshly made RNAs from the RNAs that were present before we added the 5-EU to the cell
501 culture medium. By adapting this method for yeast, we could visually verify, by fluorescence, that
502 yeast spores actively transcribe during dormancy and determine the amounts of RNA produced
503 by the dormant spores, relative to one another, during a 24-hour incubation in 5-EU (Fig. 4D). To
504 our knowledge, this is one of the first demonstrations of active transcription in dormant yeast
505 spores with a single-cell resolution, as previous studies used either indirect or bulk assays that
506 required lyses of many spores (Brengues *et al,* 2002). We used several methods to verify that our
507 method worked in yeast spores (Appendix Figs S22-S23).
508
509
510 **Total transcription rate in dormant spores is virtually uncorrelated with germination ability**
511 To apply the adapted method to the GFP-inducible spores, we incubated the spores in PBS with
512 both doxycycline and 5-EU for 24 hours. Hence, the total RNA level accumulated in a day is a
513 proxy for the total transcription rate of a dormant spore. We found a statistically significant but
514 weak, positive correlation (R=0.24) between the spores' total transcription rate (i.e., accumulated
515 RNA level) and their GFP inducibility (Fig. 4E). This, in turn, means that the total transcription rate
516 poorly correlates with the spore's chance of germinating. Thus, the total transcription rate cannot
517 explain why the GFP inducibility so precisely determines the germination ability for each glucose
518 concentration. Yet, the fact that a 24-hour exposure to thiolutin kills nearly all spores (Fig. 4B)
519 suggests that some aspect of transcription during dormancy - other than the total amount of
520 transcription - should strongly correlate with the GFP inducibility and, in turn, with the germination
521 ability for each glucose concentration. Since RNA polymerases I, II, and III are required and
522 central machineries for transcription, we next sought to address whether the amount of each RNA
523 polymerase in a dormant spore strongly correlates with its GFP inducibility.
524
525
526 **Amount of RNA polymerases I-III are strong predictors of GFP inducibility and germination**
527 **ability**
528 To measure the amounts of RNA polymerase (RNAP) I, II, and III in individual spores through live
529 time-lapse microscopy, we genetically engineered two yeast strains. One strain produced Rpb3 -
530 a subunit of RNAP II - as a protein fused to the fluorescent protein, mCherry (Fig. 4F). The other
531 strain produced Rpc40 - a subunit of both RNAP I and RNAP III - as a protein fused to mCherry
532 (Fig. 4G). Both strains also have the GFP-inducing synthetic circuit (Fig. 3A). For these strains,
533 the mCherry fluorescence was entirely confined to the nuclei of both spores and vegetative yeasts

534 (Appendix Fig S24). Hence, the mCherry levels accurately reflected the amounts of each RNAPs
535 in spores. After sporulating these two strains, we incubated the resulting spores in PBS with
536 doxycycline for 24 hours to induce their *GFP*. We then used a microscope to measure both the
537 GFP and mCherry (RNAP) levels in individual spore bags. In one of the strains, mCherry level
538 represents the level of RNAP II, which transcribes all the coding genes and also produces some
539 non-coding RNAs. In the other strain, mCherry level represents the amount of RNAP I and RNAP
540 III combined, both of which produce only non-coding RNAs such as ribosomal RNAs. Strikingly,
541 the amount of RNAP II strongly (R=0.64) and positively correlated with the GFP inducibility (Fig.
542 4F). The combined amount of RNAPs I and III also strongly (R=0.63) and positively correlated
543 with GFP inducibility (Fig. 4G). Hence the amounts of both classes of RNA polymerases - one for
544 transcribing mostly coding genes (RNAP II) and one for transcribing non-coding genes (RNAPs I
545 and III) - are strong predictors of GFP inducibility. We also used single-molecule RNA FISH (Youk
546 *et al,* 2010; Raj *et al,* 2008) to quantify the amount of 18S ribosomal RNA (18S rRNA) - a ribosomal
547 subunit - in dormant spores that we fixed after the 24 hours of doxycycline. We then determined
548 how the amount of 18S rRNA in spores correlated with the GFP inducibility. We found that the
549 amount of 18S rRNA in spores barely correlated with their GFP inducibility (R=0.19). Since the
550 amount of 18S rRNA is a proxy for the amount of ribosomes, this result suggests that the amounts
551 of ribosomes in a dormant yeast spore is virtually uncorrelated - and at most weakly correlated -
552 with GFP inducibility and thus with germination ability. This is consistent with transcription, rather
553 than translation, being the dominant process for surviving dormancy (Fig. 4B).
554
555 Since GFP inducibility strongly and positively correlates with the amounts of all three
556 RNAPs and germination ability strongly and positively correlates with GFP inducibility, we would
557 expect that the amount of RNAPs in a dormant spore should also strongly and positively correlate
558 with the spore's germination ability as well. To verify this for RNAP II - we will focus on RNAP II
559 from now on instead of RNAPs I and III because RNAP II transcribes all coding genes such as
560 *GFP* as well as some non-coding genes - we took the spores with Rpb3 fused to mCherry and
561 then incubated them in a low concentration of glucose (0.0015%). Then, using the same method
562 that we used for measuring the germination landscape (Fig. 3E), we determined how likely it is
563 that a spore bag with a given amount of RNAP II germinates after receiving the 0.0015%-glucose.
564 We found that spore bags with more RNAP II indeed were more likely to germinate (Fig. 4I).
565
566 Taken together, the results so far establish a triangular relationship in which a strong,
567 positive correlation exists between any two of the following three quantities in dormant yeast

568 spores (Fig. 4J): GFP inducibility without nutrients, amount of RNAPs (RNAP II level or level of
569 RNAPs I and III combined), and the germination ability for each glucose concentration.
570
571
572 **Dormant spores gradually lose GFP inducibility as they age**
573 With the triangular relationship, we now have a molecular view - through RNAP II level - and a
574 functional view - through GFP inducibility - for studying how dormant yeast spores age, which is
575 what we originally set out to study. To age the dormant spores, we incubated the wild-type and
576 GFP-inducible spores in either water or minimal medium without any glucose for days or weeks
577 at 30 ^{O}C. In this "ageing experiment", we found that the number of dormant (i.e., alive) spores -
578 the ones that we could germinate when we gave them a 2%-glucose - decreased by similar rates
579 over several weeks regardless of the strain type and regardless of whether we kept the spores in
580 water or minimal medium (Fig. 5A). Specifically, about half of the spores in the population died
581 after ~30 days without glucose and almost everyone died after ~60 days without glucose (Fig.
582 5A). Furthermore, we observed that spores needed more time to germinate if we kept them longer
583 without nutrients (Appendix Fig S25), suggesting that germinating is becoming more difficult as
584 the dormant spores age.
585
586 To measure the GFP inducibility in these ageing spores, we took some of the spores from
587 the ageing population, incubated them with doxycycline in PBS for 24 hours (Appendix Fig S26),
588 measured the resulting GFP levels in these spore bags with a microscope (Fig. 5B and Appendix
589 Fig S27), and thus determined how the GFP inducibility changed over the 80 days in PBS without
590 nutrients (Appendix Fig S27). Since we also observed dead spores in the ageing population, we
591 could also measure the dead spores' GFP inducibility with doxycycline on different days. The GFP
592 inducibility of dead spores remained at near zero (near background fluorescence value)
593 regardless of when the spores had died (Fig. 5B - grey points). The GFP inducibility of dormant
594 spores, when averaged over every dormant spore bag, gradually decreased and approached the
595 near-zero GFP inducibility of the dead spores that were in the same ageing population over tens
596 of days (Fig. 5B - blue points). The dormant spores' GFP inducibility eventually reached a barely
597 detectable level - matching the dead spores' GFP inducibility - after ~80 days. By this time, nearly
598 all spores had died (Appendix Fig S27). Together, these results show that dormant spores indeed
599 lose their GFP inducibility over time as they age and approach their deaths. Crucially, in
600 accordance with our revised hypothesis, we did not find any dead spores with a detectable GFP
601 inducibility. Hence dormant yeast spores lose their GFP inducibility *before* dying.

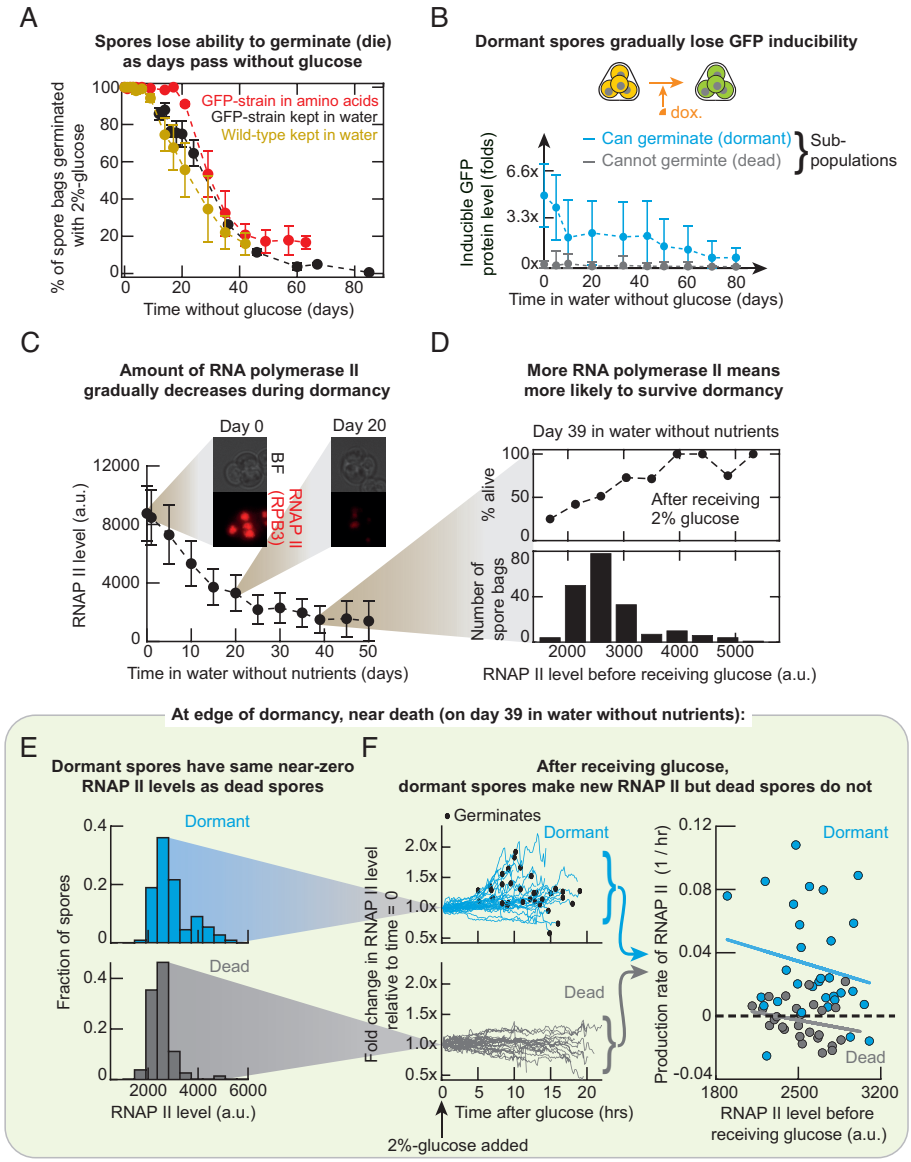

Figure 5 - Dormant spores gradually have less RNA polymerase II and GFP inducibility as they age, eventually losing all gene expressing ability just before dying.

(A) Percentage of wild-type and GFP-inducible spore-bags (Fig. 3A) that germinate due to a saturating glucose concentration (2%) that they encounter after ageing for 0 - 85 days in water (grey and black) or minimal medium with essential amino acids (red) at 30 ^{O}C. *n* = 3; error bars are s.e.m. **(B)** Mean GFP inducibility of dormant (blue) and dead (grey) spore bags that were incubated in water without nutrients at 30 ^{O}C over ~80 days (also see Appendix Figs S27-S28). Blue data points are the average GFP-levels from the histograms shown in Appendix Fig S27. Grey data points are averages of the dead spore bags' histograms shown in Appendix Fig S27. *n*=3, error bars show the average standard deviation from three biological replicates. *n* =3. **(C)** Average RNAP II level of spore bags incubated in water for 50 days at 30 ^{O}C. *n*=3, error bars represent the average standard deviation in a population, each population having at least 100 spore bags (see Appendix Figs S28-S29). **(D)** Top: Percentage of spore bags, having the same RNAP II level on 39th day of ageing in water (as in (A)), germinating after receiving a 2%-glucose. Percentages are averaged over all spore bags with the same binned RNAP II level (corresponding histogram shown below). *n* = *193* spore bags. **(E)** RNAP II levels of dormant (alive) spore bags in blue and of dead spores in grey; both from the same population on 39th day of ageing in water (as in (A)) before any glucose added. *n* = 111 spore bags (blue); *n* = 82 spore bags (grey). **(F)** Corresponds to histograms in (E). Blue is for dormant and grey is for dead spore bags. Line traces show, after receiving a 2%-glucose, the fold change in each spore bag's RNAP II level relative to the spore bag's initial RNAP II level (i.e., value just before we added the 2%-glucose). Black dots represent moments of germination. *n* = 38 for blue and *n* = 32 for grey. Data points in the rightmost plot show the RNAP II production rate in each spore bag profiled in the line traces. For each spore bag, we fitted an exponential function to its RNAP II level over time (one of the line traces). Average production rate of RNAP II in dormant spore bags (average over all blue points) is a positive value, 0.031 / hour and in dead spore bag (average over all grey points) is a negative value, -0.0030 / hour. For a t-test on the difference between the two values: p-value = 1.4 x 10^{-5}. Linear regression line for dormant (blue) is R = -0.19 and for dead (grey) is R = -0.23.

603 Moreover, the fact that the oldest dormant spores have barely detectable GFP inducibility, just
604 like the dead spores, suggest that GFP inducibility is nearly all lost moments before death.
605
606
607 **Dormant spores gradually have less copies of RNA polymerase II as they age**
608 To further validate that dormant spores lose nearly all GFP inducibility just before dying, we sought
609 to measure the RNAP II level in ageing spores since GFP inducibility strongly and positively
610 correlates with the RNAP II level in spores. We repeated the ageing experiment but now with the
611 spores that had the fluorescent RNAP II. By taking out aliquots of these spores from an ageing
612 population and then using a microscope to measure the RNAP II levels in each of these aged
613 spore bags, we found that spores' RNAP II level decreased as they aged. Moreover, after ~50
614 days of ageing, almost all the spores had barely detectable RNAP II levels which were near the
615 background fluorescence value (Fig. 5C and Appendix Figs S28-S29).
616
617
618 **More RNA polymerase II means more likely to survive dormancy**
619 After a month of ageing, more than ~50% of the spores were dead. This was the case, for
620 example, on day 39 of ageing in water. We aliquoted a subset of the aged spores on day 39.
621 Then, we used a microscope to measure the RNAP II levels in each of the aliquoted spore bags.
622 Afterwards, we incubated these spores in minimal medium with a 2%-glucose and then, with time-
623 lapse microscopy, determined which spore bags germinated (i.e., were alive) and which ones did
624 not germinate (i.e., were dead). Spores that had more RNAP II had a higher chance of being alive
625 than spores with less RNAP II (Fig. 5D). In particular, we found that nearly 50% or more spores
626 with the lowest observed RNAP II levels were dead. These spore bags had near background
627 fluorescence level of ~2000-3000 fluorescence units (Fig. 5D). Since GFP inducibility positively
628 correlates with the RNAP II level, this finding complements the observation that spores lose
629 almost completely their GFP inducibility just before dying, as also expected from the triangular
630 relationship (Fig. 4J).
631
632
633 **Aged spores at the edge of dormancy before dying have nearly depleted RNAP II and have**
634 **strained gene-expressing ability**
635 On the 39th day of ageing, many spores with barely detectable RNAP II levels (2000-3000
636 fluorescence units in Fig. 5D) were dead but many others also with the same barely detectable

637 RNAP II levels were alive, albeit with less than a 50% chance (Fig. 5D). This 39-days-old
638 population is thus ideal for understanding the last moments of dormancy - dormancy just before
639 death. With this population, we sought to understand what actually distinguishes the dead from
640 dormant spores when both have comparable, barely detectable RNAP II levels (i.e., 2000-3000
641 fluorescence units in Fig. 5D). We examined two histograms of RNAP II levels: one for dead
642 spores (Fig. 5E - lower, grey histogram) and the other for dormant spores (Fig. 5E - upper, blue
643 histogram). The two histograms show many alive and dead spores having the nearly identical,
644 low range of values for RNAP II level (Fig. 5E). After measuring the RNAP II levels in individual
645 spore bags, we incubated both the dead and dormant spore bags together in a minimal medium
646 with a 2%-glucose and then tracked the RNAP II levels in individual spore bags with time-lapse
647 microscopy. We found that, after receiving the 2%-glucose, the dead spores did not make any
648 detectable levels of new RNAP II (Fig. 5F - grey traces and points) whereas the dormant spores
649 did make observable amounts of new RNAP II until they germinated (Fig. 5F - blue traces and
650 points). Moreover, these dormant spores often started to make new RNAP II shortly before buds
651 appeared (i.e., before they germinated) (Fig. 5F). They also, on average, took longer times to
652 germinate (Appendix Fig S30). Strikingly, these aged dormant spores, near death, did not produce
653 any detectable levels of new RNAP II for the first 5 to 10 hours after receiving the 2%-glucose
654 (Appendix Fig S30). After this "lag time", they began producing new RNAP II and then germinated
655 shortly afterwards.
656
657 These results paint a picture in which dormant spores with barely detectable amounts of
658 RNAP II are struggling through gene-expression and germination after receiving a 2%-glucose,
659 as expected intuitively from dormant spores that are near death. This picture also makes sense
660 given that RNA polymerases, including RNAP II, are required to make more RNAPs I-III and that
661 the aged, nearly dead spores have barely detectable amounts of RNAP II. Crucially, these results
662 establish that dead spores, which have virtually undetectable GFP inducibilities during dormancy
663 (Fig. 5B), also have virtually undetectable gene-expressing abilities after they receive a saturating
664 amount (2%) of glucose. This is supported by the fact that RNAP II - required for expressing all
665 coding and some non-coding genes - is at barely detectable levels in the aged spores that are
666 near death (Fig. 5E) and that a 2%-glucose does not induce gene expressions that lead to the
667 making of a new RNAP II (Fig. 5F).
668
669
670

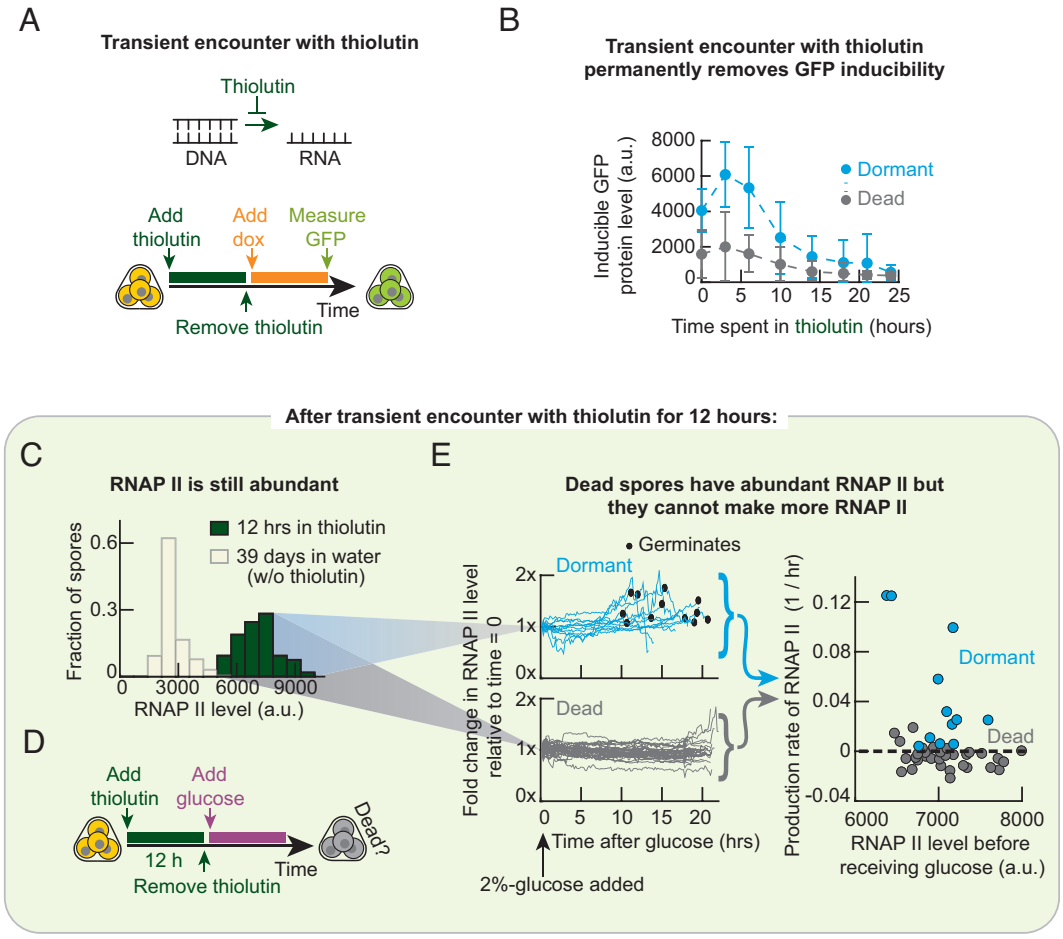

Figure 6 - Stopping transcription for a day causes dormant spores with abundant RNA polymerases to lose all gene-expressing ability and thus die.
(A) Protocol for (B-C) (See "Protocol for Figs. 6(A-C)" in Methods). **(B)** For protocol in (A), mean GFP inducibility of dormant (blue) and dead (grey) spore bags as a function of amount of time spent in thiolutin. *n*=3, error bars represent the standard deviation among spore bags in a population, averaged over three populations. **(C)** RNAP II level of spore bags after 12 hours of thiolutin (green; protocol in (A)) or after 39 days of ageing in water without drugs (light yellow; data from Fig. 5C). **(D)** Protocol for (E) (See "Protocol for Fig. 6E" in Methods). **(E)** Corresponds to green histogram in (C). Blue is for dormant and grey is for dead spore bags. Line traces show, after receiving a 2%-glucose, the fold change in each spore bag's RNAP II level relative to the spore bag's initial RNAP II level (i.e., value just before we added the 2%-glucose). Black dots represent moments of germination. *n* = 12 for blue and *n* = 42 for grey. Data points in the rightmost plot show the RNAP II production rate in each spore bag profiled in the line traces. For each spore bag, we fitted an exponential function to its RNAP II level over time (one of the line traces). Average RNAP II production rate in dormant spore bag (average over all blue points) is a positive value, 0.044 / hour and in dead spore bag (average over all grey points) is a negative value, -0.0045 / hour. For a t-test on the difference between the two values: p-value = 6.5 x 10^{-3}.

Transiently inhibiting transcription during dormancy permanently decreases GFP inducibility

We found that ageing spores gradually lose the GFP inducibility. In other words, older dormant spores, when forced by doxycycline to express *GFP* without nutrients, yield less steady-state GFP levels. Although dormant spores have less RNAP II as they age (Fig. 5C), it is not obvious that less RNAP II should lead to less steady-state GFP level after 24 hours of doxycycline. This is all the more since we showed above that the GFP level reaches a steady-state value because the

spores stop producing GFP, despite the doxycycline not degrading and still being present at the saturating concentration at the end of the 24-hour incubation (Appendix Figs S13-S14). To understand why spores lose the GFP inducibility as they age, we sought a clearer understanding of gene expression in general, which occurs during dormancy as we have shown with the 5-EU labeling of RNAs (Fig. 4E).

As a start, we incubated the GFP-inducible spores in water with thiolutin for various hours (between 0 and 24 hours). Afterwards, we washed away the thiolutin and then added doxycycline for 24 hours to induce their GFP expression (Fig. 6A). At the end of the 24-hr incubation with doxycycline, we measured the GFP inducibility of each spore bag. After measuring the GFP-inducibility, we incubated the spores in a 2%-glucose to determine which ones were dormant (alive) and which were dead. In this experiment, we found that the GFP inducibility of dead spore bags always remained at the minimally observable values, as expected (Fig. 6B - grey points). The GFP inducibility of dormant spore bags gradually decreased as the hours spent in thiolutin increased, until the GFP inducibility reached barely detectable levels after ~15 hours in thiolutin (Fig. 6B - blue points). Hence, removing thiolutin did not restore the spore's GFP inducibility back to the level that it had before it encountered thiolutin. In other words, transiently inhibiting global transcription for ~12 hours or more has permanently (irreversibly) reduced the GFP inducibility. Along with this irreversible decrease in GFP inducibility, we observed that thiolutin accelerated deaths, resulting in almost all spores being dead after ~24 hours with thiolutin as opposed to the ~2 months in the previous ageing experiment without thiolutin (Fig. 5A and Appendix Fig S31A). These results establish that if dormant spores do not produce transcripts - and potentially proteins from these transcripts - during a day, then they irreversibly and completely lose GFP inducibility and die within a day. Thus transcribing - and potentially translating - during dormancy during a day is required for surviving and directly determines how much GFP the spore can make when it is forced to do so.

Spores that died due to transient inhibition of transcription do not express genes despite having abundant RNAP II

To better understand how the transcription inhibition by thiolutin causes death, we focused on spores that encountered thiolutin for 12 hours since many of them were either dead or nearly dead, thereby affording us enough dead and nearly dead spores to compare. During the 12-hours of thiolutin, the spores' production of RNAP II itself would be stopped. Indeed, these spores had

712 ~30% less RNAP II after the thiolutin treatment than they had before the treatment. They also had
713 ~30% less RNAP II after the thiolutin treatment than the spores that we kept in water for 12 hours
714 without thiolutin (Appendix Fig S31). But these thiolutin-treated spores still had abundant RNAP
715 IIs, nearly 2-3 times more than 39-day-old spores that we kept in water without thiolutin (Fig. 6C).
716 Surprisingly, however, ~50% of the spores were dead after the 12-hours of thiolutin, which we
717 discovered by incubating the spores in a 2%-glucose after washing away the thiolutin (Appendix
718 Fig. S31A and Fig. 6D). In comparison, without thiolutin, we would need to wait ~39 days for ~50%
719 of the ageing spores to be dead (Appendix Fig. S32).
720
721 To test whether the RNAP II-abundant spores that died during the 12 hours of thiolutin
722 could express genes after receiving a 2%-glucose, we used time-lapse microscopy to track the
723 RNAP II levels in both dead and dormant spores after washing away the thiolutin and then
724 incubating the spores in minimal medium with a 2%-glucose (Fig. 6E - left half). As with the spores
725 that aged for 39 days without thiolutin (Fig. 5F), the thiolutin-treated dormant spores made new
726 RNAP II after receiving the 2%-glucose, typically taking ~5-10 hours before producing detectable
727 amounts of new RNAP II (Fig. 6E - blue curves and points) just like the 39-day-old spores with
728 little RNAP II. As for the spores that died from the thiolutin treatment, we did not observe any of
729 them producing noticeable amounts of new RNAP II during the ~20 hours of incubation in the 2%-
730 glucose, despite all of them having abundant RNAP IIs (Fig. 6E - grey curves and data).
731
732 Taken together, the thiolutin experiments (Figs. 6B-E) establish that dead spores can have
733 many copies of RNAP II and yet a 2%-glucose may not induce gene expression in them, including
734 those necessary for producing RNAP II. These results support the idea that spores need to
735 produce some transcripts, within several hours (~12 hours as a conservative estimate), in order
736 to maintain GFP inducibility - and thus for a 2%-glucose to be able to induce gene expression in
737 them - and hence for the spores to survive. Moreover, the fact that transiently inhibiting
738 transcription permanently reduces GFP inducibility (Fig. 6B) strongly suggests that during the
739 transcription inhibition, spores lost copies of key molecules that are required for gene expression
740 and that the spores could not fully replenish these molecules after transcription became
741 permissible again. In other words, the experiments suggest that the production rates for these
742 molecules is lower than their degradation rates during dormancy. This is also consistent with the
743 fact that dormant yeast spores eventually die if left they are left alone in water without any drugs
744 (Fig. 5A) - there is a net loss of the molecules because, during dormancy, the production rates for
745 the molecules are lower than the degradation rates for the molecules.

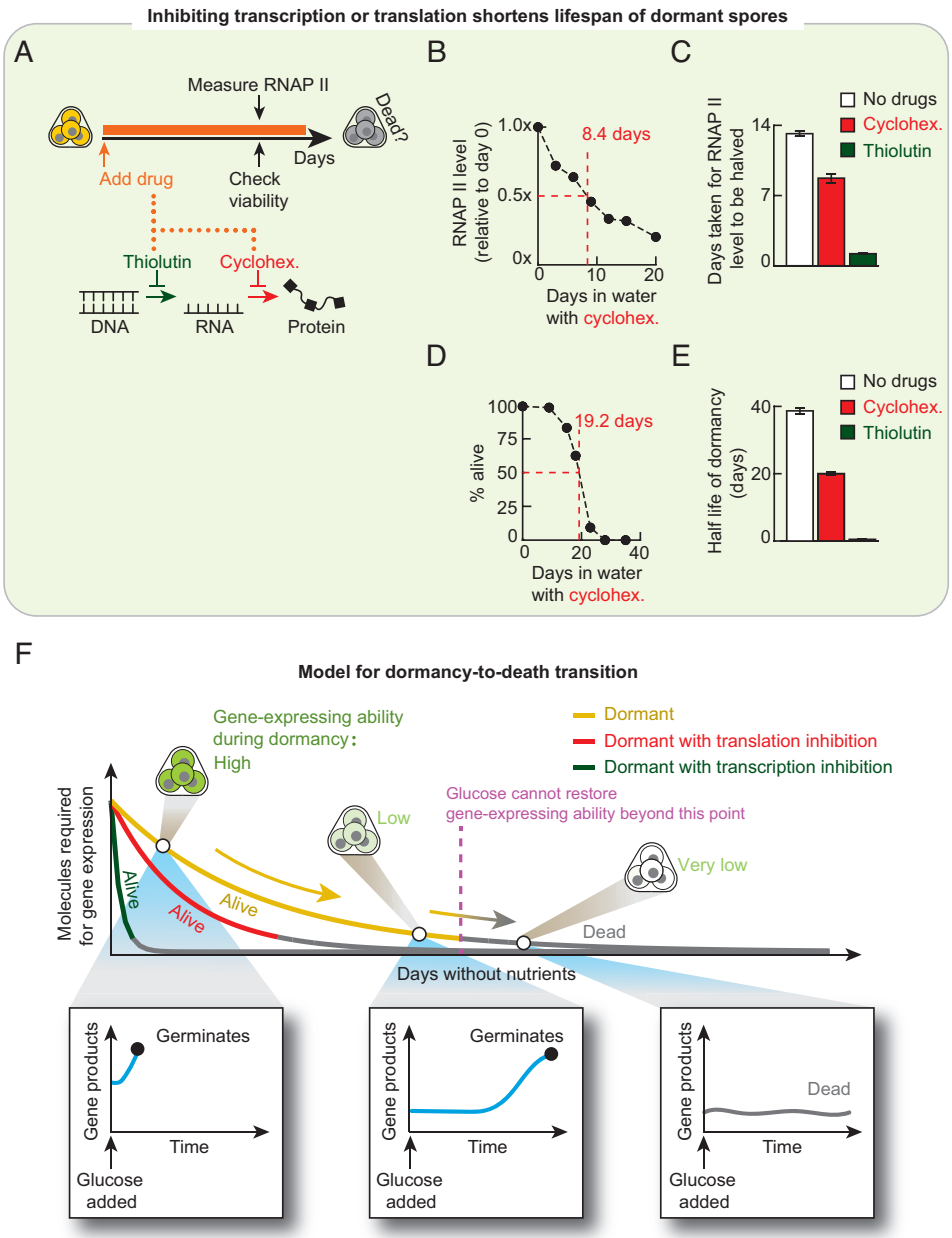

Figure 7 - Gradual loss of molecules that are required for gene expression underlies ageing and eventual death of dormant yeast spores.

(A) Protocol for (B-E). See "Protocol for Fig. 7(B-E) in Methods". Two drugs: Thiolutin inhibits transcription and cycloheximide inhibits translation. **(B)** Experiment in (A) with cycloheximide. For one representative population with cycloheximide (other replicates and data for thiolutin are in Appendix Fig S32). RNAP II level relative to its day 0 value, averaged over all dormant spore bags in a population. We used a linear interpolation determine the time taken for the halving of RNAP II level (8.4 days). **(C)** Experiment in (A). Time taken for the RNAP II level to decrease by half in dormant spores without drugs (white), with cycloheximide (red), and with thiolutin (green). For spores with thiolutin, we extrapolated this value from the measurements because, even when every spore had died after 2 days of thiolutin, average RNAP II level was still more than half its initial level (Appendix Fig S31). *n*=3; error bar are s.e.m. **(D)** Experiment in (A). For one representative population with cycloheximide (other replicates and data for thiolutin are in Appendix Fig S32). Time taken for half of the spore population to die ("half-life of dormancy"), estimated by linear interpolation (19.2 days). **(E)** Experiment in (A). Time taken for half of the spore population to die for populations without drugs (white), with cycloheximide (red), and with thiolutin (green). *n*=3; error bar are s.e.m. **(F)** Putting all our data together leads to a conceptual model of how spores age during dormancy and eventually die: key molecules required for gene expression, including RNA polymerases, are gradually lost during dormancy because spores cannot produce them faster than they degrade. These molecules eventually reach below some threshold amounts after which the spore has permanently lost the ability to express genes, meaning that even ample glucose cannot restore the gene-expressing ability (inset at bottom right). Since, after receiving glucose, spores must express genes in order to build a new cell that will bud off the spore (i.e., germinate), the permanent loss of gene expressing ability at the final moments of dormancy marks the spore's death (grey parts of the curves in the top plot).

747 **Globally inhibiting gene expression shortens spores' lifespans**
748 To end our study, we tested the idea proposed above by estimating the production and
749 degradation rates of key molecules that are necessary for gene expression during dormancy. To
750 achieve this at a systems-level - without having to identify all such molecules and then measure
751 their production and degradation rates - we continuously inhibited transcription and translation
752 while ageing the spores that have the fluorescent RNAP II for ~2 months in water with either a
753 saturating concentration of cycloheximide or thiolutin. On various days, we took out an aliquot of
754 spores from the ageing population, washed away the drug from the aliquoted population,
755 measured the RNAP II level in these spores, and then incubated them in minimal medium with a
756 2%-glucose to check how many were still alive (i.e., dormant) (Fig. 7A). From these
757 measurements, we determined the rate at which a spore's ability to express genes during
758 dormancy decreases - due to the net loss of key molecules required for gene expression - by
759 measuring the net loss rate of RNAP II as a proxy (Figs. 7B-C). The RNAP II level averaged over
760 all dormant spores decreased by half after ~8.4 days of ageing in cycloheximide (translation
761 inhibitor) (Fig. 7B and Fig. 7C - red bar). As a comparison, the RNAP II level averaged over all
762 dormant spores decreased by half after ~14 days if the spores were left to age in water without
763 any drugs (Fig. 7C - white bar). Hence, inhibiting translation accelerates the net loss of RNAP II
764 by ~1.5 times compared to natural (drug-free) dormancy. Moreover, with cycloheximide, 50% of
765 the spore population were dead after ~19 days (Fig. 7D and Fig. 7E - red bar) whereas, without
766 any drugs, half of the spore population were dead after ~38 days (Fig. 7E - white bar). The
767 approximately 2-fold difference caused by cycloheximide in both the rate of RNAP II loss and the
768 half-life of dormancy supports the idea that dormant spores age and eventually die because they
769 gradually lose enough of the molecules that are required for gene expression, including RNAP II
770 , and that spores cannot replenish these after receiving a 2%-glucose.
771
772 The fact that cycloheximide accelerates the net loss of RNAP II proves that RNAP II
773 production, which requires transcription and translation, occurs during dormancy. Cycloheximide
774 prevents spores from producing RNAP II and thus accelerates the RNAP II loss. By how much it
775 is accelerated tells us the production rate of RNAP II during dormancy. We found that dormant
776 spores in water without any drugs produced RNAP II at an extremely slow pace, having a
777 characteristic production time of ~26 days (Appendix Fig S33). In comparison, after receiving a
778 2%-glucose but before germinating, spores produced new RNAP II 10-50 times faster than they
779 did during dormancy (i.e., before receiving the 2%-glucose) (Fig. 5E and Appendix Fig S33). The
780 extremely slow production of RNAP II during dormancy supports the idea that spores do not

781 replenish the molecules required for production, such as RNAP II, fast enough for preventing their
782 net losses. As RNAP II is certainly required for expressing coding genes and thus for germination
783 (i.e., for building a new cell after receiving a 2%-glucose), the net loss of RNAP II ensures that
784 dormant spores will eventually die if left alone. Our measurements on RNAP II also strongly
785 suggest that the molecules required for gene expression, which are lost on the order of at most
786 several hours during the thiolutin treatment (Fig. 6B), require transcription for them to be made
787 and are relatively unstable compared to macromolecular machineries such as RNAP II that are
788 also required for gene expression. In fact, since the RNAP II fluorescence arises from a single
789 protein, Rpb3, fused to mCherry, the slow degradation of RNAP II also means that smaller
790 proteins likely also degrade over days, not hours, during dormancy. Taken together, these results
791 suggest that key transcripts, which are required for gene expression, are lost on the order of at
792 most several hours during the transcription inhibition. In accordance with this view, spores
793 retained more than half of their original RNAP II level after a day thiolutin (Fig. 7C) yet the number
794 of surviving spores were halved after just ~12 hours (Fig. 7E).
795
796
797 **Model for dormancy-to-death transition**
798 Taken together, our experiments suggest a model of how dormant spores gradually approach
799 their deaths (Fig. 7F). In this model, when yeast spores enter dormancy sometime after
800 sporulation (Brengues *et al,* 2002; Thacker *et al,* 2011), they initially possess high amounts of key
801 molecules that are necessary for gene expression which include, but are not limited to, RNA
802 polymerases. Spore bags would vary in the initial amounts of these key molecules - leading to
803 different germination abilities among them for each glucose - but all of them initially have
804 sufficiently high amounts of the molecules to let them germinate to a 2%-glucose. During
805 dormancy, retaining the ability to express genes is crucial for being able to germinate and thus
806 for survival because a spore must build a new cell after glucose appears (and thus express genes
807 that code for the building blocks of the new cell). The molecules required for gene expression
808 become less abundant as time passes without any nutrients because spores cannot replenish
809 these molecules faster than the rate at which they degrade. The resulting net loss of these
810 molecules over time leads to the spores gradually losing the ability to express genes such as
811 those involved in making RNAP II. The spores also become less able to germinate as days pass
812 by, requiring more glucose to guarantee that they germinate as they age (Fig. 3G). An aged spore
813 that has lost the gene-expressing ability can neither build a new cell when a 2%-glucose appears
814 - so it cannot germinate - nor can it make the lost molecules that are crucial for regaining the

gene-expression ability when a 2%-glucose appears. Accordingly, dead spores cannot express genes including *GFP* (Fig. 5B) and those involved in the making of RNAP II when a 2%-glucose appears (Fig. 5F). Once the amounts of the molecules required for gene expression decrease below certain threshold values - note that different molecules can have different threshold values - the spore cannot germinate even with a saturating amount of glucose and is thus considered dead. Our results suggest that among the many molecules that are required for gene expression, the key molecules - the ones that degrade the fastest during dormancy - are RNA polymerases and, likely, certain crucial transcripts which may be non-coding transcripts. Further studies, however, are required to definitively show that such transcripts play a role and identify them.

DISCUSSION

Our study began by posing two broad, related questions: how an organism whose life has almost ceased - including dormant organisms - remain alive and how we can monitor its approach to death given that it has faint signatures of intracellular dynamics that are difficult to discern. We addressed key aspects of these two questions in the case of dormant yeast spores while also raising new questions that invite future investigations. A conceptual difficulty in addressing these questions lies in the phrase, "almost ceased". Prior to our work, how slowly intracellular processes such as gene expression occur in dormant yeast spores has been unclear as firm numbers were missing. With single-cell level measurements, we identified key quantities that are relevant for studying ageing during dormancy: probability of germinating (which we called "germination ability"), the ability to express genes during dormancy when forced to so (proxied by the "GFP inducibility"), and the amount of RNAP polymerases inside the spores (Figs. 1-4). After identifying these, we put firm numbers on them and showed how their decays over time collectively represent the process of ageing during dormancy for yeast spores (Figs. 5-7). We found that the RNAP II production and degradation take days whereas they take minutes in vegetative yeasts. We rigorously quantified the germination ability for every glucose concentration and found that it decays over days. With the GFP-inducing synthetic circuit, we quantified the ability of a dormant spore to express genes without nutrients when forced to do so and found that it also decays over days. Finally, we measured the lifespan of dormancy and found that one can shorten it from months - for spores kept in water without any drugs at 30 ^{O}C - to either hours (by inhibiting transcription during dormancy) or days (by inhibiting translation during dormancy). We revealed, with a single-cell resolution, how these quantities decayed over time up to the last moments of dormancy (i.e., just before the yeast spores die), and then determined when a yeast spore is no

longer dormant but dead. Crucially, we found that these quantities are linked to one another and therefore we can study the dormancy-to-death transition by monitoring several different quantities. Of these, the key relationship that we uncovered is the ability to express genes being directly linked to the ability to germinate: a yeast spore cannot germinate when glucose appears after it has sufficiently lost the ability to express genes. After all, a yeast spore would need to express genes to build a new cell that will bud off the spore (i.e., germinate). By linking the different quantities and measuring the extremely slow production rates - compared to degradation rates - of gene expression machineries such as RNAP II, we showed that a complete loss of gene expressing ability is inevitable for a yeast spore. These measurements led us to a new conceptual model for how yeast spores gradually lose dormancy (Fig. 7F). One way to monitor the gradual loss of dormancy is by measuring the gradually decreasing ability of the yeast spore to express a generic gene when forced to do so without nutrients (e.g., GFP inducibility). For this reason, we propose here a concept of "amount of dormancy". The amount of dormancy gradually decreases in a yeast spore and once it goes below a certain amount, the spore dies because it cannot express genes even when glucose appears.

There are several proxies for the amount of dormancy, such as the GFP inducibility and the amount of RNAP II. But one class of molecules, which we did not directly identify and measure here, are key transcripts that we argue are likely important for maintaining gene-expressing ability in yeast spores. While RNA polymerases are, as expected, required and important for maintaining gene-expression ability during dormancy, we have shown that ample RNA polymerases and ribosomes themselves are insufficient for yeast spores to maintain gene-expressing ability during dormancy. In fact, we found that after several hours of transcription inhibition, yeast spores with ample RNAP II and ribosomes cannot express coding genes both during dormancy and after receiving a saturating concentration of glucose - these spores died. By comparing the timescale of protein degradations in spores with the timescale of deaths that occur during the transcription inhibitions, we argue that certain transcripts - perhaps non-coding transcripts - that degrade on a timescale of at most hours are required for maintaining gene-expression ability and, in turn, for keeping spores alive. We expect that future studies will investigate this interpretation. Such future studies may benefit from the method that we have, for the first time, adapted for yeast spores here: 5-Ethynyl Uridine (5-EU) labeling of new RNAs being made during dormancy.

On a related topic, our work motivates an avenue of research that is underexplored: understanding the central dogma of molecular biology in eukaryotic spores by using dormant

yeast spore as a model system. With a synthetic gene-circuit, we showed that inducing transcription and translation of a generic gene is possible in dormant yeast spores without any external energy source. We provided, to our knowledge, one of the first evidences of active gene expression inside intact, dormant yeast spores at a single-cell resolution. This approach - building synthetic circuits in dormant yeast spores - may enable us to reveal fundamental principles of eukaryotic transcription and translation in dormancy. As a starting point, our work raises a fundamental question: how does a dormant yeast spore globally repress gene expression given that its total transcription rate appears to be nearly uncorrelated with the amount of RNA polymerases that it has? We believe that a good starting point for addressing this question is our finding that *GFP* expression stops after a day because the production - either transcription or translation - stops despite the inducer (doxycycline) still being present. Moreover, we may gain additional insights into how the central dogma of molecular biology functions in dormant eukaryotic spores by further investigating the glucose-triggered gene expressions in the primed, dormant yeast spores (Fig. 2G).

While much is known about bacterial spores, particularly *B. subtilis* spores (Suel *et al,* 2007; Segev *et al,* 2012; Ablett *et al,* 1999; Parry *et al,* 2014; Sinai *et al,* 2015; Dworkin & Shah, 2010), the dormant state of yeast spores - a model for eukaryotic spores - has remained comparatively underexplored. Our discoveries on yeast spores may not apply to bacterial spores such as *B. subtilis* spores and determining the similarities and differences between dormant state of yeast spores with dormant states of bacterial spores is challenging because dormancy of both remain incompletely understood. To bridge the gap, one may adapt the approach that we introduced here: building synthetic gene circuits in bacterial spores (e.g., *B. subtilis* spores) and then studying their dormancy-to-death transition. Since eukaryotic gene regulations have many features that are distinct from those of prokaryotes, we expect that studies of gene regulation in dormant bacterial spores - another underexplored topic - along with further studies of gene regulation in dormant yeast spores will deepen our understanding of both forms of dormancy and help us elucidate the similarities and difference between the two.

We hope that our study motivates future studies that find systems-level metrics for monitoring how dormancy in a cell or multicellular organism gradually transitions to death. Collectively, such studies will likely deepen our understanding of dormancy-to-death transitions.

Materials and Methods

Protocol for Fig. 4B. We first incubated spores (strain "TT14") for 24 hours in PBS with either 10 μg/ml of thiolutin (inhibiting transcription) or 200 μg/ml of cycloheximide (inhibiting translation), or 100 μg/ml of antimycin A (inhibiting ATP production by inhibiting oxidative phosphorylation), or without any drugs. Afterwards, spores were washed 4 times with PBS and then incubated in minimal medium with 2% glucose for 24 hours.

Protocol for Fig. 4F. We simultaneously measured the GFP inducibility and RNAP II level in individual spore bags after 24 hours of incubation in PBS with 100-μg/ml doxycycline (also see "Microscope Data Analysis" section). "RNAP II level" is the mCherry fluorescence per spore bag due to the mCherry protein fused to Rpb3, a subunit of RNA polymerase II.

Protocol for Figs. 6(A-C). We incubated spores for different amounts of time in PBS with 10 μg/ml of thiolutin. Then, after washing away the thiolutin four times with PBS, we incubated the spores for 24 hours in PBS with 100 μg/ml of doxycycline. We then measured the GFP levels ("GFP inducibility") of each spore bag on a microscope. Then, with the spores still under observation in the microscope, we washed away the doxycycline three times with PBS and then incubated the spores for 24 hours in minimal medium with a 2%-glucose to assign a state (dormant or dead) to each spore bag.

Protocol for Fig. 6E. After incubating spores for 12 hours with 10 μg/ml of thiolutin, we washed away the thiolutin four times with PBS. We then place the spores under a microscope and incubated them for 24 hours in minimal medium with a 2%-glucose. Spore bags that germinated within the 24 hours of glucose were counted as dormant (alive). The ones that did not germinate during this time were counted as dead.

Protocol for Fig. 7(B-E). We incubated a population of spores (“TS8” strain) in water with either 10 μg/ml of thiolutin or 200 μg/ml of cycloheximide, or without any drugs. At various time points during the incubation, we aliquoted some of the spores from the population and then, with a microscope, measured the mCherry fluorescence of each spore bag (representing RNAP II level since these spores had mCherry protein fused to Rpb3, a subunit of RNAP II). The "RNAP II level" for each spore bag is the maximal mCherry fluorescence obtained from 36 z-stacks with a z-step

949 0.2 μm. After measuring the RNAP II level, we kept these spores under the microscope and
950 washed away the drug four times with PBS. Then, while still keeping the spore bags under the
951 microscope, we incubated them for 24 hours in minimal medium with 2%-glucose. We measured
952 the percentage of spore bags that germinated during the 24 hours (these are counted as dormant
953 (alive)).
954
955 **Drugs used in Fig. 4A.** We used thiolutin from Sigma Aldrich (CAS Number 87-11-6). For
956 storage, we aliquoted thiolutin into DMSO at a final concentration of 1 mM. We used
957 cycloheximide from Sigma Aldrich (CAS Number 66-81-9) which we maintained in DMSO at a
958 final concentration of 100 mg/mL. We used antimycin A from Sigma Aldrich (CAS Number 1397-
959 94-0) which we diluted into ethanol at 10 mg/mL.
960
961 **Strains.** The "wild-type", homozygous diploid yeast strain that we used is from Euroscarf with the
962 official strain name "20000D" and genotype as follows: *MATa/MATα his3-11_15/his3-11_15;*
963 *leu2-3_112/leu2-3_112; ura3-1/ura3-1; trp1Δ2/ trp1Δ2; ade2-1/ade2-1; can1-100/can1-100*. This
964 strain generated four genetically identical, haploid spores for each spore bag. For engineering the
965 GFP-inducible spores, we started from the haploid versions of "20000D", which were also from
966 Euroscarf. These haploid strains were "20000A" (isogenic to another standard laboratory strain
967 called "W303" with mating-type "a") and "20000B" (isogenic to W303 with mating-type "alpha").
968 The 20000A's genotype is as follows: *MATa his3-11_15; leu2-3_112; ura3-1; trp1Δ2; ade2-1;*
969 *can1-100.* The 20000B's genotype is exactly the same as 20000A's, except that it is of the
970 opposite mating type (mating type "alpha"). The GFP-expressing diploid strain, called "TT14", is
971 nearly identical to the wild-type's except for the addition of selection-marker genes that we
972 introduced during the construction of the strain. TT14 has the following genotype: (*MATa/MATα*
973 *his3-11_15/his3-11_15; leu2-3_112/leu2-3_112; ura3-1/ura3-1; ADE2/ADE2; can1-100/can1-*
974 *100; HygB/HygB*; *trp1Δ2/TRP1; URA3/ura3-1; pADH1-rtTA/pADH1-rtTA; pTET07-GFP/pTET07-*
975 *GFP*). Here, *pADH1* is the constitutive promoter for *ADH1* gene in yeast (631 bases upstream of
976 *ADH1*'s ORF), *rtTA* is the reverse tetracycline-controlled transactivator whose transcription-
977 activation domain is from the yeast's Msn2, and *pTET07* is the promoter with 7 binding sites for
978 rtTA. We sporulated TT14 to form the "GFP-inducible spores" (Fig. 3A). These constructs are
979 more fully described in a previous publication (Youk & Lim, 2014). For experiments involving the
980 metabolic labelling of RNA with 5-Ethynyl-Uridine (5-EU) (Fig. 4C), we had to obtain spores that
981 were able uptake the extracellular 5-EU for incorporation into intracellular RNA. To achieve this,
982 we engineered a diploid yeast strain, “TS3”, which is the same as TT14 - TS3 also has the same

983 GFP-inducing synthetic circuit (Fig. 3A) as TT14 - but now with additional genotype modifications
984 (*HIS3/HIS3; hENT1-ADH1/hENT1-ADH1; HSV-TKGDP/HSV-TKGDP*) for which we used a
985 construct that was previously shown to let yeast import BrdU (Viggiani & Aparicio, 2006). *hENT1*
986 is the "human Equilibrative Nucleoside Transporter", under the control of the promotor for *ADH1,*
987 *HSV* is the Herpes Simplex Virus thymidine kinase under control of the promotor for *GPD1*, and
988 *HIS3* is a selection marker gene used for the cloning. To obtain the spores whose subunits of
989 RNA polymerases were fused to mCherry (Rpb3 for RNAP II and Rpc40 for RNAP I and III) (Figs.
990 4F and 4G), we engineered two diploid strains, "TS8" and "TS9", which were the same as "TS3"
991 - TS8 and TS9 both have the GFP-inducing synthetic circuit and allow 5-EU uptake) - but now
992 with additional genotype modifications: (*RPB3-mCherry-NatR* / *RPB3-mCherry-NatR*) or (*RPC40-*
993 *mCherry-NatR* / *RPC40-mCherry-NatR).* Here "*RPB3-mCherry-NatR*" is the *RPB3* gene on the
994 yeast chromosome IX (YIL021W) fused at its 3'-end to the *mCherry* gene, using the protein-linker
995 sequence from (Sheff & Thorn, 2004). *NatR* is the Nourseothricin resistance marker inserted
996 behind the stop codon of the *RPB3-mCherry* fusion gene. "*RPC40-mCherry-NatR*" is the same
997 as the "*RPB3-mCherry-NatR"* but now with the *RPC40* gene that is on the yeast chromosome
998 XVI (YPR110C) instead of *RPB3.*
999
1000 **Strain construction.** For each yeast transformation, we integrated a single copy of an
1001 appropriate, linearized yeast-integrating plasmid at a desired genomic locus through a
1002 homologous recombination. We first introduced a promoter of *ADH1* controlling rtTA expression
1003 *pADH1-rtTA* into 20000A (wild-type haploid, mating type "a") and 20000B (wild-type haploid,
1004 mating type "alpha") at the HO locus, by inserting a linearized, singly integrating, yeast-integrating
1005 plasmid with *pADH1-rtTA* and Hygromycin-resistance gene as a selection marker. This yielded
1006 two strains, "W303r1" (From 20000A) and "W304r1" (from 20000B). We next replaced the *ade2-*
1007 *1* "ochre mutation" in W303r1 and W304r1 with a functional *ADE2* gene by a homologous
1008 recombination of *ADE2* that we obtained by PCR from the S288C reference genome (100-bp
1009 homology on both flanking sites of the PCR product). This yielded two strains, "TT2" (from
1010 W303r1) and "TT8" (from W304r1). We then inserted a *pTET07-GFP* at the *LEU2* locus in TT2
1011 and TT8 by linearizing a yeast-integration plasmid that contains *pTET07-GFP*. This yielded two
1012 strains, "TT7" (from TT2) and "TT9" (from TT8). We then introduced two constitutively expressed
1013 selection-marker genes: *URA3* into TT7 to create "TT10" and *TRP1* into TT9 marker to create
1014 "TT13". These selection markers allowed us to select the diploid strain ("TT14") that resulted from
1015 mating TT10 with TT13 by using a double drop-out medium (lacking -*ura* and -*trp*). TT14 is
1016 homozygous for *ADE2*, *pADH1-rtTA*, and *pTET07-GFP*, thus all four haploids in a TT14 spore-

1017 bag has an inducible *GFP*. For the construction of TS3 strain (EU-incorporating diploids), we
1018 transformed two haploid strains, “TT10” and “TT13”, as stated in (Viggiani & Aparicio, 2006) with
1019 a linearized fragment (digested with NheI) of the plasmid “p403-BrdU-Inc” (from (Viggiani &
1020 Aparicio, 2006) and obtained from Addgene). This plasmid contains *hENT1-ADH1*, *HSV-TKGDP*,
1021 and a constitutively expressed selection marker, *HIS3*. We linearized and then integrated the
1022 plasmid, as a single-copy, into the *HIS3* locus in the yeast genome. This resulted in two strains,
1023 “TS1” (from TT10) and “TS2” (from TT13), of opposing mating types. We mated these two to
1024 obtain a diploid strain “TS3”. To construct the strain, "TS8" (which has Rpb3 protein fused to
1025 mCherry), we transformed the haploids strains, TS1 and TS2, by integrating mCherry-NatR linker
1026 at the *RPB3* locus in the yeast genome. We obtained the mCherry-NatR linker taking a plasmid
1027 “HypI” (used in (Laman Trip & Youk, 2020) and derived from “pkt150” (Sheff & Thorn, 2004)) and
1028 then amplifying the mCherry-NatR linker through PCR. This resulted in two strains, "TS4" (from
1029 TS1) and "TS5" (from TS2), which had opposite mating types. We mated them to obtain the diploid
1030 strain, "TS8". For constructing the diploid strain, "TS9" (which has Rpc40 protein fused to
1031 mCherry), we transformed the haploids strains "TS1" and "TS2" to obtained two strains, "TS6"
1032 (from TS1) and "TS7" (from TS2), which had opposite mating types. We mated them to obtain the
1033 diploid strain, "TS9".
1034
1035 **Yeast transformations and mating.** We transformed yeasts with the standard, lithium-acetate-
1036 based method. In short, log-phase cells were resuspended in 0.1M of lithium acetate in 1x TE,
1037 together with the DNA to be inserted by homologous recombination at a desired location within
1038 the yeast genome and herring ssDNA. We then added PEG3350, 10X TE and 1 M of lithium
1039 acetate to the yeast culture so that we had final concentrations of 40% PEG 3350, 1X TE, and
1040 0.1 M of LiOAc. We then incubated the culture at 42 °C for 30 minutes. We pelleted the resulting
1041 cells by centrifuging and then washed them with water. Afterwards, we plated the cells on agar
1042 plates with dropout media to select from transformed yeasts. We used a standard method of
1043 mating yeasts. Namely, we mated yeasts of opposite sexes by inoculating a 500 µL of YPD
1044 medium with two colonies - one from each sex. We then incubated the culture overnight in 30 °C
1045 on a benchtop shaker (Eppendorf Mixmate) that agitated the culture at 300 rpm. We then spread
1046 100 µL of the culture on double-dropout agar plates which selected for the diploids that resulted
1047 from successful mating.
1048
1049 **Spore formation (sporulation).** We used a standard protocol for sporulating yeasts. In short,
1050 we first grew diploid yeasts (homozygous diploid wild-type or GFP-inducible strains) to saturation

1051 overnight. We then transferred these cells to a "pre-sporulation media" (i.e., YPAc: consists of
1052 Yeast Peptone media with a 2% potassium acetate) which we then incubated for 8 hours at 30°C.
1053 We subsequently transferred the diploid yeasts to a "sporulation medium" (i.e., 2% potassium
1054 acetate) and left them to sporulate for 5 days at 20 °C, while rotating as a liquid culture in a tube.
1055 Afterwards, we transferred the resulting spores to water and stored them at 4°C. Through
1056 measurements, we found that we could store these spores for several months without loss of
1057 viability (i.e., 2%-glucose still germinates ~100% of the spore bags).
1058
1059 **Microscope sample preparation.** We performed all microscope imaging with 96-well glass-
1060 bottom plates (5242-20, Zell-Kontakt). Prior to each microscope imaging, we pre-treated the
1061 glass-bottom by incubating it for 20 minutes with 0.1 mM of concanavalin A (ConA, C2010, Sigma-
1062 Aldrich). We washed the ConA and then typically added 1 μL of spores in 200 μL of minimal
1063 medium per well. The plates were then centrifuged at 1000 rpm for 1 minute to sediment and
1064 attach all spore bags to the glass-bottom. We then performed the microscope imaging.
1065
1066 **Microscope data acquisition.** We used Olympus IX81 inverted, epifluorescence, wide-field
1067 microscope. For each time-lapse movie, we collected images once every 10 minutes for every
1068 field of view. The temperature during microscope imaging was maintained by an incubator cage
1069 (OKO Lab) that enclosed the microscope. We acquired each image with an EM-CCD Luca R
1070 camera (Andor) and IQ3 software (Andor). We used a wide-spectrum lamp (AMH-600-F6S,
1071 Andor) for exciting fluorescent proteins.
1072
1073 **Microscope data analysis.** We processed the microscope images with ImageJ and MATLAB
1074 (Mathworks). To measure the times taken to germinate (and to count spore bags that germinated),
1075 we looked for the first haploid spore that formed a bud for each ascus (spore bag). We segmented
1076 the spore bags by using the Sobel filtering of the brightfield images to create a mask. We extracted
1077 fluorescence values inside the mask. We corrected for the background fluorescence, for each
1078 ascus one by one, by subtracting the average background in a 50-pixel area that surrounded each
1079 ascus. For GFP fluorescence (measuring GFP inducibility) and mCherry fluorescence (measuring
1080 RNAP I-III levels through Rpb3-mCherry or Rpc40-mCherry), we computed the fluorescence per
1081 spore by taking the maximal intensity from 36 z-stacks with a z-step size of 0.2 μm per spore bag.
1082
1083 **Hexokinase-based assay to measure glucose concentrations.** 10 hours after adding a low
1084 concentration of glucose to germinate some of the spores in a 1-mL minimal medium in a 24-well

1085 microscopy plate (5242-20, Zell-Kontakt), we took 800 μL of the supernatant to measure the
1086 concentration of glucose in it. We determined the glucose concentration by using a hexokinase-
1087 based glucose assay kit (Glucose (HK) Assay, G3293, Sigma-Aldrich) that is based on converting
1088 glucose through hexokinase and $NADP^+$ dependent glucose-6-phosphate-dehydrogenase.
1089
1090 **RNA-seq on un-germinated spores.** We collected un-germinated spores by first collecting 1 mL
1091 samples of spores that were incubated in a 0.002%-glucose at 0, 16, 48, and 96 hours after the
1092 incubation began. To isolate the un-germinated spores, we treated the 1-mL samples with
1093 zymolyase (786-036, G-Biosciences). Zymolyase lysed vegetative cells that formed from
1094 germinated spores, thus ensuring that we only collected RNA from un-germinated spores for
1095 sequencing. We then extracted the RNA from the leftover, un-germinated spores RiboPure Yeast
1096 Kit (Ambion, Life Technologies) as described by its protocol. Next, we prepared the cDNA library
1097 with the 3' mRNA-Seq library preparation kit (Quant-Seq, Lexogen) as described by its protocol.
1098 Afterwards, we loaded the cDNA library on an Illumina MiSeq with the MiSeq Reagent Kit c2
1099 (Illumina) as described by its protocol. We analyzed the resulting RNA-Seq data as previously
1100 described (Trapnell *et al,* 2012): We performed the read alignment with TopHat, read assembly
1101 with Cufflinks, and analyses of differential gene-expressions with Cuffdiff. We used the reference
1102 genome for *S. cerevisiae* from ensembl. We used the transcriptional modules listed in Appendix
1103 Table S1 for grouping the relevant genes into transcriptional modules.
1104
1105 **Fixing and permeabilizing spores.** Spores were fixed and made permeable - to let fluorophores
1106 into the spores for 5-EU labeling of RNAs and single-molecule RNA FISH - according to the
1107 standard protocols for vegetative *S. cerevisiae*. An important modification to these protocols is
1108 that to digest the spore wall, we extended the typical incubation in zymolyase to 2 hours during
1109 which the spores were constantly agitated at 300 rpm in 30 ^{O}C. We used this fixation and
1110 permeabilization treatment for performing single-molecule RNA FISH (Fig. 4H) and 5-EU labeling
1111 of RNAs (Fig. 4C-D).
1112
1113 **FISH probes.** We designed single-molecule FISH probes for detecting 18S rRNA (Fig. 4H) with
1114 the Stellaris® FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA):
1115 www.biosearchtech.com/stellarisdesigner. The probes were coupled to CAL Fluor Red 610
1116 (Biosearch Technologies, Inc.).
1117

1118 **Single-molecule RNA FISH.** We used the standard protocol for single-molecule RNA FISH in
1119 yeast, detailed in “Protocol for S.cerevisiae from Stellaris RNA FISH” (Biosearch Technologies,
1120 Inc., Petaluma, CA) and also in (Youk *et al,* 2010; Raj *et al,* 2008).
1121
1122 **Metabolic (5-Ethynyl Uridine) labelling of freshly synthesized RNA with click reaction.** We
1123 used click chemistry to bind fluorophores to the 5-Ethynyl Uridine (5-EU) labeled RNAs in spores.
1124 To do so, we followed the protocol for mammalian cells, from “Click-iT® Plus Alexa Fluor® picolyl
1125 azide toolkits” (Thermo Fisher) but with the following modifications so that the protocol would work
1126 in yeast spores: (1) all reactions and washing steps were done in a 1.5-mL Eppendorf tube with
1127 a centrifugation speed of 800 x G; (2) spore fixation and permeabilization were performed as
1128 mentioned above; (3) total volume for the click reaction was 50 μL; (3) for the click reaction, fixed
1129 spores were at OD ~ 0.02; (4) the click-reaction cocktail was incubated for 1 hour and 30 minutes.
1130 All components (Ethynyl-Uridine, Fluorophore-Azide, and reagents for the click reaction) were
1131 from the "Click-iT® Plus Alexa Fluor® picolyl azide toolkits" (Thermo Fisher). For simultaneously
1132 imaging 5-EU bound RNAs with GFP proteins inside spores (Fig. 4E), we used Picolyl Azide
1133 Alexa 594 from JenaBioscience. Here, the ratio of CuSO4 to the copper protectant was set to
1134 1:20 in order to simultaneously detect the fluorescence from both GFP and Alexa 594.

Supporting Information

Appendix Figures S1-S33

Appendix Table S1

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Author Contributions:

T.M. and H.Y. conceived the project and designed the experiments. T.A. and M.A.B. performed the experiments and data analyses for Fig. 1. T.M., T.A. and M.A.B, performed the experiments and data analyses for Fig. 2. T.M. performed all the experiments and data analyses for Figs. 3 to 7. H.Y. provided overall guidance. T.M. and H.Y. wrote the manuscript.

Conflict of interest:

The authors declare that they have no competing interests.

Data availability:

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information files. The data that support the findings of this study are available from the corresponding author upon reasonable request. The RNA-Seq data in Fig. 2G is available on NCBI GEO with accession number #.

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Figure captions

Figure 1 - Glucose germinates only a fraction of yeast spores.

(A) Conventional test to determine whether a spore bag (i.e., ascus) containing four haploid spores, in the absence of any external nutrients (blue box), is dormant or dead. Green box: outcome if dormant. Red box: outcome if dead.

(B) Left: Filmstrip of a time-lapse movie in which a 2%-glucose was added at the beginning of the movie (10 minutes between frames). A spore bag is counted as having germinated at the moment that at least one budding cell emerges from the spore bag (white arrows). Right: Time taken by each "wild-type" spore bag to germinate in the time-lapse movie. n = 137 spore bags from a representative time-lapse movie.

(**C)** Percentage of wild-type spore bags that germinated as a function of time after adding glucose to the minimal medium. Different colors represent different glucose concentrations (from 0.0002% to 2%). n = 3; error bars are s.e.m.

(D) Average time taken to germinate (blue circles) and the total percentage of spore bags that germinated (red squares), both 16 hours (960 minutes) after receiving glucose (i.e., the plateau values for each color in Fig. 1C). Both are functions of glucose concentration. n = 3; error bars are s.e.m.

Figure 2 - Un-germinated spores, primed by a low glucose concentration, germinate faster upon encountering more glucose a few days later.

(A) Wild-type spores are first incubated in a low glucose concentration ("1st [glucose]") before we add more glucose later to increase the glucose concentration to "final [glucose]".

(B) For experiment in (A), number of spore bags germinated within each binned time. First glucose concentration is 0.0005% (from 0 to 16 hours) (green bars) and the final concentration is 0.002% (from 16 to 32 hours) (orange bars). n = 143 spore bags (representative data).

(C) For the experiment in (A), percentage of spore bags that germinated as a function of time since the first glucose addition (1st and final concentrations as indicated). More glucose added 1000 minutes after the first glucose (purple vertical line). n = 3; error bars are s.e.m.

(D) For the experiment in (A), average time taken for a spore bag to germinate - denoted $\Delta\tau$ in bottom panel of (C) - after stepping up the glucose concentration. We varied the first glucose concentration, but the final concentration was always 2%. n = 3; error bars are s.e.m.

1365 **(E)** Spore bags that do not germinate after encountering the first glucose are "primed" to
1366 germinate faster upon encountering more glucose.
1367 **(F)** For the experiment in (A), average time ($\Delta\tau$) taken for a spore bag to germinate after stepping
1368 up the glucose concentration (final glucose concentration is 2%), plotted as a function of the first,
1369 low glucose concentration. Different colors represent different times at which we added more
1370 glucose after the first addition: 16 hours (yellow), 48 hours (blue) and 96 hours (red). "Relative
1371 $\Delta\tau$" is the average time $\Delta\tau$ divided by the $\Delta\tau$ of spore bags that were incubated in minimal media
1372 without any glucose (0%) for the same duration of time (after which they received a 2%-glucose).
1373 *n* = 3; error bars are s.e.m.
1374 **(G)** Heat map showing transcriptome-wide changes in un-germinated spores at 0, 16, 48, and 96
1375 hours after being primed by a 0.002%-glucose (via RNA-Seq). Appendix Table S1 lists the genes
1376 for each transcriptional module (each row in the heat map). Also see Appendix Fig S8. For each
1377 transcriptional module, we first divided the expression level of each gene in that module by its
1378 expression level at 0-hours - this yields "normalized expression level" for that gene for both primed
1379 and unprimed spores (unprimed spores were in minimal media without glucose for 0, 16, 48, and
1380 96 hours). We averaged these values over all genes in a transcriptional module to obtain
1381 "normalized expression (primed)" and "normalized expression (unprimed)" for each transcriptional
1382 module. Colors represent ratio of these two values, averaged over three biological replicates (*n* =
1383 3).
1384
1385
1386 **Figure 3 - Inducing GFP expression during dormancy with synthetic circuit shows that**
1387 **spores that can realize higher GFP level (higher GFP inducibility) are more likely to**
1388 **germinate (higher germination ability) for any glucose concentration.**
1389 **(A)** Synthetic gene-circuit that constitutively expresses a transcription factor, rtTA (with ADH1-
1390 promoter) and an inducible promoter *(*TET-promoter) controlling *GFP* expression. Increasing
1391 doxycycline increases GFP expression.
1392 **(B)** Engineered spore bags (shown in (A)) transcribe and translate GFP in water (without any
1393 nutrients) with 25 μg/ml of doxycycline (top row) and in a saline solution (PBS) with 50 μg/ml of
1394 doxycycline (bottom row). Snapshots of GFP expression shown 22 hours after adding
1395 doxycycline**.**
1396 **(C)** GFP levels of individual spore bags (grey curves) over time (measured every 10 minutes with
1397 a wide-field epifluorescence microscope) after incubation in PBS with 10 μg/ml of doxycycline
1398 (top panel: *n* = 104 spore bags) and 100 μg/ml of doxycycline (bottom panel: *n* = 150 spore bags).

1399 **(D)** Engineered spore bags (shown in (A)) were first incubated for 22 hours in either PBS without
1400 any doxycycline or with 100 μg/ml of doxycycline. Next, we transferred them to minimal medium
1401 with various glucose concentrations. Plot shows the total percentage of the engineered spore
1402 bags that germinated (measured 20 hours after incubating with glucose) for spores pre-incubated
1403 in PBS without doxycycline (black points) and in PBS with 100 μg/ml of doxycycline (orange
1404 points). *n* = 3; error bars are s.e.m.
1405 **(E)** Top: Percentage of spore bags with the same GFP level ("GFP inducibility") from (D) that
1406 germinated after receiving a 0.001%-glucose. Percentages are averaged over all spore bags with
1407 the same binned GFP level (in the histogram below). Representative data shown (*n* = 145 spore
1408 bags in a population).
1409 **(F)** Germination landscape: colors represent the probability that a spore bag with a particular
1410 steady-state GFP level ("GFP inducibility") germinates for each glucose concentration (i.e., data
1411 in top panel of (E) represents a single row of this heat map). To measure each pixel, as in the
1412 experiment described in (D), we incubated spore bags in PBS with 100 μg/ml of doxycycline for
1413 22 hours before adding glucose ([glucose] as indicated along the rows). Columns indicate GFP
1414 inducibility of a spore bag, measured at 22 hours after adding the doxycycline. Each pixel is an
1415 average over 3 replicate populations (*n* = 3).
1416 **(G)** Given a spore bag, its GFP inducibility is a read-out of the probability to germinate
1417 ("germination ability") for each glucose concentration and the minimal glucose concentration
1418 required to guarantee germination. Spore bags with lesser gene-expressing abilities without
1419 nutrients require more glucose to germinate.
1420
1421
1422 **Figure 4 - More RNA polymerases I-III means higher germination ability and GFP**
1423 **inducibility**
1424 **(A)** Protocol for (B). See "Protocol for Fig. 4B" in Methods. Thiolutin inhibits transcription.
1425 Cycloheximide inhibits translation. Antimycin A inhibits ATP synthesis by inhibiting oxidative
1426 phosphorylation.
1427 **(B)** For experiment in (A), percentage of spore bags that germinated. *n*=3; error bar are s.e.m.
1428 **(C)** Method to detect all RNAs being made in yeast spores with a single-cell resolution. Spores
1429 were incubated for 24 hours in PBS with 1 mM of 5-Ethynyl Uridine (5-EU; denoted U*) that
1430 incorporates into freshly made RNAs. We fix the spores afterwards and let fluorophore (Alexa
1431 488) enter the spores and bind U* (see Methods). "Total RNA level" of a spore bag is the total
1432 fluorescence from all the 5-EU labeled RNAs.

(D) Microscope images show a spore bag (from strain “TS3”) after either incorporating 5-EU as described in (C) (top two images) or, as a control, following the protocol in (C) but without the 5-EU (bottom two images) (also see Appendix Fig S23A). Scale bar: 2 μm. Top histogram: total RNA level per spore bag with 5-EU (i.e., Alexa 488 fluorescence; *n* = 103 spore bags). Bottom histogram: fluorescence per spore bag in control population (i.e., without 5-EU; *n* = 95 spore bags). Also see Appendix Fig. S22A.

(E) Each dot is from a single spore bag ("TS3" strain) with GFP-inducing circuit (Fig. 3A). For each spore bag, we measured its GFP protein level ("GFP inducibility") and total RNA level (5-EU fluorescence) after incubating the spores for 24 hours in PBS with both 100-μg/ml doxycycline and 1 mM of 5-EU (see (C)). *n* = 245 spore bags. Alexa 594 fluorophore attached to 5-EU (see Methods). Green line: linear regression with R=0.24 and Pearson p-value = 0.00018.

(F) See "Protocol for Fig. 4F" in Methods. GFP inducibility per spore bag (each dot, "TS8" strain) measured as in (E) but now with live time-lapse without the 5-EU. "RNAP II level" is the mCherry fluorescence per spore bag due to the mCherry protein fused to Rpb3, a subunit of RNA polymerase II. *n* = 182 spore bags; Red line: linear regression with R=0.64 and Pearson p-value = 3.02×10^{-22}. Scale bar = 2 μm.

(G) Same protocol as in (F) but with "TS9" spores. "RNAP I & III level" is the mCherry fluorescence per spore bag due to the mCherry protein fused to Rpc40, a subunit of both RNAP I and RNAP III. *n* = 185 spore bags; Red line: linear regression with R=0.63 and Pearson p-value = 6.6×10^{-22}. Scale bar = 2 μm.

(H) Same protocol as in (F) but with "TT14" spores fixed after 24 hours of incubation in PBS with 100-μg/ml doxycycline. 18s rRNA level is from CAL Fluor Red 610 fluorescence emitted by single-molecule FISH probes bound to 18S rRNAs (see Methods). *n* = 213 spore bags; Red line: linear regression with R = 0.19 and Pearson p-value = 0.005.

(I) Bottom: RNAP II levels of spore bags ("TS8" strain) in a population, measured as in (F). Top: As a function of the RNAP II level (binned in the histogram), percentage of spore bags that germinated after receiving a 0.0015%-glucose, averaged over all spore bags with the same binned RNAP II level. *n* = 80 spore bags.

(J) Triangular relationship. Any pair of the following three are positively correlated: Germination ability for each glucose concentration (purple), GFP inducibility (green), and amounts of RNA polymerases I-III.

Figure 5 - Dormant spores gradually have less RNA polymerase II and GFP inducibility as they age, eventually losing all gene expressing ability just before dying.

(A) Percentage of wild-type and GFP-inducible spore-bags (Fig. 3A) that germinate due to a saturating glucose concentration (2%) that they encounter after ageing for 0 - 85 days in water (grey and black) or minimal medium with essential amino acids (red) at 30 ^{O}C. *n* = 3; error bars are s.e.m.

(B) Mean GFP inducibility of dormant (blue) and dead (grey) spore bags that were incubated in water without nutrients at 30 ^{O}C over ~80 days (also see Appendix Figs S27-S28). Blue data points are the average GFP-levels from the histograms shown in Appendix Fig S27. Grey data points are averages of the dead spore bags' histograms shown in Appendix Fig S27. *n*=3, error bars show the average standard deviation from three biological replicates. *n* =3.

(C) Average RNAP II level of spore bags incubated in water for 50 days at 30 ^{O}C. *n*=3, error bars represent the average standard deviation in a population, each population having at least 100 spore bags (see Appendix Figs S28-S29).

(D) Top: Percentage of spore bags, having the same RNAP II level on 39th day of ageing in water (as in (A)), germinating after receiving a 2%-glucose. Percentages are averaged over all spore bags with the same binned RNAP II level (corresponding histogram shown below). *n = 193* spore bags.

(E) RNAP II levels of dormant (alive) spore bags in blue and of dead spores in grey; both from the same population on 39th day of ageing in water (as in (A)) before any glucose added. *n* = 111 spore bags (blue); *n* = 82 spore bags (grey).

(F) Corresponds to histograms in (E). Blue is for dormant and grey is for dead spore bags. Line traces show, after receiving a 2%-glucose, the fold change in each spore bag's RNAP II level relative to the spore bag's initial RNAP II level (i.e., value just before we added the 2%-glucose). Black dots represent moments of germination. *n* = 38 for blue and *n* = 32 for grey. Data points in the rightmost plot show the RNAP II production rate in each spore bag profiled in the line traces. For each spore bag, we fitted an exponential function to its RNAP II level over time (one of the line traces). Average production rate of RNAP II in dormant spore bags (average over all blue points) is a positive value, 0.031 / hour and in dead spore bag (average over all grey points) is a negative value, -0.0030 / hour. For a t-test on the difference between the two values: p-value = 1.4×10^{-5}. Linear regression line for dormant (blue) is R = -0.19 and for dead (grey) is R = -0.23.

Figure 6 - Stopping transcription for a day causes dormant spores with abundant RNA polymerases to lose all gene-expressing ability and thus die.

(A) Protocol for (B-C) (See "Protocol for Figs. 6(A-C)" in Methods).

(B) For protocol in (A), mean GFP inducibility of dormant (blue) and dead (grey) spore bags as a function of amount of time spent in thiolutin. *n*=3, error bars represent the standard deviation among spore bags in a population, averaged over three populations.

(C) RNAP II level of spore bags after 12 hours of thiolutin (green; protocol in (A)) or after 39 days of ageing in water without drugs (light yellow; data from Fig. 5C).

(D) Protocol for (E) (See "Protocol for Fig. 6E" in Methods).

(E) Corresponds to green histogram in (C). Blue is for dormant and grey is for dead spore bags. Line traces show, after receiving a 2%-glucose, the fold change in each spore bag's RNAP II level relative to the spore bag's initial RNAP II level (i.e., value just before we added the 2%-glucose). Black dots represent moments of germination. *n* = 12 for blue and *n* = 42 for grey. Data points in the rightmost plot show the RNAP II production rate in each spore bag profiled in the line traces. For each spore bag, we fitted an exponential function to its RNAP II level over time (one of the line traces). Average RNAP II production rate in dormant spore bag (average over all blue points) is a positive value, 0.044 / hour and in dead spore bag (average over all grey points) is a negative value, -0.0045 / hour. For a t-test on the difference between the two values: p-value = 6.5×10^{-3}.

Figure 7 - Gradual loss of molecules that are required for gene expression underlies ageing and eventual death of dormant yeast spores.

(A) Protocol for (B-E). See "Protocol for Fig. 7(B-E) in Methods". Two drugs: Thiolutin inhibits transcription and cycloheximide inhibits translation.

(B) Experiment in (A) with cycloheximide. For one representative population with cycloheximide (other replicates and data for thiolutin are in Appendix Fig S32). RNAP II level relative to its day 0 value, averaged over all dormant spore bags in a population. We used a linear interpolation determine the time taken for the halving of RNAP II level (8.4 days).

(C) Experiment in (A). Time taken for the RNAP II level to decrease by half in dormant spores without drugs (white), with cycloheximide (red), and with thiolutin (green). For spores with thiolutin, we extrapolated this value from the measurements because, even when every spore had died after 2 days of thiolutin, average RNAP II level was still more than half its initial level (Appendix Fig S31). *n*=3; error bar are s.e.m.

(D) Experiment in (A). For one representative population with cycloheximide (other replicates and data for thiolutin are in Appendix Fig S32). Time taken for half of the spore population to die ("half-life of dormancy"), estimated by linear interpolation (19.2 days).

(E) Experiment in (A). Time taken for half of the spore population to die for populations without drugs (white), with cycloheximide (red), and with thiolutin (green). *n*=3; error bars are s.e.m.

(F) Putting all our data together leads to a conceptual model of how spores age during dormancy and eventually die: key molecules required for gene expression, including RNA polymerases, are gradually lost during dormancy because spores cannot produce them faster than they degrade. These molecules eventually reach below some threshold amounts after which the spore has permanently lost the ability to express genes, meaning that even ample glucose cannot restore the gene-expressing ability (inset at bottom right). Since, after receiving glucose, spores must express genes in order to build a new cell that will bud off the spore (i.e., germinate), the permanent loss of gene expressing ability at the final moments of dormancy marks the spore's death (grey parts of the curves in the top plot).

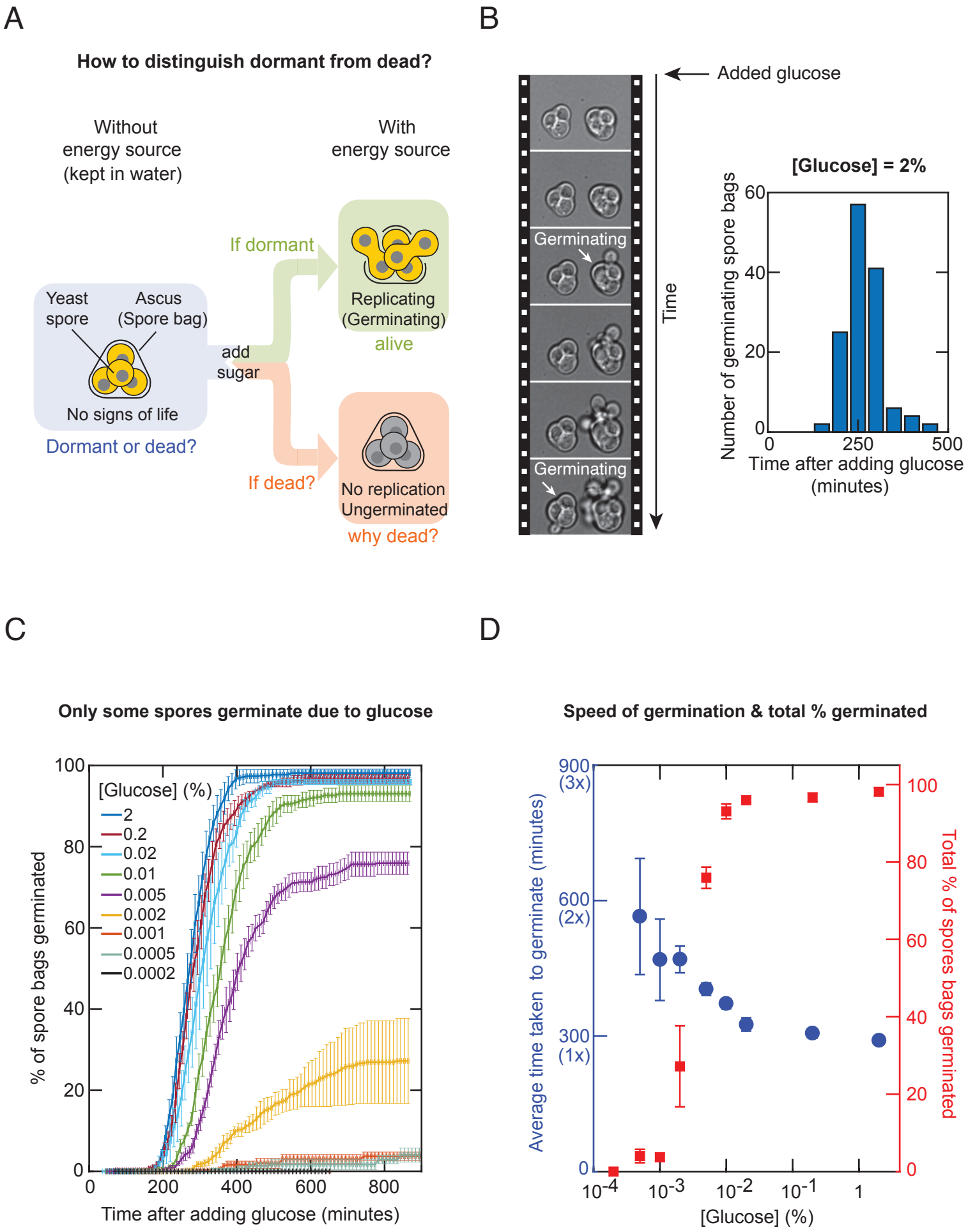

A
How to distinguish dormant from dead?
Without energy source (kept in water)
With energy source
Yeast spore
Ascus (Spore bag)
No signs of life
Dormant or dead?
add sugar
If dormant
Replicating (Germinating)
alive
If dead?
No replication Ungerminated
why dead?
B
Added glucose
Germinating
Germinating
Time
[Glucose] = 2%
Number of germinating spore bags
Time after adding glucose (minutes)
C
Only some spores germinate due to glucose
[Glucose] (%)
2
0.2
0.02
0.01
0.005
0.002
0.001
0.0005
0.0002
% of spore bags germinated
Time after adding glucose (minutes)
D
Speed of germination & total % germinated
Average time taken to germinate (minutes)
Total % of spores bags germinated
[Glucose] (%)

Fig. 1

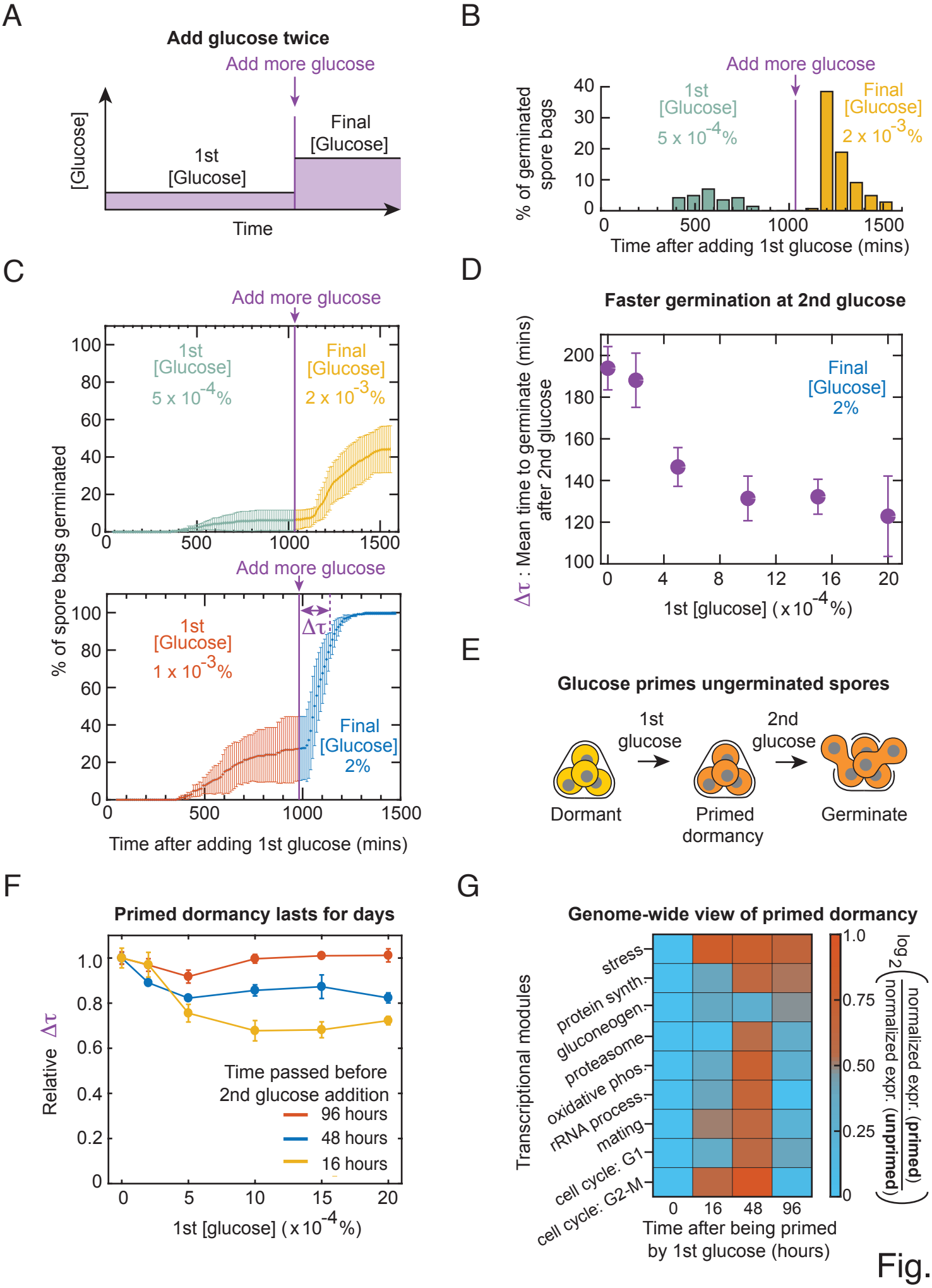
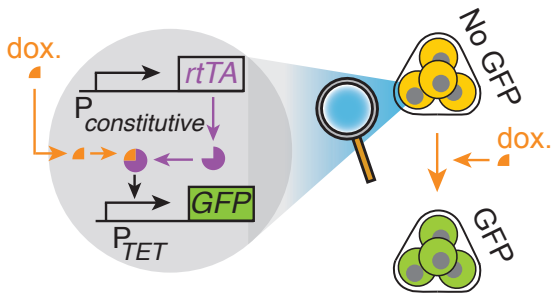

A
Add glucose twice
Add more glucose
[Glucose]
1st [Glucose]
Final [Glucose]
Time
B
Add more glucose
% of germinated spore bags
1st [Glucose] 5 x 10^-4 %
Final [Glucose] 2 x 10^-3 %
Time after adding 1st glucose (mins)
C
Add more glucose
% of spore bags germinated
1st [Glucose] 5 x 10^-4 %
Final [Glucose] 2 x 10^-3 %
Add more glucose
Δτ
1st [Glucose] 1 x 10^-3 %
Final [Glucose] 2%
Time after adding 1st glucose (mins)
D
Faster germination at 2nd glucose
Δτ : Mean time to germinate (mins) after 2nd glucose
Final [Glucose] 2%
1st [glucose] (x 10^-4 %)
E
Glucose primes ungerminated spores
1st glucose
2nd glucose
Dormant
Primed dormancy
Germinate
F
Primed dormancy lasts for days
Relative Δτ
Time passed before 2nd glucose addition
96 hours
48 hours
16 hours
1st [glucose] (x 10^-4 %)
G
Genome-wide view of primed dormancy
Transcriptional modules
stress
protein synth.
gluconeogen.
proteasome
oxidative phos.
rRNA process.
mating
cell cycle: G1
cell cycle: G2-M
Time after being primed by 1st glucose (hours)
log2 (normalized expr. (primed) / normalized expr. (unprimed))

Fig. 2

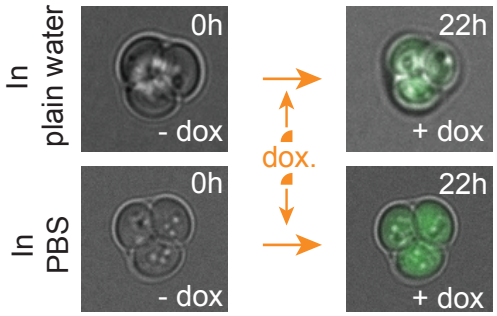
A

Synthetic circuit to test arbitrary gene induction in spores



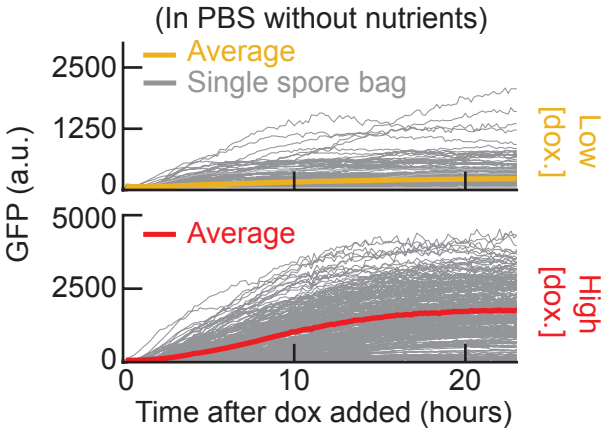
B

Gene expression is possible in spores without any external energy source



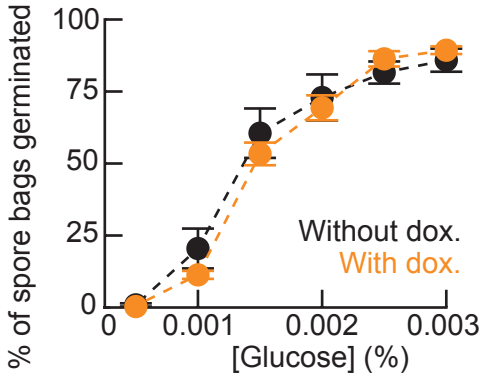
C

Spore bags vary in ability to produce GFP



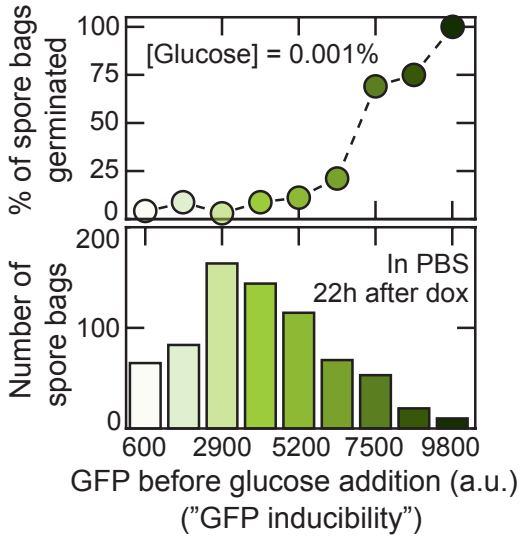
D

Inducing GFP does not change % of spore bags that germinate



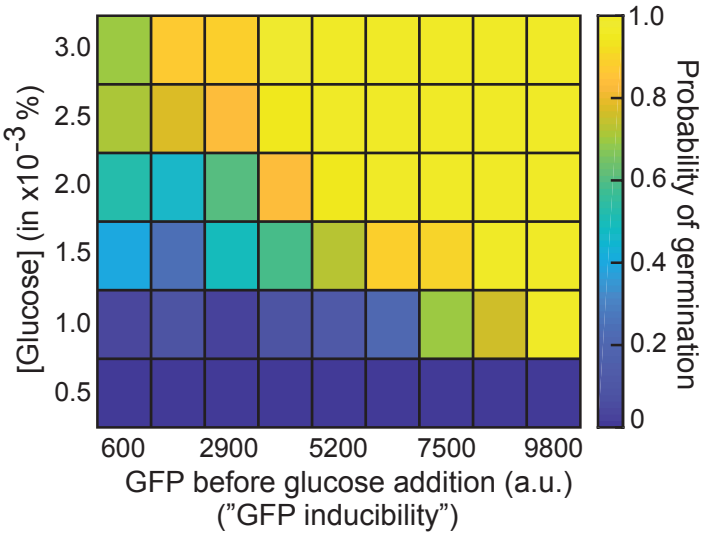
E

Spore bags with higher GFP inducibility are more likely to germinate



F

Germination landscape



G

GFP inducibility during dormancy quantifies ability to germinate

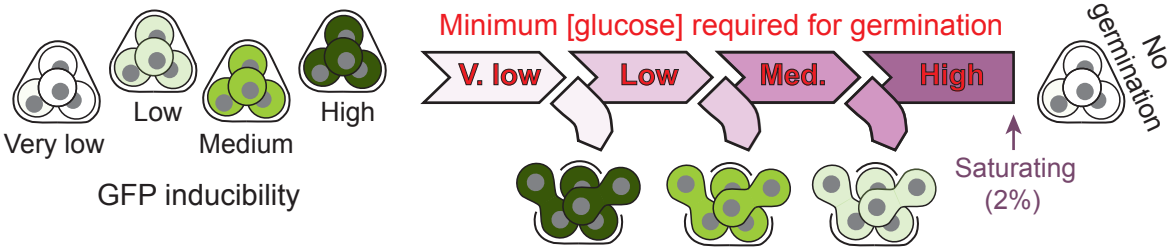


Fig. 3

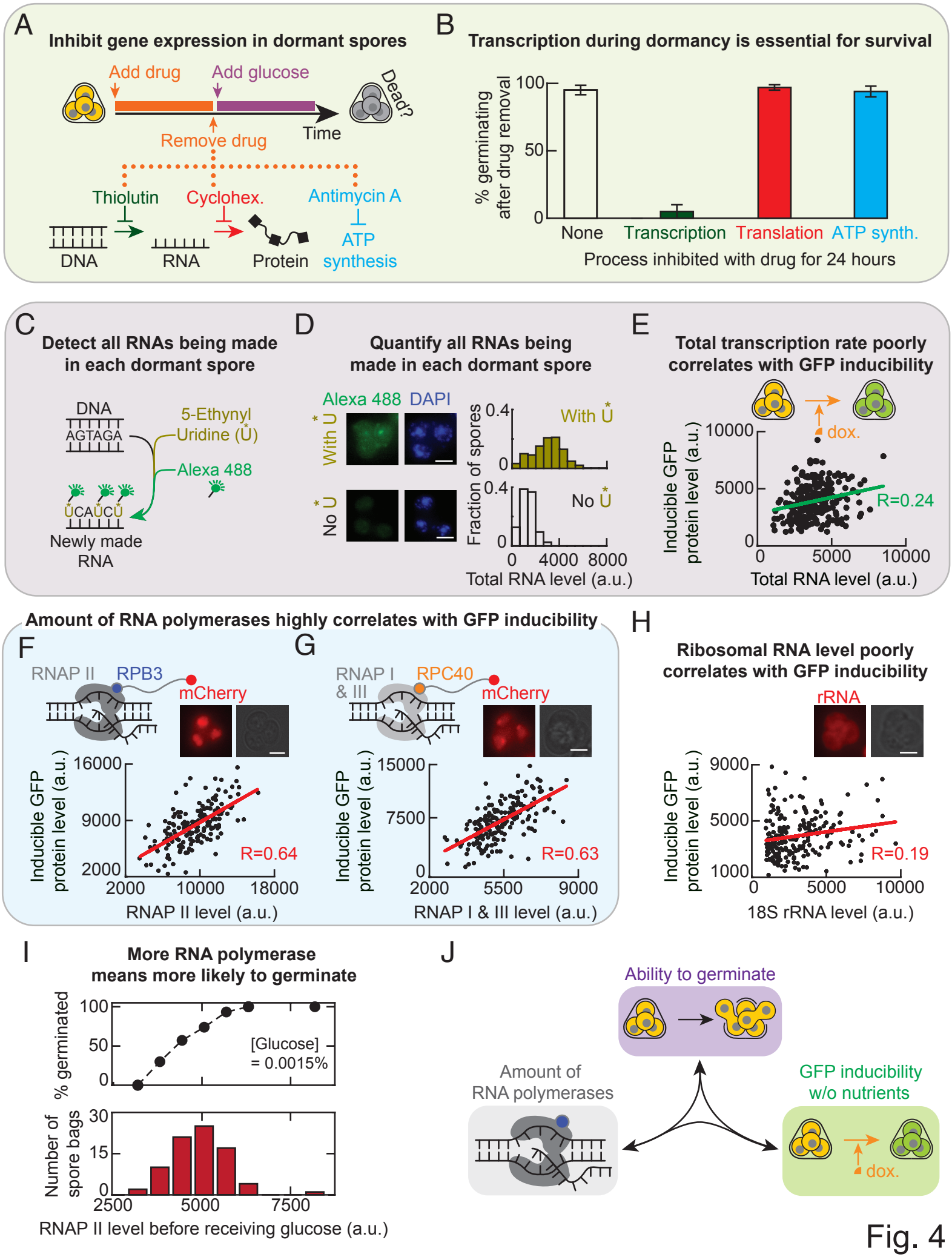

A
Inhibit gene expression in dormant spores
Add drug
Add glucose
Dead?
Time
Remove drug
Thiolutin
Cyclohex.
Antimycin A
DNA
RNA
Protein
ATP synthesis
B
Transcription during dormancy is essential for survival
% germinating after drug removal
100
50
0
None
Transcription
Translation
ATP synth.
Process inhibited with drug for 24 hours
C
Detect all RNAs being made in each dormant spore
DNA
AGTAGA
5-Ethynyl Uridine (U*)
Alexa 488
U*CAU*CU*
Newly made RNA
D
Quantify all RNAs being made in each dormant spore
Alexa 488
DAPI
With U*
No U*
Fraction of spores
0.4
0
With U*
No U*
0
4000
8000
Total RNA level (a.u.)
E
Total transcription rate poorly correlates with GFP inducibility
dox.
Inducible GFP protein level (a.u.)
10000
5000
0
R=0.24
0
5000
10000
Total RNA level (a.u.)
Amount of RNA polymerases highly correlates with GFP inducibility
F
RNAP II
RPB3
mCherry
Inducible GFP protein level (a.u.)
16000
9000
2000
R=0.64
2000
10000
18000
RNAP II level (a.u.)
G
RNAP I & III
RPC40
mCherry
Inducible GFP protein level (a.u.)
15000
7500
0
R=0.63
2000
5500
9000
RNAP I & III level (a.u.)
H
Ribosomal RNA level poorly correlates with GFP inducibility
rRNA
Inducible GFP protein level (a.u.)
9000
5000
1000
R=0.19
0
5000
10000
18S rRNA level (a.u.)
I
More RNA polymerase means more likely to germinate
% germinated
100
50
0
[Glucose] = 0.0015%
Number of spore bags
30
15
0
2500
5000
7500
RNAP II level before receiving glucose (a.u.)
J
Ability to germinate
Amount of RNA polymerases
GFP inducibility w/o nutrients
dox.

Fig. 4

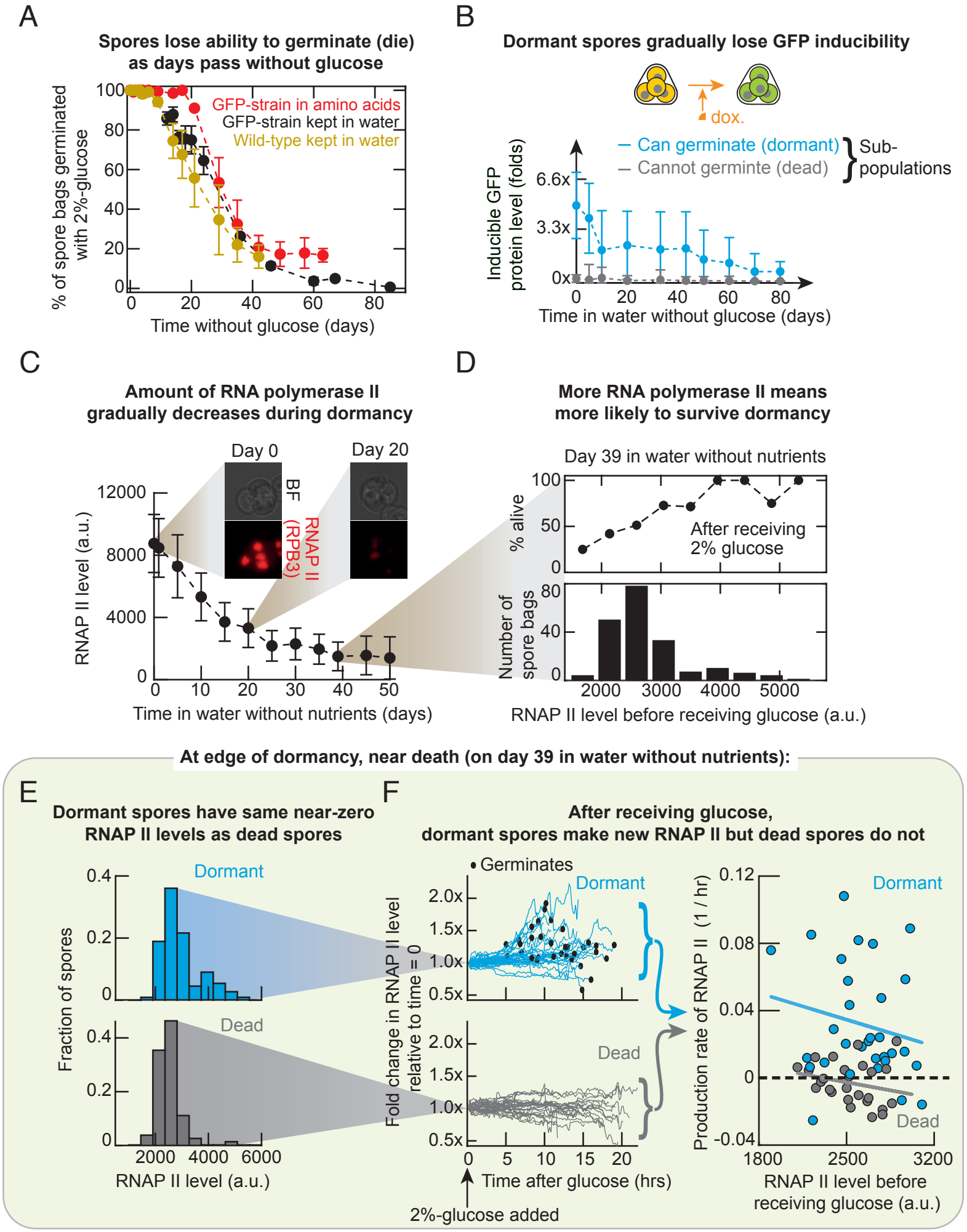

A
Spores lose ability to germinate (die) as days pass without glucose
% of spore bags germinated with 2%-glucose
GFP-strain in amino acids
GFP-strain kept in water
Wild-type kept in water
Time without glucose (days)
B
Dormant spores gradually lose GFP inducibility
dox.
Inducible GFP protein level (folds)
Can germinate (dormant)
Cannot germinte (dead)
Sub-populations
Time in water without glucose (days)
C
Amount of RNA polymerase II gradually decreases during dormancy
Day 0
Day 20
BF
RNAP II (RPB3)
RNAP II level (a.u.)
Time in water without nutrients (days)
D
More RNA polymerase II means more likely to survive dormancy
Day 39 in water without nutrients
% alive
After receiving 2% glucose
Number of spore bags
RNAP II level before receiving glucose (a.u.)
At edge of dormancy, near death (on day 39 in water without nutrients):
E
Dormant spores have same near-zero RNAP II levels as dead spores
Dormant
Dead
Fraction of spores
RNAP II level (a.u.)
F
After receiving glucose, dormant spores make new RNAP II but dead spores do not
Germinates
Dormant
Dead
Fold change in RNAP II level relative to time = 0
Time after glucose (hrs)
2%-glucose added
Production rate of RNAP II (1 / hr)
RNAP II level before receiving glucose (a.u.)

Fig. 5

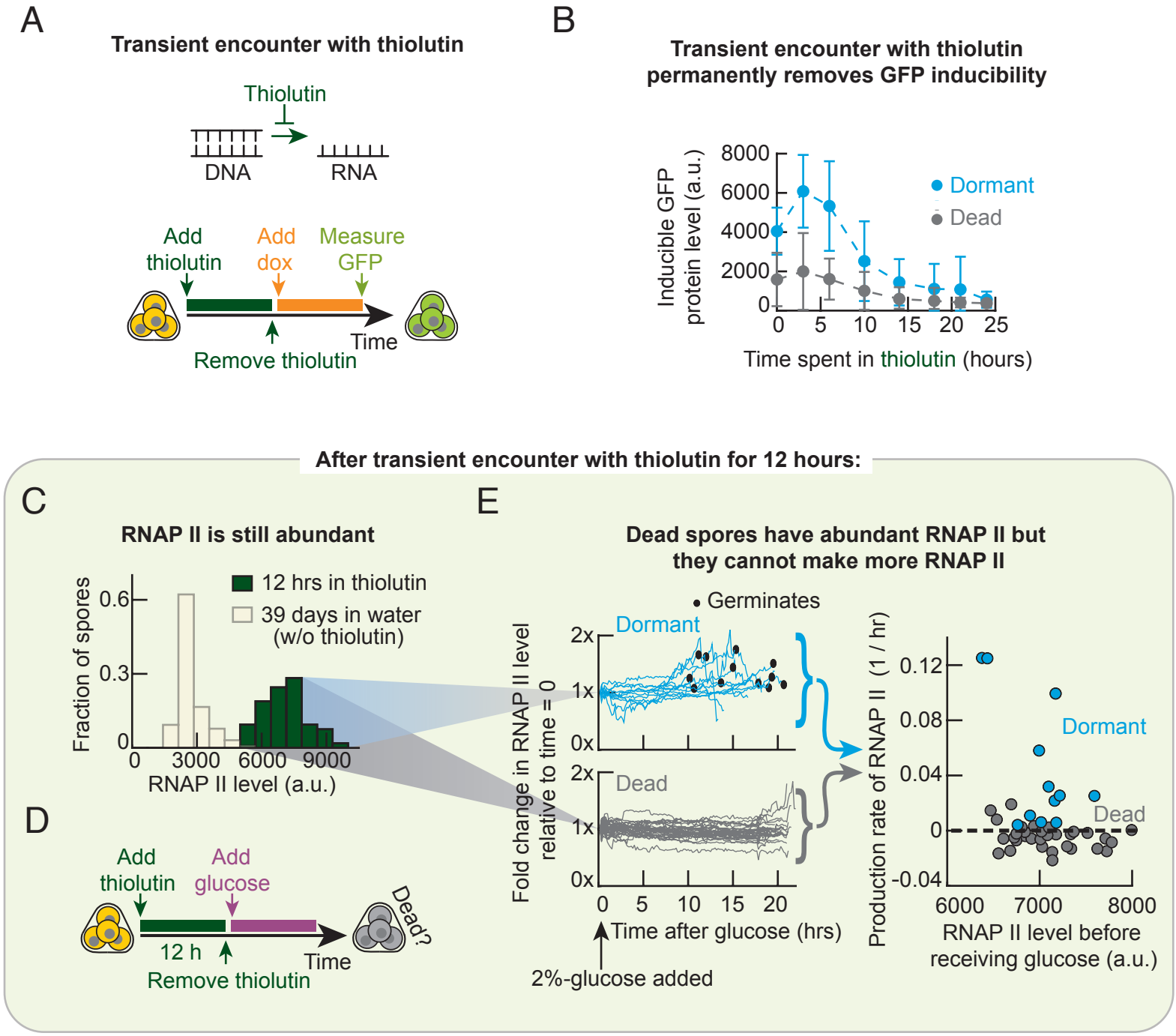
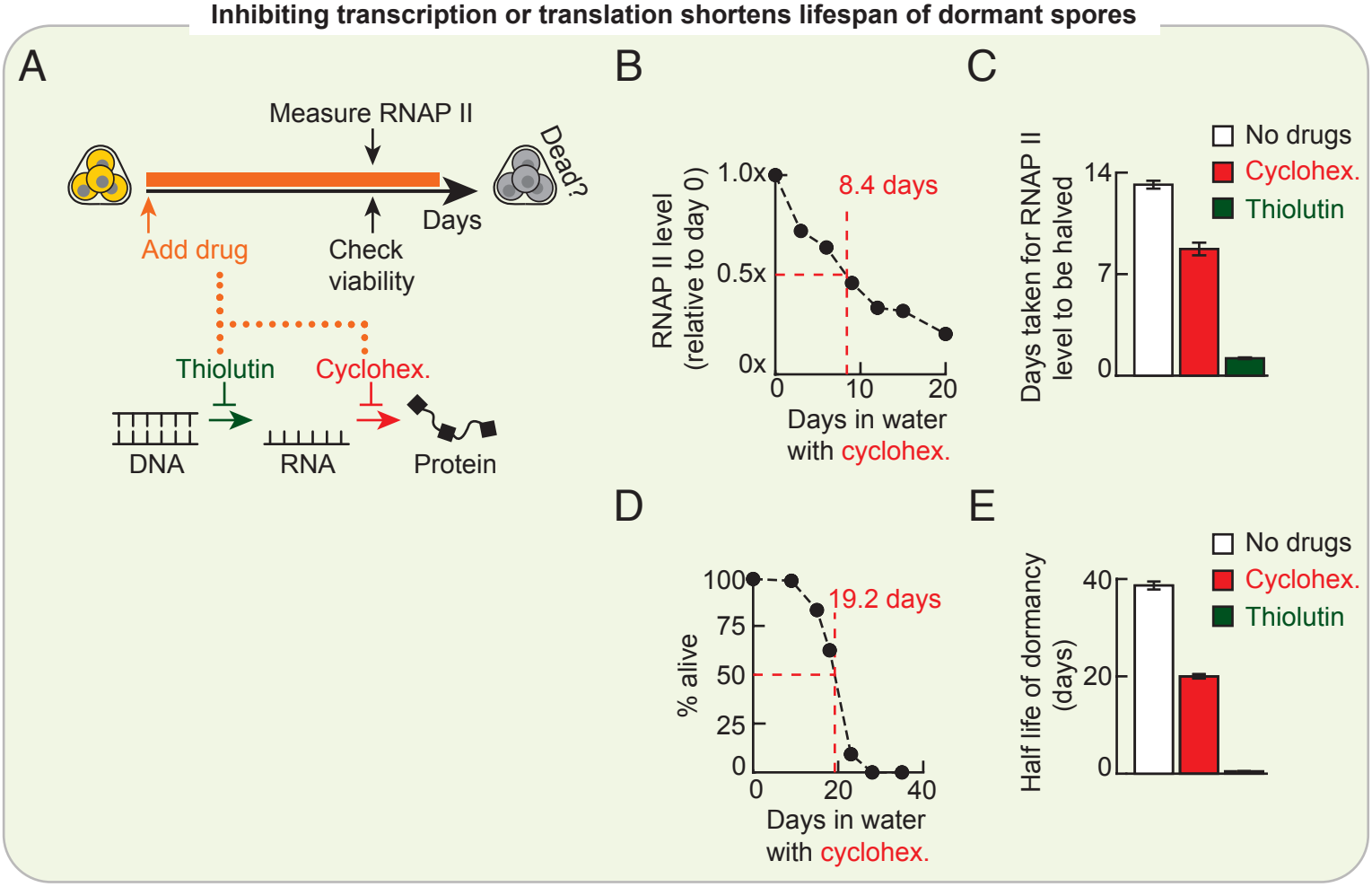

A
Transient encounter with thiolutin
Thiolutin
DNA
RNA
Add thiolutin
Add dox
Measure GFP
Time
Remove thiolutin
B
Transient encounter with thiolutin permanently removes GFP inducibility
Inducible GFP protein level (a.u.)
8000
6000
4000
2000
0
Dormant
Dead
0 5 10 15 20 25
Time spent in thiolutin (hours)
After transient encounter with thiolutin for 12 hours:
C
RNAP II is still abundant
Fraction of spores
0.6
0.3
0
12 hrs in thiolutin
39 days in water (w/o thiolutin)
0 3000 6000 9000
RNAP II level (a.u.)
D
Add thiolutin
Add glucose
12 h
Time
Remove thiolutin
Dead?
E
Dead spores have abundant RNAP II but they cannot make more RNAP II
Germinates
Fold change in RNAP II level relative to time = 0
Dormant
Dead
2x
1x
0x
0 5 10 15 20
Time after glucose (hrs)
2%-glucose added
Production rate of RNAP II (1 / hr)
0.12
0.08
0.04
0
-0.04
Dormant
Dead
6000 7000 8000
RNAP II level before receiving glucose (a.u.)

Fig. 6


Inhibiting transcription or translation shortens lifespan of dormant spores
A
Measure RNAP II
Dead?
Days
Add drug
Check viability
Thiolutin
Cyclohex.
DNA
RNA
Protein
B
RNAP II level (relative to day 0)
1.0x
0.5x
0x
8.4 days
0
10
20
Days in water with cyclohex.
C
Days taken for RNAP II level to be halved
14
7
0
No drugs
Cyclohex.
Thiolutin
D
% alive
100
75
50
25
0
19.2 days
0
20
40
Days in water with cyclohex.
E
Half life of dormancy (days)
40
20
0
No drugs
Cyclohex.
Thiolutin

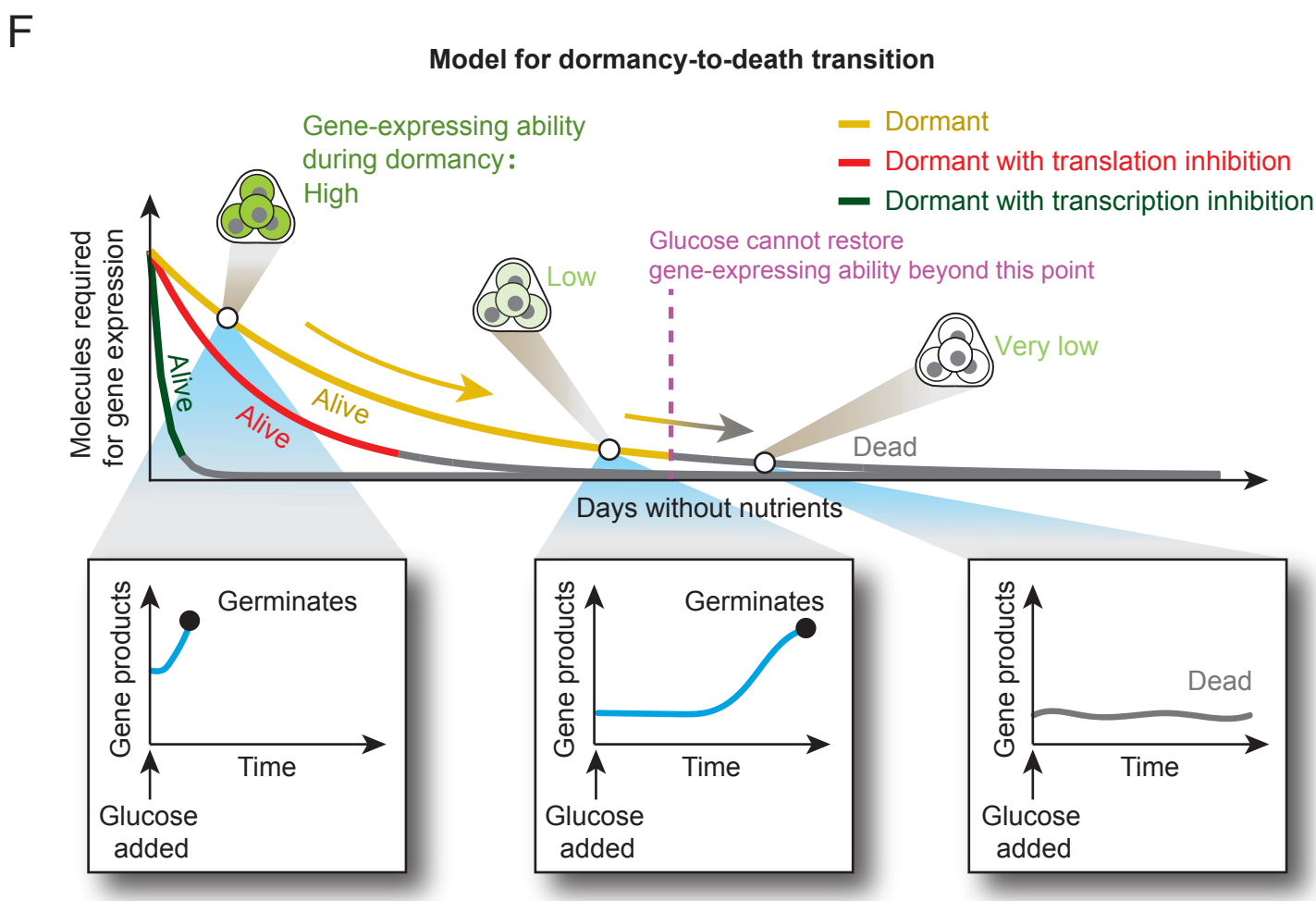

F
Model for dormancy-to-death transition
Gene-expressing ability during dormancy: High
Low
Very low
Dormant
Dormant with translation inhibition
Dormant with transcription inhibition
Glucose cannot restore gene-expressing ability beyond this point
Molecules required for gene expression
Alive
Alive
Alive
Dead
Days without nutrients
Gene products
Germinates
Time
Glucose added
Gene products
Germinates
Time
Glucose added
Gene products
Dead
Time
Glucose added

Fig. 7