# Dormancy as a spectrum measuring spore's proximity to death and to replicative life

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**ABSTRACT**

**How organisms with their lives ceased stay alive and what sets their lifespans are fundamental questions relevant for microbial spores. Starved microbes can form spores whose metabolism and gene-expressions are active for hours-to-days but nearly cease upon entering dormancy. Although dormant spores can wake-up (germinate) when nutrients reappear, they die - cannot germinate - after prolonged nutrient-absences. Previous studies identified several factors that affect spore revival. But how these and as-yet-unknown intracellular factors collectively encode a dormant spore's lifespan remains poorly understood. Here we reveal an easy-to-measure, systems-level metric - a quantity that combines many intracellular factors - that accurately predicts dormant yeast-spores' lifespans by establishing dormancy as a quantity that, without nutrients, decreases at a predictable rate, thereby revealing how dormant spores approach death. We discovered different glucose-concentrations germinating distinct percentages of yeast-spores, with low glucose-concentrations priming un-germinated spores to accelerate their germinations when more glucose appears hours-to-days later. Using a synthetic circuit, we quantified dormant spores' gene-expressing ability without nutrients - a systems-level metric - whose value determines a minimum glucose-concentration required for guaranteeing germination and, for glucose concentrations below it, probability of germinating. Dormant spores' gene-expressing ability predictably decreases over days-to-months, causing glucose-concentration required for germination to increase with predictable rates until going beyond a saturating value - spore's moment of death. By introducing "dormancy spectrum" - a ruler that measures spore's proximity to death (lifespan) and to replicative life (germination capacity) - and finding dormancy's systems-level indicators, we unveiled hidden dynamics of dormant spores approaching death.**

## INTRODUCTION

When starved of nutrients, microbes can enter dormancy - a state of "ceased life" - by first forming spores (*1-10*). Once formed, spores are not necessarily dormant right away. Instead, they can take between a few hours to about a week to enter dormancy. During this time, spores can be metabolically active and express various genes (*11-13*). For example, before entering dormancy, *Bacillus subtilis* spores undergo a week of "ageing" during which they actively degrade or produce appropriate transcripts to tailor their dormancy to the environmental temperature (*11*). As another example, before entering dormancy, *Saccharomyces cerevisiae* (budding yeast) spores express genes - such as those involved in completing the spore wall - for a few hours or days before turning off their expressions to enter dormancy (*12,13*). After entering dormancy, spores are thought to have greatly reduced metabolism and vanishingly low, if any, genome-wide expression levels (*1,11,12*). Supporting this view are recent discoveries of proteins and messenger RNPs (e.g., mRNAs bound to translational machineries) becoming inactive by aggregating into macromolecular structures inside dormant yeast and fungal spores (*14,15*). The resulting solid-like, glassy cytoplasm - being packed with these aggregates - greatly hinders proteins' movements and enzymatic activities (*16-21*). Taken together, previous findings support the widely-accepted view that dormant spores have nearly ceased all their activities (*1*), with further investigations required to quantitatively clarify how "nearly" ceased they truly are. Given that dormant spores appear lifeless and that, as time passes by without nutrients, they eventually die - meaning that nutrients can no longer revive them - an intriguing and underexplored question is how spores transition over time from being dormant to being dead (i.e., what sets dormancy's lifetime?). This question is relevant for better understanding how a ceased life is different from being dead and addressing it may reveal deep insights into dormancy - which remains poorly understood as emerging discoveries show - and the meaning of cellular death. We sought to address this question with yeast spores.

To address our question, we first reasoned that a dormant spore's lifespan is likely encoded by abundances 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 (*11,22-28*). 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 (*23*). 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 spore's lifespan, which can be months or decades (*29*) 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 all of those factors' abundances change (decrease) over time within a dormant spore, and then deducing when the spore dies, thereby determining all combinations of intracellular factors and their abundances that are necessary for sustaining dormancy. But this approach remains elusive because, for one, it requires using a current snapshot of numbers (i.e., abundances 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 discovered a single, easy-to-measure, systems-level metric - one that emerges from many intracellular factors working together - that quantitatively reveals how a yeast spore without nutrients gradually loses its dormancy and dies. Thus, we establish here a view of dormancy as a quantity that a spore loses over time with a measurable rate. 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 replicative life and that those that do not germinate are primed - they undergo accelerated germinations if they encounter more glucose hours-to-days later. These phenomena, which were previously unnoticed, led us to investigate why only a fraction of genetically identical, dormant yeast-spores in a population germinate despite having ample glucose. To investigate this, we used a synthetic circuit to induce - in dormant spores without any nutrients (i.e., in plain water) - an expression of a gene (*GFP*) that plays no role in germination. We found that a dormant spore's ability to express a gene without nutrients - a quantifiable systems-level quantity that embodies multiple factors (e.g., ribosomes, RNA polymerases) and measured by GFP - defines the spore's capacity to germinate. Namely, it determines the minimum glucose-concentration needed to guarantee that the spore germinates and, for each glucose concentration below this amount, a probability of germinating. Then by measuring how fast a dormant spore loses its gene-expressing ability (i.e., its capacity to germinate) without nutrients - the rate of losing dormancy - we determined that yeast spores lose dormancy in a predictable manner, allowing us to look at how "green" a spore can be now to determines when it will die hours-to-months ahead of its actual death. Taken together, these results establish a concept of "dormancy spectrum" - dormancy as a ruler - for yeast spores. A dormant yeast-spore's position on this ruler, set by its ability to express a gene without nutrients, simultaneously measures its proximity to death (i.e., lifespan) and to replicative life (i.e., ability to

germinate). As the spore loses this ability, it slides across the ruler towards the end that marks its death. While establishing this picture, we uncovered dormant spores' gene-expression dynamics that starkly differ from that of vegetative cells, which raises underexplored questions regarding gene regulations in dormant spores. Our work may guide future studies that investigate how dormant microbes and mammalian cells gradually lose their dormancy and die. Moreover, by providing insights into how dormant spores die, our work may also help in improving ways to kill infectious spores that are currently causing costly problems in health and agriculture (*30,31*).

## RESULTS

### Not all genetically identical spores germinate despite encountering ample glucose

We began our study by re-examining the conventional test for determining whether a yeast spore is dormant or dead, which involves giving ample glucose to yeast spores and then observing whether they germinate or not (*12,32*) (Fig. 1a). 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. But the test 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 between a spore that died *while* it was being formed versus a spore that was alive (dormant) after forming but died *during* its dormancy (and why and when the death occurred). To re-examine this test, we first asked if all yeast spores can indeed germinate after receiving ample glucose (Fig. 1a). By starving them of nutrients, a laboratory-standard ("wild type") homozygous diploid yeasts formed genetically identical, haploid spores. Specifically, each diploid cell formed a single "spore bag" (i.e., ascus) that contained four genetically identical haploid spores (Fig. 1a). We incubated a population of these spore bags in a minimal medium which has all the essential amino acids and - as a modification to the conventional test (Fig. 1a) - supplemented by a less-than-saturating concentration of glucose rather than the usual, saturating concentration (2%). We used a wide range of glucose concentrations that spanned a 10,000-fold range, from 0.0002% to 2%. For each glucose concentration, we used a wide-field microscope to observe individual spore bags and count how many of them germinated - that is, how many spore bags contained at least one spore that germinated (i.e., replicated) - as a function of time after we added glucose (Fig. 1b and Supplementary Fig. 1). We focused on spore bags instead of individual spores that are within each spore bag because we sought to assess whether a diploid cell successfully formed at least

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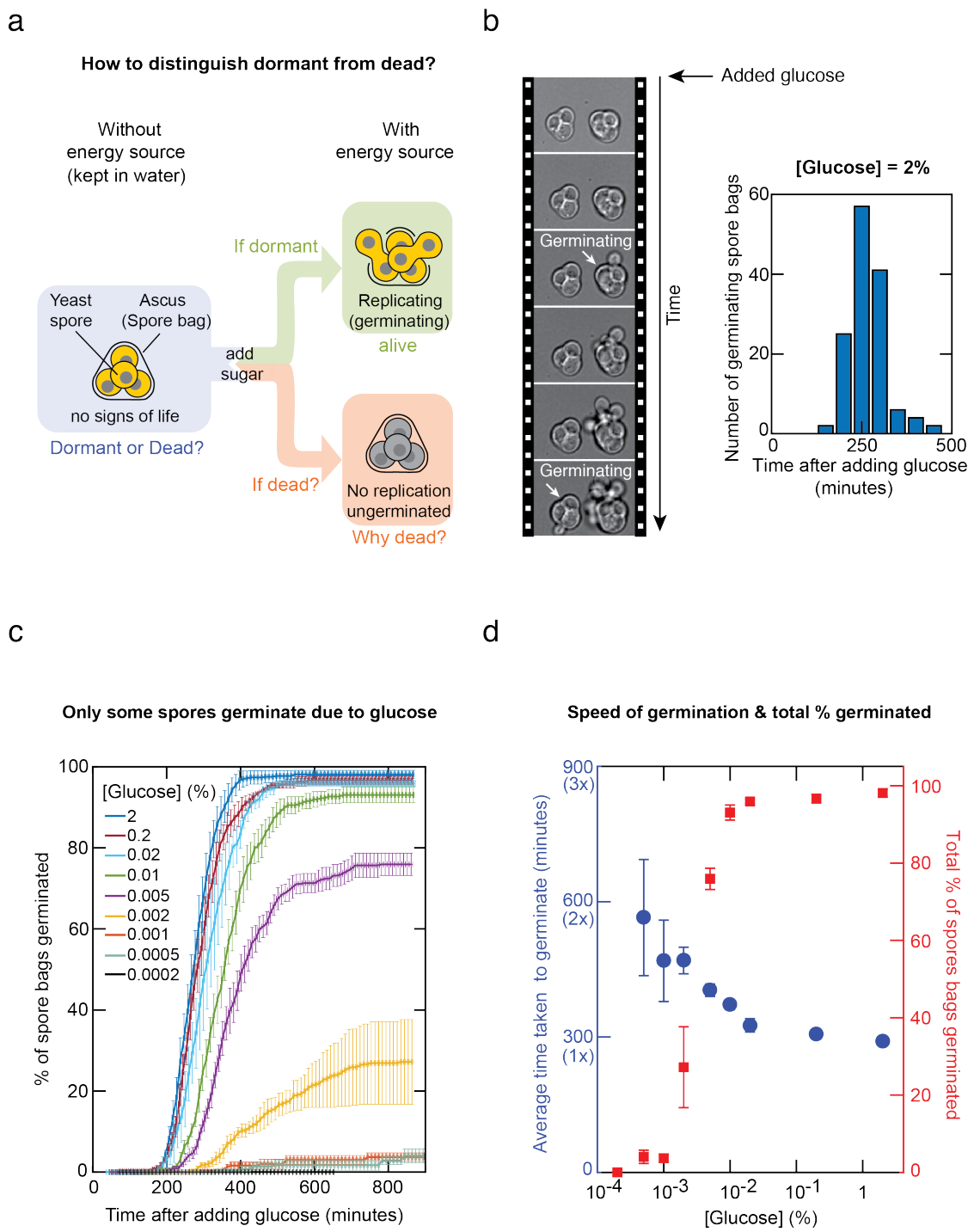


**Figure 1 | Glucose germinates only a fraction of yeast spore bags.**
**a,** Conventional test to determine whether a spore bag (i.e., ascus) with four haploid spores, in the absence of any external nutrients (blue box), is dormant or dead. Green box: Outcome if dormant. Red box: Outcome if dead. **b,** Left: Filmstrip of a time-lapse movie in which a 2%-glucose is added at the beginning of the movie. 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 function of time after adding a fixed concentration of 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) at 16 hours (960 minutes) after adding 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.

one spore that replicates after glucose reappears. With a saturating glucose concentration (2%), nearly every spore bag in the population germinated (Fig. 1c). But with lower glucose concentrations (i.e., less than ~0.01%), a noticeable percentage of spore bags in the population (i.e., ~10% or more) did not germinate regardless of how many hours we waited after adding the glucose (Fig. 1c). The percentage of spore bags that germinated followed a sharp, step-like (sigmoidal) function of the glucose concentration (Fig. 1d - red points) with the step-up located at a glucose-concentration of ~0.003% (i.e., at this concentration, ~50% of the spore bags germinate). In contrast, the average time taken to germinate weakly depended on the glucose concentration, increasing by at most 2-folds despite a 10,000-fold decrease in the glucose concentration from 2% to 0.0002% (Fig. 1d – blue points), indicating that glucose weakly affects the speed of germination. Importantly, the germinations did not stop because the spores ran out of glucose for any of the glucose concentrations that we used because when we measured the glucose concentrations in the media after no more germinations occurred (i.e., ~10 hours (~600 minutes) after adding glucose for all glucose concentrations), we found that there was always a large fraction of the original glucose left in the media and, importantly, that the glucose concentration hardly decreased for the very low glucose concentrations that we used (e.g., 0.002% and 0.001%) (Supplementary Fig. 2). Moreover, we observed that the wild-type diploid, vegetative cells - the same cells that formed the spores - could replicate multiple times even at the lowest glucose concentration (i.e., 0.0002%) (Supplementary Fig. 3), meaning that even the lowest glucose concentration was ample enough for a cell to divide multiple times. These results establish that yeast spores do not necessarily germinate in an environment with ample glucose.

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

As an answer to why some spores do not germinate 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 and thus will not germinate even after encountering more glucose. The other possibility was that the un-germinated spore bags were still able to germinate and thus not dead. To distinguish these two possibilities, we repeated the above experiments but now by adding glucose in two steps (Fig. 2a). First, we gave a relatively low concentration of glucose to the spores. We then waited, typically ~16 hours (~1000 minutes), by which point no more germinations occurred. Then, we added more glucose to increase the total glucose concentration and then observed if any more germinations subsequently occurred. We found that some of the spore bags that did not germinate after receiving the first glucose germinated after

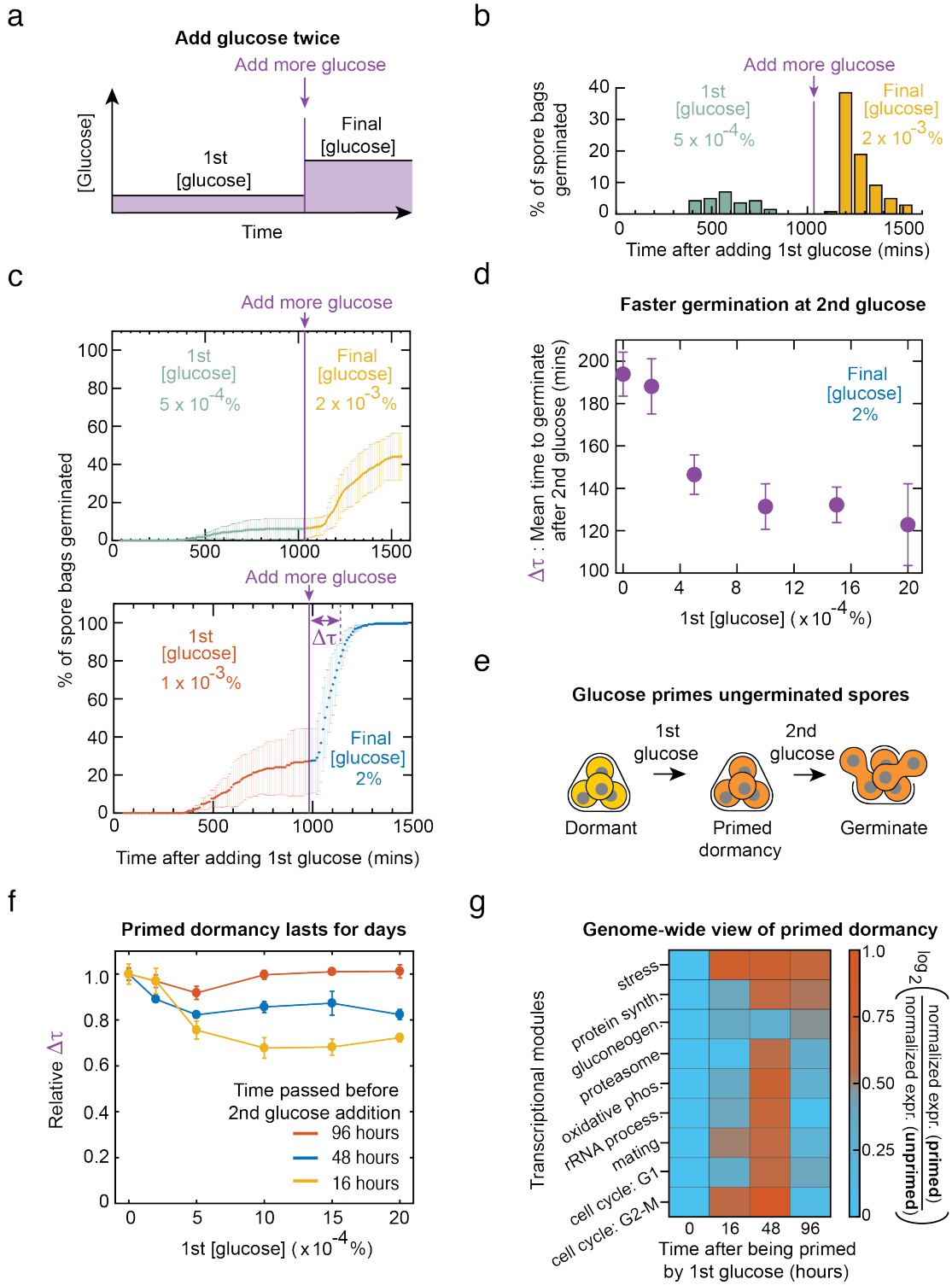


**Figure 2 | Un-germinated spores, primed for days by a low glucose concentration, germinate faster upon encountering more glucose.**

**a,** Wild-type spores are first incubated in a low glucose-concentration before we add more glucose at a later time to increase the glucose concentration. **b,** Time taken by each spore bag to germinate for the experiment in (a). 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 experiment in (a), percentage of spore bags that germinated as function of time after glucose addition in two steps (concentrations as indicated). Second glucose added at 1000 minutes (purple vertical line). $n$ = 3; error bars are s.e.m. **d,** Average time taken for a spore bag to germinate in the experiment shown in (a), due to the second added glucose (denoted **Δτ** in bottom panel of (c)). The final glucose concentration was varied and the final concentration was 2%. $n$ = 3; error bars are s.e.m.

**e,** Spore bags that do not germinate after encountering the first glucose-concentration are "primed" to germinate faster upon encountering more glucose. **f,** Average time taken for a spore bag to germinate ($\Delta\tau$) in the experiment shown in (a) after adding the second glucose (final glucose concentration is 2%) as a function of the first, low glucose concentration. Different colors represent different times at which the second glucose was added: 16 hours (yellow), 48 hours (blue) and 96 hours (red). "Relative $\Delta\tau$" is the average time $\Delta\tau$ divided by the $\Delta\tau$ for spore bags that were incubated in minimal media without any glucose (0%) for the same duration of time, before they received 2%-glucose. $n = 3$; error bars are s.e.m. **g,** Heat map showing transcriptome-wide changes in un-germinated, primed spores at 0, 16, 48, and 96 hours after being primed by a 0.002%-glucose (obtained with RNA-Seq). See Supplementary Table 1 for a list of genes for each transcriptional module (rows of heat map) and also see Supplementary Fig. 8. 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 primed and unprimed spores (the latter were incubated in minimal media without glucose for 0, 16, 48, and 96 hours). We then averaged these values over all genes in a given transcriptional module, yielding one value of "normalized expression (primed)" and one value of "normalized expression (unprimed)" for each transcriptional module. Colors represent ratio of these two values, averaged over three biological replicates ($n = 3$).

receiving more glucose (Fig. 2b and Supplementary Figs. 4-5). Yet, some of the spore bags still remained un-germinated after receiving the second batch of glucose if the final concentration was not the saturating 2% (Fig. 2c - top panel). For example, when the final concentration became 0.002%, after the second glucose, we still observed that nearly 60% of the spore bags remained un-germinated. Intriguingly, this 60% is close to the percentage that would not have germinated if we had given the 0.002% of glucose all at once instead of in two steps (compare Fig. 2c with Fig. 1c), hinting that each spore bag was 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 if they encounter more glucose days 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 encountered. When we added glucose in two steps so that the final concentration was 2% (Fig. 2a), we found that the spore bags took less times to germinate to the second batch of glucose than they would have if they had received the entire 2%-glucose all at once without encountering a lesser amount of glucose first (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 later, 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%, we still observed the sign of primed dormancy - a faster germination compared to the spores that were kept in minimal media without any glucose for the same amount of time - up to two days but not four days after the first glucose-addition (Fig. 2f and Supplementary Fig. 6). 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 gene-expression signatures of primed dormancy. To do so, 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 (*33*), to isolate the un-germinated spores from the surrounding vegetative cells (Supplementary Fig. 7) and 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 (*32*) that analyzed the yeast spores' transcriptome as they germinated after receiving a 2%-glucose over several hours, we grouped multiple genes together into a set, called a "transcriptional module" (*32,34*), if those genes are involved in the same process (e.g., protein synthesis) (Supplementary Table 1). We averaged the expression levels of all genes in a given module to obtain one expression-level for that module, for both the primed and un-primed spores. For six of nine transcriptional modules, we found that the primed spores had higher expression levels than the un-primed spores after 16 hours and 48 hours of incubations whereas both types of spores had nearly the same expression level after four days of incubation (Fig. 2g - last six rows and Supplementary Fig. 8). This trend mirrors the trend that we observed in the average time taken by primed spores to germinate (i.e., accelerated germinations up to 48 hours after being primed but no accelerated germinations after four days) (Fig. 2f). Two transcriptional modules showed this trend in a particularly pronounced manner. One of them is the module for mating (*35*), which the resulting haploid cells carry out after the germination (Fig. 2g - seventh row). The other is the module for transitioning from cell cycle's G2-phase to mitosis (Fig. 2g - last row and Supplementary Fig. 8A), which is a crucial final

step of germination. These results make sense for accelerating germinations. Together, these results establish that very low glucose concentrations can trigger transcriptome-wide changes in un-germinated spores to accelerate their potential, future germinations up to days later.

**Hypothesis on why only some yeast spores germinate for low glucose-concentrations**

Although we now understand how un-germinated spores respond to glucose, we have not addressed the question of what determines, in the first place, which spore bags germinate and which do not. As an answer, we hypothesized that each diploid cell forms a spore bag with a distinct "internal state". An internal state may be defined by a broad set of factors, including the amounts of ATPs or amino acids or ribosomes that are stored inside the spore bag or combinations of these or other stored molecules that serve as "starting materials" for entering replicative life. We then hypothesized that, for each glucose concentration, only some of the spore bags have the "right" internal states that allow for germination. Our experiments thus far involved giving glucose to spores *and then* observing their subsequent actions, including for primed dormancy. But in such experiments, due to all measurements occurring *after* the spores receive glucose, we cannot infer the spores' internal states that existed *before* they encountered glucose and thus we sought to manipulate the internal states without glucose. In particular, we reasoned that depleting any internal resources (e.g., ATPs or amino acids) that are stored inside spores *before* adding glucose would either decrease - or alter in more complex ways - the percentage of spore bags that germinate for each glucose concentration.

**Synthetic circuit to Induce gene-expression in dormant yeast-spores without any nutrients**

To test our hypothesis, we first built a synthetic gene-circuit in vegetative diploid yeast cells so that doxycyline - an inducer molecule - would cause the cells to produce the Green Fluorescent Protein (GFP). This synthetic circuit functioned in such a way that increasing the doxycyline concentration increased the cell's GFP production. We formed spores out of these engineered diploid cells (Fig. 3a). We reasoned that if doxycyline can induce GFP expression in these spores without any nutrients (e.g., in plain water without amino acids and glucose), then we might deplete their stored resources and thereby alter the percentage of spore bags that germinate for a given glucose concentration. But it was unclear whether it was possible to induce the expression of *GFP* or any arbitrary gene in dormant yeast-spores without nutrients in the first place. For one, if that were possible, then it is unclear why, apparently, the expression of almost all the genes in dormant

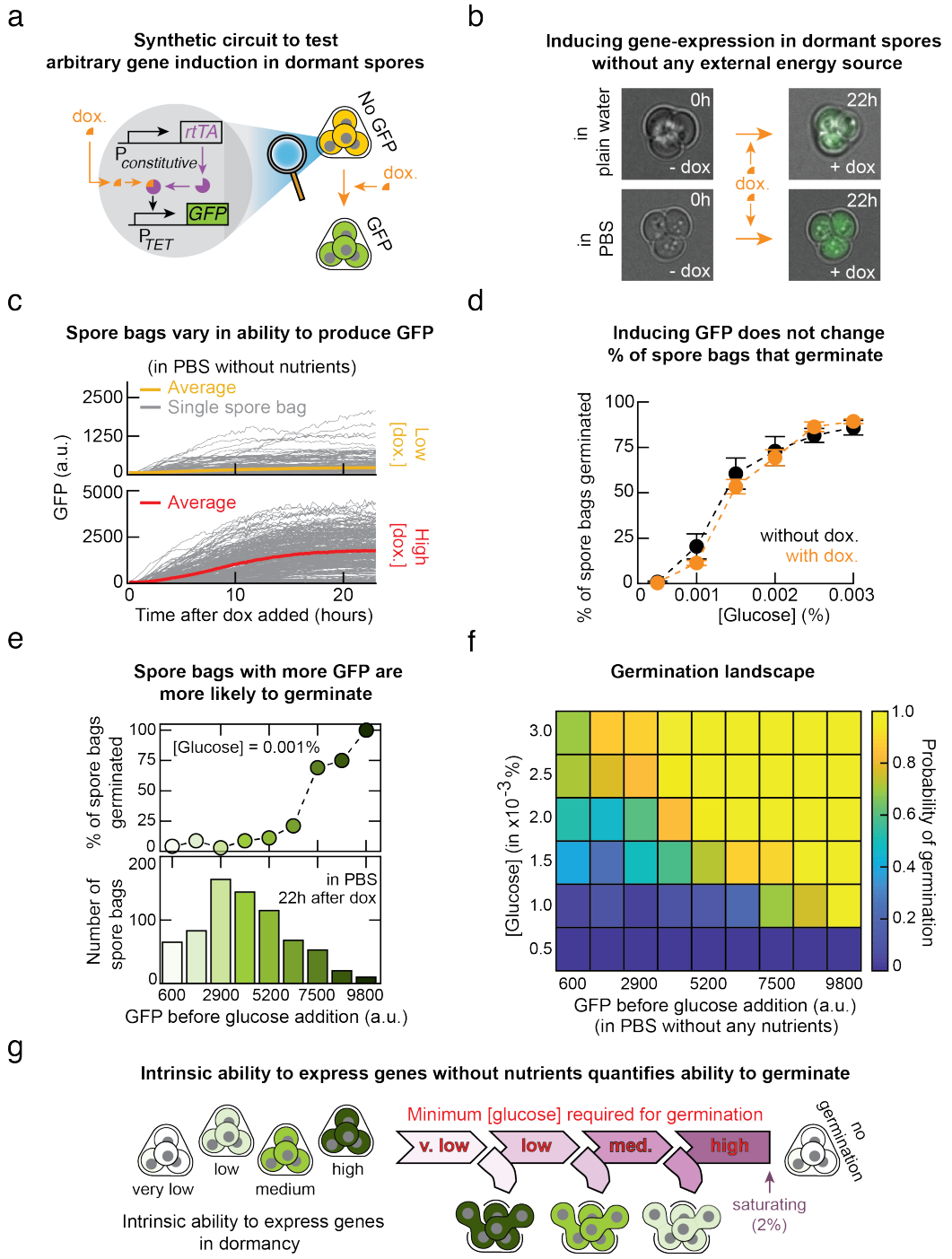


**Figure 3 | Synthetically inducing gene-expression in dormant yeast-spores without nutrients leads to germination landscape, which shows that ability to express a gene (e.g., *GFP*) without nutrients quantifies how likely a spore bag will germinate for each glucose concentration.**

**a,** 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 production. **b,** Engineered spore bags (shown in (a)) transcribe and translate GFP in plain water with 25 μg/ml of doxycycline (top row) and in a saline solution (PBS) with 50 μg/ml of doxycycline (bottom row) without nutrients. Snapshots of GFP expression shown at 22 hours after adding doxycyline**. 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 doxycyline or with 100 μg/ml of doxycycline before they were transferred to minimal media with various glucose concentrations. Plot shows the total percentage of the engineered spore bags that germinated (measured 20 hours after incubating with glucose) for those 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 (in experiment in (d)) that germinated after receiving a 0.001%-glucose. Percentages are averaged over all spore bags with the same binned GFP-level (corresponding histogram shown below). Data from a representative population of spore bags ($n$ = 145 spore bags in a population). **f,** Germination landscape: Colors represent the probability that a spore bag with a particular steady-state GFP-level 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 giving glucose concentrations indicated along the rows. Columns indicate steady-state GFP-level of a spore bag at 22 hours after adding the doxycyline. Each pixel is an average over 3 replicate populations ($n$ = 3). **g,** Given a spore bag, its steady-state GFP-level is a read-out of both its intrinsic ability to express a gene without any nutrients and the minimal glucose concentration that it needs for germination. Spore bags with lesser gene-expressing abilities without nutrients require more glucose to germinate.

yeast-spores are suppressed (*1*) since being able to induce GFP-expression would mean that there must be active RNA polymerases and ribosomes, and chromosomal regions that are accessible to them. Moreover, recent studies established that starved yeasts, dormant yeast-spores, and other dormant fungal spores have a solid-like, glassy cytoplasm that is packed with macroscopic aggregates of proteins and mRNPs that would inhibit gene-expression machineries and movement of molecules (*14,15*). By aggregating, key proteins needed for metabolism and gene-expression would be inactivated as well (*16-21*). Indeed, to date, direct observations of gene-expression dynamics in individual dormant yeast-spores without nutrients has been lacking. A previous, bulk-level (population-level) study (*12*) has shown that there are transient, constitutive expressions of two genes - *PGK1* (involved in gluconeogenesis) and *SPS100* (involved in forming spore walls during spore formation) - that turn off a few days after spore formation (i.e., during entrance into dormancy). But it remains unclear whether their expressions completely turn off or just decrease to vanishingly low, but non-zero levels a few days after spore formation. This is because these bulk-level measurements were based on finding ribosomes bound to mRNAs in lysates of populations of yeast-spores, which does not have the necessary sensitivity. Moreover, the ribosomes bound to mRNAs may be from the macroscopic aggregates that formed before the spores entered dormancy (*14,15*), which may disable translation of those ribosome-bound mRNAs (*16-21*). 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 (*12*). This finding indicates that transcription (proxied by the depleted uracil) and translation (proxied by the depleted methionine) may be possible in dormant yeast-spores, though these are indirect measurements since they did not directly visualize gene-expression dynamics inside spores. Imaging gene-induction dynamics in individual yeast-spores would provide a definitive answer that resolves these ambiguities. To that end and to test if GFP-induction was possible for testing our hypothesis for why only some spores germinate for a given glucose concentration, we incubated the engineered spores in either water or a saline solution (PBS) with only doxycyline. Surprisingly, we discovered that doxycycline fully induced

transcription and translation of GFP in these spores (Fig. 3b and Supplementary Figs. 9-10). Crucially, varying the doxycycline concentration in water and PBS tuned the spores' GFP levels over a similarly wide-range of values as in the vegetative cells with the same synthetic circuit (Supplementary Figs. 9-11). We found that both the rate of GFP-production and the final (steady-state) level of GFP widely varied among spore bags in the same population (Fig. 3c and Supplementary Fig. 12). The most striking feature, however, was that all spore bags expressed GFP very slowly – GFP levels plateaued at steady-state values only after ~20 hours of doxycyline induction whereas they plateaued after ~8 hours in vegetative cells with the same circuit (Supplementary Fig. 11). But a more puzzling discovery was that the spores' GFP-levels stabilized at steady-state values in the first place. After all, the spores were not dividing and hence their GFP - a highly stable protein - could not be diluted away by cell divisions. In replicating 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 (Supplementary Fig. 13), meaning that GFP levels reached steady state values in the dormant spores because they stopped producing GFP after a day, despite the doxycycline still being present. Together, these results provide a direct proof, within individual spores, that one can fully activate - to the level of vegetative yeasts - transcription and translation of an arbitrary gene in dormant yeast-spores without any nutrients and that their dynamics can be starkly different from that of vegetative yeasts.

**Synthetic circuit shows that activating GFP expression in dormant yeast-spores does not alter percentages of spore bags that germinate**

To test the hypothesis that depleting the spores' internal resources by expressing GFP without nutrients would hinder spore bags' ability to germinate, we incubated the spores in PBS with a high doxycycline concentration (100 μg/ml) for 24 hours so that their GFP levels would reach steady-state values (Fig. 3c and Supplementary Fig. 13). We then removed the PBS with doxycycline and transferred the spores to a minimal medium with a fixed glucose concentration. We then measured the percentage of these spore bags that germinated and, as a control, compared it with the germination percentage of spore bags that received the same glucose concentration after being incubated for 24 hours in PBS without doxycyline (Fig. 3d and Supplementary Fig. 14). For all glucose concentrations, we found that inducing GFP expression did not appreciably alter the percentage of spore bags that germinated and that it also did not

appreciably alter the average times taken for germinations (Supplementary Fig. 15). Thus, our hypothesis is incorrect - expressing GFP does not alter the spores' ability to germinate. While it may now appear that we are "back to square one" - since we still have not yet uncovered what causes only some of the spore bags to germinate for a given glucose concentration - we discovered the answer, as we will next show, by measuring the GFP levels of individual spore bags.

**Spore bag's GFP-level quantifies its probability of germinating for every glucose concentration and dormant spores' ability to express genes without nutrients**

In the above experiment, by measuring the steady-state GFP levels of individual spore bags just before they encounter glucose, we discovered that spore bags that produced more GFP were more likely to germinate (Fig. 3e and Supplementary Figs. 16-17). As an example, after encountering a 0.001%-glucose, nearly 100% of the spore bags that had the highest, steady-state GFP levels germinated whereas, in the same population, only ~10% of the spore bags with half of this GFP-level germinated. In fact, for each glucose concentration, we could precisely determine the probability of germinating for a spore bag once we knew its GFP level (Supplementary Fig. 17). We thereby established a quantitative relationship between the GFP level and the ability to germinate, rather than a qualitative relationship such as "spores that can express GFP can germinate whereas those that cannot express GFP do not germinate". Importantly, this quantitative link between the steady-state GFP-level and the probability of germinating establishes that the stochastic variability (*36,37*) in the induced GFP-expression among dormant spores is meaningful and predictive, despite GFP not having any obvious connection to the complex, multi-step process that leads to germinations. Crucially, since *GFP* is a generic gene without a functional role in germinations, it is reasonable to view the GFP-level as quantifying the spore bag's intrinsic ability to express an arbitrary, generic gene that is induced without nutrients, in accordance with how one defines the "extrinsic noise" by using cell-to-cell variability in fluorescent-protein levels (*36-38*).

**Germination landscape represents probability of germinating as function of glucose concentration and spores' ability to express genes without nutrients**

To visually represent our results, we plotted a “germination landscape” - a heat map whose color represents a probability that a spore bag with a given steady-state GFP-level germinates for each

glucose concentration (Fig. 3f and Supplementary Fig. 18). For brevity, from now on, we will refer to "steady-state GFP level" simply as "GFP level". 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 (Fig. 3f) 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 GFP level decreases), meaning that more glucose is required to guarantee a germination for a spore bag with a lesser GFP. The blue-green pixels are almost immediately below the coastline of yellow pixels, indicating that the probability of germinating, for a fixed GFP-level, 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 GFP level to have a 99%-chance of germinating (Supplementary Fig. 19). We call this concentration, given the sharpness of the nearly step-like probability function, the "minimum glucose-concentration required for germination". We determined that as a spore bag's ability to express GFP decreases, the minimum glucose-concentration required for germination increases (Fig 3g and Supplementary Fig. 19). Importantly, since inducing GFP-production does not alter the total percentage of spore bags that germinate for any glucose concentration (Fig. 3d), inducing GFP-production in a spore bag does not change (increase or decrease) its probability of germinating. The GFP level is thus merely an indicator for which spore bags are more likely to germinate and, as previously mentioned, a quantifier of an ability to express a generic gene without nutrients. Thus, we can state that spore bags with a higher ability to express a generic gene without nutrients, compared to those with a lesser ability, are more likely to germinate and require lesser glucose to germinate (Fig. 3g and Supplementary Fig. 19C).

**Hypothesis on how dormant yeast-spores die: Continual loss of gene-expressing ability gradually increases glucose-concentration required for germination until it goes above saturating level**

Having identified a quantifiable intrinsic capacity of a spore bag to germinate - the spore bag's intrinsic ability to express a generic gene - we now turn to how this capacity changes over time and whether it can reveal how and when dormant spores die. Being a dead spore means that it

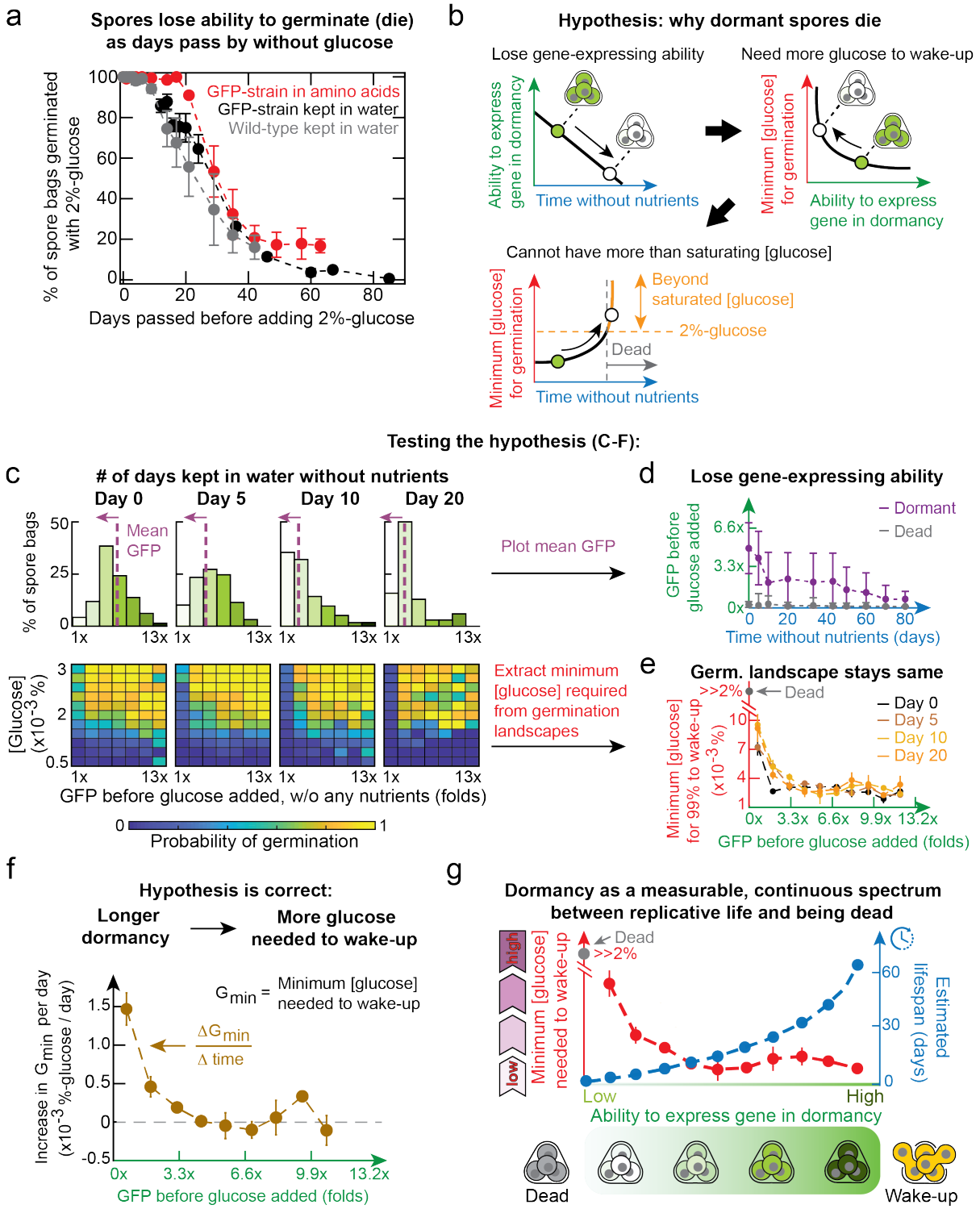


**Figure 4 | Dormancy as a quantifiable spectrum ("ruler") that measures and forecasts dormant yeast-spore's proximity to death (lifespan) and proximity to entering replicative life (germination capacity).**
**a,** Percentage of wild-type and GFP-inducible spore-bags (Fig. 3a) that germinate due to a saturating glucose-concentration (2%) that they encounter after 0 - 85 days of incubation in water (grey and black data) or minimal medium with essential amino acids (red data) at 30 $^{O}$C. $n$ = 3; error bars are s.e.m. **b,** Hypothesis on how dormancy-to-death transition gradually occurs: Dormant spore bag loses its gene-expressing ability over time (Top left), thus it needs a higher glucose concentration to germinate (Top right), which in turn causes it to require an ever-more increasing glucose concentration for germination. Eventually the glucose concentration goes above the saturating level (2%), which is impossible to obtain and thus the spore bag will never germinate (i.e., dead) (Bottom).

**c,** Top row**:** Steady-state GFP-levels of individual spore bags measured as described in Supplementary Fig. 21, after 0, 5,10 or 20 days of incubation (left to right) at 30 °C in plain water (see Supplementary Fig. 22 for data up to 80 days). Dead spore bags' GFP levels are not shown. Bottom row: germination landscape measured for these spores for 0, 5, 10 or 20 days (left to right). Each pixel's color is from averaging over 3 replicate populations (*n* = 3). **d,** Mean GFP-level of dormant (purple) and dead (grey) spore bags that were incubated in water without nutrients at 30 $^{O}$C over ~80 days (also see Supplementary Fig. 21). The purple data points are the average GFP-levels from the histograms shown in (c) and Supplementary Fig. 22. Grey data points are averages of the dead spore bags' histograms shown in Supplementary Fig. 22. *n*=3, error bars represent the average standard deviation from three biological replicates. **e,** Average, minimum glucose-concentration required for germination, '*h*' (in equation (1)) extracted from the four germination landscapes shown in (c) as a function of the gene-expressing ability (GFP-level) of dormant spore bags (see Supplementary Fig. 19 for the extraction method) incubated in water for 0 days (black), 5 days (brown), 10 days (yellow), and 20 days (orange). *n*=3, error bars are s.e.m. Dead spore bags represented by a grey point (at above saturating glucose levels). **f,** Testing the hypothesis posed in (b): $dG_{min}/dt$ in equation (1) after combining results in (d-e) (see Supplementary Fig. 23 for details). *n* = 3, error bars are s.e.m. **g,** Dormant spore bag's ability to express a gene without nutrients defines its position on the "dormancy spectrum" (bottom cartoon). The spore bag's position on the spectrum determines the minimum glucose-concentration that it needs for germination (red curve is the day-10 data in (e)) and its lifespan (blue curve is from the same mathematical analysis as in (c-f) (see Supplementary Fig. 24-25 for details)).

no longer has any capacity to germinate for any glucose concentration, including for the saturating 2%-glucose. While a 2%-glucose caused nearly every spore bag to germinate in the experiments described thus far (Fig. 1c), noticeable fractions of spore bags did not germinate even after receiving a 2%-glucose if we incubated them for days or weeks in either water or minimal medium without glucose at 30 $^{O}$C (Fig. 4a). Concretely, regardless of whether we kept the spores in water or minimal medium (which has all the essential amino acids), we found that the number of dormant (alive) spore bags - the ones that germinated after receiving a 2%-glucose - decreased by similar rates over several weeks. Specifically, about half of the spore bags in a population died after ~20 days without glucose and almost everyone died after ~60 days without glucose (Fig. 4a). Furthermore, we observed that spore bags needed more time to germinate as the number of days without nutrients increased (Supplementary Fig. 20), suggesting that a spore bag's germination ability gradually deteriorates rather than suddenly, in an all-or-none type manner, going to zero. Motivated by these observations, we hypothesized that if the germination landscape (Fig. 3f) still applies to spores that are incubated for days without nutrients, then the dormant spore bags with nearly zero GFP levels - these would have a close-to-zero ability to express genes - may not germinate even after receiving a 2%-glucose. This is because the minimum glucose-concentration required for germination keeps increasing sharply as a spore bag's gene-expressing ability (GFP-level) decreases towards zero (Supplementary Fig. 19C). Thus, we formed the following hypothesis on how dormancy might transition to death over time (Fig. 4b): Dormant spores would gradually lose their ability to express genes without nutrients due to, for example, their intracellular components naturally (thermally) degrading over time (Fig. 4b - top left panel). The consequence of this would be that a spore bag would require progressively higher concentrations of glucose to germinate (Fig. 4b - top right panel), with the required concentration eventually going above the saturating, 2% (Fig. 4b - bottom panel). At this point, the spore would

be considered dead since it can never germinate regardless of how much glucose it encounters.

**Mathematical analysis based solely on measurable quantities tests hypothesis on how dormant spores die**

The main challenge in testing the above hypothesis is that we cannot directly measure, for the same spore bag, the minimum glucose-concentration that it requires for germination (denoted $G_{min}$) on multiple different days because that would require giving glucose multiple times to the same spore bag. But each glucose-encounter may alter the spore bag's internal state and thus interrupt the natural, temporal change in $G_{min}$ that would have otherwise occurred. To circumvent this experimental limitation, we developed a mathematical analysis that only relies on directly measurable quantities to infer how a spore bag's $G_{min}$ changes over time. We defined $G_{min}$ as a mathematical function of time '$t$' and temporally changing gene-expressing ability (denoted $\text{ability}(t)$): $G_{min}(\text{ability}(t), t) = h(\text{ability}(t)) + \eta(t)$. Here, $'h(\text{ability})'$ is the average, minimum glucose-concentration required for germination by a spore bag with a particular GFP-level ('ability'), which we extract from the germination landscape (Supplementary Fig. 19c). '$\eta$' is the noise that describes how $G_{min}$ varies among spore bags of the same GFP level (i.e., averaging $G_{min}$ over all spore bags of the same GFP level yields $'h'$). Then, a spore bag's $G_{min}$ changes over time as

$$\frac{dG_{min}}{dt} = \frac{d(\text{ability})}{dt}\frac{\partial h}{\partial(\text{ability})} + \frac{d\eta}{dt} \qquad \text{(Equation 1)}$$

Measuring the three terms that appear on the right side of equation (1) would determine if the rate of change of $G_{min}$ over time (i.e., $dG_{min}/dt$) is always positive as we hypothesized (i.e., spore bag requires progressively more glucose over time to germinate). To determine $d(\text{ability})/dt$ - the rate at which the GFP-expressing ability changes over time - we incubated the GFP-inducible spores in water without nutrients for 80 days. On various days, we took out some of the spore bags, incubated them with doxycycline in PBS for 24 hours (Supplementary Fig. 21), measured their resulting GFP levels (Fig. 4c-d and Supplementary Fig. 22), and thus determined how the GFP-expressing ability changed over the 80 days (i.e.,$d(\text{ability})/dt$) (Supplementary Fig. 21). Over the 80 days, we observed the dormant spore bags' GFP levels decreasing (Fig. 4d - purple points) and approaching the GFP-levels of the dead spore bags - the ones that did not germinate to a 2%-glucose (Fig. 4a). Importantly, we found the dead spore bags' GFP levels, which were low but non-zero for many of them (Fig. 4d - grey standard-deviation bars), overlapping with the lowest GFP-levels of dormant spore bags on later days (Fig. 4d - purple

standard-deviation bars; Supplementary Fig. 22), supporting the gradual dormancy-to-death transition that we hypothesized and the idea that spores with sufficiently low, but non-zero, GFP-level may be dead. Measuring the germination landscape on various days (Fig. 4c) determined the other two terms of equation (1): $\partial h/\partial(\text{ability})$ (i.e., how the '*h*' changes with GFP level) and $\partial\eta/\partial t$ (i.e., how the noise changes over time) (Fig. 4e and Supplementary Fig. 23). We found that the germination landscapes' main feature - the value of '*h*' - virtually remained unchanged over time (Fig. 4e), meaning that $\partial\eta/\partial t$ is nearly zero. Intuitively, this means that a population of spore bags, over time, migrate from right to left (i.e., high to low GFP levels) on a fixed germination landscape.

**Mathematical analysis that links multiple measurements confirms hypothesis on how dormant spores die**

Combining all three measured terms that appear in equation (1) determined that $dG_{min}/dt$ is either positive or nearly zero for all spore bags (Fig. 4f), thus confirming our hypothesis. That is, as a spore bag's gene-expressing ability decreases, $dG_{min}/dt$ becomes more positive, meaning that the minimum glucose-concentration required for germination increases faster over time as a spore bag's gene-expressing ability gradually decreases over time (Fig. 4f). Thus, the minimum glucose-concentration required for germination eventually goes above the saturating 2%, at which point the spore bag is dead since it cannot germinate anymore. Crucially, the mathematical analysis forecasts for how many days a spore bag can survive without nutrients given its *current* GFP-level (Fig. 4g - blue curve and Supplementary Figs. 24-25). The analysis reveals that spore bags with low gene-expressing abilities will likely live for 1-5 more days whereas the ones with the highest gene-expression abilities will likely live the longest, for 50-60 more days. These results are in close agreement with our measurements of the spore casualties (Fig. 4a) in which we found virtually no survivors after 60 days without nutrients. Thus, our mathematical analysis successfully elucidated how dormancy progressively transitions to death (Supplementary Figs. 24-25). Notably, by looking at how "green" the yeast spores are now, one can determine how much longer they can live (remain dormant) before dying.

## DISCUSSION

**Dormancy spectrum: Dormancy as a systems-level quantity that decreases over time at predictable rates as dormant spores approach their deaths**

506 Our study began with two broad, related questions: how an organism whose life has ceased can
507 still remain alive and how we can monitor its approach to death given that it either completely
508 lacks any discernable intracellular dynamics (i.e., all intracellular processes have halted) or may
509 have faint intracellular dynamics that exist but are difficult to clearly measure. The difficulty in
510 addressing these questions lies in the fact that we usually associate being alive with being
511 dynamic on multiple fronts, such as being metabolically active, having active gene-expressions,
512 and possibly being motile. This is often not the case for dormant cells. A microbial spore, while it
513 can have active intracellular dynamics for hours-to-days after forming (*11-13*), eventually enters
514 dormancy during which it either completely lacks any discernible intracellular dynamics (*1*) or has
515 greatly reduced levels of dynamics that are challenging to unambiguously measure in individual,
516 dormant spores (*11,12*). In a practical sense, being alive *now* as a dormant spore means that -
517 despite appearing static - it has the potential to wake-up and re-enter replicative life if nutrients
518 were to suddenly reappear at this very moment. Thus, a brute-force approach to addressing
519 whether a given spore is dormant or dead would be to infer - if one can measure the abundances
520 of all of its key intracellular factors - whether its static contents can, in the future, achieve complex
521 dynamics - namely, restarting replicative life. Then one would monitor how these intracellular
522 factors decay over time and infer what combinations of their abundances cannot restart a
523 replicative life. But inferring such a dynamic phenotype from a static information - the abundances
524 of all the relevant molecules - is a difficult challenge that one often faces in many areas of biology.
525 In our study, we circumvented this challenge for yeast spores by taking a different approach: we
526 identified a single, systems-level metric - the dormant yeast-spore's ability to express a gene
527 without nutrients. This is a systems-level metric because the ability to express a gene depends
528 on many factors (e.g., abundances of stored ribosomes). The metric measures the dormant
529 spore's capacity for achieving a dynamic behavior - expressing a gene - when induced to do so
530 in a non-invasive manner (i.e., the spore's ability to germinate remains unchanged after we
531 measure its gene-expressing ability with a synthetic gene-induction). This systems-level quantity
532 then decays over time in a way that we could monitor, enabling us to non-invasively reveal how
533 the loss of this capacity occurs in a predictable manner, thereby allowing us to predict dormant
534 spores' lifespans based on their current gene-expressing ability and reveal a previously hidden
535 dynamics by which the dormant yeast-spores approach their deaths.
536
537 Identifying the dormant spore's ability to express a gene without nutrients as a quantity -
538 which we can think of as an "amount of dormancy" - allows us to introduce the concept of a
539 "dormancy spectrum" (Fig. 4g). We can think of the dormancy spectrum as a ruler that

540 simultaneously measures in how many hours-to-months dormant yeast-spores will die (i.e., their
541 lifespans) and how readily they can re-enter replicative life (i.e., their "proximity" to replicative life).
542 By inducing gene-expression in dormant yeast-spores and then using GFP to quantify their
543 inherent ability to express genes without nutrients, we can place dormant yeast-spores on this
544 spectrum based on their varied gene-expressing abilities and then determine how they
545 continuously slide over time along the spectrum towards their death with a predictable speed.
546 Surprisingly, despite GFP having no functional links to cell death and spore revivability, the
547 variability in GFP-levels among genetically identical yeast-spores allowed us to precisely predict
548 their lifespans and probabilities of germinating for every glucose concentration. Taken together,
549 these findings strongly indicate that key factors that globally control transcription and translation
550 (e.g., abundance of RNA polymerase in spores) determine yeast spores' germination ability. It
551 would be interesting to investigate whether one can extend the concept of dormancy spectrum -
552 which we have uncovered here for yeast spores - to dormant mammalian cells that are poorly
553 understood such as dormant cancer cells that escape treatments for years (*41*) and dormant stem
554 cells (*42*).
555
556
557 **Dormant yeast-spores potentially hedge their bets by having only some spores germinate**
558 Our discovery that ample glucose germinates only a fraction of spores in a population, highlighting
559 cell-to-cell variability and phenotypic heterogeneity (*5,8-10,23,27,36-40*) among genetically
560 identical yeast spores, relied on deviating from the common practice of giving a saturating amount
561 of glucose to yeast spores and then observing their germinations. One way to interpret the
562 fractional germinations may be to state that a population of dormant yeast-spores hedge their
563 bets by having only a fraction of its spores germinate when less-than-saturating glucose appears,
564 thereby leaving the remainder of the population as dormant spores. This would be advantageous
565 if the glucose later disappears. This bet-hedging strategy, along with the primed dormancy that
566 we discovered, may be advantageous for yeasts given that sporulating is an energetically costly
567 and time-consuming process. This idea is in accordance with previous studies that suggested
568 that microbes in soils, many of whom are dormant, may use their dormancy as a bet-hedging
569 strategy that can improve biodiversity in soils (*43*). Beyond microbes, less-than-saturating levels
570 of the hormone, gibberellin, cause only a fraction of plant seeds to germinate for reasons that are
571 not yet fully understood (*44*). As in yeast spores, it would be interesting to check if this
572 phenomenon can be linked to dormant plant seeds' inherent ability to express genes without any
573 nutrients.

**Synthetically activating gene-expression in dormant yeast-spores raise fundamental questions regarding gene-regulations**

Our study used a synthetic gene-circuit to induce transcription and translation of a generic gene in dormant yeast-spores without any external energy source. This approach motivates future studies to use synthetic circuits to examine an underexplored topic: how the central dogma of molecular biology functions in both dormant yeast-spores and other types of dormant cells that exist without any external energy-sources. As a starting point, it would be fruitful to address several fundamental questions regarding gene-regulation in dormant yeast-spores that our work raises. These include: To what extent does a dormant yeast-spore globally repress gene-expression given that it must have active RNA polymerases and ribosomes that can express *GFP* at the level of vegetative cells? Why does gene-expression, after being induced, stop as we have shown with GFP (Fig. 3c)? Addressing this last question may address why most genes are apparently silenced in dormant yeast-spores. Addressing these questions inherently requires the kind of quantitative approaches at single-cell resolutions that we used here (*45*). More generally, applying our approach - using less-than-saturating nutrients and synthetic gene-circuits - to dormant bacterial and fungal spores and plant seeds may help us better understand their dormancy (*46,47*).

**Practical implications of our work**

Finally, by better understanding how other dormant fungal and bacterial spores die, as we have done for *S. cerevisiae* spores, one may develop more effective ways to kill fungal and bacterial spores that infect agricultural crops (e.g., head blight and rust diseases) (*30*), animals (anthrax spores), and humans (spores of *C. neoformans* and *C. difficile*) (*31*). The difficulty in killing dormant spores, which partly stems from poorly understanding what causes them to die and thus how to kill them, has been a major agricultural and health problem (*30,31*). Aside from practical considerations, by finding other systems-level metrics that monitor how a dormant cell or multicellular organism gradually approaches its death - still an underexplored phenomenon - we may gain deeper insights into both dormancy and death.

605 **Methods**
606
607
608 **Strains.** The "wild-type", homozygous diploid yeast strain that we used is from Euroscarf with the
609 official strain name "20000D" and genotype as follows: *MATa/MATα his3-11_15/his3-11_15;*
610 *leu2-3_112/leu2-3_112; ura3-1/ura3-1; trp1Δ2/ trp1Δ2; ade2-1/ade2-1; can1-100/can1-100*. This
611 strain generated four genetically identical, haploid spores for each spore bag. For engineering the
612 GFP-inducible spores, we started from the haploid versions of 20000D, which were also from
613 Euroscarf. These haploid strains were "20000A" (isogenic to another standard laboratory strain
614 called "W303" with mating-type "a") and "20000B" (isogenic to W303 with mating-type "alpha").
615 The 20000A's genotype is as follows: *MATa his3-11_15; leu2-3_112; ura3-1; trp1Δ2; ade2-1;*
616 *can1-100.* The 20000B's genotype is exactly the same as 20000A's, except that it is of the
617 opposite mating type (mating type "alpha"). The GFP-expressing diploid strain, called "TT14", is
618 nearly identical to the wild-type's except for the addition of selection-marker genes that we
619 introduced during the construction of the strain. TT14 has the following genotype: (*MATa/MATα*
620 *his3-11_15/his3-11_15; leu2-3_112/leu2-3_112; ura3-1/ura3-1; ADE2/ADE2; can1-100/can1-*
621 *100; HygB/HygB*; *trp1Δ2/TRP1; URA3/ura3-1; pADH1-rtTA/pADH1-rtTA; pTET07-GFP/pTET07-*
622 *GFP*). Here, *pADH1* is the constitutive promoter for *ADH1* gene in yeast (631 bases upstream of
623 *ADH1*'s ORF), *rtTA* is the reverse tetracycline-controlled transactivator whose transcription-
624 activation domain is from the yeast's Msn2, and *pTET07* is the promoter with 7 binding sites for
625 rtTA. We sporulated TT14 to form the "GFP-inducible spores" (Fig. 3a). These constructs are
626 more fully described in a previous publication (*48*).
627
628 **Strain construction.** For each yeast transformation, we integrated a single-copy of an
629 appropriate, linearized yeast-integrating plasmid at a desired genomic locus through a
630 homologous recombination. We first introduced a promoter of *ADH1* controlling rtTA expression
631 *pADH1-rtTA* into 20000A (wild-type haploid, mating type "a") and 20000B (wild-type haploid,
632 mating type "alpha") at the HO locus, by inserting a linearized, singly integrating, yeast-integrating
633 plasmid with *pADH1-rtTA* and Hygromycin-resistance gene as a selection marker. This yielded
634 two strains, “W303r1” (From 20000A) and “W304r1” (from 20000B). We next replaced the *ade2-*
635 *1* “ochre mutation” in W303r1 and W304r1 with a functional *ADE2* gene by a homologous
636 recombination of *ADE2* that we obtained by PCR from the S288C reference genome (100-bp
637 homology on both flanking sites of the PCR product). This yielded two strains, “TT2” (from
638 W303r1) and “TT8” (from W304r1). We then inserted a *pTET07-GFP* at the *LEU2* locus in TT2

639 and TT8 by linearizing a yeast-integration plasmid that contains *pTET07-GFP*. This yielded two
640 strains, “TT7” (from TT2) and “TT9” (from TT8). We then introduced two constitutively expressed
641 selection-marker genes: *URA3* into TT7 to create "TT10" and *TRP1* into TT9 marker to create
642 "TT13". These selection markers allowed us to select the diploid strain ("TT14") that resulted from
643 mating TT10 with TT13 by using a double drop-out medium (lacking -*ura* and -*trp*). TT14 is
644 homozygous for *ADE2*, *pADH1-rtTA*, and *pTET07-GFP*, thus all four haploids in a TT14 spore-
645 bag has an inducible *GFP*.
646
647 **Spore formation (sporulation).** We used a standard protocol for sporulating yeasts. In short,
648 we first grew diploid yeasts (homozygous diploid wild-type or GFP-inducible strains) to saturation
649 overnight. We then transferred these cells to a "pre-sporulation media" (i.e., YPAc: consists of
650 Yeast Peptone media with a 2% potassium acetate) which we then incubated for 8 hours at 30°C.
651 We subsequently transferred the diploid yeasts to a "sporulation medium" (i.e., 2% potassium
652 acetate) and left them to sporulate for 5 days at 20 °C, while rotating as a liquid culture in a tube.
653 Afterwards, we transferred the resulting spores to water and stored them at 4°C. Through
654 measurements, we found that we could store these spores for several months without loss of
655 viability (i.e., 2%-glucose still germinates ~100% of the spore bags).
656
657 **Microscope sample preparation.** We performed all microscope imaging with 96-well glass-
658 bottom plates (5242-20, Zell-Kontakt). Prior to each microscope imaging, we pre-treated the
659 glass-bottom by incubating it for 20 minutes with 0.1 mM of concanavalin A (ConA, C2010, Sigma-
660 Aldrich). We washed the ConA and then typically added 1 µl of spores in 200 µl of minimal
661 medium per well. The plates were then centrifuged at 1000 rpm for 1 minute to sediment and
662 attach all spore bags to the glass-bottom. We then performed the microscope imaging.
663
664 **Microscope data acquisition.** We used Olympus IX81 inverted, epifluorescence, wide-field
665 microscope. For each time-lapse movie, we collected images once every 10 minutes for every
666 field of view. The temperature during microscope imaging was maintained by an incubator cage
667 (OKO Lab) that enclosed the microscope. We acquired each image with an EM-CCD Luca R
668 camera (Andor) and IQ3 software (Andor). We used a wide-spectrum lamp (AMH-600-F6S,
669 Andor) for exciting fluorescent proteins.
670
671 **Microscope data analysis.** We processed the microscope images with ImageJ and MATLAB
672 (Mathworks). To measure the times taken to germinate (and to count spore bags that germinated),

673 we looked for the first haploid spore that formed a bud for each ascus (spore bag). We segmented
674 the spore bags by using the Sobel filtering of the brightfield images to create a mask. We extracted
675 fluorescence values inside the mask. We corrected for the background fluorescence, for each
676 ascus one by one, by subtracting the average background in a 50-pixel area that surrounded each
677 ascus.
678
679 **Yeast transformations and mating.** We transformed yeasts with the standard, lithium-acetate-
680 based method. In short, log-phase cells were resuspended in 0.1M of lithium acetate in 1x TE,
681 together with the DNA to be inserted by homologous recombination at a desired location within
682 the yeast genome and herring ssDNA. We then added PEG3350, 10X TE and 1M of lithium
683 acetate to the yeast culture so that we had final concentrations of 40% PEG 3350, 1X TE, and
684 0.1M LiOAc. We then incubated the culture at 42 °C for 30 minutes. We pelleted the resulting
685 cells by centrifuging and then washed them with water. Afterwards, we plated the cells on agar
686 plates with dropout media to select from transformed yeasts. We used a standard method of
687 mating yeasts. Namely, we mated yeasts of opposite sexes by inoculating a 500 µl of YPD
688 medium with two colonies - one from each sex. We then incubated the culture overnight in 30 °C
689 on a benchtop shaker (Eppendorf Mixmate) that agitated the culture at 300 rpm. We then spread
690 100 µl of the culture on double-dropout agar plates which selected for the diploids that resulted
691 from successful mating.
692
693 **Hexokinase-based assay to measure glucose concentrations.** 10 hours after adding a low
694 concentration of glucose to germinate some of the spores in a 1-mL of minimal medium in a 24-
695 well microscopy plate (5242-20, Zell-Kontakt), we took 800 µl of the supernatant to measure the
696 concentration of glucose in it. We determined the glucose concentration by using a hexokinase-
697 based glucose assay kit (Glucose (HK) Assay, G3293, Sigma-Aldrich) that is based on converting
698 glucose through hexokinase and $NADP^+$ dependent glucose-6-phosphate-dehydrogenase.
699
700 **RNA-seq on un-germinated spores.** We collected un-germinated spores by first collecting 1 ml
701 samples of spores that were incubated in a 0.002%-glucose at 0, 16, 48, and 96 hours after the
702 incubation began. To isolate the un-germinated spores, we treated the 1-mL samples with
703 zymolyase (786-036, G-Biosciences). Zymolyase lysed vegetative cells that formed from
704 germinated spores, thus ensuring that we only collected RNA from un-germinated spores for
705 sequencing. We then extracted the RNA from the leftover, un-germinated spores RiboPure Yeast
706 Kit (Ambion, Life Technologies) as described by its protocol. Next, we prepared the cDNA library

707 with the 3' mRNA-Seq library preparation kit (Quant-Seq, Lexogen) as described by its protocol.
708 Afterwards, we loaded the cDNA library on an Illumina MiSeq with the MiSeq Reagent Kit c2
709 (Illumina) as described by its protocol. We analyzed the resulting RNA-Seq data as previously
710 described (*49*): We performed the read alignment with TopHat, read assembly with Cufflinks, and
711 analyses of differential gene-expressions with Cuffdiff. We used the reference genome for *S.*
712 *cerevisiae* from ensembl. We used the transcriptional modules listed in Supplementary Table 1
713 for grouping the relevant genes into transcriptional modules.

## Supplementary Information

Supplementary Figs. 1-25

Supplementary Table 1


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## Author Contributions:

H.Y. initiated the research and designed the initial experiments. T.M. subsequently conceived and developed the project with guidance from H.Y. T.M. and T.A. performed the experiments and analyzed the data with initial help from M.A.B and guidance from H.Y. All authors checked the data. T.M. and H.Y. wrote the manuscript with inputs from T.A.

## Declaration of interests:

The authors declare 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.

741 **References**

742


743 1. Rittershaus, E. S. C., Baek, S.-H., & Sassetti, C. M. The normalcy of dormancy: common
744 themes in microbial quiescence. *Cell Host Microbe* **13**, 643-651 (2013).
745

746 2. Neiman, A. M. Sporulation in the budding yeast Saccharomyces cerevisiae, *Genetics* **189**,
747 737-765 (2011).
748

749 3. Stragier, P. & Losick, R. Molecular genetics of sporulation in Bacillus subtilis. *Annu. Rev.*
750 *Genet.* **30**, 297-341 (1996).
751

752 4. Errlngton, J. Regulation of endospore formation in Bacillus subtilis. *Nat Rev Microbiol*
753 **1**:117-126 (2003).
754

755 5. Lennon, J. T. & Jones, S. E. Microbial seed banks: the ecological and evolutionary
756 implications of dormancy. *Nat Rev MIcrobiol* **9**, 119-130 (2011).
757

758 6. Nachman, I., Regev, A. & Ramanathan, S. Dissecting timing variability in yeast spores. *Cell*
759 **131**, 544-556 (2007).
760

761 7. Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O. & Herskowitz, I.
762 The transcriptional program of sporulation in budding yeast. *Science* **282**, 699-705 (1998).
763

764 8. Eldar, A. *et al.* Partial penetrance facilitates developmental evolution in bacteria. *Nature*
765 **460**, 510-514 (2009).
766

767 9. Suel, G., Garcia-Ojalvo, J., Liberman, L. & Elowitz, M. B. An excitable gene regulatory
768 circuit induces transient cellular differentiation. *Nature* **440**, 545-550 (2006).
769

770 10. Suel, G., Kulkarni, R. P., Dworkin, J., Garcia-Ojalvo, J. & Elowitz, M. B. Tunability and noise
771 dependence in differentiation dynamics. *Science* **315**, 1716-1719 (2007).
772

773 11. Segev, E., Smith, Y. & Ben-Yehuda, S. RNA dynamics in aging bacterial spores. *Cell* **148**,
774 139-149 (2012).

775
776 12. Brengues, M., Pintard, L., & Lapeyre, B. mRNA decay is rapidly induced after spore
777 germination of Saccharomyces cerevisiae. *J Biol Chem* **277**, 40505-40512 (2002).
778
779 13. Thacker, D., Lam, I., Knop, M., & Keeney, S. Exploiting spore-autonomous fluorescent
780 protein expression to quantify meiotic chromosome behaviors in Saccharomyces
781 cerevisiae. *Genetics* **189**, 423-439 (2011).
782
783 14. Laporte, D., Salin, B., Daignan-Fornier, B., & Sagot, I. Reversible cytoplasmic localization of
784 the proteasome in quiescent yeast cells. *J Cell Biol* **181**, 737-745 (2008).
785
786 15. Petrovska, I. et al. Filament formation by metabolic enzymes is a specific adaptation to an
787 advanced state of cellular starvation. *eLife* **3**, e02409 (2014).
788
789 16. Ablett, S., Darke, A. H., Lillford, P. J., & Martin, D. R. Glass formation and dormancy in
790 bacterial spores. *Int. Journal of Food Sci Tech.* **34**, 59-69 (1999).
791
792 17. Cowan, A.E., Koppel, D. E., Setlow, B., & Setlow, P. A soluble protein is immobile in
793 dormant spores of bacillus subtilis but is mobile in germinated spores: implications for spore
794 dormancy. *Proc. Natl. Acad. Sci. USA* **100**, 4209-4214 (2003).
795
796 18. Dijksterhuis, J., Nijsse, J., Hoekstra, F. A., & Golovina, E. A. High viscosity and anisotropy
797 characterize the cytoplasm of fungal dormant stress-resistant spores. *Eukaryotic Cell* **6**,
798 157-170 (2007).
799
800 19. Parry, B. R., Surovtsev, I. V., Cabeen, M. T., O'Hern, C. S., Dufresne, E. R., Jacobs-
801 Wagner, C. The bacterial cytoplasm has glass-like properties and is fluidized by metabolic
802 activity. *Cell* **156**, 183-194 (2014).
803
804 20. Joyner, R. P. et al. A glucose-starvation response regulates the diffusion of
805 macromolecules. *eLife* **5**, e09376 (2016).
806
807 21. Munder, M. C. et al. A pH-driven transition of the cytoplasm from a fluid- to a solid-like state
808 promotes entry into dormancy. *eLife* **5**, e09347 (2016).

809
810 22. Dworkin, J. & Shah, I. M. Exit from dormancy in microbial organisms. *Nat Rev Microbiol* **8**,
811 890-896 (2010).
812
813 23. Mutlu, A et al. Phenotypic memory in Bacillus subtilis links dormancy entry and exit by a
814 spore quantity-quality tradeoff. *Nat Communications* **9**:69 (2018).
815
816 24. Donnini, C., Puglisi, P. P., Vecli, A. & Marmiroli. N. Germination of Saccharomyces
817 cerevisiae ascospores without trehalose mobilization as revealed by in vivo 13C nuclear
818 magnetic resonance spectroscopy. *J. Bacteriol.* **170**, 3789-3791 (1988).
819
820 25. Geijer, C., Pirkov, I., Vongsangnak, W., Ericsson, A., Nielsen, J., Krantz, M. & Hohmann, S.
821 Time course gene expression profiling of yeast spore germination reveals a network of
822 transcription factors orchestrating the global response. *BMC Genomics* **13**:554 (2012).
823
824 26. Herman, P. K., & Rine, J. Yeast spore germination: a requirement for Ras protein activity
825 during re-entry into the cell cycle. *EMBO J.* **16**, 6171-6181 (1997).
826
827 27. Sturm, A. & Dworkin, J. Phenotypic diversity as a mechanism to exit cellular dormancy.
828 *Current Biology* **25**, 2272-2277 (2015).
829
830 28. Sinai, L., Rosenberg, A., Smith, Y., Segev, E. & Ben-Yehuda, S. The molecular timeline of
831 a reviving bacterial spore. *Mol Cell.* **57**, 695-707 (2015).
832
833 29. Nagtzaam, M. P. M., & Bollen, G. J. Long shelf life of Talaromyces flavus in coating
834 material of pelleted seeds. *Eur. J. Plant Pathol.* **100**, 279-282 (1994).
835
836 30. Sakulkoo, W. et al. A single fungal MAP kinase controls plant cell-to-cell invasion by the rice
837 blast fungus. *Science* **359**, 1399-1403 (2018).
838
839 31. Dyer, C., Hutt, L. P., Burky, R., & Joshi, L. T. Biocide resistance and transmission of
840 Clostridium difficile spores spiked onto clinical surfaces from an American health care facility.
841 *Appl Environ Microbiol* doi:10.1128/AEM.01090-19 (2019).
842

843 32. Joseph-Strauss, D., Zenvirth, D., Simchen, G. & Barkai, N. Spore germination in
844 Saccharomyces cerevisiae: global gene expression patterns and cell cycle landmarks,
845 *Genome Biol.* **8**:R241 (2007).
846
847 33. Coluccio, A., Bogengruber, E., Conrad, M. N., Dresser, M. E., Briza, P. & Neiman, A. M.
848 Morphogenetic pathway of spore wall assembly in Saccharomyces cerevisiae. *Eukaryot*
849 *Cell,* **3**, 1464-1475 (2004).
850
851 34. Ihmels, J., Friedlander, G., Bergmann, S., Sarig, O., Ziv, Y. & Barkai, N. Revealing modular
852 organization in the yeast transcriptional network. *Nat. Genet.* **31**, 370-377 (2002).
853
854 35. Houser, J. R., Ford, E., Nagiec, M. J., Errede, B. & Elston, T. C. Positive roles for negative
855 regulators in the mating response of yeast. *Mol Syst Biol* **8**:586 (2012).
856
857 36. Padovan-Merhar, O. & Raj, A. Using variability in gene expression as a tool for studying
858 gene regulation. *Wiley Interdiscip Rev Syst Biol Med.* **5**, 751-759 (2013).
859
860 37. Balazsi, B., Van Oudenaarden, A. & Collins, J. J. Cellular decision making and biological
861 noise: From microbes to mammals. *Cell* **144**, 910-925 (2011).
862
863 38. Elowitz, M. B., Levine, A. J., Siggia, E. D., & Swain, P. S. Stochastic gene expression in a
864 single cell. *Science* **297**, 1183-1186 (2002).
865
866 39. Şimşek, E., & Kim, M. Power-law tail in lag time distribution underlies bacterial persistence.
867 *Proc Natl Acad Sci USA* doi:10.1073/pnas.1903836116 (2019).
868 40. Coates, J., Park, B., Le, D., Simsek, E., Chaudhry, W., & Kim, M. Antibiotic-induced
869 population fluctuations and stochastic clearance of bacteria. *eLife* e32976 (2018).
870 41. Marx, V. How to pull the blanket off dormant cancer cells. *Nat. Methods* **15**, 249-252 (2018).
871
872 42. Luo, Y et al. Single-cell transcriptome analyses reveals signals to activate dormant neural
873 stem cells. *Cell* **161**, 1175-1186 (2015).
874

875 43. Jones, S. E. & Lennon, J. T. Dormancy contributes to the maintenance of microbial
876 diversity. *Proc Natl Acad Sci USA* **107**, 5881-5886 (2010).
877

878 44. Yamaguchi, S. Gibberellin metabolism and its regulation, *Annu. Rev. Plant Biol.* **59**, 225-
879 251 (2008).

880 45. Ringrose, L. & Howard, M. Dissecting chromatin-mediated gene regulation and epigenetic
881 memory through mathematical modelling. *Current Opinion in Systems Biology* 3, 7-14
882 (2017).
883
884 46. Chen, M. & Penfield, S. Feedback regulation of COOLAIR expression controls seed
885 dormancy and flowering time. *Science* **360**, 1014-1017 (2018).
886
887 47. Ietswaart, R., Rosa, S., Wu, Z., Dean, D. & Howard, M. Cell-size-dependent transcription of
888 FLC and its antisense long non-coding RNA COOLAIR explain cell-to-cell expression
889 variation. *Cell Systems* **4**, 622-635 (2017).
890
891 48. Youk, H. & Lim, W. A. Secreting and sensing the same molecule allows cells to achieve
892 versatile social behaviors. *Science* **343**, 1242782 (2014).

893 49. Trapnell, C. *et al*, Differential gene and transcript expression analysis of RNA-seq experiments
894 with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562-578 (2012).

## Figure captions

**Figure 1 | Glucose germinates only a fraction of yeast spore bags.**

**a,** Conventional test to determine whether a spore bag (i.e., ascus) with 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 is added at the beginning of the movie. 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 function of time after adding a fixed concentration of 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) at 16 hours (960 minutes) after adding 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 for days by a low glucose concentration, germinate faster upon encountering more glucose.**

**a,** Wild-type spores are first incubated in a low glucose-concentration before we add more glucose at a later time to increase the glucose concentration.

**b,** Time taken by each spore bag to germinate for the experiment in (a). 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 experiment in (a), percentage of spore bags that germinated as function of time after glucose addition in two steps (concentrations as indicated). Second glucose added at 1000 minutes (purple vertical line). $n$ = 3; error bars are s.e.m.

**d,** Average time taken for a spore bag to germinate in the experiment shown in (a), due to the second added glucose (denoted $\Delta\tau$ in bottom panel of (c)). The final glucose concentration was varied and the final concentration was 2%. $n$ = 3; error bars are s.e.m.

**e,** Spore bags that do not germinate after encountering the first glucose-concentration are "primed" to germinate faster upon encountering more glucose.

928 **f,** Average time taken for a spore bag to germinate ($\Delta\tau$) in the experiment shown in (a) after
929 adding the second glucose (final glucose concentration is 2%) as a function of the first, low
930 glucose concentration. Different colors represent different times at which the second glucose was
931 added: 16 hours (yellow), 48 hours (blue) and 96 hours (red). "Relative $\Delta\tau$" is the average time
932 $\Delta\tau$ divided by the $\Delta\tau$ for spore bags that were incubated in minimal media without any glucose
933 (0%) for the same duration of time, before they received 2%-glucose. *n* = 3; error bars are s.e.m.
934 **g,** Heat map showing transcriptome-wide changes in un-germinated, primed spores at 0, 16, 48,
935 and 96 hours after being primed by a 0.002%-glucose (obtained with RNA-Seq). See
936 Supplementary Table 1 for a list of genes for each transcriptional module (rows of heat map) and
937 also see Supplementary Fig. 8. For each transcriptional module, we first divided the expression
938 level of each gene in that module by its expression level at 0 hours - this yields "normalized
939 expression level" for that gene for primed and unprimed spores (the latter were incubated in
940 minimal media without glucose for 0, 16, 48, and 96 hours). We then averaged these values over
941 all genes in a given transcriptional module, yielding one value of "normalized expression (primed)"
942 and one value of "normalized expression (unprimed)" for each transcriptional module. Colors
943 represent ratio of these two values, averaged over three biological replicates (*n* = 3).
944
945
946 **Figure 3 | Synthetically inducing gene-expression in dormant yeast-spores without**
947 **nutrients leads to germination landscape, which shows that ability to express a gene (e.g.,**
948 ***GFP*) without nutrients quantifies how likely a spore bag will germinate for each glucose**
949 **concentration.**
950 **a,** A synthetic gene-circuit that constitutively expresses a transcription factor, rtTA (with ADH1-
951 promoter) and an inducible promoter (TET-promoter) controlling GFP expression. Increasing
952 doxycycline increases GFP production.
953 **b,** Engineered spore bags (shown in (a)) transcribe and translate GFP in plain water with 25 μg/ml
954 of doxycycline (top row) and in a saline solution (PBS) with 50 μg/ml of doxycycline (bottom row)
955 without nutrients. Snapshots of GFP expression shown at 22 hours after adding doxycyline**.**
956 **c,** GFP levels of individual spore bags (grey curves) over time (measured every 10 minutes with
957 a wide-field epifluorescence microscope) after incubation in PBS with 10 μg/ml of doxycycline
958 (top panel: *n* = 104 spore bags) and 100 μg/ml of doxycycline (bottom panel: *n* = 150 spore bags).
959 **d,** Engineered spore bags (shown in (a)) were first incubated for 22 hours in either PBS without
960 any doxycyline or with 100 μg/ml of doxycycline before they were transferred to minimal media
961 with various glucose concentrations. Plot shows the total percentage of the engineered spore

962 bags that germinated (measured 20 hours after incubating with glucose) for those pre-incubated
963 in PBS without doxycycline (black points) and in PBS with 100 μg/ml of doxycycline (orange
964 points). *n* = 3; error bars are s.e.m.
965 **e,** Top: Percentage of spore bags with the same GFP-level (in experiment in (d)) that germinated
966 after receiving a 0.001%-glucose. Percentages are averaged over all spore bags with the same
967 binned GFP-level (corresponding histogram shown below). Data from a representative population
968 of spore bags (*n* = 145 spore bags in a population).
969 **f,** Germination landscape: Colors represent the probability that a spore bag with a particular
970 steady-state GFP-level germinates for each glucose concentration (i.e., data in top panel of (e)
971 represents a single row of this heat map). To measure each pixel, as in the experiment described
972 in (d), we incubated spore bags in PBS with 100 μg/ml of doxycycline for 22 hours before giving
973 glucose concentrations indicated along the rows. Columns indicate steady-state GFP-level of a
974 spore bag at 22 hours after adding the doxycyline. Each pixel is an average over 3 replicate
975 populations (*n* = 3).
976 **g,** Given a spore bag, its steady-state GFP-level is a read-out of both its intrinsic ability to express
977 a gene without any nutrients and the minimal glucose concentration that it needs for germination.
978 Spore bags with lesser gene-expressing abilities without nutrients require more glucose to
979 germinate.
980
981
982 **Figure 4 | Dormancy as a quantifiable spectrum ("ruler") that measures and forecasts**
983 **dormant yeast-spore's proximity to death (lifespan) and proximity to entering replicative**
984 **life (germination capacity).**
985 **a,** Percentage of wild-type and GFP-inducible spore-bags (Fig. 3a) that germinate due to a
986 saturating glucose-concentration (2%) that they encounter after 0 - 85 days of incubation in water
987 (grey and black data) or minimal medium with essential amino acids (red data) at 30 $^{O}$C. *n* = 3;
988 error bars are s.e.m.
989 **b,** Hypothesis on how dormancy-to-death transition gradually occurs: Dormant spore bag loses
990 its gene-expressing ability over time (Top left), thus it needs a higher glucose concentration to
991 germinate (Top right), which in turn causes it to require an ever-more increasing glucose
992 concentration for germination. Eventually the glucose concentration goes above the saturating
993 level (2%), which is impossible to obtain and thus the spore bag will never germinate (i.e., dead)
994 (Bottom).

**c,** Top row**:** Steady-state GFP-levels of individual spore bags measured as described in Supplementary Fig. 21, after 0, 5,10 or 20 days of incubation (left to right) at 30 °C in plain water (see Supplementary Fig. 22 for data up to 80 days). Dead spore bags' GFP levels are not shown. Bottom row: germination landscape measured for these spores for 0, 5, 10 or 20 days (left to right). Each pixel's color is from averaging over 3 replicate populations (*n* = 3).

**d,** Mean GFP-level of dormant (purple) and dead (grey) spore bags that were incubated in water without nutrients at 30 $^{O}$C over ~80 days (also see Supplementary Fig. 21). The purple data points are the average GFP-levels from the histograms shown in (c) and Supplementary Fig. 22. Grey data points are averages of the dead spore bags' histograms shown in Supplementary Fig. 22. *n*=3, error bars represent the average standard deviation from three biological replicates.

**e,** Average, minimum glucose-concentration required for germination, '*h*' (in equation (1)) extracted from the four germination landscapes shown in (c) as a function of the gene-expressing ability (GFP-level) of dormant spore bags (see Supplementary Fig. 19 for the extraction method) incubated in water for 0 days (black), 5 days (brown), 10 days (yellow), and 20 days (orange). *n*=3, error bars are s.e.m. Dead spore bags represented by a grey point (at above saturating glucose levels).

**f,** Testing the hypothesis posed in (b): $dG_{min}/dt$ in equation (1) after combining results in (d-e) (see Supplementary Fig. 23 for details). *n* = 3, error bars are s.e.m.

**g,** Dormant spore bag's ability to express a gene without nutrients defines its position on the "dormancy spectrum" (bottom cartoon). The spore bag's position on the spectrum determines the minimum glucose-concentration that it needs for germination (red curve is the day-10 data in (e)) and its lifespan (blue curve is from the same mathematical analysis as in (c-f) (see Supplementary Fig. 24-25 for details)).

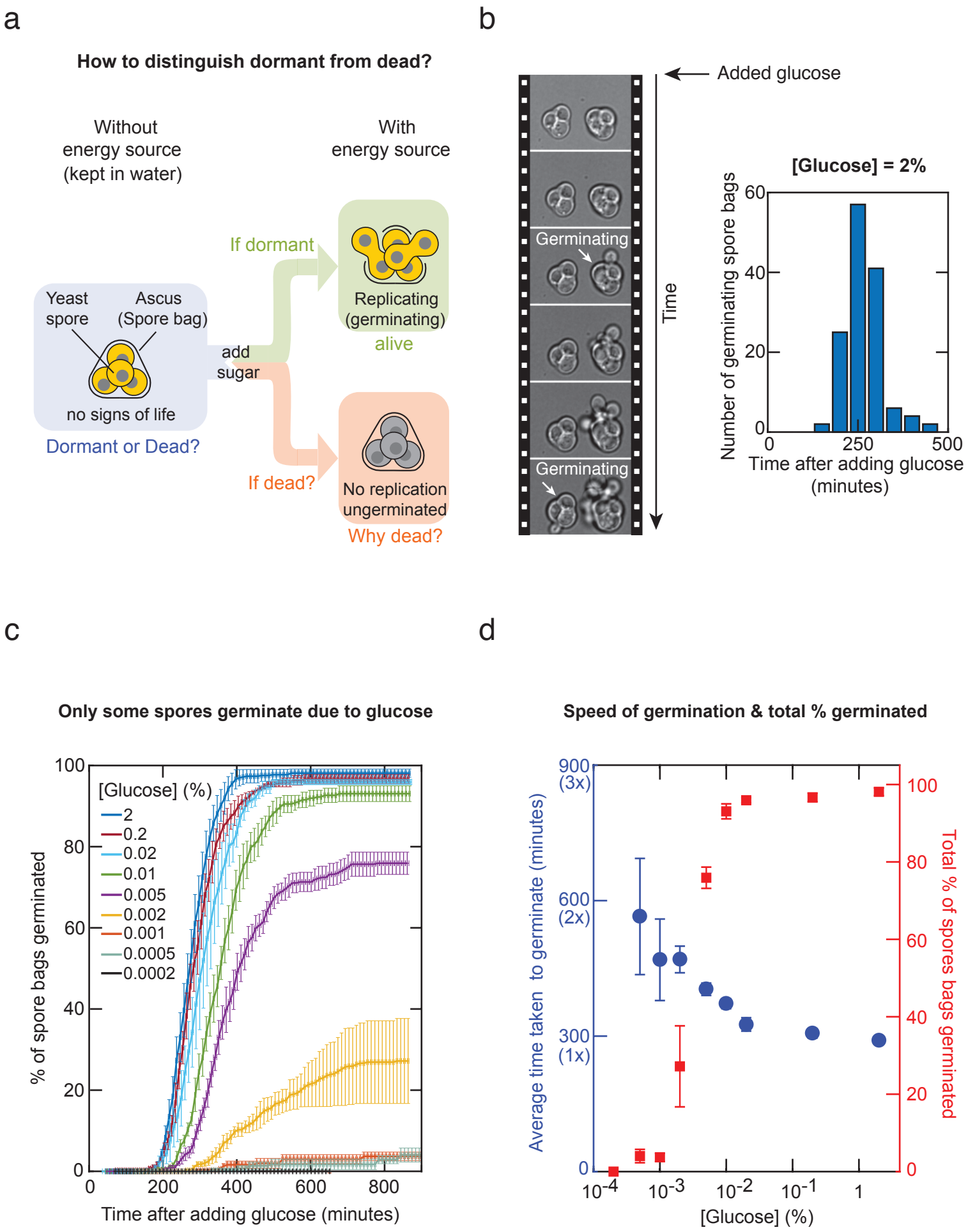

a
How to distinguish dormant from dead?
Without energy source (kept in water)
With energy source
Yeast spore
Ascus (Spore bag)
no signs of life
Dormant or Dead?
add sugar
If dormant
Replicating (germinating)
alive
If dead?
No replication ungerminated
Why dead?
b
Added glucose
Germinating
Germinating
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

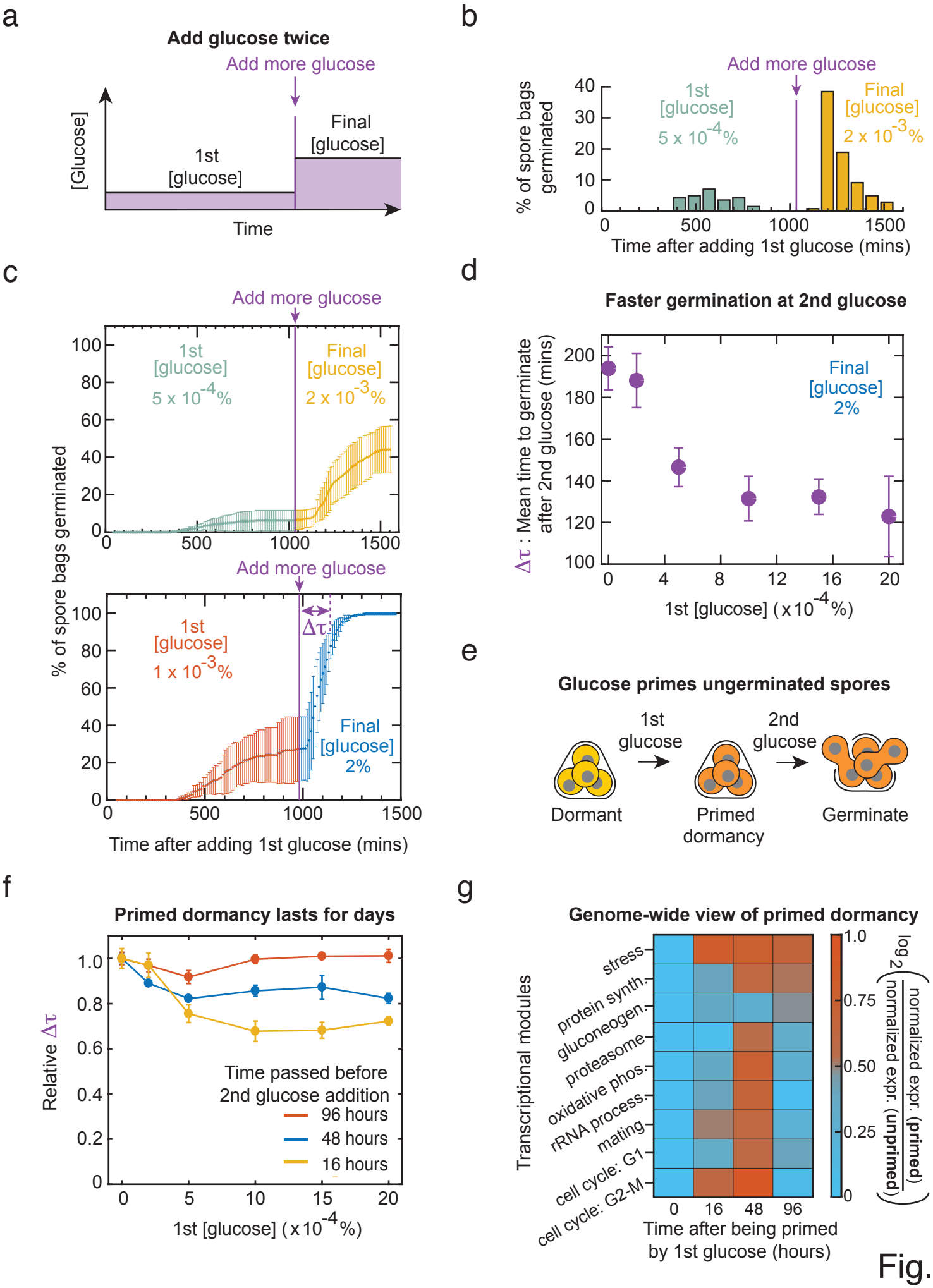
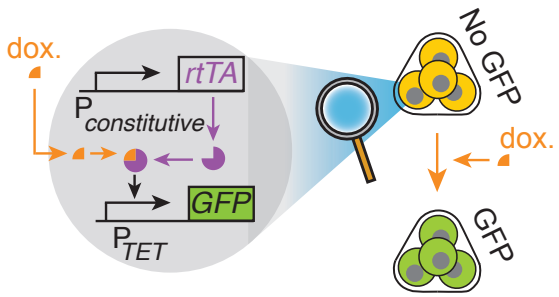

a
Add glucose twice
Add more glucose
[Glucose]
1st [glucose]
Final [glucose]
Time
b
Add more glucose
% of spore bags germinated
1st [glucose] 5 x 10^-4 %
Final [glucose] 2 x 10^-3 %
Time after adding 1st glucose (mins)
c
Add more glucose
1st [glucose] 5 x 10^-4 %
Final [glucose] 2 x 10^-3 %
Add more glucose
Δτ
1st [glucose] 1 x 10^-3 %
Final [glucose] 2%
% of spore bags germinated
Time after adding 1st glucose (mins)
d
Faster germination at 2nd glucose
Final [glucose] 2%
Δτ : Mean time to germinate after 2nd glucose (mins)
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

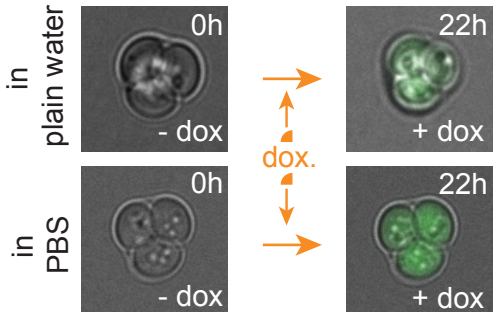
a

**Synthetic circuit to test arbitrary gene induction in dormant spores**



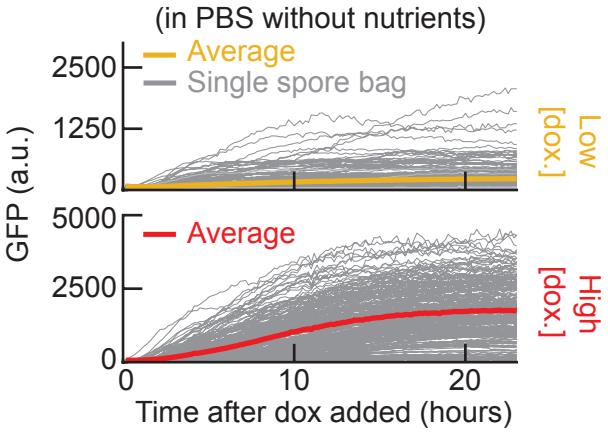

b

**Inducing gene-expression in dormant spores without any external energy source**



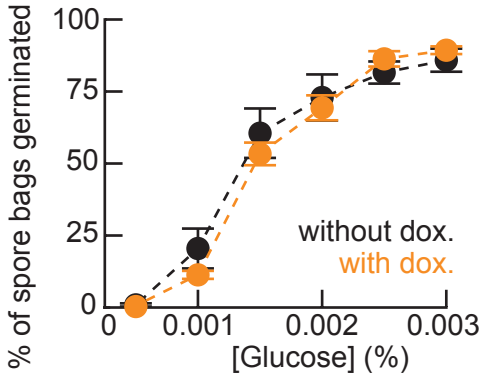

c

**Spore bags vary in ability to produce GFP**



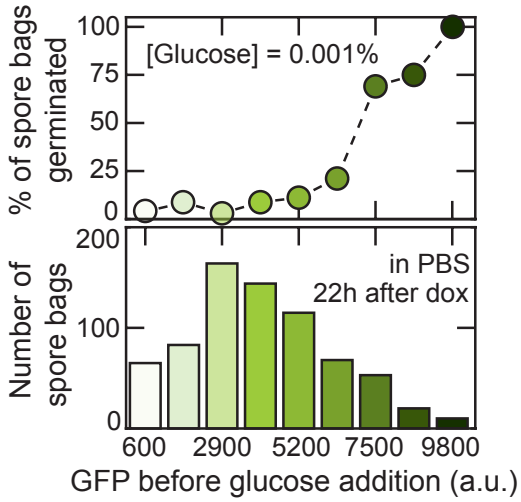

d

**Inducing GFP does not change % of spore bags that germinate**



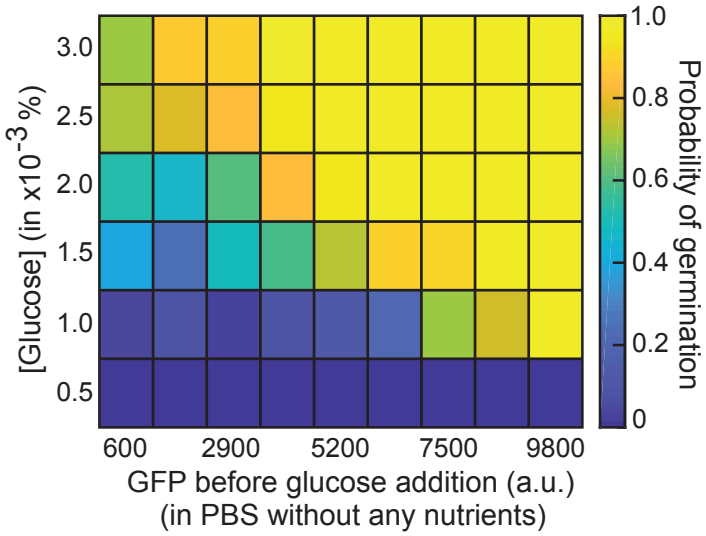

e

**Spore bags with more GFP are more likely to germinate**




f

**Germination landscape**




g

**Intrinsic ability to express genes without nutrients quantifies ability to germinate**

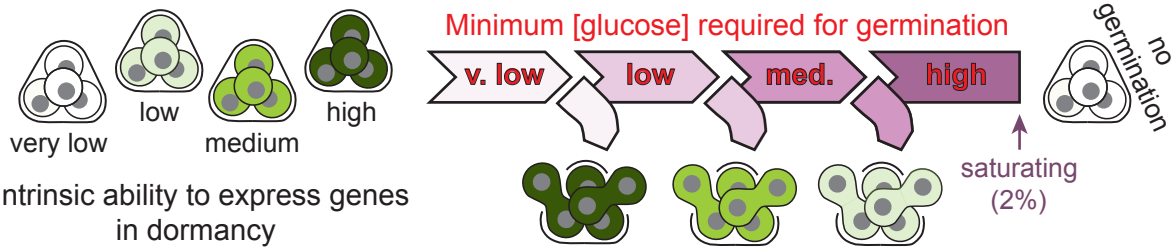



Fig. 3

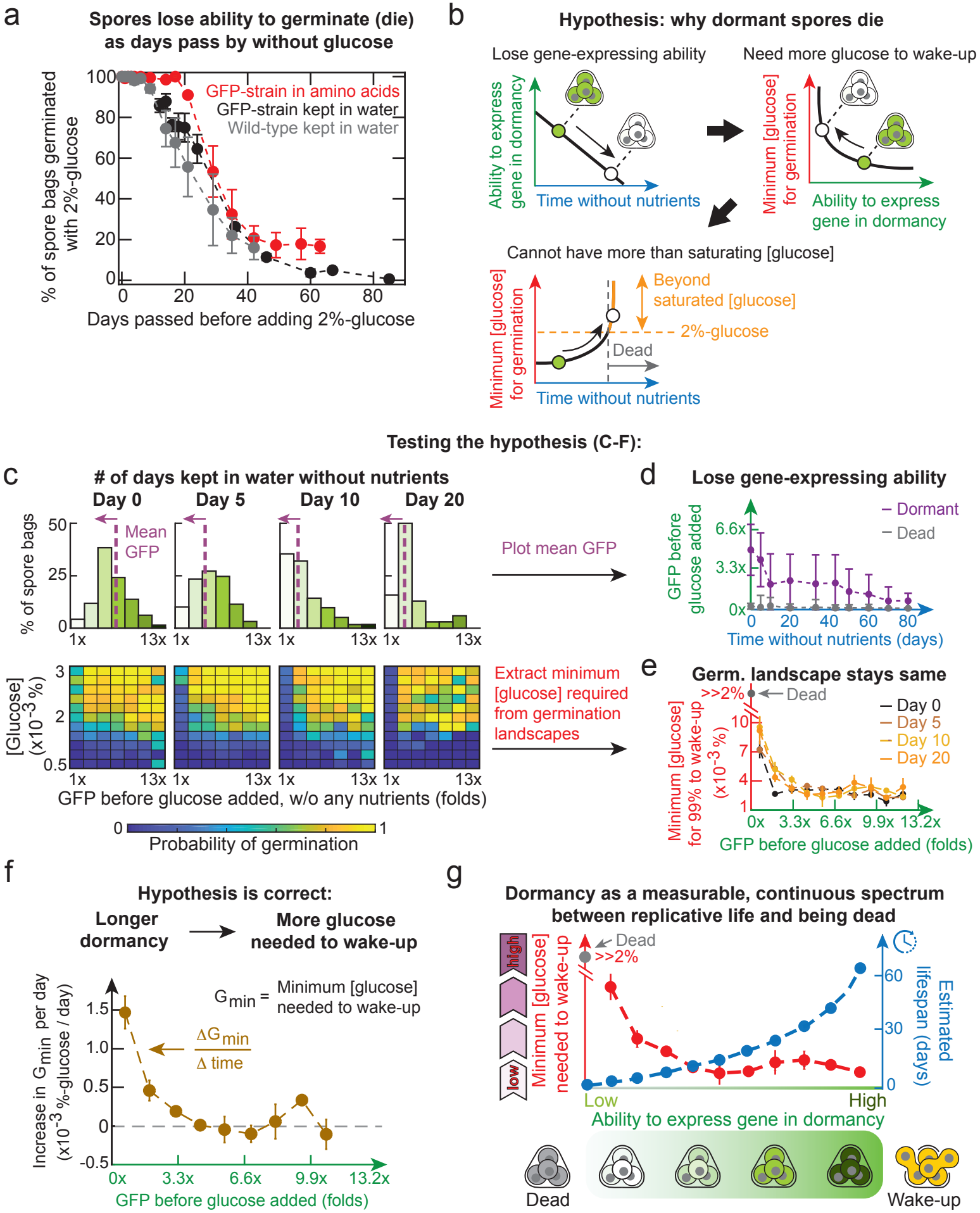

a
Spores lose ability to germinate (die) as days pass by without glucose
% of spore bags germinated with 2%-glucose
Days passed before adding 2%-glucose
GFP-strain in amino acids
GFP-strain kept in water
Wild-type kept in water
b
Hypothesis: why dormant spores die
Lose gene-expressing ability
Need more glucose to wake-up
Ability to express gene in dormancy
Time without nutrients
Minimum [glucose] for germination
Ability to express gene in dormancy
Cannot have more than saturating [glucose]
Beyond saturated [glucose]
2%-glucose
Dead
Minimum [glucose] for germination
Time without nutrients
Testing the hypothesis (C-F):
c
# of days kept in water without nutrients
Day 0
Day 5
Day 10
Day 20
Mean GFP
% of spore bags
Plot mean GFP
[Glucose] (x10^-3 %)
Extract minimum [glucose] required from germination landscapes
GFP before glucose added, w/o any nutrients (folds)
Probability of germination
d
Lose gene-expressing ability
Dormant
Dead
GFP before glucose added
Time without nutrients (days)
e
Germ. landscape stays same
>>2%
Dead
Day 0
Day 5
Day 10
Day 20
Minimum [glucose] for 99% to wake-up (x10^-3 %)
GFP before glucose added (folds)
f
Hypothesis is correct:
Longer dormancy
More glucose needed to wake-up
G_min = Minimum [glucose] needed to wake-up
ΔG_min / Δ time
Increase in G_min per day (x10^-3 %-glucose / day)
GFP before glucose added (folds)
g
Dormancy as a measurable, continuous spectrum between replicative life and being dead
high
low
Minimum [glucose] needed to wake-up
Dead
>>2%
Estimated lifespan (days)
Low
High
Ability to express gene in dormancy
Dead
Wake-up

Fig. 4

# Supplementary Information

## Dormancy as a spectrum measuring spore's proximity to death and to replicative life

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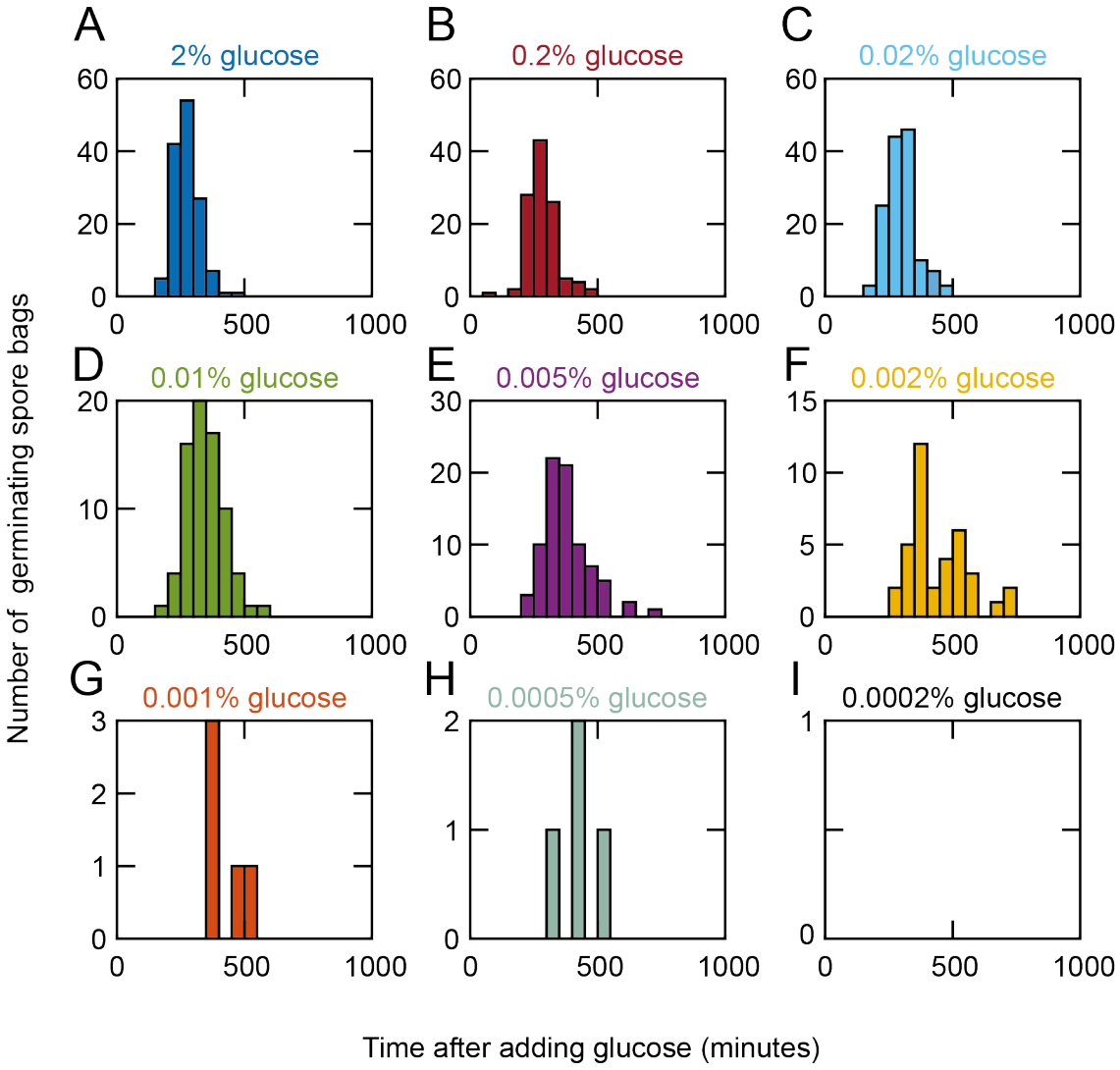
**This PDF file includes:**

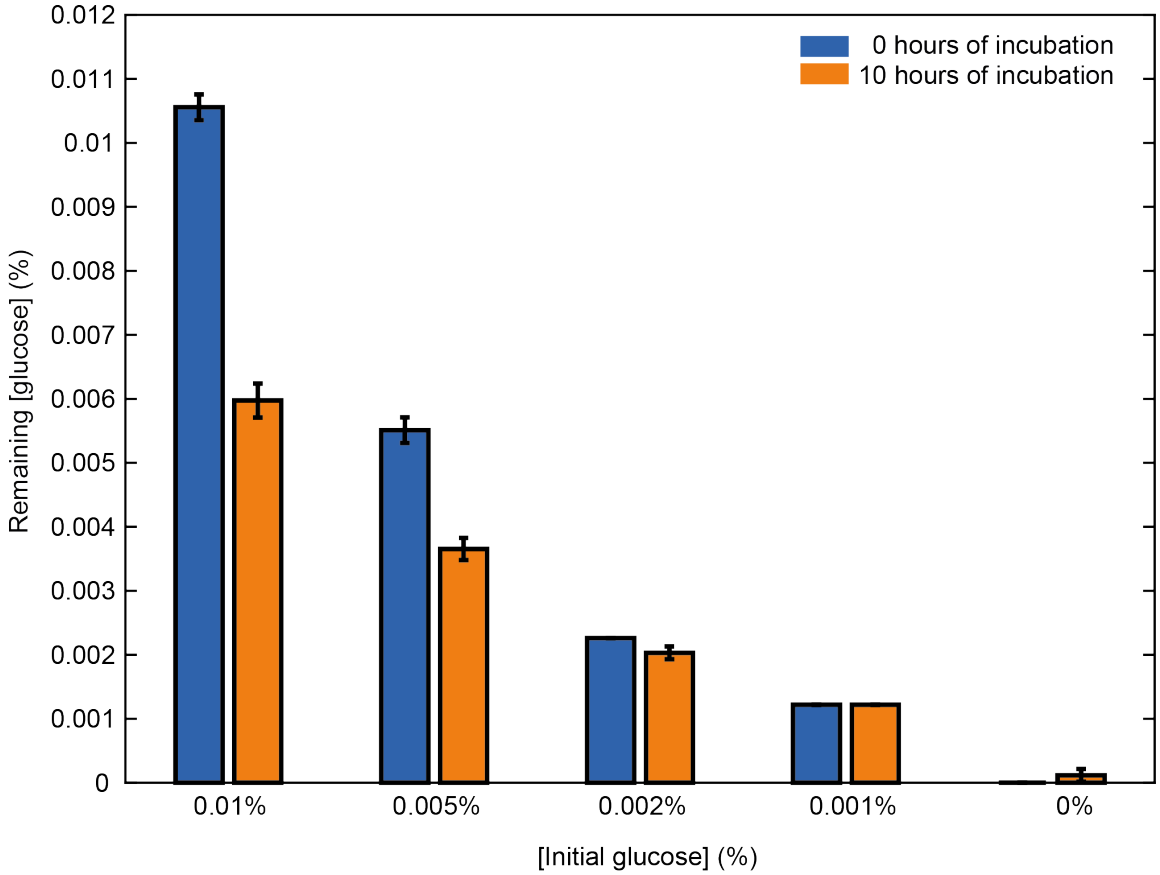
- **Supplementary Figures 1-25**
- **Supplementary Table 1**

## Supplementary Figures



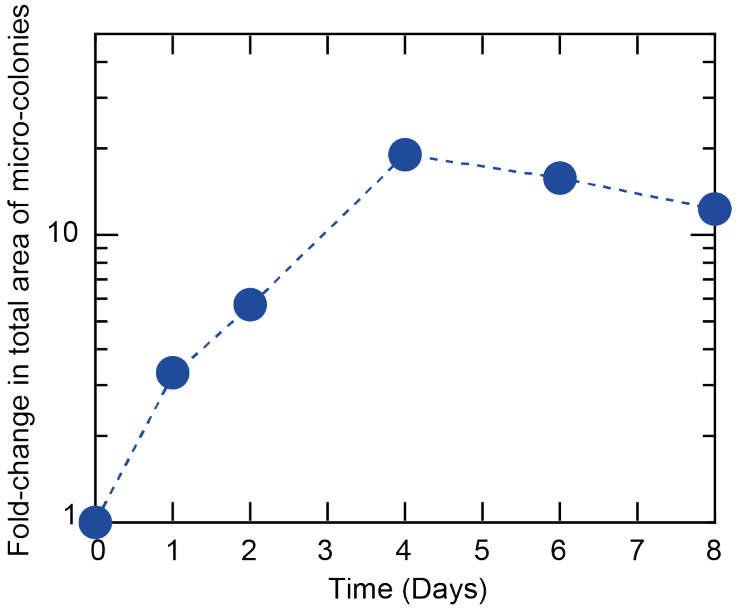

**Supplementary Figure 1 | Representative histograms showing how many wild-type spore bags typically germinated for each glucose concentration (complements Fig. 1b-c)**. We incubated the spores in minimal media (which contains all essential amino acids and nitrogenous bases) that we supplemented with a desired concentration of glucose (denoted above each histogram). We then used a wide-field microscope to observe individual spore bags over time and noted how many spore bags germinated as a function of time after we added glucose. For each glucose concentration, the number of spore bags that we analyzed for the histograms shown here are: **(A)** 2%-glucose, $n$ = 137 spore bags; **(B)** 0.2%-glucose, $n$ = 117 spore bags; **(C)** 0.02%-glucose, $n$ = 142 spore bags; **(D)** 0.01%-glucose, $n$ = 78 spore bags; **(E)** 0.005%-glucose, $n$ =

103 spore bags; **(F)** 0.002%-glucose, *n* = 190 spore bags; **(G)** 0.001%-glucose, *n* = 106 spore bags; **(H)** 0.0005%-glucose, *n* = 95 spore bags; **(I)** 0.0002%-glucose, *n* = 50 spore bags (note that none of these spore bags germinated at this glucose concentration).

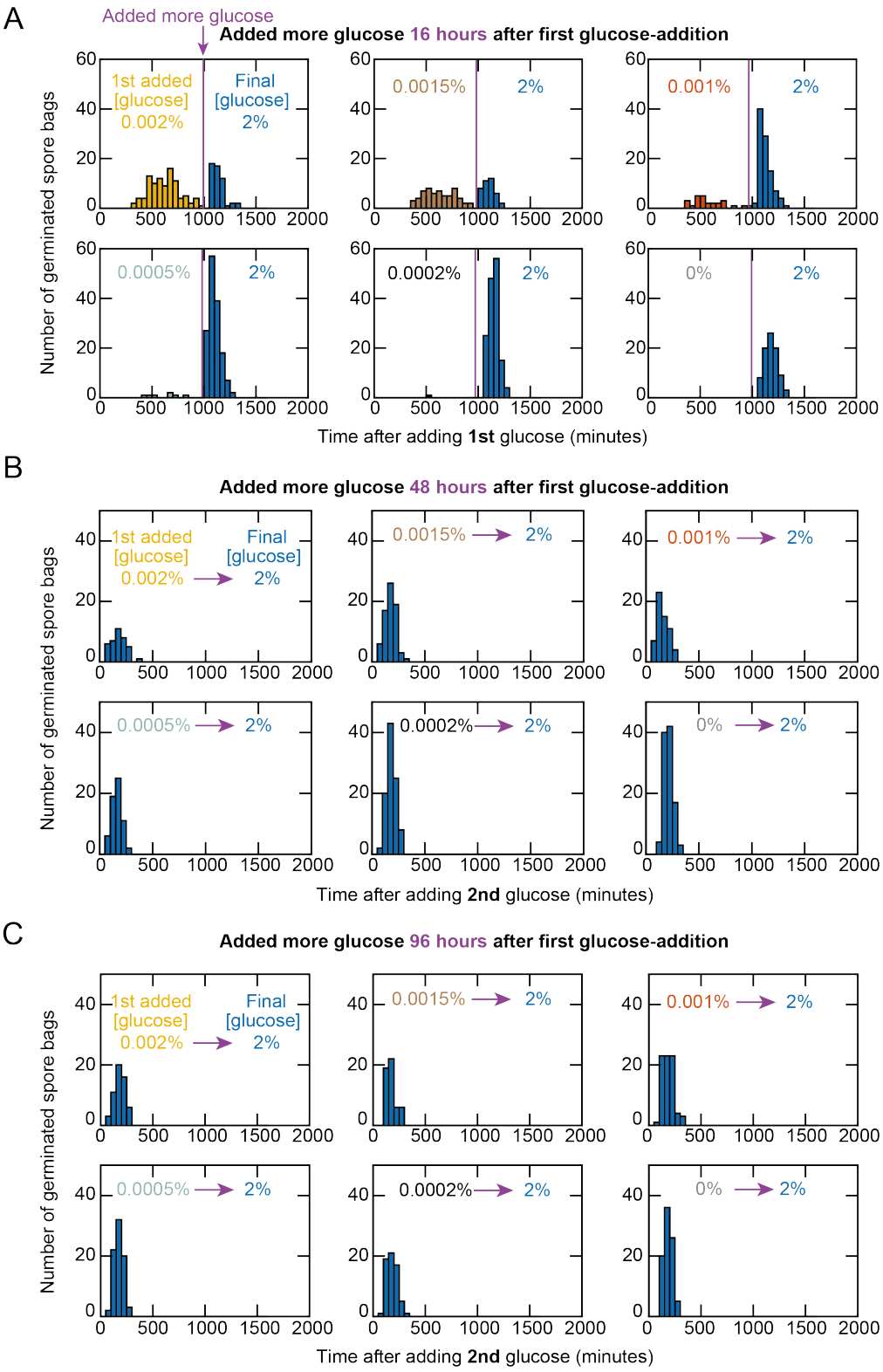



**Supplementary Figure 2 | Running out of glucose is not the reason for only a fraction of spore bags germinating for non-saturating glucose-concentrations (complements Fig. 1c-d).** Before incubating the wild-type spores in a minimal medium with glucose (experiment shown in Fig. 1c), we measured the initial glucose-concentration in the medium (blue bars - "0 hours of incubation") with a hexokinase-based assay (see Materials and Methods). In all the glucose concentrations that we studied (from 0.0002% to 2%), all germinations have either stopped or were about to stop after ~10 hours (~600 minutes) of incubation. This is evidenced by the plateauing of all the curves - representing the % of spore bags germinated as a function of time for various initial glucose-concentrations - beginning at around 600 to 700 minutes in Fig. 1c. We sought to determine whether the germinations were stopping due to vegetative cells, which result from germinated spores, having consumed appreciable amounts of glucose during their continuous cell divisions (vegetative yeasts divided once every 2-3 hours at these glucose concentrations). We measured the remaining glucose-concentration after ~10 hours of incubation for various initial glucose-concentrations (orange bars - "10 hours of incubation"). For the relatively high initial glucose-concentrations (e.g., 0.01% and 0.005% shown here), germinated

spores and the resulting vegetative cells depleted nearly half of the initial glucose-concentration after 10 hours. But for the relatively low initial glucose-concentrations (e.g., 0.002% and 0.001% shown here), the germinated spores and the resulting vegetative cells have not consumed appreciable amounts of glucose - the glucose-concentration remained nearly unchanged after 10 hours. These results show that the germinations do not stop at any of the glucose-concentrations that we studied (Fig. 1c) because the spores ran out of glucose. This is also true for the relatively high glucose-concentrations (e.g., 0.01% and 0.005%) since these conditions still had high amounts of glucose remaining after 10 hours - these remaining glucose-concentrations were still higher than some the low initial glucose-concentrations (e.g., 0.002% and 0.001%) which were enough to germinate spores. For all bars shown: $n$ = 3, error bars are s.e.m.

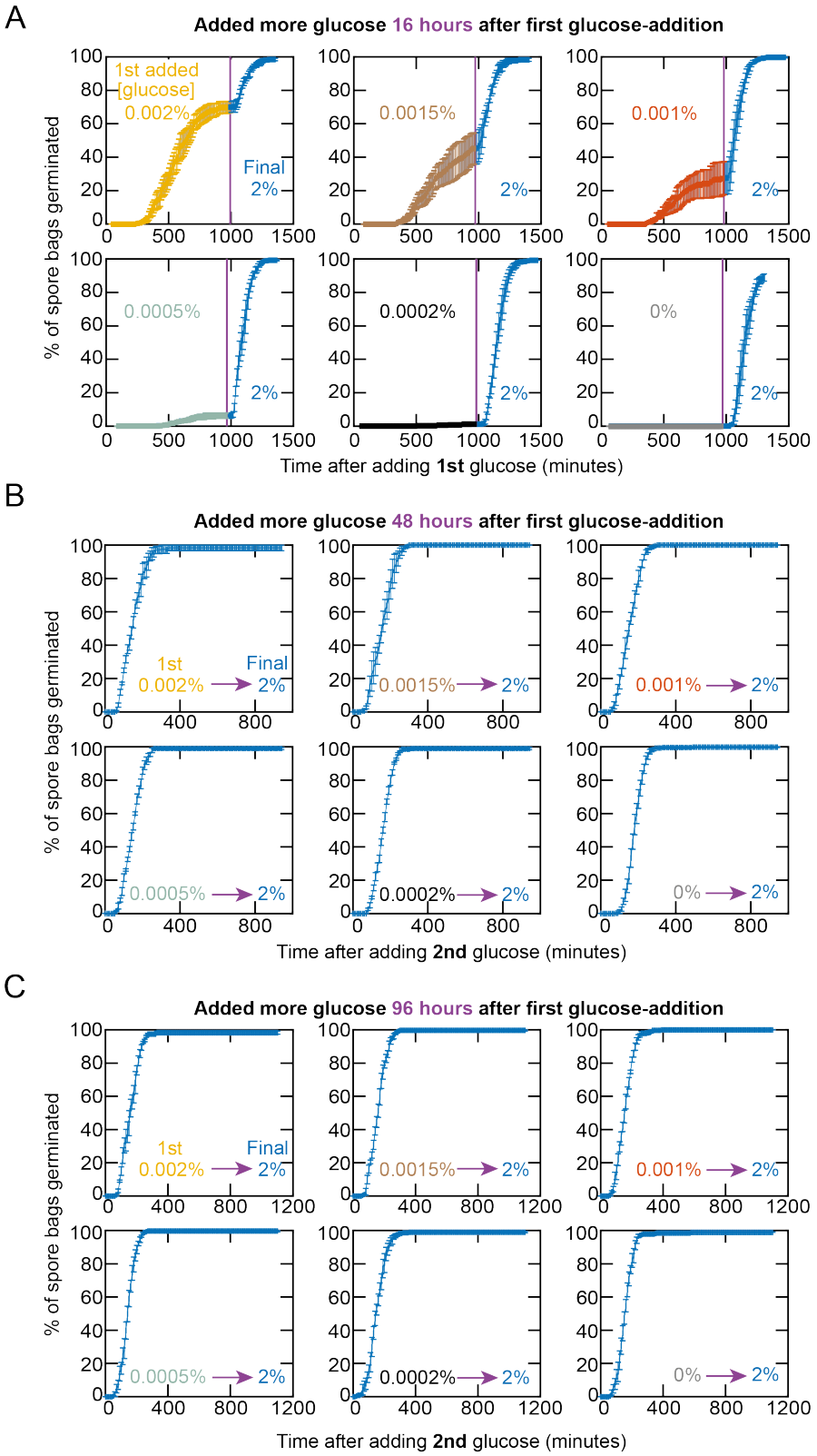



**Supplementary Figure 3 | A vegetative yeast cell can divide multiple times even with the lowest glucose-concentration (0.0002%) that we used, which could not germinate any spores (complements Fig. 1c-d).** We sought to test if the lowest concentration of glucose that we used in Fig. 1c-d, which was 0.0002% and could not germinate any spores, was enough to allow divisions of vegetative, diploid wild-type cells that formed the spore bags. We incubated the vegetative, diploid wild-type cells in a minimal medium with 0.0002% glucose and used a wide-field microscope to observe them over 8 days. We found that these cells could divide multiple times. Specifically, we took pictures of 10 micro-colonies on different days and measured the area of each micro-colony over time. We then combined all their areas into a single number, on each day, and thus determined the fold-change in the total (combined) area of the colonies over time (i.e., the combined area of all micro-colonies on each day divided by the combined area of all the micro-colonies on day 0). On the fourth day, the colonies stopped growing, at which point the total area had increased by ~19-fold compared to the initial total area (corresponds to ~4.2 divisions). As a control, we incubated the vegetative cells in a minimal medium without any glucose, which led to a lesser, transient growth that stopped after two days (data not shown). These results establish that 0.0002% of glucose is enough to sustain multiple divisions of a single, isolated, vegetative wild-type cell despite it not germinating any spores.



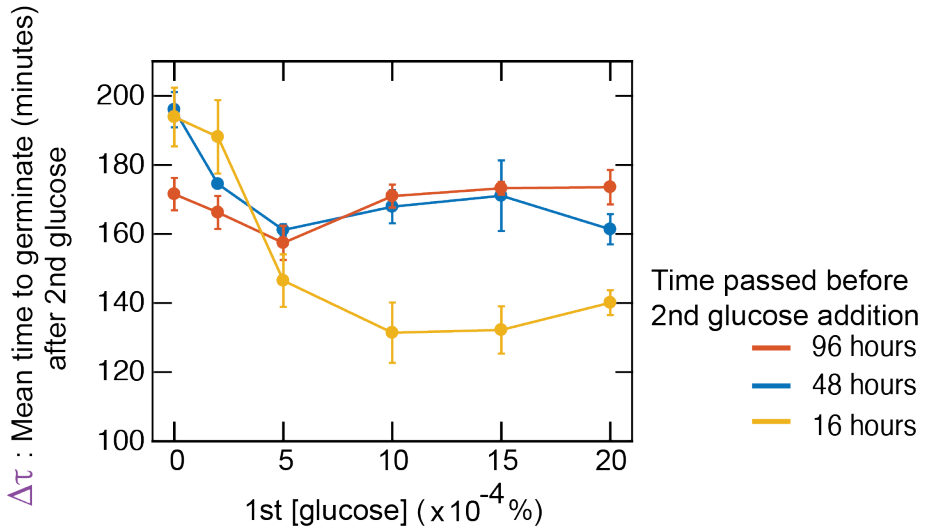

**Supplementary Figure 4 | Representative histograms showing how many wild-type spore-bags typically germinate when we add glucose in two steps as shown in Fig. 2a**

**(complements Fig. 2b).** In the experiment described in Fig. 2a, we first incubated the wild-type spores in a minimal medium with a relatively low concentration of glucose. We used a wide-field microscope to observe the spores and count the number of spore bags that germinated as a function of time. A set number of hours after incubating the spores in the low glucose-concentration (16 hours in **(A)**, 48 hours in **(B)**, and 96 hours in **(C)**), we added more glucose to the medium and then counted how many more spore bags germinated as a result - these spore bags did not germinate when we gave them the first, low concentration of glucose. Shown here are typical histograms from these experiments. **(A)** Adding more glucose after 16 hours (~1000 minutes) of incubation in the lower glucose-concentration (indicated within each of the histograms) so that, after adding the second batch of glucose, the final glucose-concentration was 2% (saturating concentration). In each histogram, the colored bars that appear to the left of the purple vertical line show germinations that occur in the first glucose-concentration while the blue bars that are to the right of the purple vertical line show germinations that occur after adding more glucose. Time here is the time after adding the first batch of glucose. The number of spore bags that we analyzed for each histogram are (including those that did not germinate and thus not shown as bars in the histograms): $n$ = 208 (top left); $n$ = 126 (top center); $n$ = 126 (top right); $n$ = 220 (bottom left); $n$ = 150 (bottom center); $n$ = 100 (bottom right). **(B)** Showing histograms only for the germinations that occur after adding the second batch of glucose (48 hours (2 days) after adding the first batch of glucose) so that the final glucose-concentration was 2%. Time here is the time after adding the second batch of glucose. We do not show the germinations that occur before adding the second batch of glucose because they resemble the data show in (A). The first glucose-concentrations are indicated in each histogram. The number of spore bags that we analyzed for each histogram are (including those that did not germinate and thus not shown as bars in the histograms): $n$ = 41 (top left); $n$ = 49 (top center); $n$ = 60 (top right); $n$ = 63 (bottom left); $n$ = 98 (bottom center); $n$ = 106 (bottom right). **(C)** Showing histograms only for the germinations that occur after adding the second batch of glucose (96 hours (4 days) after adding the first batch of glucose) so that the final glucose-concentration was 2%. Time here is the time after adding the second batch of glucose. We do not show the germinations that occur before adding the second batch of glucose because they resemble the data show in (A). The first glucose-concentrations are indicated in each histogram. The number of spore bags that we analyzed for each histogram are (including those that did not germinate and thus not shown as bars in the histograms): $n$ = 58 (top left), $n$ = 53 (top center); $n$ = 77 (top right); $n$ = 79 (bottom left); $n$ = 65 (bottom center); $n$ = 88 (bottom right).

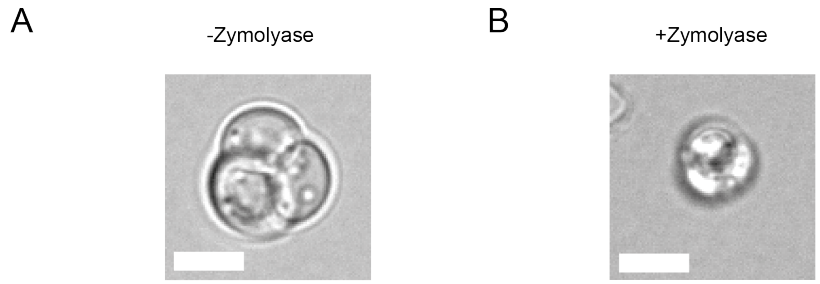



**Supplementary Figure 5 | Percentage of wild-type spore-bags that germinated as a function of time in experiments in which we add glucose in two steps as shown in Fig. 2a**

**(summarizes data shown in Supplementary Fig. 4 and complements Fig. 2c). (A)** Added more glucose after 16 hours (~1000 minutes) of incubation in the lower glucose-concentration (indicated within each of the histograms) so that, after adding the second batch of glucose, the final glucose-concentration was 2% (saturating concentration). Summarizes the histograms shown in Supplementary Fig. 4A by plotting the percentage of the wild-type spore-bags that have germinated after some time (i.e., cumulative percentage of germinations as a function of time). Time here is the time after adding the first batch of glucose. Purple line indicates when we added the second batch of glucose that raised the glucose-concentration to a saturating value (2%). The first glucose-concentration (to the left of the purple vertical line) is indicated in each histogram. **(B)** Summarizes the histograms shown in Supplementary Fig. 4B. Data shown only for the germinations that occur after adding the second batch of glucose (48 hours (2 days) after adding the first batch of glucose) so that the final glucose-concentration was 2%. We do not show the germinations that occur before adding the second batch of glucose because they resemble the data show in (A). Time here is the time after adding the second batch of glucose. **(C)** Summarizes the histograms shown in Supplementary Fig. 4C. Data shown only for the germinations that occur after adding the second batch of glucose (96 hours (4 days) after adding the first batch of glucose) so that the final glucose-concentration was 2%. We do not show the germinations that occur before adding the second batch of glucose because they resemble the data show in (A). Time here is the time after adding the second batch of glucose. In all plots (A-C): $n$ = 3 and error bars are s.e.m.

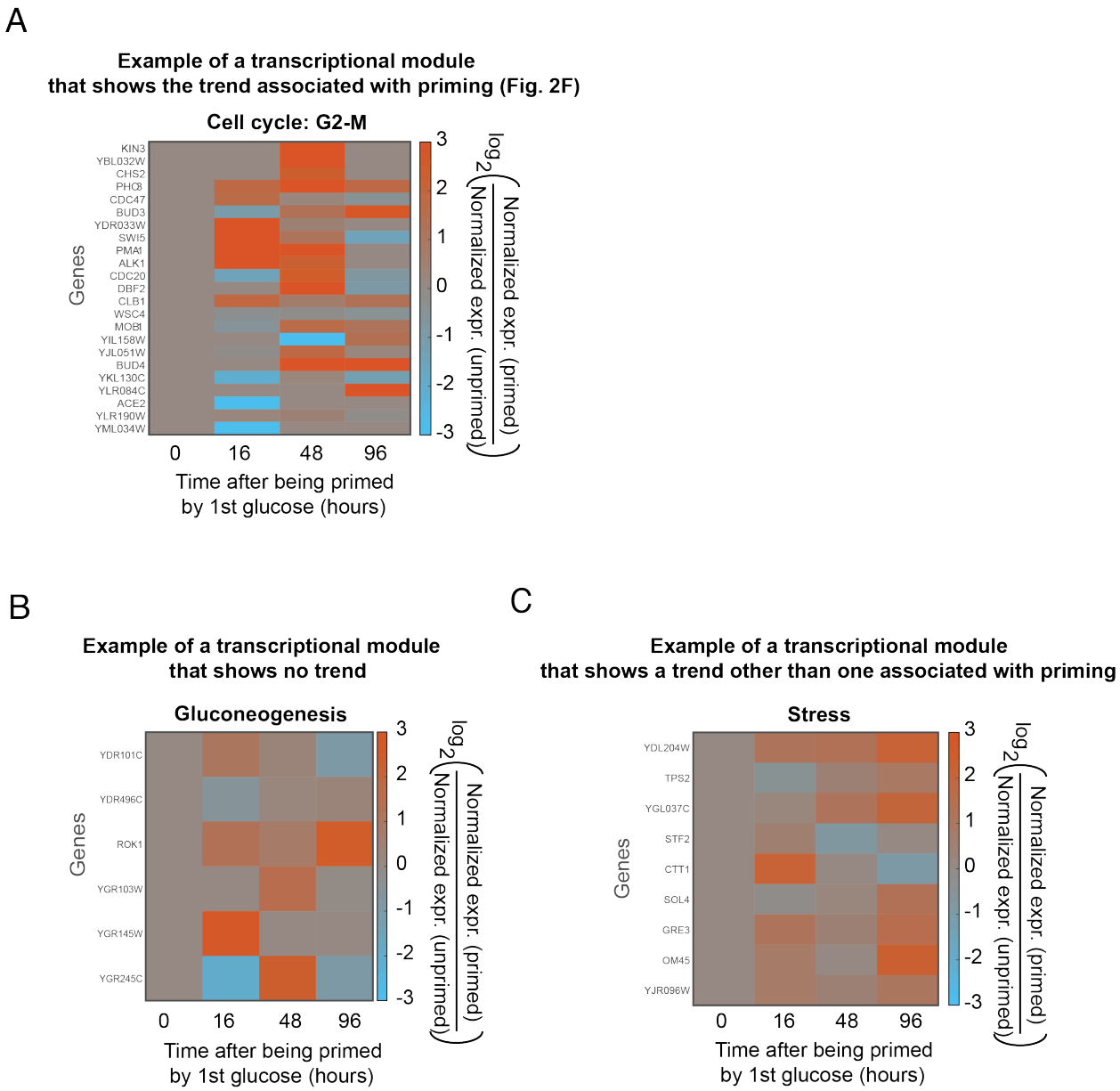



**Supplementary Figure 6 | Average time taken by wild-type spore-bags to germinate, as a function of the first, low glucose-concentration that primes the spores, in experiments in which we add glucose in two steps (shown in Fig. 2a) (complements Fig. 2f).** We gave glucose in two steps to wild-type spore-bags as indicated in Fig. 2a. We first added a relatively low glucose-concentration (indicated along the horizontal axis in the plot). After 16 hours (yellow) or 48 hours (blue) or 96 hours (red), we added more glucose to raise the total glucose-concentration to 2% (saturating level). The mean time ($\Delta\tau$) plotted along the vertical axis represents the time after adding the second batch of glucose. These are the same data as the ones plotted in Fig. 2f but now shown in different units - the average time ($\Delta\tau$) is now in minutes whereas Fig. 2f shows, for each color, the $\Delta\tau$ after dividing it by the average time taken by the spore bags that did not see any glucose (0%-glucose condition) before receiving the second batch of glucose. $n$ = 3; error bars are s.e.m.



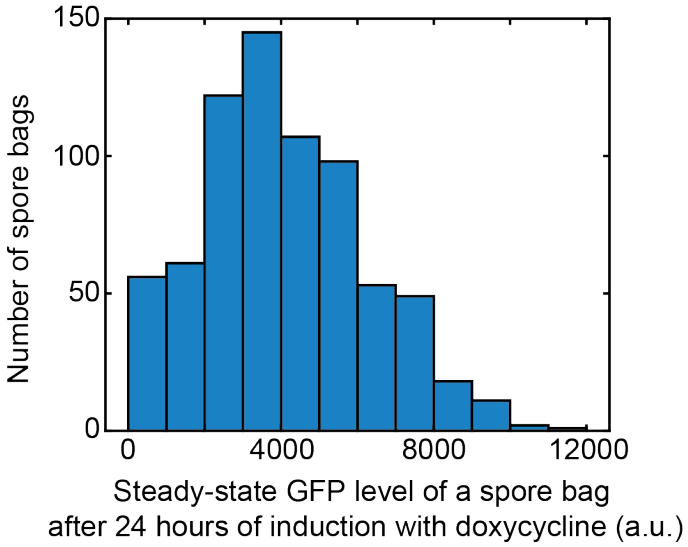

**Supplementary Figure 7 | Zymolyase leaves un-germinated spore-bags intact while lysing vegetative yeasts that result from germinated spores (complements Fig. 2g).** In a mixture of spore-bags and vegetative yeasts, one typically isolates the spore bags by using zymolyase, which lyses vegetative yeasts but not spore bags due to the spore bags' thick, protective outer walls. For this reason, typical (but not all) sporulation procedures (i.e., procedures for forming spores from diploid yeasts) involve adding zymolyase at the end to isolate spore bags and kill off any diploid yeasts that failed to form spore bags. We did not use zymolyase at the end of our sporulation procedure because we typically had high yields of spore bags and, more importantly, zymolyase hurts the spore bags by causing them to lose their protective walls (seen in (B)). We did not want to hurt the spore bags in our experiments. Since our experiments involved using a microscope to track individual spore bags, we could always distinguish vegetative cells from spores. Thus, we did not need to add zymolyase at the end of our sporulation procedure (zymolyase is necessary for population-level experiments in which one does not track individual spore bags). But we used zymolyase to isolate primed, un-germinated spores (Fig. 2e) from the vegetative cells that resulted from the spore bags that did germinate. To be sure, we checked by microscopy that zymolyase indeed lysed vegetative cells and left behind only un-germinated spore bags. **(A)** A representative microscope-image that shows un-germinated spore bags in the absence of zymolyase (scale bar, 5 μm). **(B)** A representative microscope-image that shows intact, un-germinated spore bags after the zymolyase treatment (not the same field of view as (A)). As seen here, spore bags appear smaller after encountering zymolyase than they did before they encountered zymolyase because zymolyase partially degrades their protective walls (note the lack of white-outline in (B) that exists around the spore bag in (A). But the spores are still intact and kept together as one unit inside a bag, as seen here (scale bar, 5 μm). After adding

zymolyase, we immediately proceeded to the next step, in which we lysed the spores to extract their RNAs for RNA-seq (Fig. 2g and Supplementary Fig. 8).

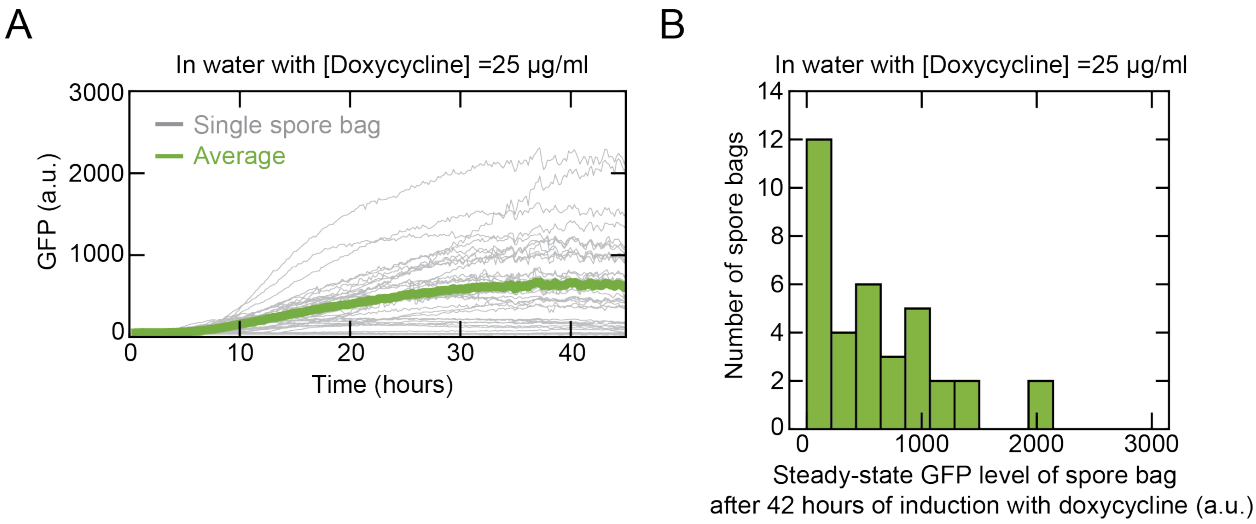



**Supplementary Figure 8 | Genome-wide view of primed dormancy (representative transcriptional modules shown; complements Fig. 2g).** As described in the main text and in Materials and Methods, we performed a transcriptome (RNA-seq) analysis of spores that did not germinate after encountering a low glucose-concentration. This plot showcases details of RNA-seq analyses that led to the summarized results shown in Fig. 2g. Our analysis relies on an insightful previous work by Joseph-Strauss et al. (2007) (*10*) that identified a list of transcriptional modules by studying yeast spores that germinated after receiving a saturating concentration (2%) of glucose (list of genes that they found for each transcriptional module, which we used here, is in Supplementary Table 1). In the three heat maps shown here, the colors represent normalized

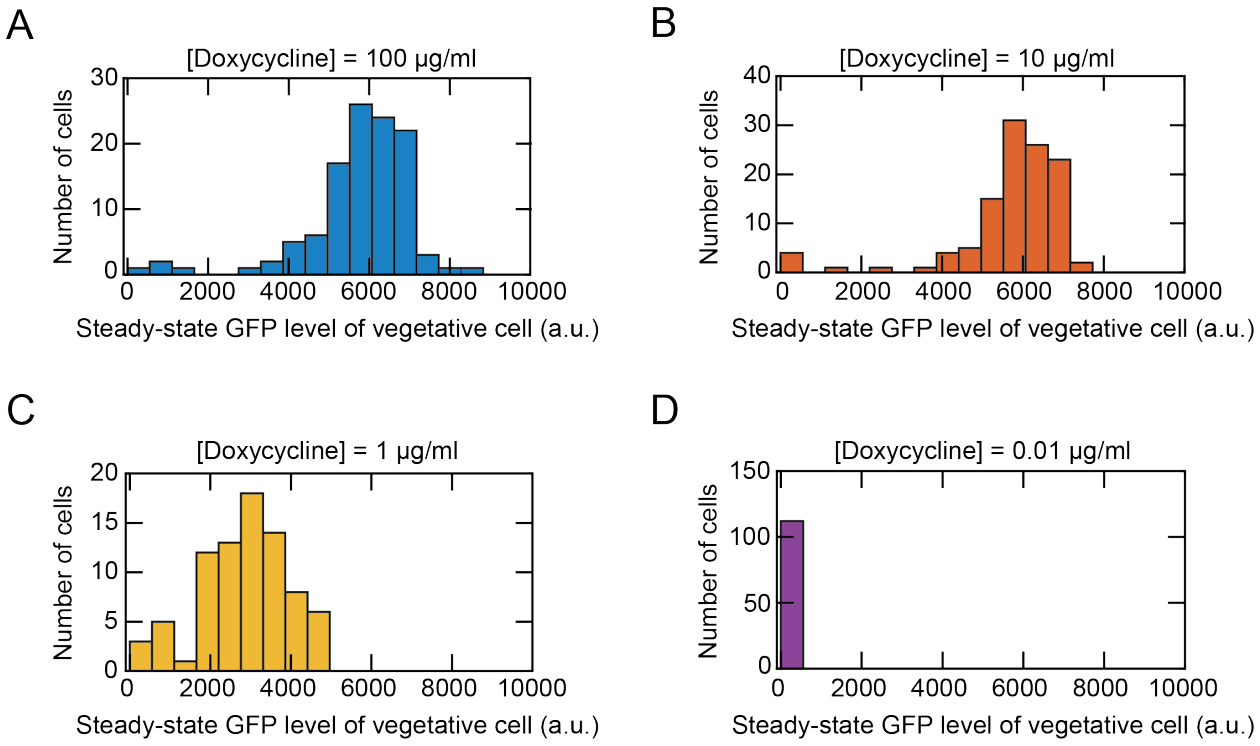
gene-expression levels for individual genes within each transcriptional module whereas Fig. 2g shows a single, normalized gene-expression for an entire transcriptional module at each time point that we obtained by averaging the expression levels of all genes in that module. "Normalization" for (A-C) means that we divided the expression level of a given gene for spores that received a low concentration of glucose - which did not germinate them - by the expression level of the same gene for spores that were kept in minimal media without glucose for the same amount of time as the spores that received the glucose (for Fig. 2g, we normalized in the same way except that we used the average expression level of a module instead of individual genes). Here we show representative transcriptional modules that reveal how studying individual genes can give a different perspective from the one provided by averaging over all genes in a module (Fig. 2g). **(A)** Normalized gene-expression profiles of the “Cell-cycle: G2-M” module (list of genes in Supplementary Table 1). When we average the expression levels of all genes in this module for each time point (Fig. 2g), we observe a clear temporal trend that qualitatively mirrors the temporal trend in the average time taken by the primed spores to germinate (Fig. 2f) - namely, the normalized gene-expression level for the module (Fig. 2g) is elevated after 16 hours and 48 hours but decays away after 96 hours. Interestingly, we observe the same trend for a number of individual genes in this module, with slight differences in timing (i.e., heat map here shows some genes having their expression level peaking at 16 hours while others do so at 48 hours). **(B)** Normalized gene-expression profiles of the “gluconeogenesis” module (list of genes in Supplementary Table 1). When we average the expression levels of all genes in this module for each time point (Fig. 2g), we observe no clear trend. However, when we study the expression levels of individual genes in this module, as shown here, we observe diverging trends (i.e., some genes have a red pixel while others have a green pixel at the same time point), explaining the absence of any observable trends when we average over all genes to get a single expression-level for this module (Fig. 2g). **(C)** Normalized gene-expression profiles of the “stress” module (list of genes in Supplementary Table 1). When we average the expression levels of all genes in this module for each time point (Fig. 2g), we observe a clear trend (i.e., elevated expression-level over time) that is qualitatively different from the temporal trend in the average time taken by the primed spores to germinate (Fig. 2f). When we study the expression levels of individual genes in this module, as shown here, we see a homogeneous expression profile (i.e., all genes have red pixels at and after 16 hours - no temporal undulations in the expression levels over time).



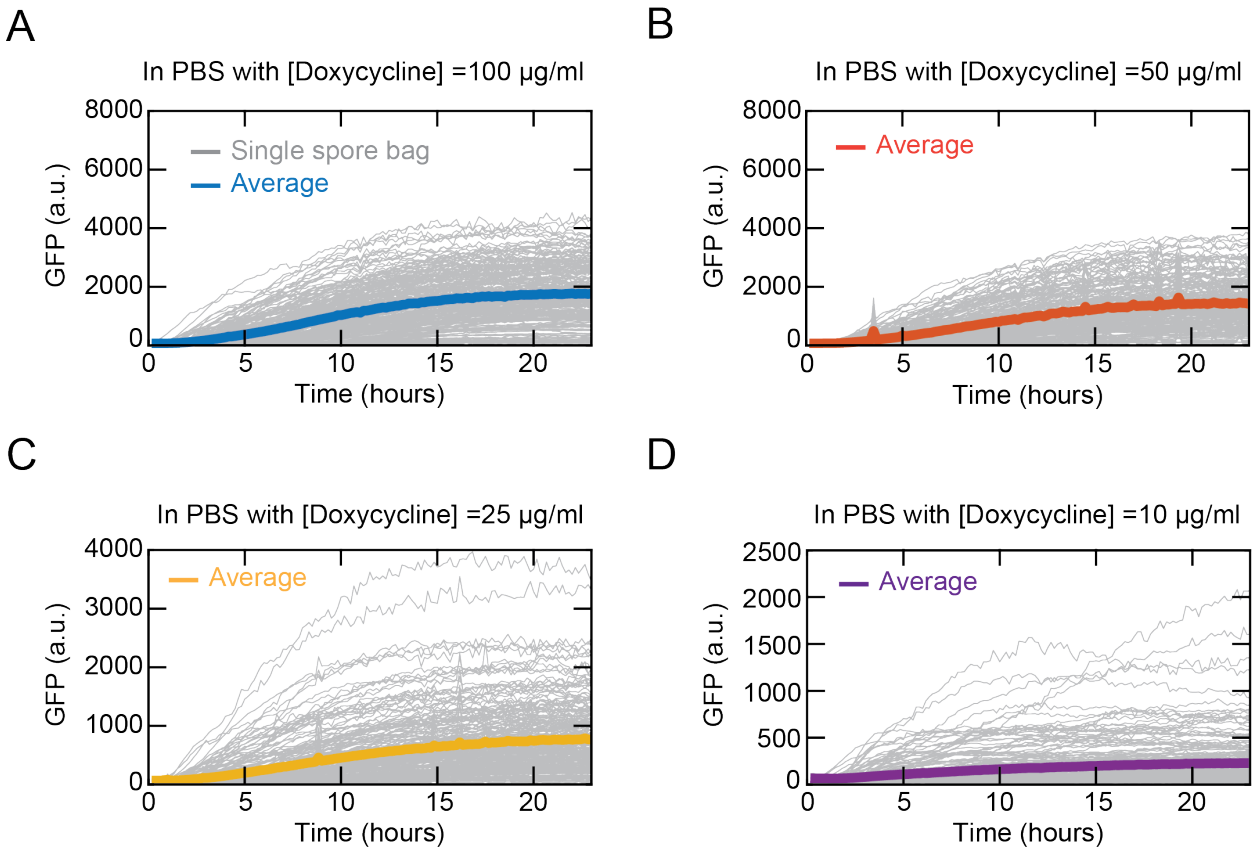

**Supplementary Figure 9 | GFP expression by dormant spores without any nutrients (in PBS) (complements Fig. 3c).** We incubated a population of GFP-inducible spores (Fig. 3a) in a saline solution (PBS) with 100 μg/ml of doxycycline for 24 hours. By the end of the 24-hour incubation, spore bags' GFP levels reach steady-state values (Fig. 3c). At the end of the 24-hour incubation, we measured the steady-state GFP level of each spore bag in the population with a wide-field, epifluorescence microscope and plotted the distribution of their GFP levels here ($n$ = 723 spore bags counted). To measure the GFP level of a single spore bag, we computed the average intensity of all the pixels that belonged to a single spore bag in a microscope image in the GFP-channel. This average is the steady-state GFP level of a spore bag which we plotted here for multiple spore bags in a population. Here, we also subtracted the background fluorescence value so that a GFP level of zero represents a spore bag with no GFP (i.e., it has the same fluorescence as the background).




**Supplementary Figure 10 | Induction of GFP expression is possible for spores that are in water without any nutrients (no amino acids and no glucose) (complements Fig. 3b). (A)** GFP level of individual spore bags (grey curves) over time during 42 hours of incubation in Milli-Q water (dd$H_2O$) with 25 µg/ml of doxycycline (*n* = 36 spore bags). Green curve is the GFP-level averaged over all the spore bags. We see here that the spore bags require about 30 to 40 hours to produce steady-state levels of GFP in water whereas they require ~20 hours to do so in PBS with the same doxycycline concentration (see Supplementary Fig. 12C). **(B)** Steady-state GFP-levels of individual spore bags after 42 hours of incubation in Milli-Q water with 25 µg/ml of doxycycline. These results show that spores, in plain water without any nutrients (no amino acids and no glucose), can still highly express GFP - as high as vegetative yeasts (compare (B) with Supplementary Fig. 11C). Interestingly, while spore bags in PBS reach half of their saturating GFP levels after 10 hours of induction (see Fig. 3c or Supplementary Fig. 12), we see here that the spore bags in water take ~30 to 40 hours to reach steady-state GFP levels. In particular, as seen in (A), spore bags in water have nearly undetectable levels of GFP even ~10 hours after encountering doxycycline. On the other hand, for the same doxycycline concentration, the average steady-state GFP-levels are similar between spore bags in water and spore bags in PBS.

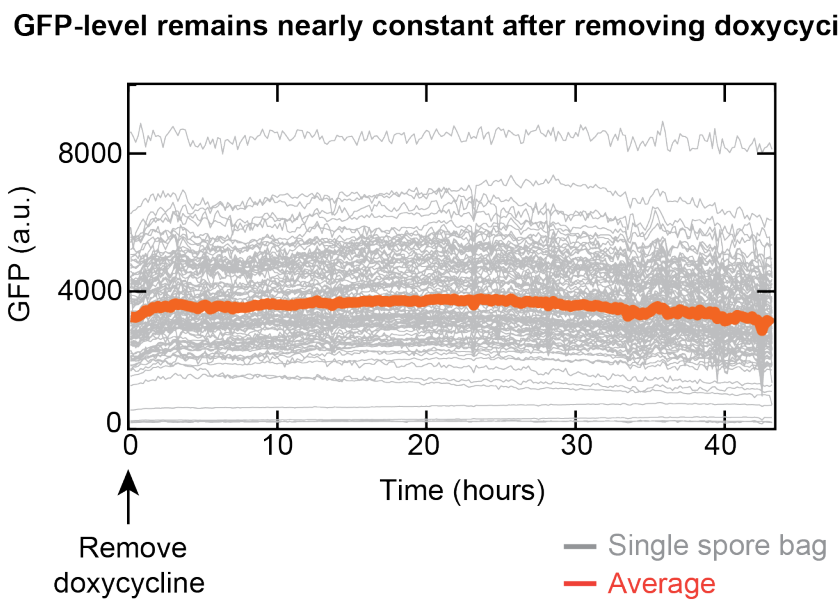



**Supplementary Figure 11 | Steady-state GFP levels of vegetative (replicating) cells that have the same synthetic gene-circuit as the GFP-inducible spores (Fig. 3a) (for comparison with the steady-state GFP levels of spore bags) (complements Fig. 3c).** We sought to compare how the GFP levels of spores compare with the GFP levels of replicating cells that have the same gene circuit. Shown here are the steady-state GFP-levels of individual, replicating, diploid cells that have the same GFP-inducing gene-circuit as the GFP-inducible spores (Fig. 3a). In fact, we sporulated these cells to form the GFP-inducible spores. We measured these GFP levels after 8 hours of incubation in minimal media with a 2%-glucose and **(A)** 100 μg/ml of doxycycline ($n$ = 112 cells), or **(B)** 10 μg/ml of doxycycline ($n$ = 113 cells), or **(C)** 1 μg/ml of doxycycline ($n$ = 80 cells), or **(D)** 0.01 μg/ml of doxycycline ($n$ = 108 cells). Data obtained by using wide-field epifluorescence microscopy as in Supplementary Fig. 9. By comparing these GFP levels of diploid, replicating cells with the GFP levels of spores (Supplementary Fig. 9), we see that even without nutrients, spores can produce GFP at levels that are similar to those of replicating cells. The two main differences between the spores and vegetative cells is that (1) the spores without nutrients requires more time (~24 hours) to reach steady-state GFP levels where as the vegetative cells require only ~ 8 hours to reach steady-state GFP levels and that (2) spores need more doxycycline (100 μg/ml) than the vegetative cells (1 μg/ml) to reach similar GFP levels.

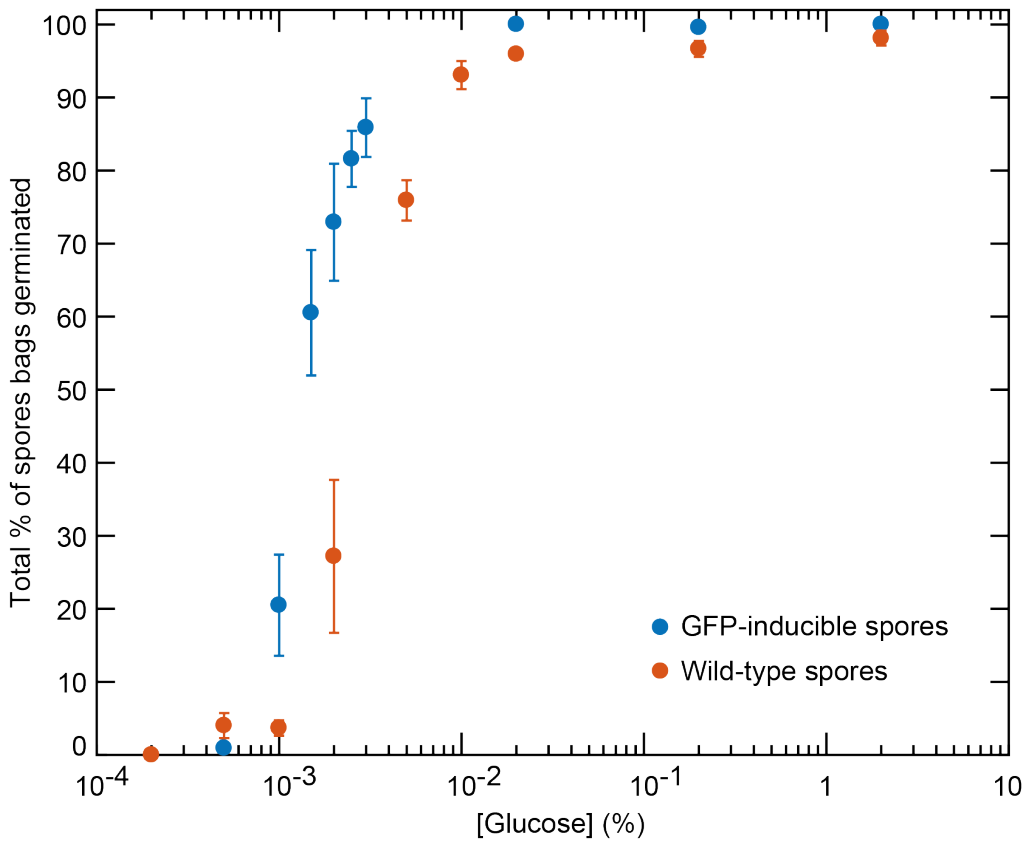



**Supplementary Figure 12 | GFP expression without nutrients as function of time for different doxycycline concentrations.** GFP level of a single spore bag (grey curve) and population-level average (solid colored curves) over time while incubated in PBS (i.e., without any nutrients such as glucose and amino acids). We incubated the GFP-inducible spores in PBS with a set doxycycline-concentration (indicated above each graph) and then used a wide-field, epifluorescence microscope to measure the GFP levels of each spore bag for the next 22 hours. As seen in the grey curves plateauing over time, the GFP level of each spore bag reached a steady-state value by the end of the 22-hours of imaging. **(A)** [Doxycyline]=100 μg/ml, $n$ = 233 spore bags; **(B)** [Doxycyline] = 50 μg/ml, $n$ = 194 spore bags; **(C)** [Doxycycline] = 25 μg/ml, $n$ = 217 spore bags; **(D)** [Doxycyline] = 10 μg/ml, $n$ = 206 spore bags. Here, we measured the GFP levels continuously over time with a microscope for 22 hours to obtain the kinetics of GFP production whereas in Supplementary Fig. 9, we performed one-time measurement on a microscope at the end of the 22 hours of incubation. We observed that spores could achieve higher GFP levels for the same doxycycline concentration if we incubated them in a continuously mixing liquid medium for 22 hours (Supplementary Fig. 16) rather than in a stationary liquid

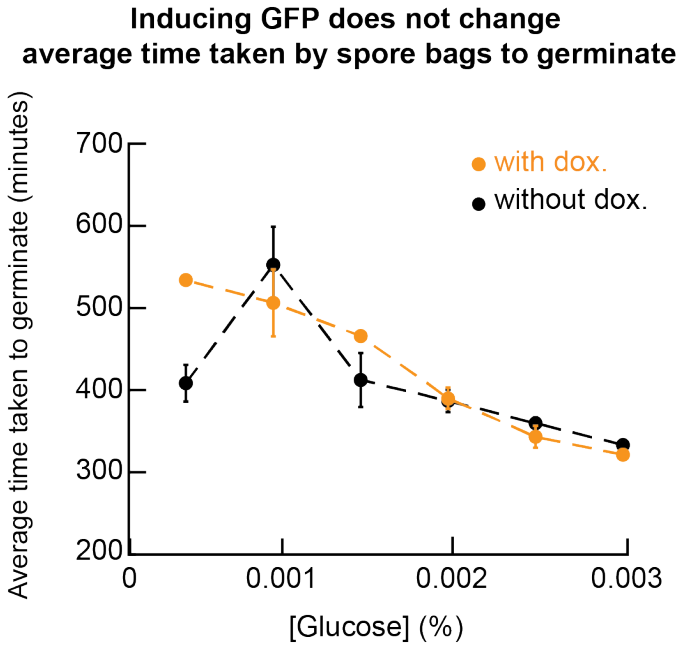
medium inside a microscope well for 22 hours as shown here, due to the difference in culturing conditions.



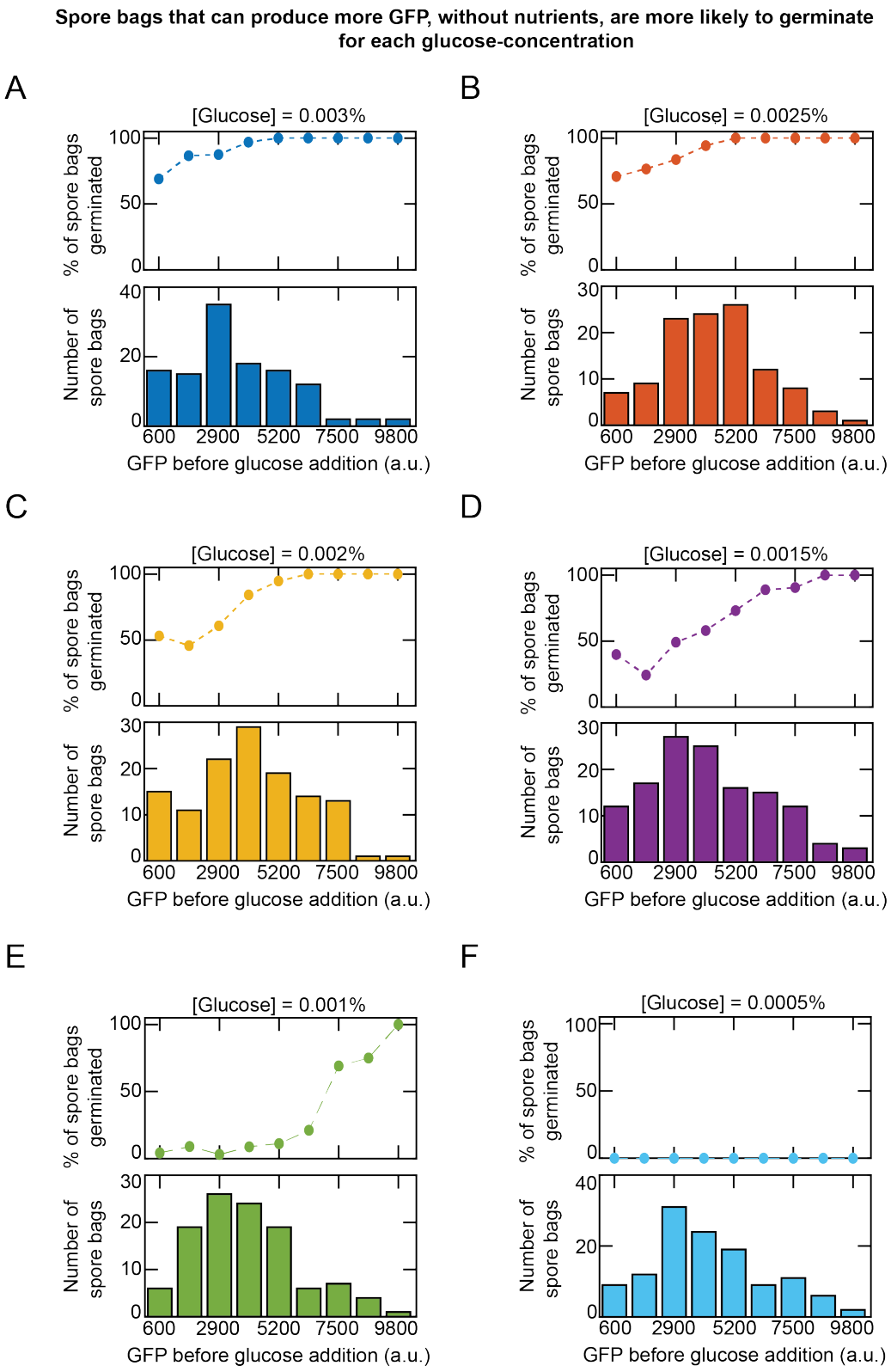

**Supplementary Figure 13 | GFP level of a spore bag remains nearly constant after removing doxycycline.** GFP-levels of individual spore bags (grey curves) during 42 hours in PBS without doxycycline (*n* = 101 spore bags). The red curve shows the average GFP-level for these spore bags over time. We first induced GFP expression in these spores for 24 hours with 100 μg/ml of doxycycline. At the end of the 24-hour incubation, the GFP levels reached their steady-state values (Fig. 3c and Supplementary Fig. 12). Then, we removed the doxycycline and washed away any residual doxycycline with PBS several times. We then incubated these spores in PBS at 30 $^{O}$C (start of this incubation marks "0 hours"). By doing so, we sought to understand why the GFP-levels reach steady-state values given that spores are not dividing to dilute away their accumulated copies of GFP - a vegetative cell's GFP level would reach a steady-state value because the production rate of GFP matching the dilution rate of GFP (dilution by cell divisions). If a spore bag's GFP level reached a steady-state value because of its GFP-production rate matching the GFP-degradation rate, then stopping the production of GFP by removing the doxycycline should cause decreases in its GFP-level. This is because GFP can then only degrade stochastically (thermally) and it cannot be replenished. As seen in the grey curves and the red curve, we did not observe any significant decreases in the GFP levels during the 42-hours that followed the removal of doxycycline. Thus, the reason that the GFP levels reached steady-state values is not because of the GFP-production rate matching the GFP-degradation rate during the 24 hours of induction with doxycyline. In fact, we see here that the GFP-degradation rate is nearly zero inside the spores. Thus, we can conclude that it is the eventual stopping of GFP-production, while doxycycline is still present, that causes the GFP-levels to reach steady-state values during the 24-hours of induction.




**Supplementary Figure 14 | Comparing efficiency of germination for wild-type spores with that of GFP-inducible spores (complements Fig. 3d).** The GFP-inducible spores (Fig. 3a), aside from *GFP*, has additional genes (selection markers such as amino-acid biosynthesis genes) that we inserted during the yeast transformations (i.e., *ADE2, TRP1, URA3*). To see how these selection markers, as some of them pertain to amino-acid biosynthesis, might affect the percentage of spore bags that germinate for a given glucose-concentration, we gave different concentrations of glucose to the GFP-inducible spores (without any doxycycline, shown as blue data points) and compared the percentage of these spore bags that germinated with the percentage of wild-type spore bags that germinated (red data points) for the same glucose-concentration. As shown here, more GFP-inducible spores germinate than the wild-type spores for the same glucose-concentration but the overall trend is the same for both types of spores. $n$ = 3; error bars are s.e.m.

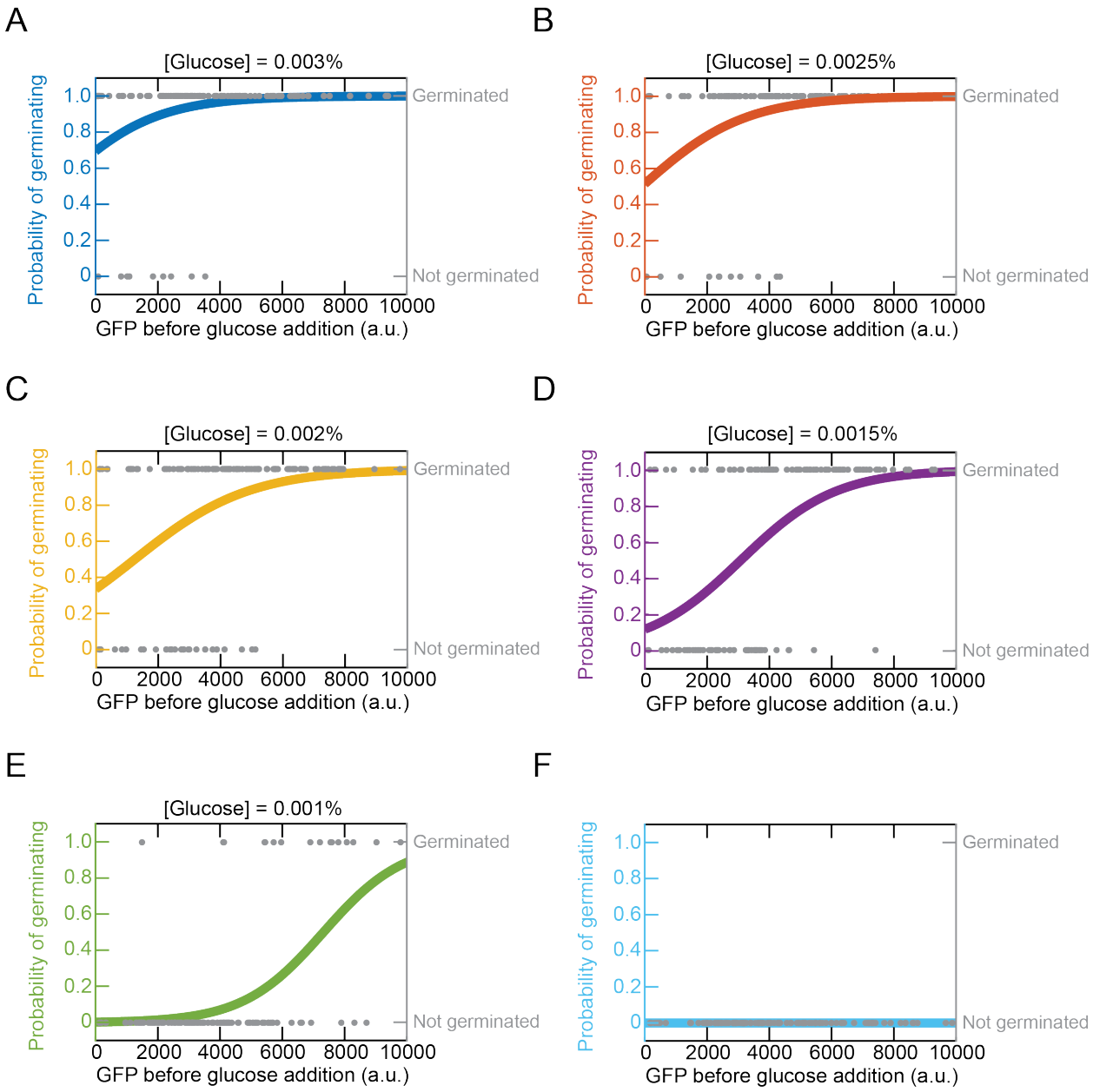



**Supplementary Figure 15 | Inducing GFP production does not appreciably alter the average time taken by spore bags to germinate (complements Fig. 3d).** Same experiments and data as in Fig. 3d but now showing the average time taken by the GFP-inducible spore bags to germinate for low glucose-concentrations. Orange data points are for the GFP-inducible spores (Fig. 3a) that we first incubated with doxycycline for ~24 hours prior to receiving glucose (thus these spores have steady-state GFP levels prior to receiving glucose) and the black data points are for the GFP-inducible spores that did not receive any doxycyline (thus these spores have not produced GFP prior to receiving glucose). There is virtually no difference between the two conditions. Only for the lowest glucose concentration shown here, we see some differences in the average time taken for germination between the two conditions. This is due to small-number fluctuations (i.e., almost no spore bag germinates at such a low glucose concentration; we observed at most one or two spore bag germinating, if any, out of hundreds (c.f. Supplementary Fig. 1H)). Although not shown here, inducing GFP expression also does not appreciably alter the average time taken to germinate at much higher glucose concentrations than shown here (up to 2%-glucose) and it also does not appreciably alter the percentage of spore bags that germinate at each glucose concentration (shown in Fig. 3d). $n$ = 3; error bars are s.e.m.



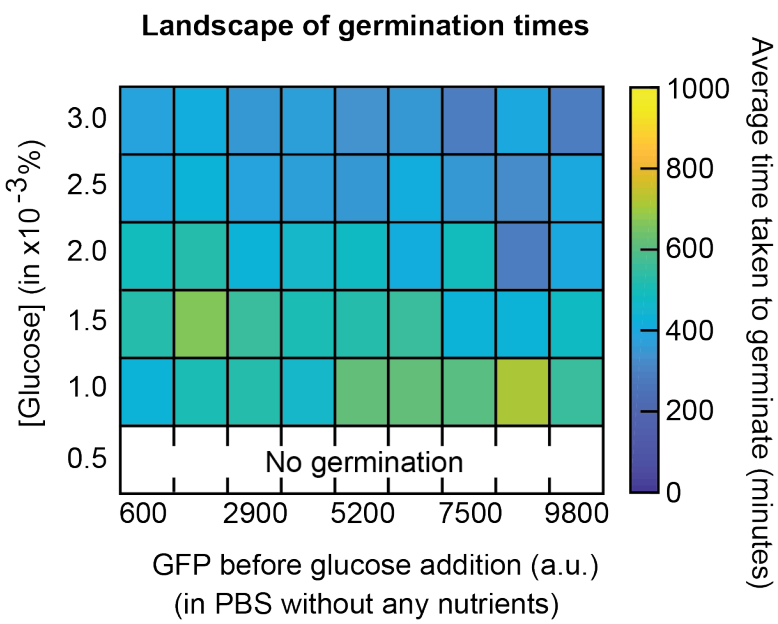

**Supplementary Figure 16 | Spore bags that can express more GFP without any nutrients (in PBS) are more likely to germinate for each glucose-concentration (complements Fig.**

**3e).** As liquid cultures inside rotating tubes, we incubated the GFP-inducible spores (Fig. 3a) in PBS first with 100 μg/ml of doxycycline for 22 hours. The spore bags' GFP levels reached steady-state values which we measured after transferring the spores onto microscope-imaging wells. After measuring the GFP levels at the end of the 22-hour incubation in this way, we removed the PBS containing doxycycline and then replaced it with a minimal medium that contained a relatively low concentration of glucose (indicated above each panel). We then measured, for each glucose concentration, how many of the spore bags that had similar GFP levels (i.e., GFP levels that fall within a binning range shown in the histograms above) germinated. **(A)** 0.003%-glucose, $n$ = 118 spore bags; **(B)** 0.0025%-glucose, $n$ = 113 spore bags. **(C)** 0.002%-glucose, $n$ = 125 spore bags. **(D)** 0.0015%-glucose, $n$ = 131 spore bags; (**E)** 0.001%-glucose, $n$ = 112 spore bags; and **(F)** 0.0005%-glucose, $n$ = 124 spore bags. The data shown here are for one of three biological replicates. We used all three biological replicates to construct the germination landscape in Fig. 3f. We used the procedure outlined here to measure all germination landscapes (Fig. 3f and in Fig. 4). We observed that spores could achieve higher GFP levels for the same doxycycline concentration if we incubated them in a continuously mixing liquid medium for 22 hours, as we did here, rather than in a stationary liquid medium inside a microscope well for 22 hours (Supplementary Fig. 12), due to the difference in culturing conditions. To be consistent, we used the method shown here (rotating liquid cultures) to obtain all germination landscapes and all the main conclusions in our study (e.g., probability of germinating as a function of GFP). We only cultured the GFP-inducible spores in microscope-imaging wells and continuously imaged them for 22 hours to show the kinetics of GFP-expression over time (only for Supplementary Figs. 10, 12, and 13 and Fig. 3c) but not for deriving the probabilities of germinating as a function of GFP levels. Importantly, our study's main conclusions, such as those expressing more GFP are more likely to germinate, are unaffected by the method of culturing GFP-inducible spores since these conclusions rely on relative levels of GFP rather than on the absolute levels of GFP.

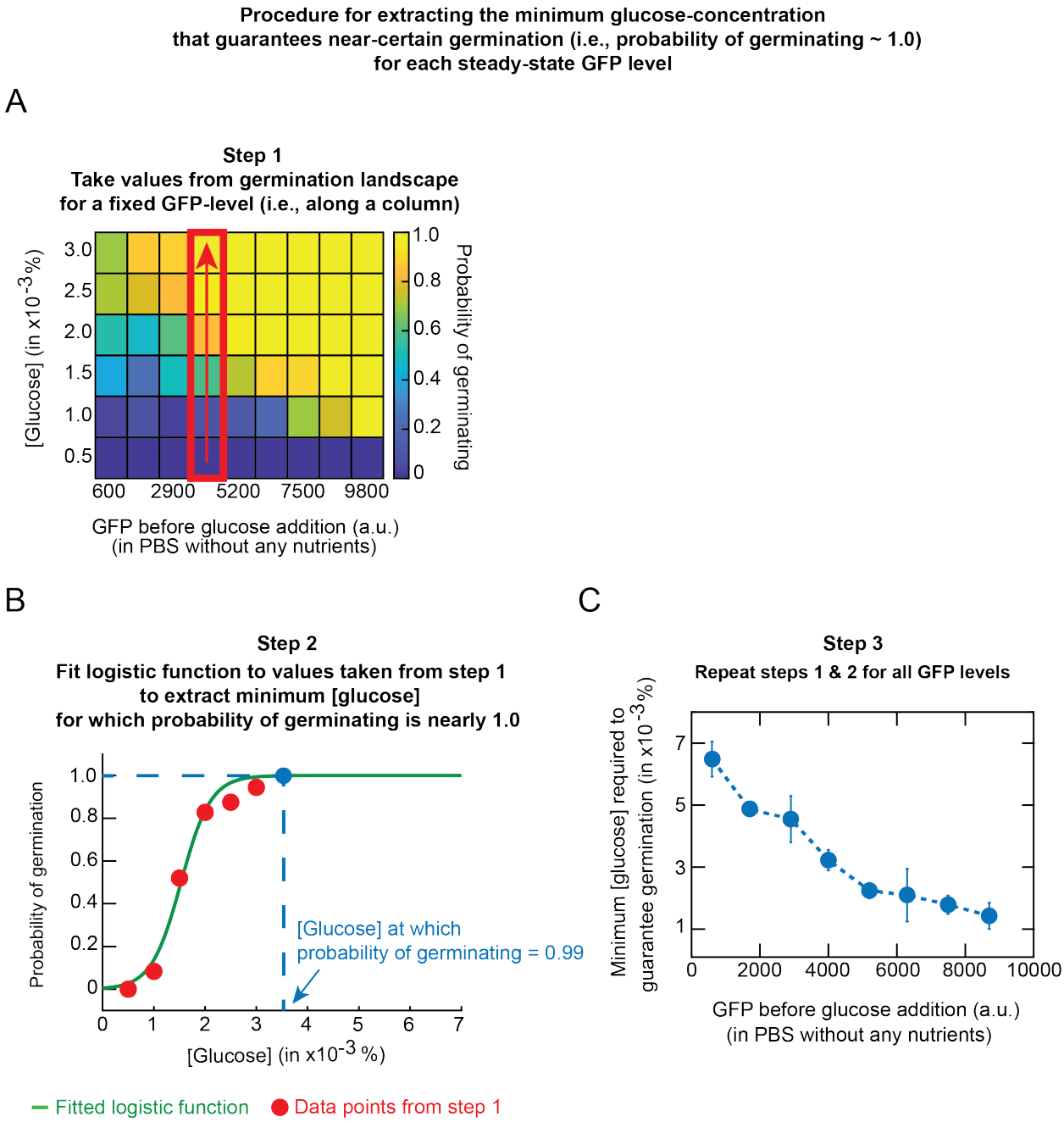



**Supplementary Figure 17 | Statistical test with a logistic regression-fit establishes that spore bags that can express more GFP without nutrients are more likely to germinate for each glucose-concentration (complements Supplementary Fig. 16 and the germination landscape in Fig. 3f).** Same experiments as in Supplementary Fig. 16 (i.e., GFP-inducible spores incubated for ~22 hours in PBS with 100 μg/ml of doxycycline, then measuring the steady-state GFP-levels of individual spore bags, and then finally adding glucose at concentrations as indicated above each panel (A-F) to determine which spore bags germinated). We used the same data as in Supplementary Fig. 16 except that now, we plot the data differently. Each grey data

point represents a single spore bag. For each spore bag, whose steady-state GFP-level we measured before adding glucose, we assigned it a "1" if it germinated or a "0" if it did not germinate after receiving the specified concentration of glucose. We then plot these grey data points (1 or 0) as a function of the GFP-level of each spore bag in the panels shown above (A-F). Afterwards, we performed a logistic regression on the grey data points, for each glucose-concentration, by fitting a logistic function, $p(x) = \frac{1}{1+e^{-(\beta_0+\beta_1 x)}}$, for the probability $p(x)$ that a spore bag with a steady-state GFP-level of $x$ germinates with a specified glucose-concentration. We used MATLAB's built-in "mnrfit" script to perform the logistic-regression fits (colored curves for each panel (A-F)). With the logistic function $p(x)$, testing a statistical link - that is, showing that there is a positive correlation between the GFP-level of a spore bag and its probability to germinate for a given glucose-concentration - is equivalent to testing whether the *x* (GFP-level before the spore bag receives glucose) is a sufficient predictor of the observed probability to germinate. We have done this by computing the p-value associated with the Wald test on the fit parameter $\beta_1$ which multiplies the *x* in $p(x)$. For every glucose-concentration, we found that the p-values were either below or equal to 0.01, meaning that the steady-state GFP-level of a spore bag indeed is a sufficient predictor for that spore bag's probability of germinating at the given glucose-concentration. Specifically, we found: **(A)** for a 0.003%-glucose: p-value ≈ 0.01, $\beta_1$ = -0.00063 $\pm$ 0.00049, *n* = 118 grey data points (92% germinated); **(B)** for a 0.0025%-glucose, p ≈ 0.002; $\beta_1$ = -0.0006 $\pm$ 0.00037, *n* = 113 grey data points (89% germinated); **(C)** for a 0.002%-glucose, p-value ≈ 3 x $10^{-5}$; $\beta_1$ = -0.00054 $\pm$ 0.00026, *n* = 125 grey data points (77% germinated); **(D)** for a 0.0015%-glucose, p-value ≈ 4 x $10^{-7}$; $\beta_1$ = -0.00066 $\pm$ 0.00025, *n* = 131 grey data points (61% germinated); **(E)** for a 0.001%-glucose, p-value ≈ 2 x $10^{-5}$; $\beta_1$ = -0.00077 $\pm$ 0.00035, *n* = 118 grey data points (14% germinated); and **(F)** for a 0.005%-glucose, we did not observe any germinations in this data set. We have shown data from just one biological replicate here as a representative data set.



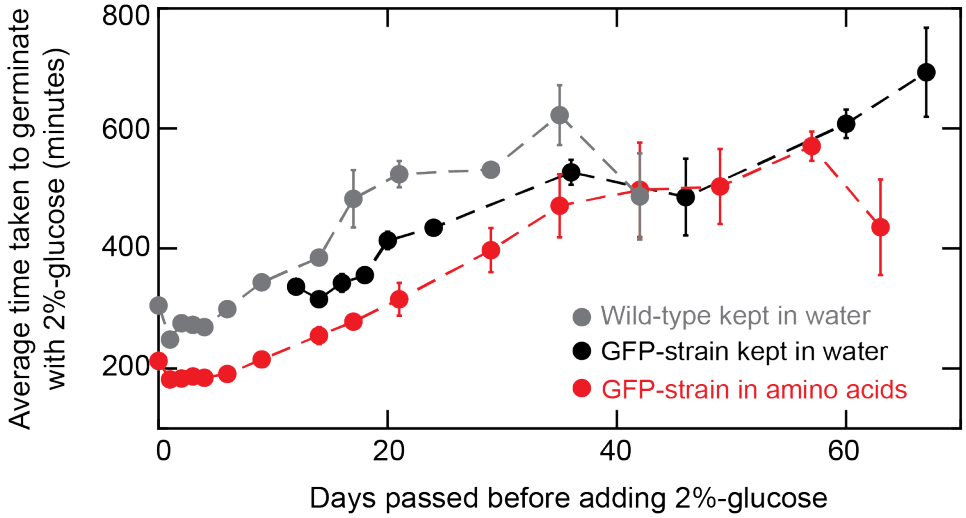

**Supplementary Figure 18 | Average time taken to germinate depends weakly on the steady-state GFP-levels that spore bags have without any nutrients (complements Fig. 3f).** Each color represents the average time taken by a spore bag to germinate as a function of the glucose-concentration that it encounters and its steady-state GFP-level before it receives any glucose (result of GFP-induction with 100 μg/ml of doxycycline for 22 hours in PBS). Each color represents an average from three different populations of the GFP-inducible spores (from the same three biological replicate-populations as in Fig. 3f). For the lowest row, which represents a 0.0005%-glucose, the average time taken by a spore bag to germinate is undefined because we did not observe any spores germinating with this very low glucose-concentration. The lack of any dramatic changes in the shading of the colors across the pixels indicates that the average time taken by a spore bag to germinate depends weakly on the glucose concentration - a result that mirrors our earlier observation that the average time taken to germinate by the wild-type spores is also nearly independent of the glucose concentration (Fig. 1d). Crucially, we see here that the average time taken to germinate is nearly independent of a spore bag's steady-state GFP-level in PBS (as indicated by the absence of any clear changes in the shading of the colors across the pixels within a given row).



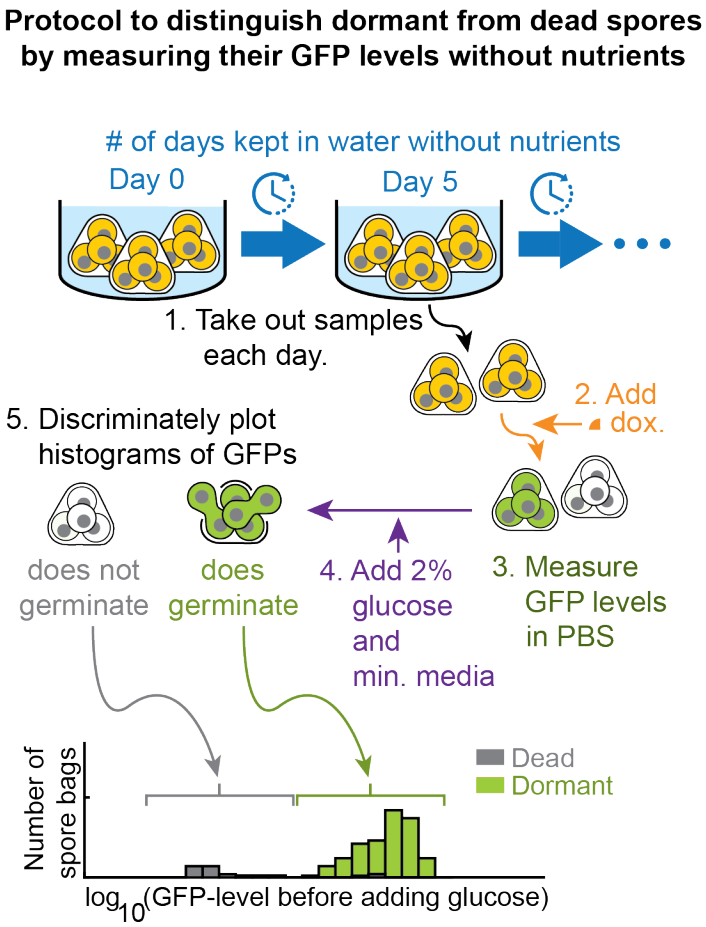

**Supplementary Figure 19 | Three-step procedure shown here establishes that the minimum glucose-concentration that is required for guaranteeing that a spore bag will germinate (i.e., probability of germinating ~ 0.99) decreases as the spore bag's ability to express a generic gene without nutrients increases (complements Fig. 3f). (A)** The germination landscape (copy of Fig. 3f) groups the GFP-levels of spore bags into bins (columns of the heat map), with each bin (column) thus representing a defined range of GFP-levels. For each column of the germination landscape, we read-off the probability to germinate from each pixel (by moving

up within the red box as shown in the figure). **(B)** We plot the values that we read-off from each pixel as a function of the glucose-concentration (red data points). We then fit a logistic function that has the same mathematical form as in Supplementary Fig. 17 but now with a different meaning: $p(x) = \frac{1}{1+e^{-(\beta_0+\beta_1 x)}}$. Here, $p(x)$ is the probability that a spore bag with the GFP-level specified in (A) germinates after encountering a glucose-concentration equal to $x$ (green curve) - note that the $x$ in Fig. S17 represented a spore bag's steady-state GFP-level. Here we chose the logistic function for its simplicity. From the fitted logistic function (green curve), we can extract the value of $x$ (blue point) for which $p(x) \approx 0.99$ (i.e., the glucose-concentration for which the probability of germinating is 0.99). We chose 0.99 because choosing "1" will yield an artificially high value of $x$ given that the logistic function $p(x)$ asymptotically approaches 1 without ever reaching it. **(C)** Repeated the procedure in (A) and (B) for each column of the germination landscape yields the plot shown here: the minimum glucose-concentration that is required to guarantee that a spore bag will germinate (i.e., probability of germination ~ 0.99) if the spore bag's steady-state GFP-level in PBS is as specified in (A). The data points here are averages from three biological replicates (*n* = 3) and the error bars are s.e.m.

**Spores take progressively longer times to germinate as time passes without nutrients**

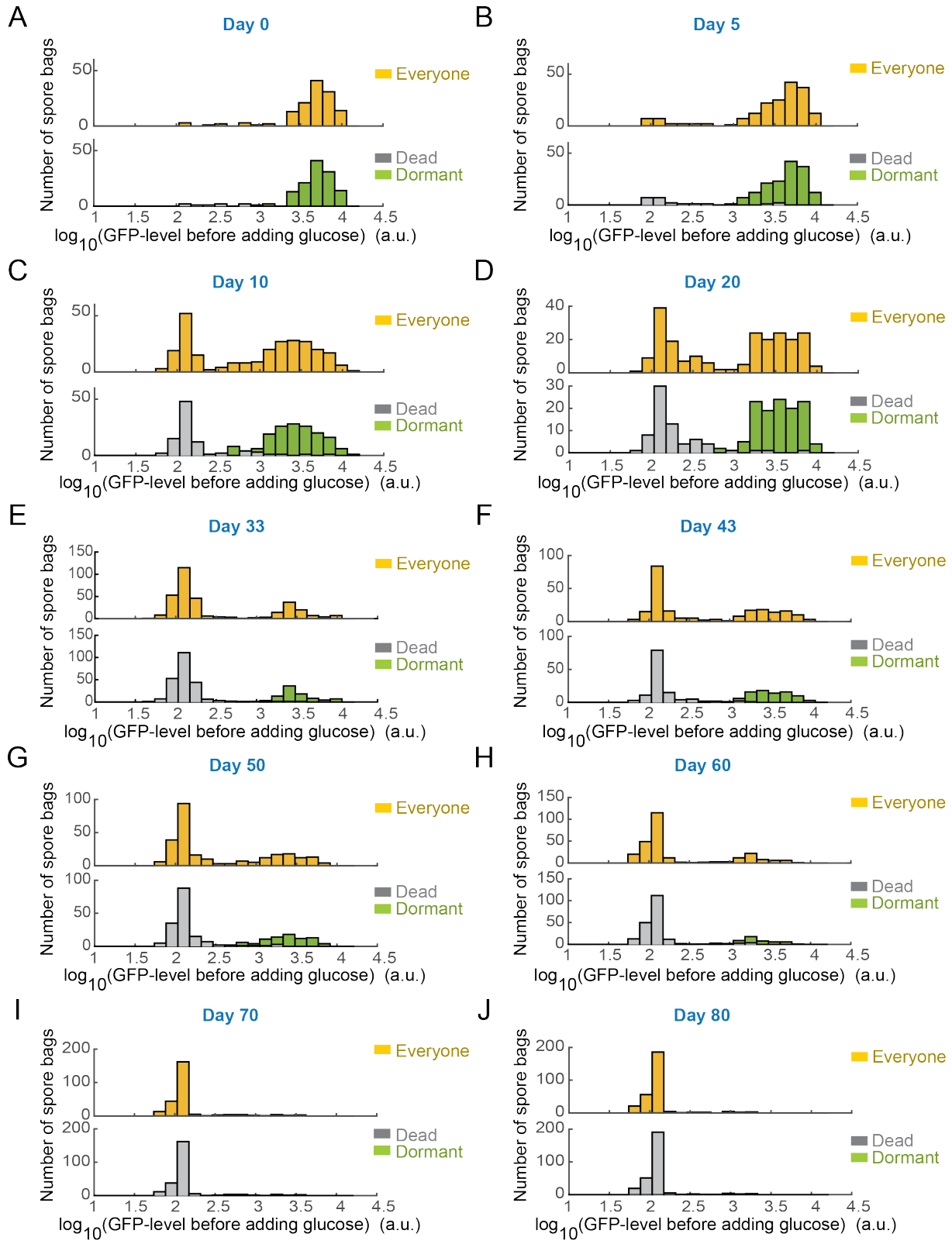



**Supplementary Figure 20 | Dormant spores gradually take longer times to germinate as days pass by without any glucose (complements Fig. 4a).** Same experiments as in Fig. 4a but now plotting the average time taken by the GFP-inducible (black and red) and the wild-type (grey) spore bags to germinate due to a 2%-glucose, as a function of the number of days that they were incubated in either water (grey and black) or minimal media with essential amino acids (red) before receiving the 2%-glucose. In all cases, we that a spore bag, on average, requires more time to germinate the longer it is incubated in water or minimal media without any glucose. Importantly, we see that having the spores incubated in minimal media (which contains all the essential amino acids) does not majorly reduce the average time taken to germinate compared to having the spores incubated in water without any amino acids (compare red with black). Intriguingly, after 60 days (by which point almost all spores are dead according to Fig. 4a), the average time taken for germination is approximately 600 minutes, which is nearly equal to the average time taken by the wild-type spore bags to germinate on day 0 after encountering a 0.0005%-glucose - the smallest glucose-concentration for which we could observe germinations (see Fig. 1d). This suggests that both cases - one being a prolonged (~60-day) incubation without any nutrients and the other being a very low glucose-concentration - are probing spores at the "limits" of germination capabilities. $n$ = 3; error bars are s.e.m.



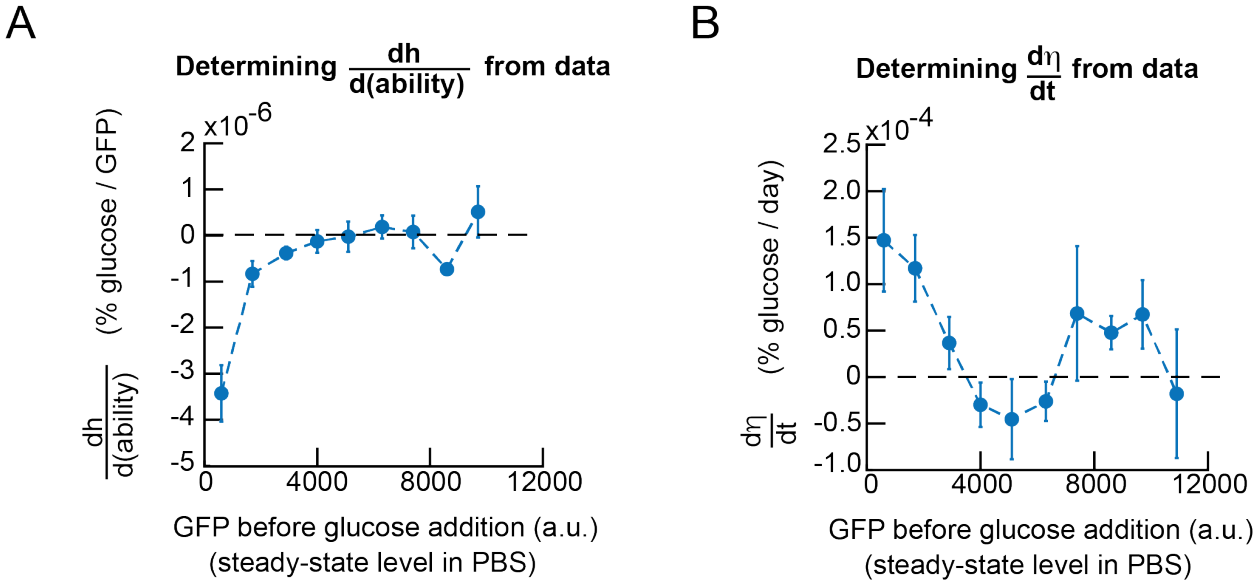

**Supplementary Figure 21 | Protocol for Supplementary Fig. 22, which distinguishes dormant from dead spores by measuring their GFP levels without nutrients on different days of incubation in water without any nutrients at 30 $^{o}$C (complements Fig. 4c-f).** Protocol for measuring how the ability to express a generic gene (GFP) without nutrients changes over time in spore bags incubated in water for many days. The procedure shown here allows us to prove that spore bags lose their ability to express GFP (express a generic gene) and that as a result, the spore bag dies (i.e., once the ability-level reaches "zero"). There is a subtle procedural detail that we did not explain in the main text. By solely measuring the GFP-levels of individual spore bags after inducing them with doxycycline, we cannot distinguish between the values that correspond to dead or non-dead (still dormant) spores. This ambiguity is problematic because we want to demonstrate a cause-and-effect: the ability to express genes decreases *before* death. Since the GFP-level of dead spores is always close to zero (as defined by the relationship in Fig. 4b), by not excluding the GFP-level of dead spores in our analysis, we would then see that the mean GFP-level of the population would decrease over time, simply because spores are dying over time (Fig. 4a). Thus, after inducing GFP expression with doxycycline without nutrients, we

add a 2%-glucose and then observe which spore bags germinate. We then plot the GFP-levels of the spore bags that germinate due to the 2%-glucose in the histograms in Supplementary Fig. 22 and discriminate them from the GFP-levels of dead spore-bags (the ones that do not germinate with the 2%-glucose). In other words, we plot the GFP levels of dormant spore bags in Supplementary Fig. 22 and discriminate them from the GFP levels of dead spore bags.

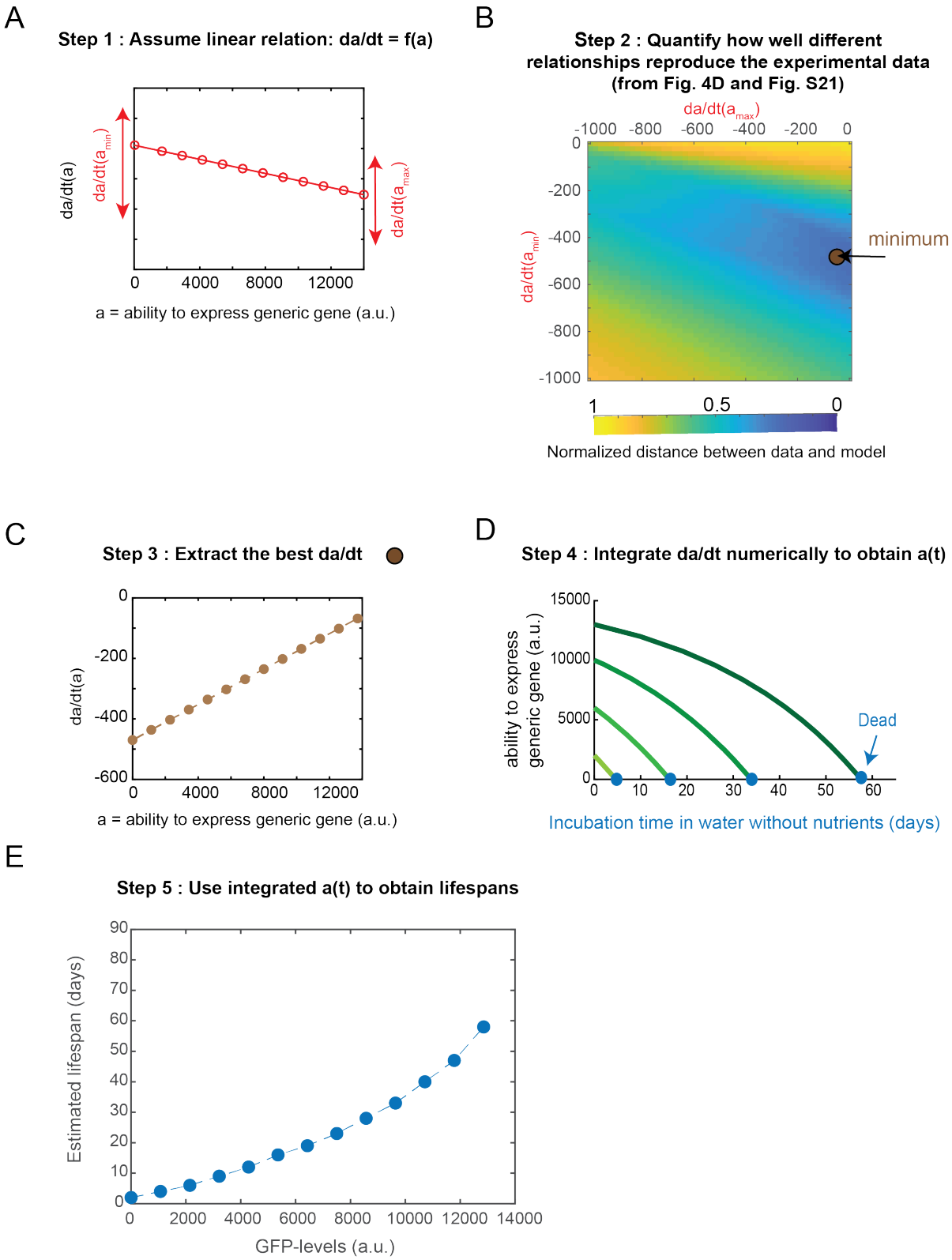



**Supplementary Figure 22 | Dormant spores lose their gene-expressing ability before dying (complements Fig. 4c-f).** With the procedure in Supplementary Fig. 21, we measured the GFP-

levels of each dormant spore-bag and dead spore-bag after incubating them in PBS with 100 µg/ml of doxycycline for 24 hours, on each of the incubation days in water without nutrients (from day 0 to day 80 of incubation in water without nutrients at 30 $^{O}$C). Yellow bars represent the entire population of spore bags; green bars represent dormant (alive) spore bags - these germinated after receiving a 2%-glucose; grey bars represent dead spores - these did not germinate after receiving a 2%-glucose. **(A)** Day 0: *n* = 225 spore bags in total (yellow bars), *n* = 221 dormant spore-bags (green bars); *n* = 14 dead spore-bags (grey bars). **(B)** Day 5: *n* = 180 spore bags in total (yellow bars), *n* = 158 dormant spore bags (green bars); *n* = 22 dead spore-bags (grey bars). **(C)** Day 10: *n* = 270 spore bags in total (yellow bars); *n* = 95 dormant spore-bags (green bars); *n* = 175 dead spore-bags (grey bars). **(D)** Day 20: *n* = 335 total spore-bags (yellow bars); *n* = 101 dormant spore-bags (green bars); *n* = 234 dead spore-bags (grey bars). **(E)** Day 33: *n* = 241 total spore-bags (yellow bars); *n* = 120 dormant spore-bags (green bars); *n* = 121 dead spore-bags (grey bars). **(F)** Day 43: *n* = 240 total spore-bags (yellow bars); *n* = 99 dormant spore-bags (green bars); *n* = 141 dead spore-bags (grey bars). **(G)** Day 50: *n* = 288 total spore-bags (yellow bars); *n* = 100 dormant spore-bags (green bars); *n* = 188 dead spore-bags (grey bars). **(H)** Day 60: *n* = 287 total spore-bags (yellow bars); *n* = 65 dormant spore-bags (green bars); *n* = 222 dead spore-bags (grey bars). **(I)** Day 70: *n* = 265 total spore-bags (yellow bars); *n* = 27 dormant spore-bags (green bars); *n* = 238 dead spore-bags (grey bars). **(J)** Day 80: *n* = 304 total spore-bags (yellow bars); *n* = 15 dormant spore-bags (green bars); *n* = 289 dead spore-bags (grey bars). By plotting the GFP-levels in base-10 logarithm, as we do here, we can see that the GFP levels of dead spore-bags (grey bars) are clearly distinct from those of dormant spore-bas (green bars).

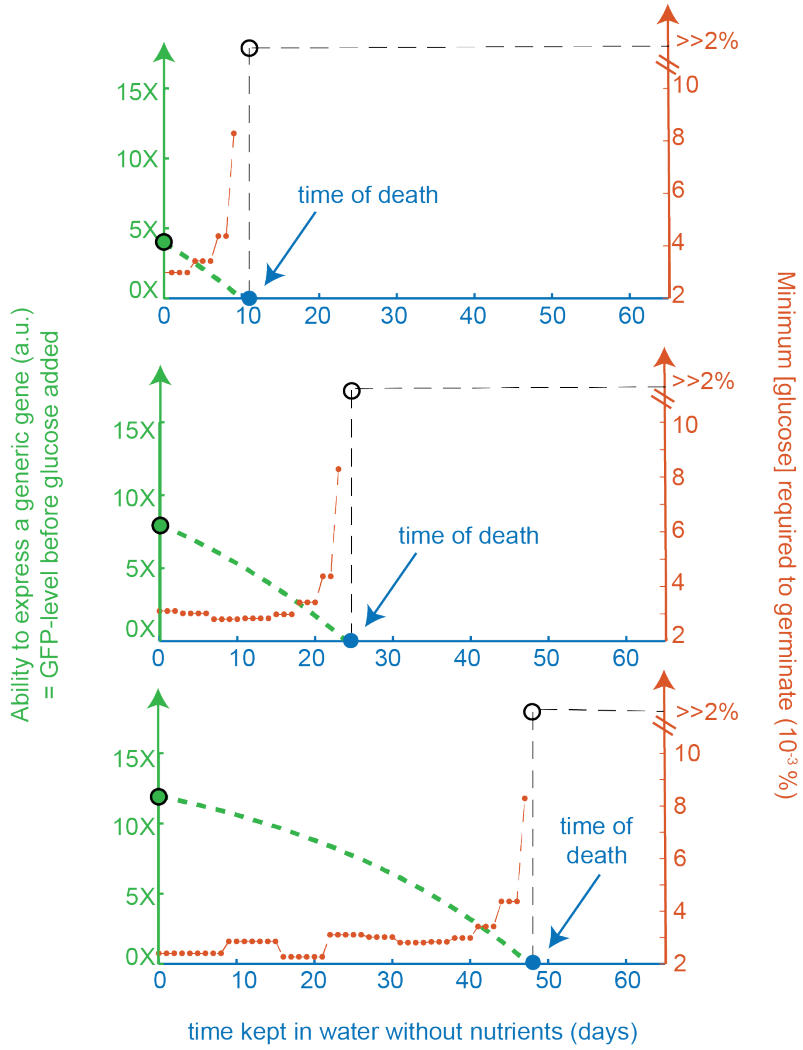



**Supplementary Figure 23 | Extracting the values of $\partial h/\partial(\text{ability})$ and $d\eta/dt$ from measurements (complements Fig. 4f)**. **(A)** To determine $\partial h/\partial(\text{ability})$, we first averaged the values of *h* that are given in Fig. 4e over all the different days (0 ~ 20 days of incubation without nutrients). This yielded, for a fixed GFP-level, the average value of *h* (averaged over time): $< h >$. Then, we computed $\Delta < h >/\Delta(ability)$, which is equal to $\partial h/\partial(\text{ability})$ and is plotted here (*n* = 3; error bars are s.e.m). Averaging $\partial h/\partial(\text{ability})$ over all values of GFP levels yields 5 x $10^{-7}$. **(B)** We extracted $d\eta/dt$ from the measurements by averaging $\Delta < h >/\Delta t$ over time (from 0 to 20 days of incubation without nutrients). The result, $< \Delta < h >/\Delta t >$, is what we call $d\eta/dt$ and is plotted here as a function of GFP level (*n* = 3; error bars are s.e.m). Averaging $d\eta/dt$ over all GFP-levels yields 3 x $10^{-5}$.




**Supplementary Figure 24 | Mathematical analysis based solely on measurements (Fig. 4d-f) reveals relative lifespans of spore bags based on their gene-expressing abilities (GFP**

**levels) (complements Fig. 4g).** The procedure outlined here shows how the mathematical analysis (based on equation 1), which uses solely measured quantities, determines the lifespans of individual spore bags based on their current GFP-levels (i.e., gene-expressing abilities). For brevity, we denote a spore bag's GFP-level by "$a$". **(A)** To determine $\partial a/\partial \mathrm{t}$, we assumed it to be a function f($a$) that is linear in $a$ and is parametrized by two values: $\partial a/\partial \mathrm{t}\,(a_{max})$ and $\partial a/\partial \mathrm{t}\,(a_{min})$. **(B)** On each day of incubation in water without nutrients (as in Supplementary Fig. 21), we used f($a$) to simulate histograms of GFP levels that we would observe for dormant spore bags (same as the yellow bars in Supplementary Fig. 22 but simulated). We then computed the "distance" between the simulated histograms and the experimentally measured histograms (Supplementary Fig. 22). Specifically, if $h_{data}(a_i)$ represents a simulated histogram with $a_i$ being the simulated GFP-levels, then at each time *t*, the distance between the simulated and measured histograms is $d_t = \sum_i |h_{data}\,(a_i) - h_{model}(a_i)|$ . The total distance over time, as a normalized sum, is then $d = (\sum_{t=1}^{n} d_t)/n$, with $n$ being the number of time points that we used. With this definition, $d$ being close to zero means that the proposed f($a$) almost perfectly reproduces the experimental histograms shown in Supplementary Fig. 22 whereas $d$ being close to one means that the proposed f($a$) fails to reproduce the data in Supplementary Fig. 22. We computed the $d$ for a wide range of two-tuple values ($\partial a/\partial \mathrm{t}\,(a_{max})$, $\partial a/\partial \mathrm{t}\,(a_{min})$), which we represent as a heat map shown here. From this heat map, we determined the value ($\partial a/\partial \mathrm{t}\,(a_{max})$, $\partial a/\partial \mathrm{t}\,(a_{min})$) that minimizes the distance $d$ (brown point on the heatmap, *d* = 0.23). **(C)** The choice of f($a$) that most closely reproduces the data (i.e., histograms in Supplementary Fig. 22), determined by steps 1 and 2 (A and B). **(D)** Inferred *a*(t) - the gene-expressing ability decreasing over time - for spore bags with three different starting GFP-levels (from bottom to top green curve: $a(t = 0)$= 2000, 6000, 10000, and 13000). We obtained these curves by integrating $\partial a/\partial \mathrm{t}$ that is shown in (C) over time. Spore bag is pronounced “dead” when $a(t)$ reaches zero (blue points). **(E)** A spore bag's inferred lifespan as a function of its initial GFP level ($a(t = 0)$), determined by extracting the time *t* at which $a(t) = 0$.




**Supplementary Figure 25 | Trajectories of dormant spore bags approaching their deaths (complements Figs. 4b and 4g).** With data in Fig. 4e and Supplementary Fig. 24, we inferred, as a function of time $t$ and for individual spore bags, $G_m(t)$ - the minimum glucose required for germination - and a(t) - the gene-expressing ability. Shown here are three example trajectories (for three spore bags) that each start with distinct gene-expressing abilities (GFP levels): $a(t=0)$= 4000 (top), 8000 (middle), and 12000 (bottom). We obtained a(t) (green curves) by integrating da/dt over time as in Supplementary Fig. 24. We obtained $G_m(t)$ (red curves) by using $h(a)$ - average minimum glucose concentration required for germination (in equation (1)) - that we obtained from the Day-10 data in Fig. 4e (also in Fig. 4g). These inferred trajectories, based solely on measured values, show why dormant spores die: The ability to express a generic gene keeps decreasing over time, which in turn keeps increasing the glucose concentration required for germination until it eventually goes above the saturating level of 2% (white circle). Starting at

this point in time (blue circle), the spore bag cannot germinate anymore regardless of the glucose concentration that it encounters and is thus pronounced dead.

**Supplementary Table 1 | List of genes for each transcriptional module (from Joseph-Strauss et al. *Genome Biol.* (2007)(*32*)) (complements Fig. 2g and Supplementary Fig. 8).**

A transcriptional module is a group of genes that are involved in a related cellular process (e.g., stress response). An insightful work by Joseph-Strauss et al. (2007) (*32*) has identified a list of transcriptional modules by studying yeast spores that germinated after receiving a saturating concentration (2%) of glucose. We performed transcriptome (RNA-seq) analysis of un-germinated spores that were primed by a low glucose-concentration (Fig. 2g and Supplementary Fig. 8). For this analysis, we used the transcriptional modules listed below - we looked at the specific genes listed below for each transcriptional module. This list is from Joseph-Strauss et al. *Genome Biol.* (2007) (*32*).

| **Transcriptional module** | **Genes in each module** |
|---|---|
| Stress | YDL204W, TPS2, YGL037C, STF2, CTT1, SOL4, GRE3, OM45, YJR096W, YKL091C, TFS1, YLR251W, YLR252W, TSL1, YML128C, PGM2, YMR250W, YNL274C |
| Protein synthesis | RPL19A, RPS8A, RPL17A, RPS18B, RPS10A, URP1, CRY1, YS29B, RPL43A, YDL082W, YDL083C, SOS2, SOS1, RPS18A, RPS13C, RPL45, RPL15A, RP51B, YDR450W, RPL27B, RPL35B, RPL15B, RPS24EA, RPS8B, RPL17B, RPS26B, RPL32, RPL30A, RPL6A, CYH2, SUP44, SSM2, RPL9A, RPS31A, YGR034W, RPL16A, RPS28A, RPL30B, YST1, RPL14B, URP2, RPL4A, RPL27, MAK18, RPS7A, RPL5A, YIL052C, RPL13, UBI1, RPS25B, TIF2, YJL177W, RPS24A, RPS5, RPS7B, RPL14A, YKL056C, RPS27A, RPL17, RPS25, TIF1, UBI2, RPL4B, RPL13A, YST2, YLR061W, GRC5, UBI3, RPL35A, RPS33B, YLR325C, RPS31, YLR388W, RP10A, RPL16B, YML024W, YML026C, RP10B, YL16A, BEL1, YMR142C, YMR242C, RPL9B, RP23, YNL096C, RPL41A, RPS3, SSB2, RP28B, RPS16A, RPLA2, RPS21, RP28A, RPS16B, RPL25, TCM1, RPS30, RPS33A, RPL37B, YOR293W, RPL18A1, RPS12, EGD1, YPL079W, YPL090C, RPL37A, SSM1, YPR102C, RPS28B, YBR084CA, YER056CA, YFR031CA |
| Gluconeogenesis | YCR010C, ICL1, YFL030W, YGR067C, HXT5, YIL057C, MBR1, YKL187C, JEN1, PCK1, IDP2, FBP1, CYB2, YMR107W, YMR206W, MLS1, YNL194C, YNL195C, GAC1, LEE1, PXA1, YPR030W |

| | |
|---|---|
| Proteasome | PRE7, YBR062C, YBR173C, YTA5, RPN5, RPN4, YTA2, RPN8, PRE1, SUN2, PUP3, MPR1, YFR010W, PRE4, NIN1, SCL1, SUG1, UFD1, PRE9, PHB2, PUP2, ARC15, PRE3, CAP1, SBA1, YTA3, YKT6, YKR011C, YLR387C, YLR421C, GLO1, PRE8, PRE5, YNL155W, PRE6, CRL13, RPN7, PRE10, PRE2, RPN6 |
| Oxidative phosphorylation | PET9, COR1, ATP1, ATP3, YBR183W, YBR230C, ATP16, COX9, INH1, SDH4, ATP5, ATP17, QCR7, RIP1, YER053C, COX15, QCR6, COX4, COX13, CBP4, YGR182C, QCR9, COX6, QCR8, CYC1, MIR1, ATP2, ATP7, MDH1, HAP4, SDH3, SDH1, MCR1, SDH2, COX12, YLR294C, ATP14, COX8, NDI1, COX7, PBI2, COX5A, POR1, YNL100W, CIT1, CYT1, ATP4, ATP15, YPR020W, QCR2, YHR001WA |
| rRNA processing | YDR101C, YDR496C, ROK1, YGR103W, YGR145W, YGR245C, DRS1, PWP1, YLR222C, DBP9, YLR409C, YML093W, HAS1, YNL132W, YNL174W, YNL182C, YOR206W |
| Mating | PRM9, YAR033W, FUS3, YBL062W, FIG1, YBR156C, YBR158W, YBR223C, YBR225W, YBR226C, FUS1, KAR4, YCL074W, YCL075W, YCL076W, RVS161, FIG2, PCL2, RDI1, AFR1, YDR124W, ECM18, YDR241W, YDR249C, PAM1, YDR309C,YDR340W, STE14, MFA1, YER187W, STE2, YFL027C, YFL047W, AGA2, YGL052W, PRM8, IME4, YGL223C, GPA1, STE12, YHR097C, CHS7, PRM2, YIL060W, YIL080W, YIL082W, YIL083C, PRM5, YJL107C, PRM10, FAR1, ASG7, PGU1, GFA1, PGM1, PMU1, HYM1, YKL221W, KTR2, YLR042C, MID2, SST2, PRP39, PRM6, KAR5, CIK1, FUS2, YNL042W, MSG5, INP52, CHS1, YNL208W, PRM1, ERG24, AGA1, YOL095C, YOR129C, YOR343C, PRM4, PRM3, YPL193W, KAR3, YML048WA, YIL082WA, YMR304CA |
| Cell cycle: G1 | RFA1, SEN34, HTB2, HTA2, YBL009W, POL12, HHF1, HHT1, YBR070C, YBR071W, RDH54, RFC5, POL30, YBR089W, YCL022C, YCL024W, YCL061C, HCM1, MCD1, YDL018C, DUN1, YDL163W, CDC9, ASF2, MSH6, PDS1, HTB1, HTA1, YDR279W, GIN4, YDR528W, MNN1, PMI40, RNR1, RAD51, SSU81, ADK2, SMC1, CLB6, YGR151C, RSR1, YGR221C, YHR110W, YHR127W, SPO16, YHR154W, YHR173C, IRR1, YIL132C, SRO4, SMC3, HPR5, ASF1, RFA3, YJL181W, SWE1, POL32, PRI2, MIF2, HSL1, YKL108W, RAD27, YKR077W, YKR090W, KIM2, |

| | |
|---|---|
| | SPA2, YLL022C, STU2, YLR049C, CDC45, YLR183C, TUB4, SPH1, YOX1, OGG1, CTF18, YMR144W, SPT21, CLN1, HHF2, HHT2, PMS1, POL1, SPC98, YNL166C, BNI4, POL2, YIF1, TOF1, RFA2, YNR009W, YOL007C, YOL017W, MSH2, BUB3, DHS1, CDC21, YOR114W, YOR144C, NIP29, HHO1, RAD53, SVS1, IPL1, BBP1, CLN2, YPL267W, RLF2, CLB5, DPB2 |
| Cell cycle: G2-M | KIN3, YBL032W, CHS2, PHO3, CDC47, BUD3, YDR033W, SWI5, PMA1, ALK1, CDC20, DBF2, CLB1, WSC4, MOB1, YIL158W, YJL051W, BUD4, YKL130C, YLR084C, ACE2, YLR190W, YML034W, SUR7, YML058W, YML119W, CDC5, YMR032W, YMR215W, YNL057W, YNL058C, YOL070C, HST3, YOR315W, YPL141C, KIP2, IQG1, CLB2, YCR024CA |