Keeping still life away from death

Functions and dynamics of gene expression during dormacy of Saccharomyces cerevisiae spores

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Keeping still life away from death

Functions and dynamics of gene expression during dormancy of *Saccharomyces cerevisiae* spores

Dissertation

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Front & Back: Theo Maire

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Summary

While life is present everywhere on earth, each individual species can only grow and proliferate in a specific set of conditions. Moreover due to inherent fluctuation of the environment, individual organisms are often periodically confronted to stressful conditions that prohibit growth, reproduction and increase mortality. One of the most common strategy to cope with these harmful conditions is to shield and quietly wait for the storm to finish. Indeed upon change of the environment, many organisms enter "dormancy", whereby they differentiate into a distinct resting form with additional protection, storage and with greatly reduced internal activity (dormant state). Although relying on widely different molecular basis, specialized dormant stage are found in virtually every group of living organisms, including endospores of bacteria, spores of fungi, seeds of plants, cysts of protists and diapaused eggs of animals. Even after sometimes years of apparent inactivity, dormant organisms can resume growth and reproduction upon sudden improvement of environmental conditions.

How such organisms manage to keep the potential to resume growth while having a nearly ceased activity? Over the last 20 years, scientists have uncovered various physiological and molecular mechanisms that control entry and exit of dormancy. However a basic understanding of what exactly happens during dormancy, i.e how to survive while stopping nearly all internal activity, is still missing. Two reasons are likely responsible for that knowledge gap. A first conceptual obstacle is a general view that since by definition nothing much happens during dormancy, nothing important happens. A second technical obstacle is the lack of sensitive enough instruments to quantify the "nearly ceased" activity during dormancy. As a result, very few studies have established the concrete link between a vanishing internal activity and the ability to survive during dormancy in a single experimental system.

In this thesis we propose to focus on dormant Saccharomyces cerevisiae yeast spores to specifically investigate the links between gene expression and the ability to survive during week-long dormancy.

In **Chapter 1** we introduce dormant yeast spores in the wider context of dormancy in the living world. We show that dormancy is mostly studied in plants, animals and prokaryotes and that yeast spores represent a great opportunity to learn

about dormancy of unicellular eukaryotes, for which dormant spores and cysts are widespread phylogenetically and likely of major ecological and clinical importance. Previous studies demonstrated that in the absence of nutrients, dormant yeast spores do express genes and can survive for several weeks. However the role of that dormant gene expression to support survival has not been explored.

As function of their internal state and the environment, a dormant yeast spore can have three distinct fates : germinate (i.e. resume replication), die (i.e. cannot resume replication) or stay dormant. In this thesis we propose to study the role of gene expression by successively focusing on these three fates.

In **Chapter 2**, we focus on interplay between gene expression and germination. First we show dormant yeast spores varies in their ability to germinate, meaning that despite ample glucose concentrations, not all spores germinate. Using single spore imaging and RNA-seq, we show that un-germinated spores are "primed", meaning they initiate germination program at the transcriptome level, and germinate faster upon addition of higher glucose concentration. We then investigate what underpins a spore's ability to germinate. By inducing GFP expression in dormant spores before they encounter glucose, we show that the ability to express GFP positively correlates with the ability to germinate. Then by inhibiting gene expression, we show that transcription is necessary for maintaining the ability to germinate during dormancy. Finally we show that the amount of RNA polymerases in dormant spores, proteins necessary for transcription, positively correlate with both the ability to germinate and the ability to express GFP.

In **Chapter 3**, we focus on the link between gene expression and the decay to death of dormant spores. In the absence of external nutrients, dormant yeast spores gradually lose their ability to germinate over several weeks. Building on results of Chapter 2, we show that as spores are losing their ability to germinate, both the amount of RNA polymerases and the ability to express GFP gradually decays over a similar time-scale. By inhibiting translation over months in dormant spores, we show that even if proteins are stables for days, RNA polymerases are less produced than degraded, yielding an inevitable net-loss and ultimately causing dormant spores to die when transcription becomes impossible. We then propose that this decay of molecule necessary for maintaining gene expression sets the time-scale of spore dormant-to-death transition.

In **Chapter 4**, we focus on studying gene expression when spores stay dormant. Based on previous results of chapter 2 and 3 showing that one can induce gene expression during dormancy, we sought to characterized dynamics of expression at single spore level. By combining single molecule RNA FISH, time-lapse microscopy and mathematical modeling, we show that transcription is likely occurring in a discontinuous way, randomly alternating between short period of fast and

intense activity and hour-long periods of inactivity. We then show that upon induction, two physically distant genes activate and de-activate simultaneously, suggesting mechanism controlling transcription globally rather than locally. Finally based on pair measurement of endogenous or induced genes mRNA and modelling, we provide evidence that hour-long random activation/de-activation of transcription during dormancy might be happening genome-wide.

In conclusion, we propose a minimal model that links the rates of RNA and protein production/degradation to the time-scale of survival of dormant yeast spores. We stress out that future studies un-ambiguously quantifying RNA decay kinetics in dormant spores are crucial to validate the current version of this model. Finally we emphasize that methods used in this thesis, such as inducing a single exogenous gene or inhibiting gene expression during dormancy, would likely be relevant to investigate the role and dynamic of gene expression during dormancy of other organisms. By providing new tools and concepts through the example of dormant yeast spores, our work paves the way for discovering new quantitative principles underpinning dormancy.

Samenvatting

Hoewel het leven overal op aarde aanwezig is, kan elke individuele soort alleen groeien en zich vermenigvuldigen onder een specifieke reeks omstandigheden. Bovendien worden individuele organismen door inherente fluctuatie van de omgeving vaak periodiek geconfronteerd met stressvolle omstandigheden die groei, voortplanting en verhoogde sterfte belemmeren. Een van de meest gebruikelijke strategieën om met deze schadelijke omstandigheden om te gaan, is afschermen en rustig wachten tot de storm voorbij is. Inderdaad, bij verandering van de omgeving gaan veel organismen in "slaaptoestand", waarbij ze differentiëren in een aparte rustvorm met extra bescherming, opslag en met sterk verminderde interne activiteit (slapende toestand). Hoewel ze berusten op een sterk verschillende moleculaire basis, worden gespecialiseerde slapende stadia aangetroffen in vrijwel elke groep levende organismen, inclusief endosporen van bacteriën, sporen van schimmels, zaden van planten, cysten van protisten en eieren van dieren in diapauze. Zelfs na soms jaren van schijnbare inactiviteit, kunnen slapende organismen de groei en reproductie hervatten bij een plotselinge verbetering van de omgevingsomstandigheden.

Hoe slagen dergelijke organismen erin om het potentieel om de groei te hervatten te behouden terwijl ze een bijna gestaakt activiteit hebben? In de afgelopen 20 jaar hebben wetenschappers verschillende fysiologische en moleculaire mechanismen blootgelegd die het binnenkomen en verlaten van de rustperiode regelen. Een basisbegrip van wat er precies gebeurt tijdens de rustperiode, d.w.z. hoe te overleven terwijl bijna alle interne activiteit wordt gestopt, ontbreekt nog steeds. Twee redenen zijn waarschijnlijk verantwoordelijk voor die kennisklоof. Een eerste conceptueel obstakel is een algemene opvatting dat aangezien er per definitie niet veel gebeurt tijdens de rustperiode, er niets belangrijks gebeurt. Een tweede technisch obstakel is het ontbreken van voldoende gevoelige instrumenten om de "bijna gestaaktäctiviteit tijdens de rustperiode te kwantificeren. Als gevolg hiervan hebben zeer weinig studies het concrete verband aangetoond tussen een verdwijnende interne activiteit en het vermogen om te overleven tijdens de rustperiode in een enkel experimenteel systeem.

In dit proefschrift stellen we voor om ons te concentreren op slapende Saccharomyces cerevisiae gistsporen om specifiek de verbanden tussen genexpressie en het vermogen om te overleven tijdens een weeklange rustperiode te onderzoeken.

In **Hoofdstuk 1** introduceren we slapende gistsporen in de bredere context van kiemrust in de levende wereld. We laten zien dat kiemrust voornamelijk wordt bestudeerd bij planten, dieren en prokaryoten en dat gistsporen een geweldige kans bieden om meer te weten te komen over de kiemrust van eencellige eukaryoten, waarvoor slapende sporen en cysten fylogenetisch wijdverbreid zijn en waarschijnlijk van groot ecologisch en klinisch belang zijn. Eerdere studies hebben aangetoond dat slapende gistsporen, in afwezigheid van voedingsstoffen, genen tot expressie brengen en enkele weken kunnen overleven. De rol van die slapende genexpressie om overleving te ondersteunen is echter niet onderzocht.

Als functie van hun interne toestand en de omgeving, kan een slapende gistspore drie verschillende lotsbestemmingen hebben: ontkiemen (d.w.z. hervatten replicatie), sterven (d.w.z. kunnen replicatie niet hervatten) of slapend blijven. In dit proefschrift stellen we voor om de rol van genexpressie te bestuderen door achtereenvolgens te focussen op deze drie lotgevallen.

In **Hoofdstuk 2** richten we ons op de wisselwerking tussen genexpressie en kieming. Eerst laten we zien dat slapende gistsporen variëren in hun vermogen om te ontkiemen, wat betekent dat ondanks ruime glucoseconcentraties, niet alle sporen ontkiemen. Met behulp van beeldvorming met enkele sporen en RNA-seq laten we zien dat niet-gekiemde sporen "geprimed"zijn, wat betekent dat ze een kiemprogramma op transcriptoomniveau initiëren en sneller ontkiemen bij toevoeging van een hogere glucoseconcentratie. Vervolgens onderzoeken we wat ten grondslag ligt aan het vermogen van een spore om te ontkiemen. Door GFP-expressie in slapende sporen te induceren voordat ze glucose tegenkomen, laten we zien dat het vermogen om GFP tot expressie te brengen positief correleert met het vermogen om te ontkiemen. Door genexpressie te remmen, laten we zien dat transcriptie nodig is om het vermogen om te ontkiemen tijdens de rustperiode te behouden. Ten slotte laten we zien dat de hoeveelheid RNA-polymerasen in slapende sporen, eiwitten die nodig zijn voor transcriptie, positief correleert met zowel het vermogen om te ontkiemen als het vermogen om GFP tot expressie te brengen.

In **Hoofdstuk 3** richten we ons op het verband tussen genexpressie en het verval tot de dood van slapende sporen. Bij afwezigheid van externe voedingsstoffen verliezen slapende gistsporen geleidelijk hun vermogen om te ontkiemen gedurende enkele weken. Voortbouwend op de resultaten van Hoofdstuk 2, laten we zien dat naarmate sporen hun vermogen om te ontkiemen verliezen, zowel de hoeveelheid RNA-polymerasen als het vermogen om GFP tot expressie te brengen geleidelijk afneemt over een vergelijkbare tijdschaal. Door de translatie gedurende maanden in slapende sporen te remmen, laten we zien dat zelfs als eiwitten dagenlang stabiel zijn, RNA-polymerasen minder worden geproduceerd dan afgebroken, wat een onvermijdelijk nettoverlies oplevert en er uiteindelijk toe leidt dat slapende sporen afsterven wanneer transcriptie onmogelijk wordt. Vervolgens stellen we voor dat

dit verval van het molecuul dat nodig is voor het in stand houden van genexpressie, de tijdschaal bepaalt van de sluimerende-naar-dood-overgang van sporen.

In **Hoofdstuk 4** richten we ons op het bestuderen van genexpressie wanneer sporen slapend blijven. Gebaseerd op eerdere resultaten van hoofdstuk 2 en 3, die aantoonden dat men genexpressie kan induceren tijdens de rustperiode, hebben we geprobeerd de dynamiek van expressie op enkelsporig niveau te karakteriseren. Door single-molecule RNA FISH, time-lapse microscopie en wiskundige modellering te combineren, laten we zien dat transcriptie waarschijnlijk op een discontinue manier plaatsvindt, willekeurig afgewisseld tussen korte periodes van snelle en intense activiteit en urenlange perioden van inactiviteit. We laten dan zien dat na inductie worden twee fysiek ver verwijderde genen gelijktijdig geactiveerd en gedeactiveerd, wat een mechanisme suggereert dat de transcriptie globaal in plaats van lokaal regelt. Tot slot, gebaseerd op paarmeting van endogene of geïnduceerde genen mRNA en modellering, leveren we bewijs dat een uur durende willekeurige activering/de-activering van transcriptie tijdens de rustperiode genoom-breed kan plaatsvinden.

Concluderend stellen we een minimaal model voor dat de snelheid van RNA en eiwitproductie/afbraak koppelt aan de tijdschaal van overleving van slapende gistsporen. We benadrukken dat toekomstige studies die de kinetiek van RNA-verval in slapende sporen ondubbelzinnig kwantificeren, cruciaal zijn om de huidige versie van dit model te valideren. Ten slotte benadrukken we dat methoden die in dit proefschrift worden gebruikt, zoals het induceren van een enkel exogeen gen of het remmen van genexpressie tijdens de rustperiode, waarschijnlijk relevant zouden zijn om de rol en dynamiek van genexpressie tijdens de rustperiode van andere organismen te onderzoeken. Door nieuwe hulpmiddelen en concepten te bieden door middel van het voorbeeld van slapende gistsporen, maakt ons werk de weg vrij voor het ontdekken van nieuwe kwantitatieve principes die ten grondslag liggen aan de kiemrust.

Preface : Decoding still life

Figure 0.1: Vanitasstilleven, Pieter Claesz, Haarlem 1625. Oil painting exposed in Franz Haals museum,Haarlem, The Netherlands. Description from : "On a table are a burning candle, a watch, an anemone, a skull and a letter on which only the words 'Haarlem and Georg...' can be distinguished. These objects are carefully chosen for their meaning. They all refer to the relativity of life. The candle is nearly burned out, indicating the passing of time, and an anemone is quickly wilting. Death is imminent". Image from https://commons.wikimedia.org/wiki/File:Pieter_Claeszoon-_Vanitas_-_Still_Life_(1625,_29,5_x_34,5_cm).JPG

A burning candle, a watch, a wilting flower, a letter, a skull and a cracked walnut. Putting all these inanimate objects on a that 17th century oil painting is nothing but innocent. Each object carries a codified message : the watch is wide open to invite us to look at time passing, the candle and the flower are about to fade away, they create the impression that something is about to end, and the shining skull occupies one third of the picture, forcing us to face death. Altogether these objects allow Pieter Claezs to convey a universal message : we should not forget that life is transient and that as time passes, life always inevitably end up by death. While such message about the fatality of death is not original, conveying it only with a combination of symbolic inanimate objects was a specificity of 17th century dutch artists. This even became a unique genre called "Vanitas Still lifes", which is also the name of the painting we just described (Fig.0.1).

Now, let us imagine that you did not read the previous paragraph or are not familiar with the artistic convention of 17th century dutch paintings. When looking at that specific painting you will be able to appreciate its aesthetics, but you would not have access to the actual meaning. Understanding such a painting requires knowing the code that links inanimate objects to their symbolic value. Without the code, we barely get the impression that nothing happens, because we are just in front of objects standing still.

In the present thesis, we will aim at decoding a different form of still life. Not inanimate objects on a painting, but rather a form "life being still" that exists in nature, what biologists called "dormant" living organisms. These can typically be found in winter by looking under a rock, digging in the soil or diving in the sediment at the bottom of a lake. Most of them look like a small oval seed, ranging from microns to a few centimeters. Strikingly, they look quiet, showing no growth, movement or proliferation. Thus, they do not display any of the familiar signs of life, so that the uneducated eye will have a hard time to distinguish them from the dirt they reside in. However, appearances can be deceiving. Indeed, these dormant organisms are not like dirt because upon sudden increase of temperature, humidity or food availability, they will wake up and start to grow and reproduce, giving rise to a new generation of bacteria, animals or plants. Dormancy is a temporary state, during which organisms survive long periods of stress by staying still, waiting for the storm to pass.

Despite a growing interest for dormancy, scientists mostly focused on understanding how organisms either enter or exit dormancy, leaving a major mystery unsolved : how come organisms with nearly ceased activity keep the capacity to reconstitute either a tree, a shrimp, a mushroom or a bacterial colony on demand? In this thesis we aim at directly investigating the link between the nearly ceased activity and the

ability to survive during dormancy.

In order to decode the mystery of dormancy, we focus our efforts on experimenting on a specific system : dormant yeast spores. Yeasts are single cell organism of about 10 microns that are astonishingly good at doing one thing : produce more yeasts cell by fermenting sugar. For long, human have leverage that properties for two services : alimentation (fermentation of wine, beer and bread) and fundamental research on cell biology (cell-cycle and glucose metabolism). Less familiar is that budding yeasts, like many fungi, form spores when they starve from nutrients. Once spores are formed, yeasts turn dormant and can survive without nutrients for weeks. But then what is the interest of studying dormancy in yeast and not in another organisms? We can give two essential reasons.

First, dormancy has been historically studied in plants and bacteria, but rarely in a free living unicellular eukaryote organism such as yeast. Unicellular eukaryotes are organisms that resemble cells of animals or plants (they have a nucleus), but have the life-style of bacteria (they live as single-cell and multiply quickly). Moreover unicellular eukaryotes are extremely diverse and known to form a plethora of dormant forms, called spores or cysts, many of which are resistant form of parasites causing disease in crops or human (e.g. Giardiasis). Therefore, by studying dormancy of yeast spores, we hope to establish a model system for dormancy of unicellular eukaryotes, which could be help fundamental research (cell biology,ecology and evolution of eukaryotes) as well as concrete application (finding how to overcome the resistance of dormant parasites).

Second, although S.cerevisiae yeasts have been an historical crucial model organism for cell biology, very little is known about dormant yeasts spores. Intensively studying how S.cerevisiae yeasts divide and grow has allowed scientists to uncover fundamental molecular mechanisms that happened to be relevant for cell biology of many animals including humans. As a side result, a plethora of methods and tools have been developed to study yeasts quantitatively and thus can be easily exported to dormant yeasts spores.

In the present thesis, we will seize that double opportunity (technical and conceptual) by combining quantitative experiments and mathematical modeling to discover mechanisms of dormancy of yeast spores.

1

Introduction

This thesis is about dormancy, a temporary period in certain organism's life where internal activities are drastically reduced. During dormancy organisms do not move, grow or reproduce and yet they remain alive. Dormancy is thought to help organisms survive stressful periods, because organisms turn dormant upon deterioration of the environment, and exit dormancy when it improves. Indeed, some organisms are known to be able remain dormant for months to hundreds of years before resuming growth and reproduction when conditions finally improve.

Puzzled by this surprising behavior, scientists have dissected molecular mechanisms underpinning organisms entry or exit of dormancy. They uncovered that being dormant relies on drastic metabolic and morphological changes, tightly controlled by specific signalling pathways that match precise environmental cues. Recently, there has been growing interest for theoretically studying the impact of dormancy on evolutionary dynamics (Lennon et al. 2021). However, the period of dormancy itself, i.e. after entry and before exit, during which organisms extend their lifespan by being still, remain poorly understood mechanistically. To us, there is clearly a (dormant) elephant in the room :

How can organisms remain alive and being nearly inactive at the same time?

Indeed, artificially stopping the activity of many organisms including humans would kill them quickly. Therefore, this ability to drastically reduce activity while surviving is far from being universal to all living organisms. Likely, it is rather an adaptation that relies on specific biophysical principles.

In this thesis, we propose to start uncovering these principles by performing experiments on a specific model organism : **dormant yeast spores**. Yeasts are unicellular fungi of about $10\mu m$, that reproduce by mitosis in 90 minutes if sufficient nutrients are present, but can form spores by meiosis if starved. If kept in water without nutrients, yeast spores stay dormant. Indeed, studies have shown that yeast spores have greatly reduced gene expression but can survive for weeks to months (Brengues et al. 2002). However the link between the reduced activity and survival

has not been established yet. In this thesis, we specifically ask :

What is the interplay between reduced gene expression and survival during dormancy of yeast spores?

Before presenting our results, we will introduce what is the current state of knowledge about dormancy across all living world, in order to widely contextualize our work on dormant yeast spores. Admittedly, this is quite an ambitious challenge since dormancy is loosely defined and encompass widely different organisms and mechanisms, from plant seed to bacterial endospores. Here we propose a "bottom-up" approach, which consists in starting from a gallery of concrete examples, all accepted to be "dormant". Then we will gradually increase in level of abstraction until we can accurately compare yeast spores with other dormant forms.

The reader in a hurry can skip that part and directly jump to the second part of the introduction (1.2) that directly addresses what is sufficient to contextualize our work on dormant yeast spores.

Alternatively for the very curious reader, we recommend this recent review with synthesis element and evolutionary perspective (Lennon et al. 2021) or this book (Kaplan-levy et al. 2010) that regroups other examples or dormancy we will not mention here (e.g. hibernation or bud dormancy in plants). Our own introduction was based partially based on ideas and examples extracted from these two references.

1.1. Overview of dormancy in the living world

1.1.1. Challenged to survive growth-prohibiting periods

Life is present everywhere on earth, but individual species can not proliferate anywhere. Indeed individuals from a given species can only grow and reproduce within its own combination of environmental conditions. For instance *Saccharomyces cerevisiae* (budding yeast) only grow and replicate in liquid or humid surface between 10 to 38 degrees Celsius if there is sufficient carbon source like glucose, nitrogen bases and various vitamins. In nature we find these yeast-ideal conditions on grapes and bark of trees in temperate regions.

Within their life-time, individual organisms are sometimes departed from these ideal conditions, either because physically moved (by wind, water current or other organisms...) or because the local environment changes (season, modification by other organisms). For yeast cells, this would happen typically when they exhausted local resources since they cannot move away.

As scientist or simple curious observer, we are inherently biased to neglect these growth-prohibiting periods, because our attention and interest naturally prefers action to inaction. However, the absence of external activity does not equate to an absence of activity. A first question to go beyond our natural bias would be the following :

How often in their life time are organisms typically confronted to growth-prohibiting periods?

Obviously, the answer varies a lot between species. However, for micro-organisms that reproduce quickly, the answer is "certainly most of the time". We will now give two arguments, one based on pure reasoning and one based on direct empirical evidence.

First, by reasoning on a simple question : "what happens if fast-growing microorganisms such as yeasts are always in conditions that allow them to proliferate?" A single yeast cell weighs approximately $5.10^{-11} g$ (Łabędź et al. 2017), and can replicate every 90 to 120 minutes in ideal conditions. Now let us assume yeasts are always in these ideal growth-permissive conditions, so that the population readily doubles every 120 minutes. After only 93 generations and one week, a single yeast yields a population that have the same weight as the total biomass on earth (**BarOn2018**). The absurdity of this "constant growth" scenario highlights that fast-growing microorganisms are likely in conditions where they are not reproducing.

Second, by directly looking at the metabolic state of microorganisms in their nat-

ural habitat. A recent review recapitulated several of this measurement made by counting the number of inactive vs active bacteria cells in various ecosystems such as soil, mud or marine water (Lennon et al. 2011). Strikingly they estimated that in soil, up to 80% of bacteria were metabolically inactive, i.e. non dividing. This likely indicates that in soil, these bacteria are more often confronted to growth-prohibiting rather than growth-permissive conditions.

Given the likely prevalence of growth-prohibiting periods, we could speculate that many organisms are particularly adapted to it. Indeed, a myriad of organisms are known to be adapted to cope with such growth-inhibiting periods by entering a state called "dormancy". According to the dictionary, dormancy is a "state of reduced metabolic activity adopted by many organisms under conditions of environmental stress or, often, as in winter, when such stressful conditions are likely to appear." However in scientific literature, the word dormant or dormancy (i.e. the period when an organism is dormant) refers to a huge diversity of phenomenon and organisms, from bacteria to plant seeds.

Therefore, we will now confront ourselves with real-life examples of "dormancy" and try to extract what exactly these examples of dormancy have in common. Specifically we will aim to comparing dormancy of yeast spores to other types of dormancy.

1.1.2. Being dormant with five concrete examples

Akinete, oospore, hypnozygote, conidia, diapaused egg, sclerotium, dinocyst, hypnospore, true dormant seed, endospore. When looking for example of dormant stage of organisms, we are quickly facing a zoo of words and associated definitions. Biology roots in the patient observation and careful description of the huge diversity of life forms and behaviors surrounding us. Attributing a specific name to a structure or a process is often the first step between genuine contemplation and rigorous understanding. Therefore, the profusion of terms associated to a concept like dormancy simply indicates how widespread and ubiquitous it is.

We will now compare dormant yeast spore bag (*S.cerevisiae*) to 4 concrete examples : a dormant cyst (*G. intestinalis*), bacterial endospore (*B.subtillis*), a diapaused egg (*B. poppei*) and a "truly dormant" seed (*A. thaliana*) (Fig.1.1). These were chosen to quickly span different organisms, ecology and type of dormancy.

Dormant spores of a unicellular fungus Upon starvation of nutrients, especially from fermentable carbon source such as glucose, vegetative yeast can form spores, usually composed of 4 cells packed into a thick spore wall (Fig.1.1C). This process follows a programmed sequence of morphological changes and is called sporula-

tion (Neiman 2011). If kept in water without nutrients at 30C, yeast spores do not grow or reproduce, and have a reduced gene expression activity, around 5% of vegetative cells (Brengues et al. 2002). During this dormancy, yeast spores can survive for months to years at 4C and around 1 month in water at 30C (Brengues et al. 2002). Upon addition of fresh nutrients, yeast spores resume replication within hours, in a process called "germination" (Joseph-Strauss et al. 2007).

Dormant cysts of a parasitic protist Unlike budding yeast who has been of great service for humanity since 9000 years, some micro-organisms are pathogenic agent that are a pain to eradicate. Giardi lambia is a flagellated parasitic protist (i.e. unicellular eukaryote), that reproduces in the intestine, causing a diarrheal disease known as giardiasis (Birkeland et al. 2010) Within the host's intestine, it proliferates as a motile "trophozoite" whereas it survives and disperses outside the host (for instance in moist food or fresh water) as a dormant ovoid cyst (1.1). Giardia's cysts are quadrinucleated and shielded by a thick mesh of a single sugar polymer that allow cyst to survive up to months outside the host's intestine. Cysts exit dormancy and give rise to new replicating trohpozoite and upon ingestion by a new host. Here dormancy allows the propagation between hosts, or from our point of view transmission of disease Giardiasis.

Endospores of a Firmicutes bacteria Endospores of Firmicutes bacterias are the text-book example of resistant dormant life forms can be (Kaplan-levy et al. 2010). Indeed, endospores are known to resist the most extreme conditions such as intense heat, desiccation or freezing (Mckenney et al. 2012). Notably, this extraordinary resistance mislead scientists of 19th century who were trying to decide whether life could arise spontaneously from non living matter or not (Box 1.1.2). Consequently, dormant endospores are thought to hold the record of longevity in dormant state, up to hundreds of millions of years (Vreeland et al. 2000). Indeed, while this is always ambiguous to establish, recent reports claim that they revived endospores of Bacilli spp enclosed in a saline crystal from 250 millions years(Vreeland et al. 2000). Endospores are common in Gram-positive bacteria, with Bacillus subtillis the widely studied model system (Fig.1.1D). Spores are formed from asymmetric division of rod-shape bacteria, upon various conditions like starvation. The endospore is composed of DNA surrounded by a tough spore coat, a complex multilayered structure composed of up to 70 different proteins (Mckenney et al. 2012). Endospore bacteria occupy many different ecosystems such as soil, marine and fresh water sediment (Wörmer et al. 2019). Once formed, endospores are thought to be strictly metabolically inert, which together with the spore coat confers extreme longevity and resistance. They are often referred to as "cryptobiotic" life forms. Aside from DNA and ribosomes, endospore contain high amounts of dipicolinic acid, a spore-specific chemical that is thought is help maintaining

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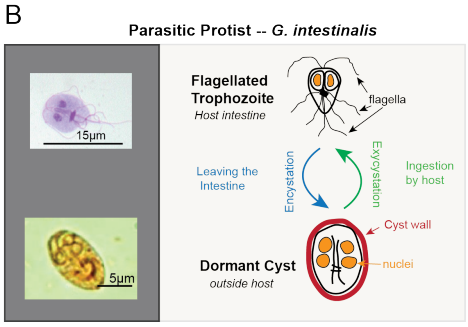
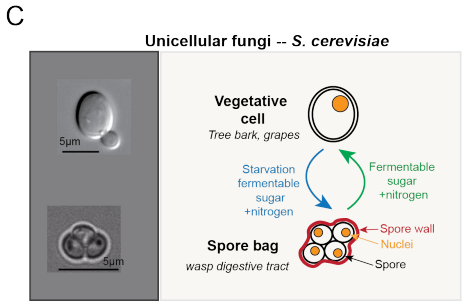
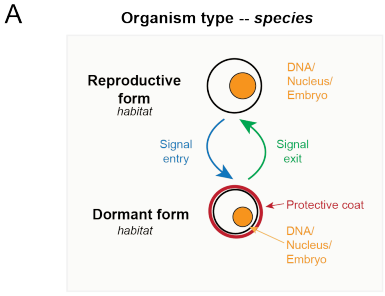
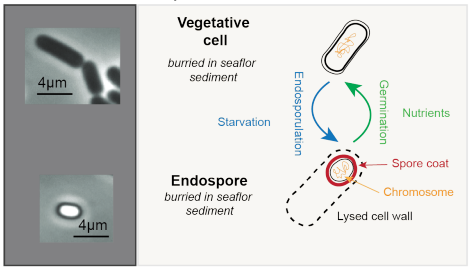
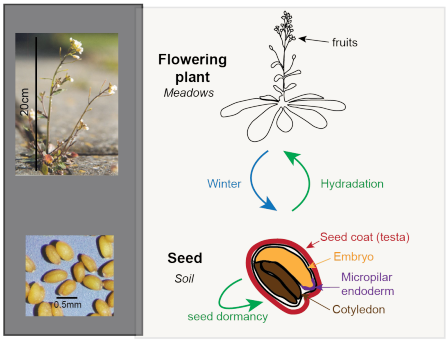
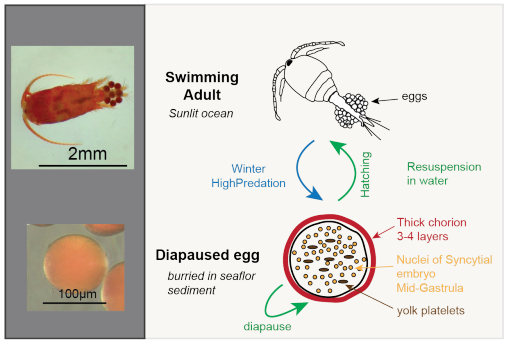

B
Parasitic Protist -- G. intestinalis
15µm
5µm
Flagellated Trophozoite
Host intestine
flagella
Leaving the Intestine
Encystation
Excystation
Ingestion by host
Cyst wall
Dormant Cyst
outside host
nuclei


C
Unicellular fungi -- S. cerevisiae
5µm
5µm
Vegetative cell
Tree bark, grapes
Starvation fermentable sugar +nitrogen
Fermentable sugar +nitrogen
Spore bag
wasp digestive tract
Spore wall
Nuclei
Spore


A
Organism type -- species
Reproductive form
habitat
DNA/ Nucleus/ Embryo
Signal entry
Signal exit
Dormant form
habitat
Protective coat
DNA/ Nucleus/ Embryo


D
Gram positive bacteria -- B. subtillis
4µm
4µm
Vegetative cell
buried in seaflor sediment
Endosporulation
Germination
Starvation
Nutrients
Endospore
buried in seaflor sediment
Spore coat
Chromosome
Lysed cell wall


F
Flowring plant -- A. thaliana
20cm
0.5mm
Flowering plant
Meadows
fruits
Winter
Hydradation
Seed
Soil
Seed coat (testa)
Embryo
Micropilar endoderm
Cotyledon
seed dormancy


E
Marine copepod -- B. poppei
2mm
100µm
Swimming Adult
Sunlit ocean
eggs
Winter HighPredation
Hatching
Resuspension in water
Diapaused egg
buried in seaflor sediment
Thick chorion 3-4 layers
Nuclei of Syncytial embryo
Mid-Gastrula
yolk platelets
diapause

dormancy (Setlow et al. 2006).

Figure 1.1 ***(preceding page)*****: Zoo of dormant organisms**. (A) Schematic view of dormancy. As function of the environment, an organism alternates between a "reproductive form" and a "dormant form". The dormant form is characterized by a reduced internal activity (orange circle is transparent), a coat protecting from external stresses (red). (B) (bottom) Giardia intestinalis dormant cysts Giardia cyst: wet smear stained with iodine, optical microscopy (source: https://www.cdc.gov/dpdx/giardiasis/index.html). (top)Giardia trophozoites stained with Giemsa; 100x magnification. wikipedia (C) *Saccharomyces cerevisiae* vegetative cells (top) yeast spore bag (bottom). Optical microscope 60X, this thesis. Here 3 spores packed within 1 spore bag. (D) Bacillus subtillus endospore (bottom) and vegetative cells (top). (C) (top) adult Bockella poppei. (bottom) light-micrographs (PN) stages of post-diapause development in B. poppei when viewed with a dissecting microscope at 100X magnification: (Reed et al. 2021). (F) Arabidopsis thaliana seeds (bottom) and mature flowering plant (top).

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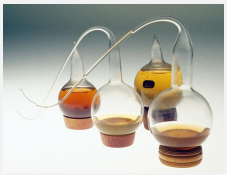
Box 1 : Dormancy and the controversy of spontaneous generation



Pasteur's Swan neck ballons

In 1860, somewhere in the French alps, at around 2000 meters, a man is disposing and opening some weird glass balloon with swan neck (see image). Louis Pasteur is about to perform an experiment that will officially settle a century-old controversy : *is life only coming from life or can it spontaneously be generated from purely mineral matter?* Pasteur is capturing air into identical balloons containing sterilized nutrients. If micro-organisms grow similarly in all balloons, it means life is generated from the inside (from non-living matter), whereas if they grow as function of the quality of the air, it means that life only comes from the outside (from living matter). After analysis, statistics are clear : balloons opened at high altitude, i.e. where the air is pure, are much less contaminated than the ones opened in other places such as Pasteur own laboratory. This argument convinced scientists at the time that spontaneous generation is impossible. All but one. Félix Archimède Pouchet firmly believes Pasteur is wrong. Indeed, he performed similar experiments, climbing up Mount Etna in Italy or Pyrenees in France. Unlike Pasteur, Pouchet always finds the same result : microscopic life always proliferate in balloons, without any influence of the quality of the air they were contaminated with. Unfortunately for him, the majority believes that Pasteur is right and that Pouchet simply does not manipulate properly and contaminates his balloons. While history showed that Pasteur was right to advocate against spontaneous generation, it also showed he was wrong to blame Pouchet for being a bad experimentalist. Indeed, everyone at the time thought that life could not survive the sterilization they both used : boiling water at 100C for several hours. Sadly for Pouchet, they were wrong. Indeed, before sterilization, Pouchet added hay to the liquid broth, hoping to provide nutrients only. He did not know that his hay contained dormant Bacillus subtillis endospores, microbes capable of survive through "sterilization". ref : (https://wsimag.com/fr/science-et-technologie/67428-la-fin-des-generations-spontanees)

Diapaused eggs of a copepod Copepods are millimeter size crustaceans living in all fresh and marine water around the globe. There are several stages from adults to eggs that can enter dormancy or "diapause" upon decrease of temperature (winter) of high predation rate (Hansen 2019, Fig.1.1E). For instance, Bockella poppei survives the winter of fresh water Antarctic lakes by producing diapaused eggs, that sink to the bottom and are buried in sediments. While most of them will

hatch by next spring, some resting eggs have been found to survive dormant in sediment for up to 200 years (Jiang et al. 2012). Diapaused eggs of B.poppei are mid-gastrula embryo (syncytium of multiple nuclei), protected by a thick chorion and stuffed with yolk platelets. (Fig.1.1E) Diapaused eggs production is characterized by an absence of morphogenesis; a greatly decreased rate of DNA, RNA, and protein synthesis; and a reduced or negligible metabolic rate (Reed et al. 2021). Because resting eggs maintain some level of metabolism (Hansen 2019),they gradually deplete their storage resources when residing in the sediments and embryos eventually die because of energy depletion (Reed et al. 2021). Unlike yeast spores, or bacterial endospores, resting eggs of Bockella poppei will not wake up from dormancy only if environment improves. Exit of dormancy is also controlled by internal cues, so that hatching occurs only after a given refractory period (Reed et al. 2021). Such delay is called "diapause".

Dormant seeds of a flowering plant Seeds are the most familiar, yet one of the most complex form of dormancy. Indeed, patiently harvesting, stocking and controlling germination of dormant seeds has allowed humanity to develop agriculture and become sedentary twelve thousands years ago. Modern science uncovered that seeds are produced by conifers and flowering plants, with closest cousin ferns producing simpler "spores". Like the ones of A.thaliana, most seeds consist of an embryo covered by living tissues (endoderm) and dead protective tissue (seed coat or testa). During maturation, the mother plant progressively reduces the water of content of seeds, ultimately forming a desiccated form, highly resistant to stresses and able to remain viable for centuries to millenia (Kaplan-levy et al. 2010). Decreasing water-content is thought to enable the cytoplasm of cells within the embryo to transition into a "glassy" state, drastically reducing molecular reaction and thus allowing long-term viability. Adding to this reduction of internal activity, seeds often display another specific adaption called "seed dormancy", defined as "temporary incapacity of a viable seed to germinate upon favorable conditions" (Finch-Savage et al. 2006). This is thought is to be caused by complex endogenous cues from the embryo itself and or from surrounding tissues, such as the "micropilar endoderm". Imposing a minimal period of dormancy prevents seeds from germinating in the middle of winter, triggered by a particularly warm day.

Quiescence and dormancy Apart from these examples, there are forms of dormancy characterized by lesser morphological changes and an absence of coupling with dispersion. At the multicellular level, plants and animals display "whole body" dormancy to overcome one dry or cold season : hibernation, brumation or aestivation in animals, vernalization of trees or bud dormancy in plants (Kaplan-levy et al. 2010). These are particular physiological states. At the cellular level, there exists a wide range of "non-replicating cells with reduced metabolic activ-

ities" phenotypes (Rittershaus et al. 2013). These are often referred to as "quiescence" or "cell-cycle arrested", such as quiescent stem cells or quiescent yeast. However, these forms of dormancy are thought to be critical in resistance to drugs treatment, such as with bacterial persisters resisting antibiotics and dormant cancer-cells resisting chemo therapies (Phan et al. 2020). In the case of *S. cerevisiae*, haploid cells can enter quiescence, usually after nutrients exhaustion in days-old liquid culture (Laporte et al. 2008). Considerably more studies have been conducted on quiescent yeast compared to yeast spores, so that the question of the comparison between these two state remains largely open (Rittershaus et al. 2013).

1.1.3. The design principle of dormancy

All the examples above are described in literature are referred to as "dormant". What do they have in common?

In all the examples above, the dormant forms share several traits, such as low internal activity, a protective coat, stored resources and specific systems to control the exit and entry of dormancy. Depending on the organisms, these different traits are implemented by different structures but with similar functions that we summarized in the following table (Table.1.1).

Trait	Structure	Function
Low internal activity	Embryo, single cell, Chromosome	• Minimize energy expenditure • Maintain viability
Protective coat	cyst wall, spore wall, endospore coat, seed testa	• Protection from external stress • Dispersion
Stored ressources	glycogen, trehalose, cotyledon, yold platelets	• Long term survival • quick regrowth
Control entry and exit	Hormones,cascades signalling,dipicolinic acid,glassy cytoplasm	• Match dormancy with growth-prohibiting periods • Quickly re-colonize

Table 1.1: Relation structure function during dormancy

All together, these different traits reveal a unifying design principle : a programmed isolation from the environment. For all living organisms, exchanging with the environment is always twofold : while the organism can extract nutrients and infor-

mation, it is also exposed to all potential damages (predation, osmotic shock etc). By turning dormant, organisms isolate themselves by abolishing the exchange with the environment. Indeed, they are adapted to be shielded from external stresses (protective coat) and are also adapted to survive with internal resource only (low internal activity from stored resources). Dormant form can be seen an autonomous entity that is adapted to remain viable without exchanging with the environment (Fig.1.4A).

Overall dormancy can be understood as **programmed isolation from environment, that couples protection from external stresses with maintenance of viability from internal resource.**

How to compare the dormancy of different organisms along that design principle? Specifically, what is the place of dormant yeast spores compared to other form of dormancy such as plant seed and bacterial endospores?

1.1.4. Opportunity to study dormant single-celled eukaryotes

One key aspect of dormancy is that it is not exclusive to any group of living organisms. Indeed, dormancy as we defined it above (programmed isolation from the environment) is phylogenetically widespread, as examples of dormant stage are present virtually in every group of organisms, from single cell bacteria to complex multicellular animals (Fig.1.3). However, as we noticed in the previous part, the underlying structures and molecular mechanisms implementing dormancy can be drastically different, so that it seems difficult to make a statement about how evolutionary conserved it is. Conceptually, we can say that dormancy is possible as soon as there is a form of cellular life that controls the exchange between the inside containing the genetic materials and the outside environment. Based on that idea, we could even speculate that dormancy is the most primitive form of cellular differentiation, since any emerging single celled life would be immediately challenged to survive growth-inhibiting periods (Tocheva et al. 2016).

Studies that systematically surveyed the presence or absence of dormancy in a given clade exists for animals (hibernation in mammals or diapaused in crustacean, see Kaplan-levy et al. 2010) but are rare for unicellular eukaryotes such as budding yeasts. A simple proxy to detect the presence of dormancy in an organism can be the presence of a morphological differentiated stage in the life cycle. Specifically in microbial eukaryotes, dormancy is often associated to the formation of spores and cysts. A recent review surveyed the presence of cysts (like the one of G.intestinalis) or spore (like the one of *S. cerevisiae*) across nine main eukaryotes groups (Schaap et al. 2018, Fig 1.3). Strikingly, spores, cysts or analogous differentiated dormant stage were found in all nine groups. This interesting analysis inspires two reflec-

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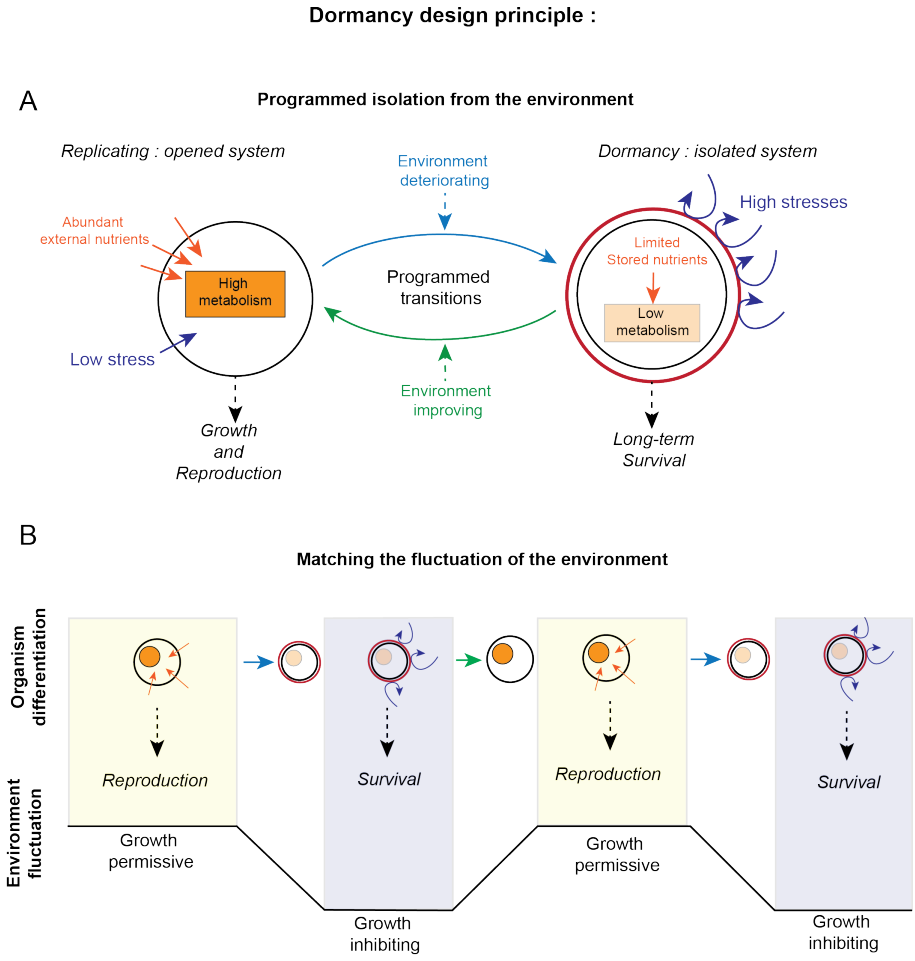


Figure 1.2: Dormancy design principle : a programmed isolation from the environment (A) Schematic view of the design principle of dormancy. Organisms alternate between two states. In the "opened", organism grow and reproduce by taking energy from the environment. Exchanging with the environment comes at the cost of being vulnerable to external stresses. In the "isolated" state, organism only survive from internal resources and are protected from external stresses. (B) The second part of the design principle of dormancy is that the isolation from the environment is programmed to match growth-inhibiting periods only. As the environment fluctuate between, organisms transits between its isolated (dormant) and opened state (replicating).

tions :

The presence of differentiated dormant stage suggests that dormancy is likely a major part of the life-cycle of unicellular eukaryotes. Therefore, gaining mechanistic

insights in the dormancy of yeast spores could be of great interest to understand the ecology and evolution of unicellular eukaryotes. Additionally, sporulation or encystation could be an interesting model system to study the origin of temporal differentation in stem eukaryotes (Schaap et al. 2018).

As noted by the authors, "often not all species within the genus have a dormant stage". Even though not all species have a dormant stage, there is a high probability that a phylogenetically close cousin has one. This fact rises tantalizing questions for species without a dormant stage such as : *How "close" are these species from having a differentiated dormant stage?* By "close" we mean that some species might have a close ancestor with a dormant stage and that it was lost recently. As a consequence, it might be easier than expected to turn certain cells into a differentiated dormant phenotype, by direct mutation or by exposure to artificial environmental cues. A corollary follow-up provocative question could be : *"Can we turn stem cells or humans cells into cysts?"* Actually recent studies mentioned the potential link between eukaryotic encystation and dormant cancer cell that are resisting chemotherapies (Baig et al. 2015). Thus, studying how single-cell eukaryotes turn and remain dormant could have surprising therapeutic and engineering applications.

1.1.5. A roadmap to quantitatively study dormancy

Since dormancy is phylogenetically widespread, in order to compare different type of dormancy we need to see beyond the specific structures and molecular mechanisms. Among the different traits of dormancy listed above (Table1.1), "protective coat", "stored ressources" and "control of entry in dormancy" are the ones that strongly depend on the differentiation into the dormant form. This differentiation process is usually complex and coupled to sexual reproduction (sporulation, encystation, endosporulation). Therefore, here we propose to use the two other traits, "Low internal activity" and the "Control of exit from dormancy" to compare dormant organisms between them. To do so, we formulate two questions :

- *What are the sufficient signals to resume activity?*
- *How reduced is the internal activity during dormancy?*

Signals to exit dormancy Improvement of the environment is not always sufficient or even necessary to trigger exit of dormancy. At one end of the spectrum we find what is called "seed dormancy" or "diapause" in some animals (insect and crustacean particularly) : even if the embryo is viable, it remains unable to resume development upon favorable conditions. This specific adaptation is controlled by complex endogenous cues that prevent "accidental waking-up" before winter has passed. At the other end of the spectrum we find "bacterial scouts",

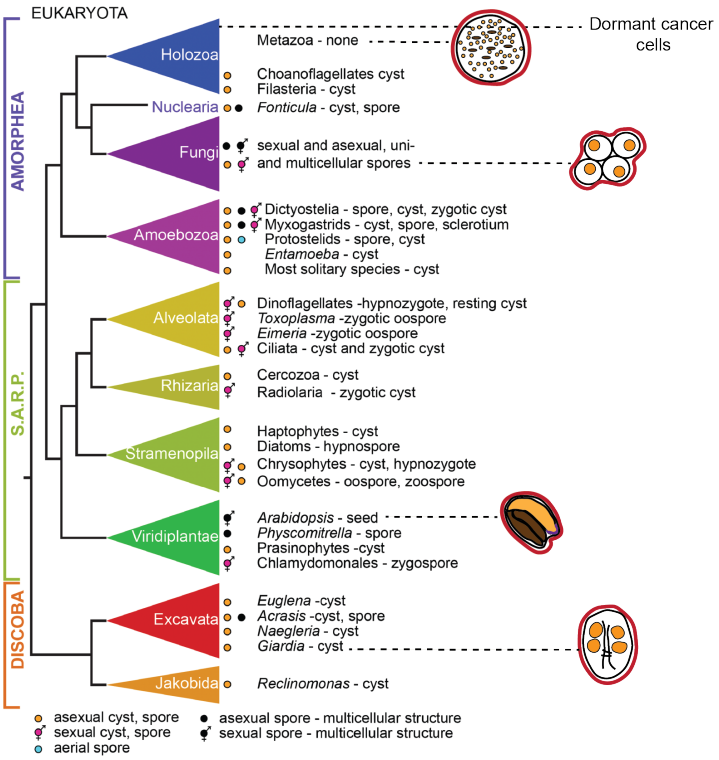


Figure 1.3: Dormancy is phylogentically common but widespread Adapted from Schaap et al. 2018 Although eukaryotes are mostly famous for being the family of large obligatory multicellular organisms such as animals, plants and fungi, they also display a huge diversity of unicellular or conditionally mutlicellular organisms, known as "protists". Throughout the tree of eukaryotes, distinct morphological stages associated to dormancy were surveyed. Here we found relevant to also add the diapaused eggs (here) typically found in metazoan, as well as the "dormant cancer cells", which are closer to cell-cycle arrested cells, but can studied in light of their proximity to other cysts and spores of protists.

that can spontaneously and randomly resume growth, independently of the environment (Buerger et al. 2012). With such bet-hedging strategy, bacteria take the risk of waking up in a stressful environment but do not have the cost of sensing it. In between these two extreme strategies lies a continuum of ways to exit dormancy. Most common case is to wake up quickly upon exogenous signal that are synonymous to growth-permissive environment. This can be light, temperature, water and passive nutrients influx, such as for for *S.cerevisiae* spores or B.subtillis endospores.

Level of activity during dormancy All dormant states are by definition characterized by a reduced internal activity. However, what are the hard numbers behind

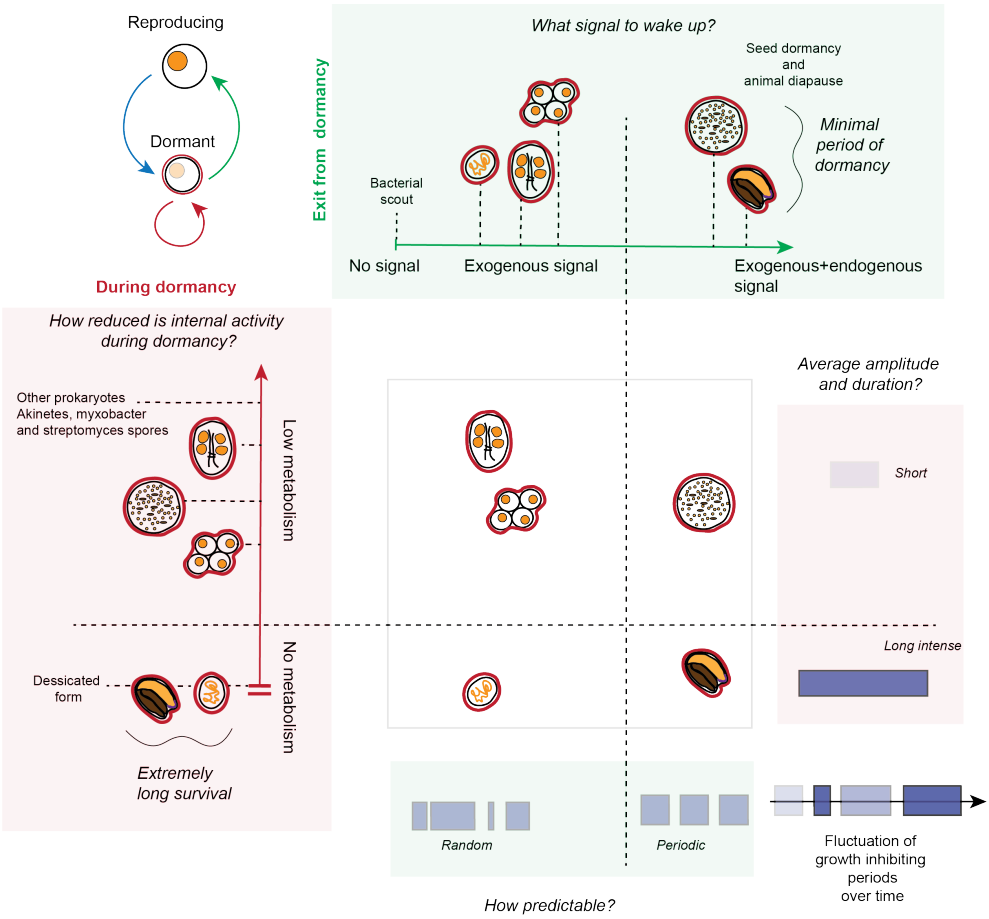


Figure 1.4: How to compare dormant organisms? (Red panel) Dormant stage are sorted as function of the internal activity during dormancy from the bottom (metabolically inert) to the top (highly active).Unlike bacillus, other groups of prokaryotes have dormant stage have a metabolically active dormant stage, such as akinetes and spores from myxomycytes. (Green panel) Dormant stage are sorted as function of the sufficient signal to exit dormancy from the left (no signal) to the right (complex interplay between endogenous and exogenous signals). In between the two extremes lies most of the microbial dormant form, which can be "easily" woken up by providing sufficient nutrients in liquid culture.(Central panel) Dormant stage of phylogenetically widespread organisms are grouped on a 2D map, combining information about the internal activity and the ability to wake-up. Cysts and spores of yeasts are in the middle group, (Right and bottom panel)

loose words such as "reduced","low" or "minimal"?, is a difficult question to address. Even though direct measurement are either technically challenging or not performed yet, dormant state are usually sorted into one of two categories. In the first extreme category, it is assumed that there is no metabolic activity at all. This means no synthesis of macromolecules and no energy production through catabolism or electron chain transport. This is what is believed to happen for bac-

terial endospores, dessicated plants seeds and generally for all dormant state that are dried (i.e. "anhydrobiosis"). In the other category are all dormant states with measurable activity, usually energetic metabolism (giardia or diapaused eggs) or gene expression (yeast spores Brengues et al. 2002). Conceptually the amount of activity is put in relation with the life-span. Indeed a minimal activity means consumption of internal resource which are by definition limited. Therefore, the rate of consumption determines the lifespan as dormant. This mechanism is thought to underpin the lifespan of diapaused eggs, yeast spores or giardia cysts. On the other hand, when the internal activity is drastically reduced, like for bacterial endospores, lifespan is thought to be determined by physical degradation of molecules constituting the dormant organism. When dried, frozen of mineralized (amber or salt), these process can be extremely slow, which confers extremely long lifespan.

Importantly, that simplified classification identifies pure strategies that often to do not correspond the actual dormancy adopted by a specific species. For instance, it has been established that although this is not the dominant strategy, a small fraction of B.subtillis endospores display "scout-like" random exit of dormancy (Dworkin et al. 2010). Similarly, several species can survive both in a dessicated (dried yeast with no activity) and in a non dessicated form (yeast spores in water, with internal activity).

Relative position of dormant yeast spores Each dormant state can be located along two axis, placing it on a planar map. Locating our five examples of dormancy reveals the specific type of dormancy. For instance plant seed combines "no metabolism" during dormancy with "Exogenous+endogenous signals" to exit dormancy, and is therefore located at the bottom right corner. Interestingly, both yeast spores and Giardia's cysts are occupies a central region or "style of dormancy", with a low but non zero metabolic activity and "simple" exit of dormancy upon exogenous signals. Strikingly, literature about dormancy historically focused on extremes, represented by endospores bacteria and plant seeds. Our work on dormant yeast spores aim at filling that gap by adding a new model system for that "intermediate" dormancy.

Hypothesis on the interplay between style of dormancy and statistics of growth-inhibiting periods Classifying different type of dormancy is a step towards mechanistic understanding, but what is the next step beyond description? One idea could be to link the type of dormancy to the statistics of how the environment fluctuates (Blath et al. 2021). Indeed, dormancy is fundamentally an adaptation to growth-inhibiting periods. Therefore we could speculate that the different styles of dormancy could result from an adaptation to the statistics of the growth inhibiting periods. To put it simply, statistics of the growth inhibiting periods can

be captured by two aspects : 1) On average how long and how stressful are the growth-inhibiting periods?, 2) How predictable are these growth inhibiting periods? (from completely random to strictly periodic).

For instance, diapause and seed dormancy can be interpreted as an adaptation to a very predicable growth inhibiting period such as winter. On the other hand, bet-hedging strategies such as random exit of dormancy can be interpreted as adaptations to a very un-predictable growth inhibiting periods.

Similarly, dormancy without metabolism can interpreted as an adaptation to extremely long and stressful growth-inhibiting periods. Whereas dormancy with metabolic activity is limited by the amount of internal resource, and therefore can be interpreted as an adaptation to shorter and less-stressful growth-inhibiting periods.

This formal classification and the corresponding hypothesis is only one proposition based on our current knowledge about dormancy. However theoretical and empirical work about dormancy itself are rare and therefore our understanding of could be drastically modified in the near-future. One priority could be to interrogate the function of the internal activity during dormancy. Specifically, what is the causal link with the lifespan or the ability to wake-up under specific environmental signals?

The work presented in this thesis participates that ambitious program by modestly investigating the functions of gene expression during the dormancy of *S. cerevisiae* yeast spores.

1.2. Mechanisms underpinning dormancy

In the previous part, we saw that in response to growth-inhibiting conditions, a myriad of organisms undergo a programmed isolation from the environment, commonly named "dormancy". Aside from being protected from external stresses, one major aspect of that isolation is that dormant organisms manage to survive autonomously without harvesting energy from the environment. In parallel it has been observed that dormant organism have nearly ceased internal activities.

What are the causal links between having a nearly ceased activity and remain viable?

Here we review typical experiments and concepts that aim at answering that question, with a focus on dormant yeast spores. We will proceed by asking three simple questions about the internal activity during dormancy :

1. What is it? (How can we accurately measure a nearly ceased activity?)
2. What is its function? (what happens if the activity is inhibited?)
3. How is it regulated? (what are the knobs to tune it?)

1

1.2.1. Probing internal activity during dormancy

As we saw previously (Fig.1.4), internal activity during dormancy is usually sorted into one of two categories : truly metabolically inert (endospores of bacteria) or low but non-zero metabolism (yeast spores).

What are the typical argument supporting such claim?

Indirect methods First, metabolic state of during dormancy is often determined indirectly. For instance in desiccated plant seed, biophysical experiments and modeling showed that below a certain water content, molecular motion is so reduced that enzymatic reactions are impossible (Buitink et al. 2008). One other indirect method is to use a drug that inhibits energetic metabolism. If the drug happens to kill the organism during dormancy, it indirectly shows that metabolism was active during dormancy (example ATPases Mycobacterium tuberculosis Rao et al. 2008). Conversely if the drug does not kill the during dormancy, it is thought that ATP metabolism is negligible.

Direct methods Robust methods to directly probe the level of internal activity are based on the fact that being metabolically active is synonymous to consuming various inorganic and organic chemicals. Therefore by measuring the consumption or incorporation of these compounds, one can efficiently measure the metabolic activity. For instance measuring oxygen uptake can be used to probe ATP production via oxydative phosphorylation . Such method allow to estimate that dormant cysts of Giardia intestinalis have a metabolism of 15% of their non-dormant stage (replicating trophozoite) (PAGET et al. 1993).

Another way to probe internal activity is to measure the incorporation of building blocks of macromolecules constituting cells. Indeed a major part of internal activity of cells consists in the synthesis of polymers like RNA and proteins from standard building blocks such as nucleic acids and amino acids. One idea is then to incubate dormant organisms together with labelled building blocks (i.e. non standard ones) and then detect their incorporation in macromolecules. If the labelled building block are incorporated, it shows that synthesis of macromolecules is happening. This idea was applied to probe the synthesis of RNA (i.e. transcription) as well as synthesis of proteins (i.e. translation) during dormancy of *S.cerevisiae* yeast spores (Brengues et al. 2002). The authors of the study used radioactively labelled nucleic acids to probe transcription ((Fig.1.5)A) and radioactively labelled amino acids to probe translation ((Fig.1.5)B). Together these results show that during dormancy of yeast spores, gene expression is measurable yet only 5% of what is typically measured for vegetative cells exposed to ample amounts of nutrients.

One additional element brought by this study was that certain mRNA in dormant spores seem to be polyadynalted, indicating they might be actively translated.

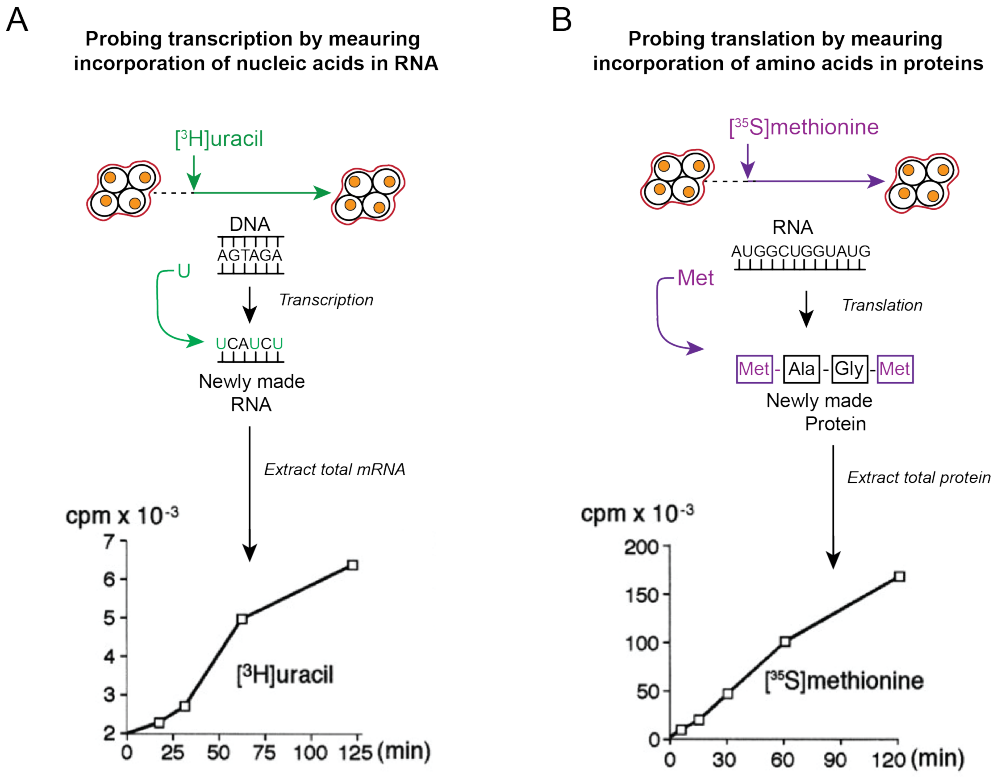


Figure 1.5: Dormant yeast spores express genes *S. cerevisiae* spores were re-suspended in water and incubated at 30C with 100$\mu Ci/ml$ of either [3H]uracil (A) or [35S]methionine (B) for the indicated time. Total RNA or proteins were extracted, and incorporation of radioactivity was determined by trichloroacetic acid precipitation. (A), incorporation of [3H]uracil reflects transcriptional activity; (B), incorporation of [35S]methionine reflects translational activity. Adapted from Brengues et al. 2002.

1.2.2. Causal links between activity and survival during dormancy

Now we will turn into a central question when we study dormancy : "how can organisms survive with nearly ceased activity?"

Dormant organisms have nearly ceased internal activity yet are still alive. But what part of that nearly ceased activity causes them to stay alive? In order to address that question, we first need to address a more concrete one :

How do we know whether an organism with "nearly ceased activity" is alive or dead?

Indeed the absence of outward activity prevents us from answering that question by simply observing an organism during its dormancy. Therefore, the only option is often to try to wake up the dormant organism by transferring it to an environment favorable to growth (with abundant food, humidity etc). If it wakes up, the organism was dormant, if it does not wake up, the organism was dead. In the case of dormant yeast spores, the test consists in incubating spores in a medium that contains essential nutrients together with saturating glucose.

With that convention to measure whether an organism is dormant or dead, one can directly measure the lifespan during dormancy, i.e. the time an organism can remain dormant before turning dead. Concretely, one simple way to estimate that lifespan is to frequently sample individuals of the same "ageing" dormant populations, and subsequently measure the percentage that are still dormant. This experiment was conducted for dormant yeast spores kept in water without nutrients, and revealed that the half-life was around 21 days (Brengues et al. 2002, Fig.1.7).

The lifespan during dormancy measured in this way is the fundamental "observable" we can use to decipher the mechanistic basis of survival during dormancy. Indeed, by adding inhibitory drugs during dormancy, we can directly measure how it shortens the lifespan and then estimate the relative importance of certain process to maintain viability during survival.

While this type of approach has been used on some dormant organisms to qualitatively probe the importance of internal process on survival during dormancy (ATP synthesis in Mycobacterium Rao et al. 2008), such simple and crucial experiments have never been conducted in dormant yeast spores.

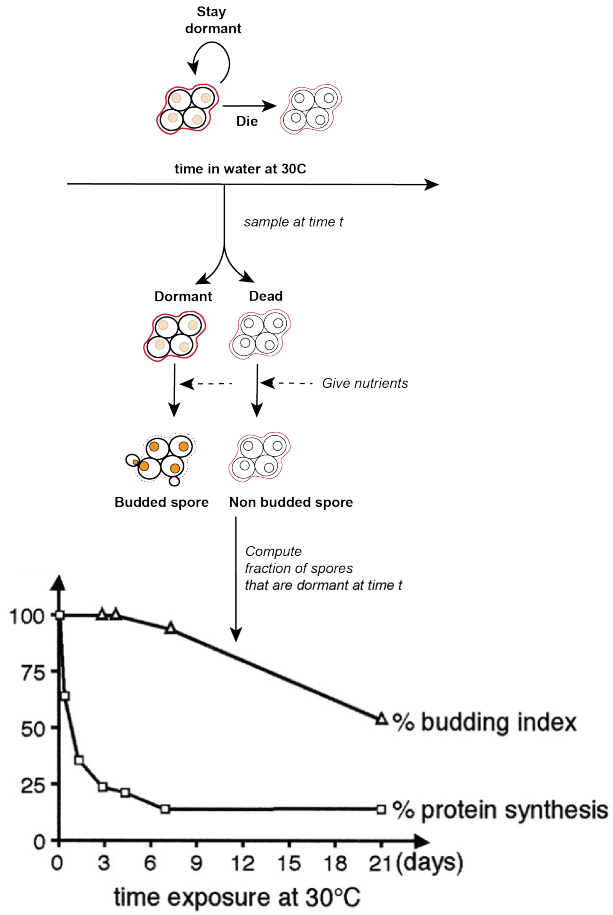


Figure 1.6: Dormant yeast spores gradually die at 30C Spores were incubated for various times in water at 30 °C, then their ability to synthesize proteins and to germinate was determined. Protein synthesis was followed by labeling spores for 1 h with [35S]methionine at the indicated times. Germination capacity was determined by transferring the spores to YPD at 30 °C and incubating them for 4 h before by measuring the budding index with an hematimeter. Graph and legend text from Brengues et al. 2002

1.2.3. Regulation of internal activity

Organisms usually turn dormant in environments where nutrients are not available, preventing growth. Thus, dormancy is by definition nutrient-depleted, which already imposes a strong limit to their internal activity. However, there has been several evidence that there exists additional endogenous mechanism, promoting shut down of internal activities.

This can be by accumulation of a specific chemical such as dipicolinc acid in endospores (Setlow et al. 2006), or the presence of a specific organ like the micropylar in plant seeds (Finch-Savage et al. 2006). Aside from structure or chemicals, researchers have identified multiple active processes that are thought to further reduce internal activity upon entry in dormancy. One first mechanism is the degradation of macromolecules such as RNA and proteins. Indeed reducing the number of mature RNA during dormancy naturally limits the protein synthesis. This has been observed in the formation of bacterial endospores (Segev et al. 2012).

One last mechanisms promoting entry in dormancy is due to modification of biophysical properties of cells inner environment. In several systems such as bacteria, plant seed (Buitink et al. 2008, and yeasts (Munder et al. 2016), it has been shown that cells undergo a "glass-like" transition upon starvation or desiccation. Concretely this means that viscosity of cellular micro-environment increases and chemical reactions slow down.

Concerning yeast spores, such phenomenon is only supported by indirect evidence. Indeed, a recent study showed that in vegetative yeasts, starvation caused a transition of the cytoplasm to a "solid-like" state where diffusion of molecules is greatly reduced (Munder et al. 2016). Moreover, the study demonstrates that lowering the cytoplasmic pH is sufficient to recapitulate that transition. Dormant yeast spores kept in water are by definition already starved, and cytoplasm pH has been shown to be more acid than vegetative cells. While direct measurement of molecular diffusion have not been conducted in dormant yeast spores, the authors report that several proteins seem to form dense clusters, similarly to what is observed for starved vegetative yeast. This result indicates that the cytoplasm of dormant yeast spores might be in a similar "solid-like" state.

Accordingly, this "solid like" transition mechanism is merely a "positive feedback loop", by which reducing the metabolic activity causes the cytoplasm to become more solid-like, which causes the metabolic activity to be reduced, etc. By being generic, this mechanism could be important to explain how cell maintain their activity to a minimal level during dormancy. However one important open question is whether this results from an adaption or just relies on a universal property of cellular physico-chemistry.

Overall the mechanistic understanding of function and regulation of metabolic activity during dormancy is still an emerging field, which is largely limited by the lack of measurement during dormancy itself. In this thesis we aim to provide such measurement of activity during dormancy and try to infer the underlying regulatory mechanisms.

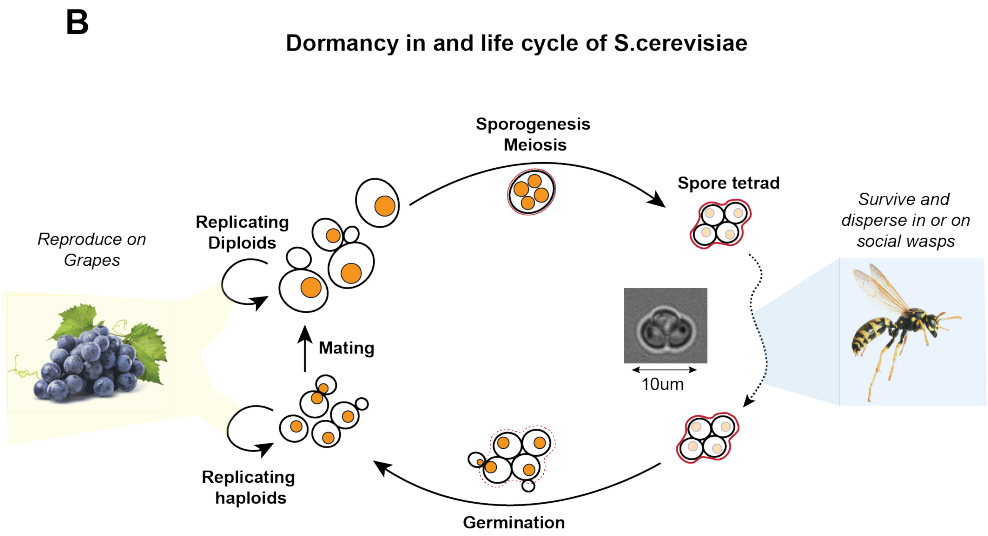


Figure 1.7: More about S.cerevisiae life cycle dormant stage Saccharomyces cerevisiae has a complex life cycle with alternance of haploid and diploids phases. Both haploids and diploids cells replicate by mitosis if sufficient nutrients are present, usually on grapes or tree bark. Haploids yeasts exits in two sexual type a and alpha and mate to form diploid cells. Upon starvation of nutrients, diploids yeast engage "sporulation", a complex process coupling meiosis with sporegenesis. Haploid spores are usually packed in tetrades in spores bag, coated with a thick spore wall. Spores are dispersed through digested tract of social wasps.

1.3. SUPPLEMENTARY INFORMATION ON *S.CEREVISIAE* SPORES

We will now briefly summarize what is known about dormant yeast spores, the main system that will be used throughout this thesis.

Saccharomyces cerevisiae yeast cells require 10-38C, liquid or humide surface with nutrients like glucose, nitrogen bases and various vitamins, to proliferate (Kaplan-levy et al. 2010). In nature we find these conditions on grapes, bark of trees in temperate regions.

Upon starvation of fermentable nutrients, diploids yeasts engage in a process known as sporulation : meiosis and production of a thick spore wall (Neiman 2011).

While still debated by specialists, yeasts spores are thought to be particularly adapted to survival in the digestive tract of insect such as wasps (Stefanini et al. 2012).

Useful reference for *S.cerevisiae* yeasts spores :

- Sporulation : Neiman 2011
- Germination : Joseph-Strauss et al. 2007.

- Ecology of spores : Stefanini et al. 2012
- Glassy cytoplasm (speculated) : Munder et al. 2016
- Gene expression during dormancy : Brengues et al. 2002
- Quiescence (haploid) in starved yeast : **Sagot2009**

1.4. Outline of the thesis

This thesis is organized around three main chapters that each cover a specific aspect of dormant spores "life", in relation with gene expression. From a temporal perspective, only three distinct things can happen to a dormant spore. It can:

1. Germinate if transferred to nutrient-rich environment.
2. Die if it stays without nutrients for too long.
3. Stay dormant if none of the two above things happen.

For each of these three fates, "resume replication", "die" and "stay dormant", we successively explore the importance of gene expression.

Chapter 2 "From dormant to replicating" and Chapter 3 "From dormant to death" are part of a single very long research article that was published in Molecular Systems Biology (Maire et al. 2020).

Chapter 4 "Gene expression dynamics during dormancy" is still a work in progress that originated from a serendipitous discovery while working on the first paper.

Chapter 5 regroups all the methods used in Chapter 2-3-4.

Chapter 6 is the global conclusion where we try to summarize and speculate on how the gene expression dynamics set the time-scale of survival during dormancy.

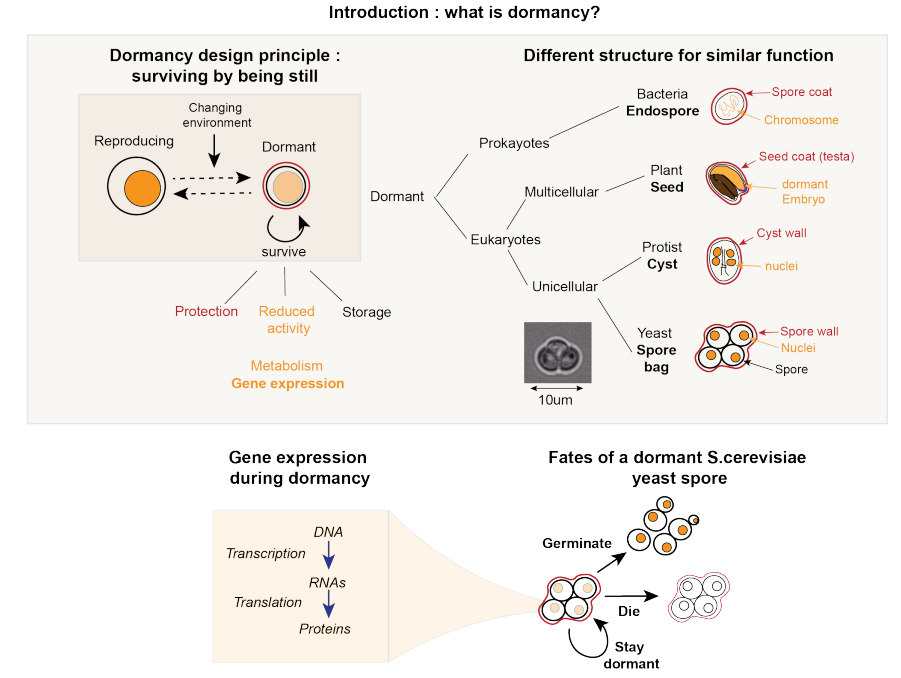
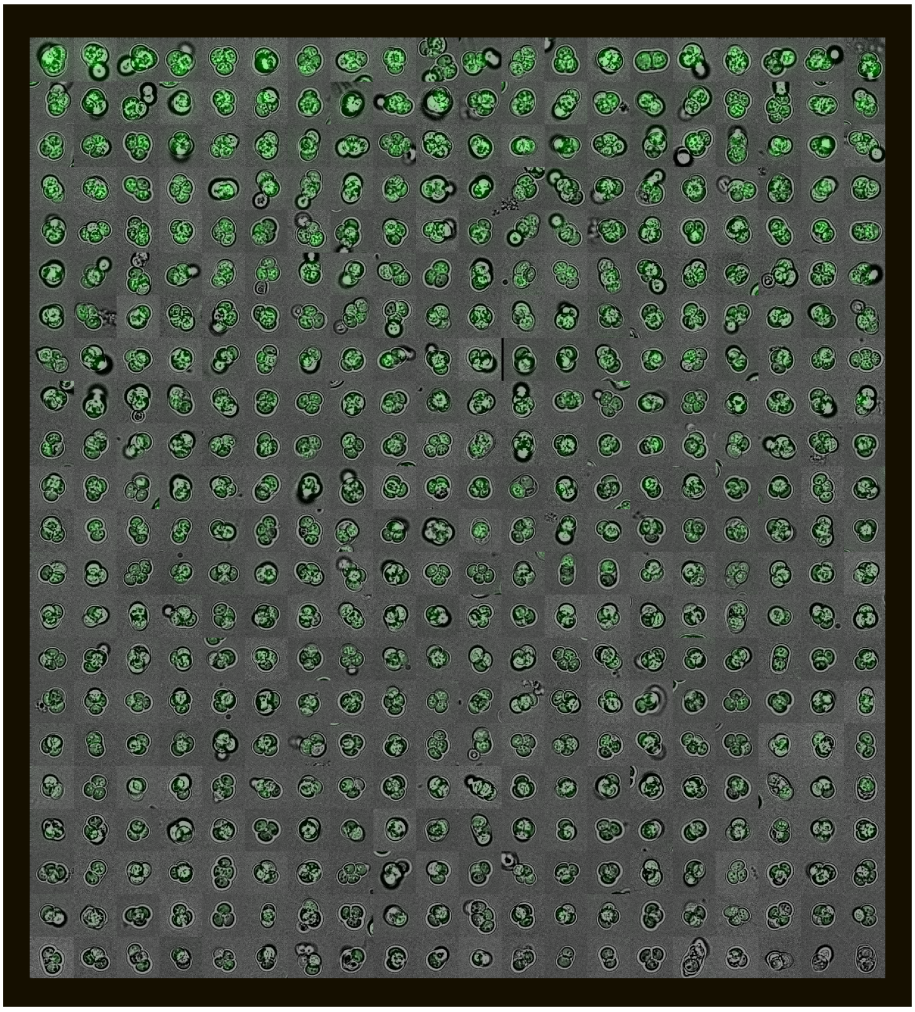


Figure 1.8: Graphic outline of the thesis

2

From dormant to replicating



Contents

2.1. Introduction

When starved of nutrients, microbes can become dormant by forming spores (Neiman 2011; Stragier et al. 1996; Errington 2003; Lennon et al. 2011; Nachman et al. 2007;Chu et al. 1998; Eldar et al. 2009; Süel et al. 2006; Süel et al. 2007). Dormancy is a state in which gene expression, metabolism, and all other cellular processes are thought to have nearly or completely ceased. But exactly to what extent each process of life has halted inside dormant spores remain ambiguous as attaching firm numbers to them has been challenging due to the barely detectable levels of activities inside dormant spores.

Entering dormancy is a multi-step process that does not necessarily end with the spore formation. In fact, after sporulation, spores of some species are not dormant right away. For example, Bacillus subtilis spores - a model for prokaryotic spores - undergoes a week-long "ageing" process during which they are metabolically active, and they actively degrade or produce key transcripts to tailor their dormancy to the environmental temperature during this process (Segev et al. 2012). The spores of budding yeast, *Saccharomyces cerevisiae* - a model for eukaryotic spores - takes between a few hours to a few days to fully enter dormancy (Brengues et al. 2002; Thacker et al. 2011). During this time, the not-yet-dormant yeast spores express various genes - such as those involved in completing the spore wall - before gradually turning off gene expressions, at the end of which the spores are considered to be dormant (Brengues et al. 2002; Thacker et al. 2011).

After entering dormancy, both bacterial and yeast spores are thought to have greatly reduced metabolism and vanishingly low, if any, genome-wide expression levels (Rittershaus et al. 2013; Segev et al. 2012; Brengues et al. 2002). Supporting this view for yeast spores are the recent discoveries of proteins and messenger RNPs (e.g., mRNAs bound to translational machineries) becoming inactive by aggregating into macromolecular structures inside dormant yeast spores and other fungal spores (Laporte et al. 2008; Petrovska et al. 2014). The resulting solid-like, glassy cytoplasm - being packed with these aggregates - greatly hinders proteins' movements and enzymatic activities (**1999**; Cowan et al. 2003; Dijksterhuis et al. 2007; Parry et al. 2014; Joyner et al. 2016; Munder et al. 2016). Taken together, previous findings support the widely accepted view that dormant yeast spores have nearly ceased all their activities (Rittershaus et al. 2013).

Although dormant yeast spores have nearly ceased activities, what distinguishes them from dead spores is that they are able to germinate and resume replicate life upon addition of nutrients. How dormant cells with nearly ceased activity keep the ability to resume replication within minutes or hours remain largely elusive and often referred to as "resuscitation" (Lennon et al. 2011). Motivated by this deep

unanswered question, several studies focused on dissecting the sequence of molecular events that occur just after addition of nutrients until the first cell replication (**Setlow2014**,Joseph-Strauss et al. 2007). Specifically in yeast spores, genome-wide assay during germination uncovered a specific transcription program that consists in two distinct parts, each controlled by specific nutrients (Joseph-Strauss et al. 2007). However these approaches take for granted the fact that dormant spores are able to germinate *before* addition of nutrients.

Here we sought to investigate what determines the ability to germinate of dormant yeast spores before addition of nutrients, i.e. while the activity is nearly ceased.

To address our question, we first reasoned that a dormant yeast spore's ability to germinate is likely encoded by amounts of its stored intracellular factors (e.g., specific proteins and RNAs) which are required for restarting replicative life. For dormant spores of some species, researchers have identified several intracellular factors that affect their revival (Segev et al. 2012; Dworkin et al. 2010; Mutlu et al. 2018; Donnini et al. 1988; Geijer et al. 2012; Herman et al. 1997; Sturm et al. 2015; Sinai et al. 2015). For example, alanine is more likely to revive B. subtilis spores that have more alanine dehydrogenase stored in them than spores that stored less of it (Mutlu et al. 2018). But it is unclear how the known and as-yet-unknown intracellular factors - perhaps the numbers of stored ribosomes and RNA polymerases - collectively affect revivability of dormant yeast or bacterial spore.

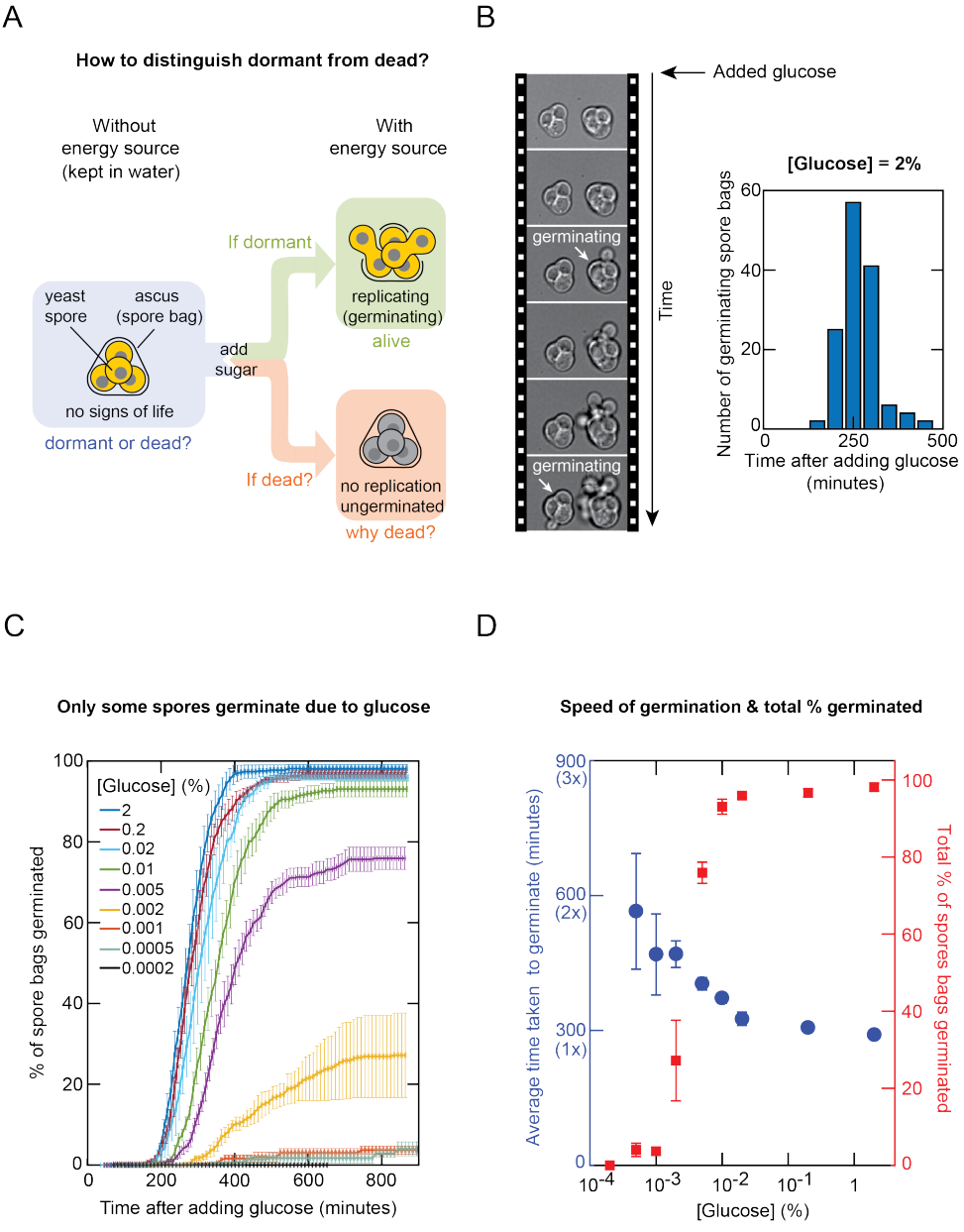
A brute-force way to address this question is identifying all intracellular factors that are necessary for sustaining spores' dormancy and allowing revival. But this approach remains elusive because, for one, it requires using a current snapshot of numbers (i.e., amounts of intracellular factors now) to predict a capacity for achieving a complex future behavior - whether the dormant spore still has an ability to wake-up if nutrients were to reappear. Addressing our question requires circumventing this difficulty. Our study begins by giving various glucose concentrations to dormant yeast spores. From this, we discovered that not all yeast spores wake-up (germinate) to re-enter a replicative life and that those that do not germinate are primed - they undergo accelerated germinations if they encounter more glucose hours-to-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 yeast spores, expression of a gene (GFP) that plays no role in germination. We found that one can induce GFP expression in dormant yeast spores that are in water without any nutrients (no extracellular amino acids and glucose) and that, surprisingly, the inducible GFP level in a dormant spore tells us the probability of that spore germinating for a given concentration of glucose. Strikingly, we found that dormant yeast spores, when induced, can express genes at levels that are as high as those of vegetative yeasts.

To our knowledge, we provide here one of the first evidences and quantifications of gene expression in intact, dormant yeast spores at a single-cell resolution.

2.2. Different ability to germinate

We began by re-examining the conventional test for determining whether a yeast spore is dormant or dead. The test involves giving ample glucose to yeast spores and then observing whether they germinate or not (Brengues et al. 2002; Joseph-Strauss et al. 2007) (Fig. 2.2A). If the spore germinates, then it is considered to have been dormant whereas if it does not, then it is considered to have been dead. This test, however, does not reveal why a spore that does not germinate is dead in the first place and when it died. For instance, the test cannot distinguish between a spore that died while it was being formed versus a spore that was alive (i.e., dormant) after forming but died during its dormancy (and why and when the death occurred). Motivated by this deficiency, we re-examined the test by asking whether all yeast spores can indeed germinate after receiving ample glucose (Fig. 2.2A). Starving a laboratory-standard ("wild type") homozygous diploid yeasts caused them to form genetically identical, haploid spores. Specifically, each diploid cell formed a single "spore bag" (i.e., ascus) that contained four genetically identical haploid spores (Fig. 2.2A). We incubated a population of these spore bags in a "minimal medium" - this has all the essential amino acids but no glucose - and, as a modification to the conventional test, supplemented the minimal medium with a less-than-saturating concentration of glucose (less than 2%) instead of the usual, 2%-glucose. 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 track 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. 2.2B and Supp.Fig2.10). 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 one spore that germinates. With a saturating glucose concentration (2%), nearly every spore bag in the population germinated (Fig. 2.2C). But with lower glucose concentrations (i.e., less than $\sim$ 0.01%), a noticeable percentage of spore bags in the population - about 10% or more - did not germinate regardless of how many hours we waited after adding the glucose (Fig. 2.2C). The percentage of spore bags that germinated changed in a sharp, step-like (sigmoidal) manner as a function of the glucose concentration (Fig. 2.2D - red points), with the change in step located at a glucose concentration of $\sim$ 0.003% (i.e., at this concentration, 50% of spore bags germinate). In contrast, the average time taken to germinate only weakly depended on the glucose concentration, increasing by at most 2-fold despite a 10,000-fold decrease in the glucose concentration from 2% to 0.0002% (Fig. 2.2D

– blue points). This indicates 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 because when we measured how much glucose was left in the minimal medium after all the germinations stopped (i.e., ∼ 600 *minutes after adding glucose for all glucose concentrations*), *we always found a large fraction of the glucose concentration hardly decreased even for the lowest starting glucose concentrations that we use type strain – the same strain as the spores – could replicate multiple times even with the lowest glucose conce*


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
% of spore bags germinated
[Glucose] (%)
2
0.2
0.02
0.01
0.005
0.002
0.001
0.0005
0.0002
Time after adding glucose (minutes)
D
Speed of germination & total % germinated
Average time taken to germinate (minutes)
Total % of spores bags germinated
[Glucose] (%)

2.3. Primed spores germinate faster

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

To explain why some spores do not germinate even with ample glucose, we considered two possibilities. One was that the spore bags that did not germinate (i.e., "un-germinated spore bags") died while trying to germinate. The other possibility was that the un-germinated spore bags were still able to germinate and thus were still alive (i.e., dormant). We distinguished these two possibilities by repeating the above experiments but now by adding glucose in two steps (Fig. 2.2A). First, we gave the spores a relatively low concentration of glucose. We then waited, typically $\sim$ 16 hours ($\sim$ 1000 minutes), after which no more germinations occurred. Then, we added more glucose to increase the total glucose concentration and then observed if this led to any more germinations. We found that some of the spore bags that did not germinate the first time were germinating after receiving more glucose (Fig. 2.2B and Supp.Fig2.13-2.14). Yet, some of the spore bags still did not germinate after receiving the second batch of glucose if the resulting, final concentration was not the saturation value, 2% (Fig. 2.2C - top panel). For example, when the final concentration was 0.002%, nearly 60% of the spore bags in the population remained un-germinated. Intriguingly, this 60% is close to the percentage that would have remained as un-germinated if we had given the 0.002%-glucose all at once instead of in two steps (compare Fig. 2.2C with Fig. 2.2C). This suggests that each spore bag may be pre-programmed to germinate for certain glucose concentrations. Accordingly, nearly every spore bag eventually germinated if the second batch of glucose increased the total glucose concentration to 2% (Fig. 2.2C - bottom panel). These results establish that spores that do not germinate after encountering ample glucose are not necessarily dead.

Figure 2.1 ***(preceding page)*****: Glucose germinates only a fraction of yeast spores. (A)** Conventional test to determine whether a spore bag (i.e., ascus) containing four haploid spores, in the absence of any external nutrients (blue box), is dormant or dead. Green box: outcome if dormant. Red box: outcome if dead. **(B)** Left: Filmstrip of a time-lapse movie in which a 2%-glucose was added at the beginning of the movie (10 minutes between frames). A spore bag is counted as having germinated at the moment that at least one budding cell emerges from the spore bag (white arrows). Right: Time taken by each "wild-type" spore bag to germinate in the time-lapse movie. n = 137 spore bags from a representative time-lapse movie. **(C)** Percentage of wild-type spore bags that germinated as a function of time after adding glucose to the minimal medium. Different colors represent different glucose concentrations (from 0.0002% to 2%). n = 3; error bars are s.e.m. **(D)** Average time taken to germinate (blue circles) and the total percentage of spore bags that germinated (red squares), both 16 hours (960 minutes) after receiving glucose (i.e., the plateau values for each color in Fig. 2.2C). Both are functions of glucose concentration. n = 3; error bars are s.e.m.

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

. To better understand why only some spore bags germinated for a given glucose concentration, we examined whether the un-germinated spore bags had any measurable response to the glucose that they first encountered. When we added glucose in two steps so that the final concentration was 2% (Fig. 2.2A), we found that the spore bags took less time to germinate, in response to the second batch of glucose, than they would have if they had received the entire 2%-glucose all at once (Fig. 2.2D). 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. 2.2D). Thus, encountering a very low amount of glucose "primes" some spores so that, upon encountering a saturating level of glucose at a later time, they would germinate faster - up to two times faster on average - compared to spores that did not previously encounter any glucose (Fig. 2.2E). Furthermore, when we primed the spores with a very low glucose concentration and then waited between 16 hours to 4 days before increasing the glucose concentration to 2%, the spores were still primed - they germinated faster compared to spores that were kept in minimal medium without any glucose for the same amount of time. They were primed for up to two days after we added the first glucose but no longer primed four days after we added the first glucose (Fig. 2.3A and Supp.Fig.2.15). Thus, primed dormancy lasts for and decays over days.

2.3.3. Transcriptome-wide view of primed dormancy

Before turning to the question of what causes only some spore bags to germinate for a given glucose concentration, we sought to uncover gene expressions that underlie the primed dormancy. We first primed the spores by incubating them with a low glucose concentration (0.002%) for either 16 hours, 1 day, 2 days, or 4 days. We then used zymolyase, as is the standard (Coluccio et al. 2004), to isolate the un-germinated spores from the surrounding vegetative cells (Supp.Fig.2.16) and then analyzed their transcriptomes with RNA-seq. As a control, we also analyzed the transcriptome of un-primed spores, which were incubated in minimal media without glucose for the same amounts of time as the primed spores. Following an insightful previous study (Joseph-Strauss et al. 2007) that analyzed the yeast spores' transcriptome over several hours after they received a 2%-glucose,

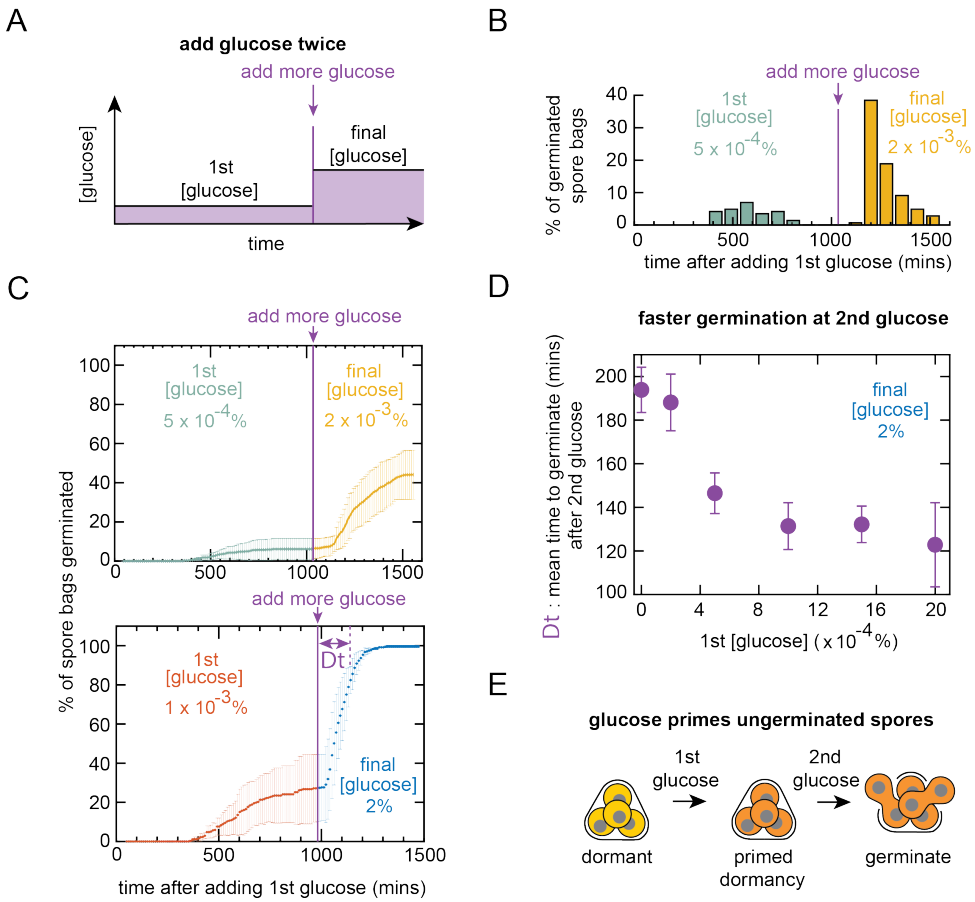


Figure 2.2: Un-germinated spores, primed by a low glucose concentration, germinate faster upon encountering more glucose a few days later. (A) Wild-type spores are first incubated in a low glucose concentration ("1st [glucose]") before we add more glucose later to increase the glucose concentration to "final [glucose]". **(B)** For experiment in (A), number of spore bags germinated within each binned time. First glucose concentration is 0.0005% (from 0 to 16 hours) (green bars) and the final concentration is 0.002% (from 16 to 32 hours) (orange bars). $n = 143$ spore bags (representative data). **(C)** For the experiment in (A), percentage of spore bags that germinated as a function of time since the first glucose addition (1st and final concentrations as indicated). More glucose added 1000 minutes after the first glucose (purple vertical line). $n = 3$; error bars are s.e.m. **(D)** For the experiment in (A), average time taken for a spore bag to germinate - denoted $\Delta\tau$ in bottom panel of (C) - after stepping up the glucose concentration. We varied the first glucose concentration, but the final concentration was always 2%.$n = 3$; error bars are s.e.m. **(E)** Spore bags that do not germinate after encountering the first glucose are "primed" to germinate faster upon encountering more glucose.

we grouped multiple genes together into a set, called a "transcriptional module" (Joseph-Strauss et al. 2007; Ihmels et al. 2002), if those genes participate in the same process (e.g., protein synthesis) (Appendix Table S1). For both the primed and un-primed spores, we averaged the expression levels of all genes in a given

module to obtain one expression value for that module. For six of nine transcriptional modules, the primed spores had higher expression values than the unprimed spores after 16 hours and 48 hours of incubations. But both types of spores had nearly the same expression value after four days of incubation (Fig. 2.3B - last six rows and Supp.Fig.2.17). This trend mirrors the trend followed by 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. 2.3A). Two transcriptional modules showed this trend in a particularly pronounced manner. One of them is the module for mating - haploid cells mate after germinating (Fig. 2G - seventh row). The other is the module for transitioning from cell cycle's G2-phase to mitosis - a crucial final step of germination (Fig. 2.3A - last row and Supp.Fig.2.17). Intuitively, these results make sense since one expects that getting ready for mitosis and mating would accelerate germinations. Together, these results establish that very low glucose concentrations can trigger transcriptome-wide changes in un-germinated spores to prime them, so that they can germinate faster when they encounter more glucose hours-to-days later.

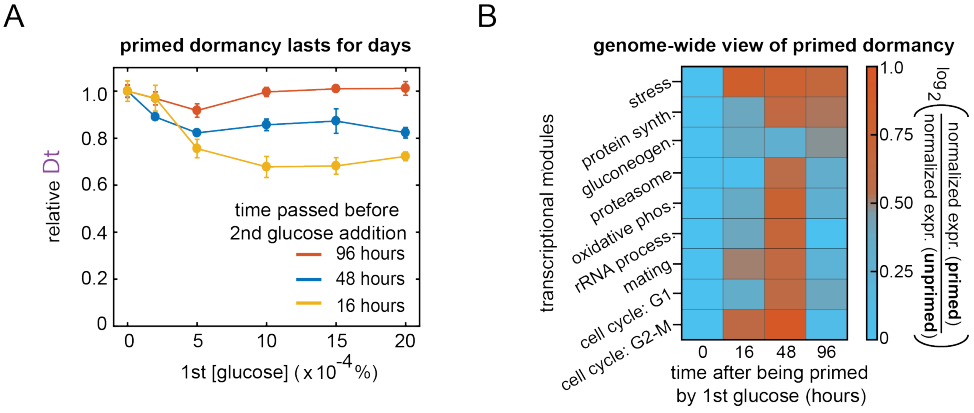


Figure 2.3: Genomic view of primed dormancy (A) For the experiment in (A), average time ($\Delta\tau$) taken for a spore bag to germinate after stepping up the glucose concentration (final glucose concentration is 2%), plotted as a function of the first, low glucose concentration. Different colors represent different times at which we added more glucose after the first addition: 16 hours (yellow), 48 hours (blue) and 96 hours (red). "Relative $\Delta\tau$" is the average time $\Delta\tau$ divided by the $\Delta\tau$ of spore bags that were incubated in minimal media without any glucose (0%) for the same duration of time (after which they received a 2%-glucose). $n = 3$; error bars are s.e.m. **(B)** Heat map showing transcriptome-wide changes in un-germinated spores at 0, 16, 48, and 96 hours after being primed by a 0.002%-glucose (via RNA-Seq). Appendix Table S1 lists the genes for each transcriptional module (each row in the heat map). Also see Appendix Fig S8. For each transcriptional module, we first divided the expression level of each gene in that module by its expression level at 0-hours - this yields "normalized expression level" for that gene for both primed and unprimed spores (unprimed spores were in minimal media without glucose for 0, 16, 48, and 96 hours). We averaged these values over all genes in a transcriptional module to obtain "normalized expression (primed)" and "normalized expression (unprimed)" for each transcriptional module. Colors represent ratio of these two values, averaged over three biological replicates ($n = 3$).

2.3.4. Hypothesis on why not every yeast spore germinates with low glucose concentrations

Although we now understand how un-germinated spores respond after receiving glucose, we have not addressed our original question: what determines, in the first place, which spore bags germinate? We hypothesized that each diploid, vegetative yeast forms a spore bag with a distinct "internal state". Many intracellular factors may define an internal state, including amounts of ATPs or ribosomes that are stored inside the spores or combinations of these and other molecules that are stored inside spores. We then hypothesized that, for each glucose concentration, only some of the spore bags have the "right" internal states that enable germination. A difficulty in testing this hypothesis is that in our experiments thus far, we gave glucose to spores and then observed their subsequent actions, including for discovering 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 since glucose likely changes the internal states. Therefore, we sought to manipulate the internal states without giving 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.

2.4. GFP expression predicts the ability to germinate

2.4.1. Synthetic circuit to induce GFP in dormant yeast spores without any nutrients

To test our hypothesis, we built a synthetic gene-circuit in vegetative diploid yeasts so that doxycycline - a small inducer molecule that easily diffuses through the yeast's cell wall - would cause the cells to produce the Green Fluorescent Protein (GFP). In vegetative yeasts, this synthetic circuit functions in such a way that increasing the doxycycline concentration increases the GFP expression. We formed spores out of these engineered diploid cells (Fig.2.4A). We reasoned that if doxycycline can induce GFP expression in these spores without any nutrients (i.e., in water), then we might deplete the stored resources inside the spores and thereby alter the percentage of spore bags that germinate for a given glucose concentration. But it was unclear whether it was even possible to induce GFP expression or any arbitrary gene in dormant yeast spores without nutrients. For one, if gene induction were possible, then it is unclear why, apparently, almost all gene expressions are suppressed in dormant yeast spores (Rittershaus et al. 2013) 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 vegetative 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 (mRNAs bound by proteins) that inhibit gene-expression machineries and movement of molecules inside the cytoplasm (Laporte et al. 2008; Petrovska et al. 2014). By aggregating, proteins needed for metabolism and gene expression would be inactivated as well (Ablett et al. 1999; Cowan et al. 2003; Dijksterhuis et al. 2007; Parry et al. 2014; Joyner et al. 2016; Munder et al. 2016). Indeed, to our knowledge, there has not yet been a direct, single-cell-level observation of gene expression occurring in yeast spores during dormancy. A previous, bulk-level (population-level) study (Brengues et al. 2002) has shown, from lysates of many yeast spores, that two genes - PGK1 (involved in gluconeogenesis) and SPS100 (involved in forming spore walls during spore formation) - are expressed but that they turn off a few days after sporulation (i.e., while the spores enter dormancy). But it remains unclear whether their expressions completely cease or remain at low, non-zero levels during dormancy. This is because these bulk-level measurements were based on finding ribosomes bound to mRNAs after lysing populations of yeast spores, meaning that the measurements cannot clearly distinguish between zero and very low expression levels.

Moreover, the ribosomes bound to mRNAs may be from the macroscopic aggregates that formed before the spores entered dormancy (Laporte et al. 2008; Petro-

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

Before testing the hypothesis posed in the previous section, we investigated whether GFP induction in dormant spores is even possible by incubating the engineered spores with doxycycline in either water or saline solution (PBS). Surprisingly, we discovered that doxycycline fully induced GFP expression in these spores (Fig.2.4B and Appendix Figs S9-S10). Varying the doxycycline concentration tuned the spores' GFP levels over the same, wide range of values as in vegetative cells that also have the synthetic circuit (Supp.Fig2.18-2.18). We found that both the rate of GFP production and the final (steady state) level of GFP widely varied among spore bags of the same population (Fig.2.4C and Supp.Fig2.21). The most striking feature was that all spore bags expressed GFP very slowly – GFP levels plateaued at steady-state values after $\sim$ 20 hours of doxycycline whereas the vegetative yeasts needed 8 hours of doxycycline for the GFP level to plateau (Supp.Fig.2.20). But a more puzzling discovery was that the spores' GFP levels stabilized at steady-state values in the first place. This is puzzling because the spores were not dividing and hence their GFP - a highly stable protein - could not be diluted away by cell divisions. In replicating (vegetative) yeasts, highly stable proteins such as GFP reach steady-state levels because their production rate matches the cell-division rate. After the spores' GFP levels reached steady-state values, we incubated the spores in PBS without doxycycline for two days during which their GFP levels remained virtually unchanged (Supp.Fig.2.22). This means that GFP levels reached steady-state values in dormant spores because the spores stopped producing GFP after a day, despite the saturating concentration of doxycycline ($100\mu g/ml$) still being present in the medium after a day, with barely detectable degradations (Supp.Fig.2.23). Together, these results provide a direct proof, with intact spores, that one can fully activate - to the same level as in vegetative yeasts - transcription and translation of an arbitrary gene such as GFP in dormant yeast spores without any nutrients. These results also establish that gene expression in dormant yeast spores can exhibit starkly different dynamics when compared to vegetative yeasts, such as vastly slower timescales and gene regulations.

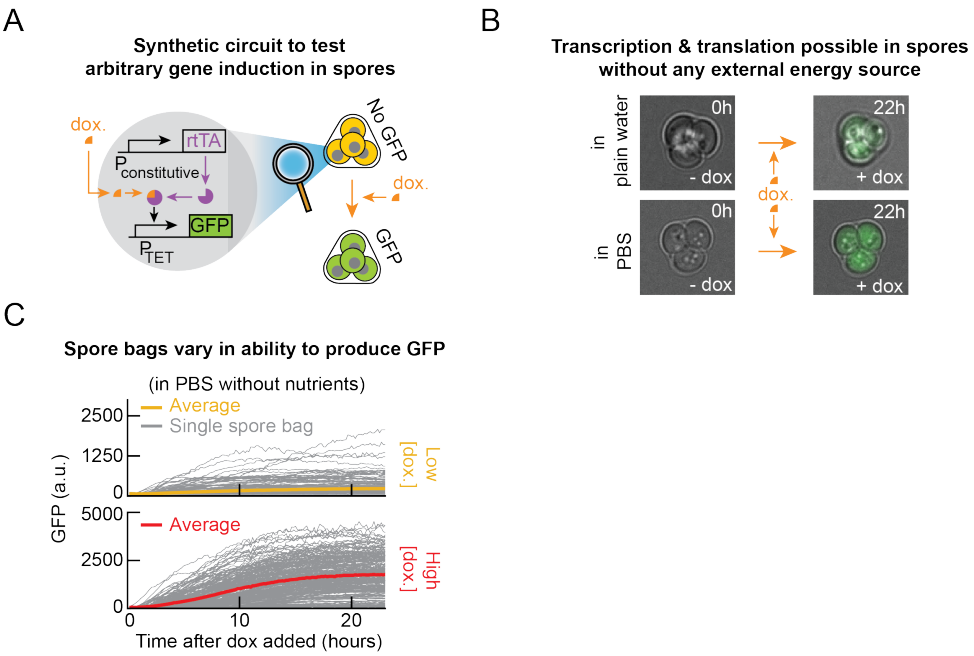


Figure 2.4: Inducing GFP expression during dormancy with synthetic circuit (A) Synthetic gene-circuit that constitutively expresses a transcription factor, rtTA (with ADH1-promoter) and an inducible promoter (TET-promoter) controlling GFP expression. Increasing doxycycline increases GFP expression. (B) Engineered spore bags (shown in (A)) transcribe and translate GFP in water (without any nutrients) with $25\mu g/ml$ of doxycycline (top row) and in a saline solution (PBS) with $50\mu g/ml$ of doxycycline (bottom row). Snapshots of GFP expression shown 22 hours after adding doxycycline. (C) GFP levels of individual spore bags (grey curves) over time (measured every 10 minutes with a wide-field epifluorescence microscope) after incubation in PBS with $10\mu g/ml$ of doxycycline (top panel: n = 104 spore bags) and $100\mu g/ml$ of doxycycline (bottom panel: n = 150 spore bags).

2.4.2. Inducing GFP expression in dormant yeast spores does not alter percentages of spore bags that germinate

Returning to our hypothesis that depleting yeast spores' internal resources through GFP induction hinders germination, we incubated spores in PBS with a saturating concentration of doxycycline ($100\mu g/ml$) for 24 hours, after which the GFP levels plateaued at steady-state values (Fig. 3C and Appendix Fig S13). We then washed away the doxycycline and then incubated the spores in a minimal medium with a fixed concentration of glucose. Then, with time-lapse microscopy, we tracked individual spore bags to determine how many spore bags germinated. For comparison, we used the same method to determine how many spore bags without GFP induction germinated - these spores were incubated for 24 hours in PBS without doxycycline and then in a minimal medium with the same concentration of glucose as the GFP-induced spores (Fig. 3D and Appendix Fig S15). For every glucose concentration that we examined, 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 by the spores to germinate (Appendix Fig S16). 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 explain in the next section, when we examined the GFP levels of individual spore bags rather than the average GFP level of the entire spore population as we have just done.

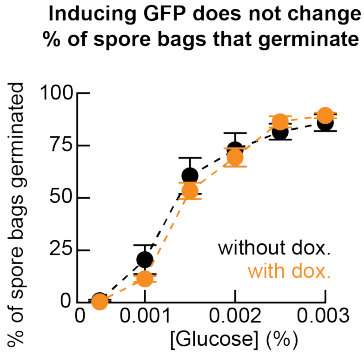


Figure 2.5: Inducing GFP does not change germination Engineered spore bags (shown in (A)) were first incubated for 22 hours in either PBS without any doxycycline or with $100\mu g/ml$ of doxycycline. Next, we transferred them to minimal medium with various glucose concentrations. Plot shows the total percentage of the engineered spore bags that germinated (measured 20 hours after incubating with glucose) for spores pre-incubated in PBS without doxycycline (black points) and in PBS with $100\mu g/ml$ of doxycycline (orange points). n = 3; error bars are s.e.m.

2.4.3. GFP level that a spore can realize (GFP inducibility) encodes the probability of germinating (germination ability) for each glucose concentration

In the previous experiment, we measured the steady-state GFP levels just before the spores encountered glucose. This led to the finding that spore bags that produced more GFP - spore bags with higher "GFP inducibility" - were more likely to germinate (Fig. 3E and Appendix Figs S17-S18). For example, after encountering a 0.001%-glucose, nearly 100% of the spore bags with the highest, steady-state GFP levels (highest GFP inducibility) germinated whereas, in the same population, only $\sim$ 10% of the spore bags with half the GFP inducibility germinated. In fact, for every glucose concentration, we could precisely determine the probability of germinating for a spore bag once we knew its GFP inducibility (Appendix Fig S18). We thereby established a quantitative relationship between the inducible GFP level and the ability to germinate. Importantly, this means that just because a spore bag can express any amount of GFP without nutrients does not mean that it will germinate after receiving a certain amount of glucose. There is no binary

relationship between the GFP expression and germination. The quantitative relationship between the GFP inducibility and the probability of germinating ("germination ability") for a given glucose concentration establishes that the stochastic variability (**Padovan2013**; **Balazsi2011**) in the induced GFP expression among dormant spores is meaningful and predictive, despite GFP not having any functional role in the complex, multi-step process that is germination.

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

To visualize our results, we plotted a "germination landscape" - a heat map whose color represents a probability that a spore bag with a given GFP inducibility germinates (i.e., germination ability) for each glucose concentration (Fig. 3F and Appendix Fig S19). In the germination landscape, yellow represents a near-certain germination (i.e., germination probability of nearly 1), green represents a germination probability of ~ 0.5, and dark blue represents a germination probability of nearly zero. The germination landscape shows a "coastline" of nearly-yellow pixels moving up towards higher rows (i.e., towards higher glucose concentrations) as one moves from right to left (i.e., as the GFP inducibility decreases), meaning that more glucose is required to guarantee a germination for a spore bag with a lesser GFP inducibility. The blue-green pixels are almost immediately below the coastline of yellow pixels, indicating that the probability of germinating, for a fixed value of GFP inducibility, is a sharp step-like function of the glucose concentration. We confirmed this by quantitatively extracting (by log-regression), from the germination landscape, the minimum glucose-concentration required for a spore with a given value of GFP inducibility to have a 99%-chance of germinating (Appendix Fig S20). We call this concentration, given the sharpness of the nearly step-like probability function, the "minimum glucose concentration required for germination". We determined that as a spore bag's GFP inducibility decreases, the minimum glucose concentration required for germination increases (Fig 3G and Appendix Fig S20). Importantly, since inducing GFP expression does not alter the total percentage of spore bags that germinate for any glucose concentration (Fig. 3D), inducing GFP expression in a spore bag does not change - neither increase nor decrease - the probability of germinating. Hence, GFP inducibility merely indicates which spore bags are more likely to germinate for a given glucose concentration but does not cause or hinder germination. Hence, for the same glucose concentration, spores with higher GFP inducibility have higher germination ability than spores with lesser GFP inducibility. Moreover, spores with a higher GFP inducibility require less glucose to guarantee that they germinate (i.e., have 100% chance of germinating) than spores with lesser GFP inducibility (Fig. 3G and Appendix Fig S20C).

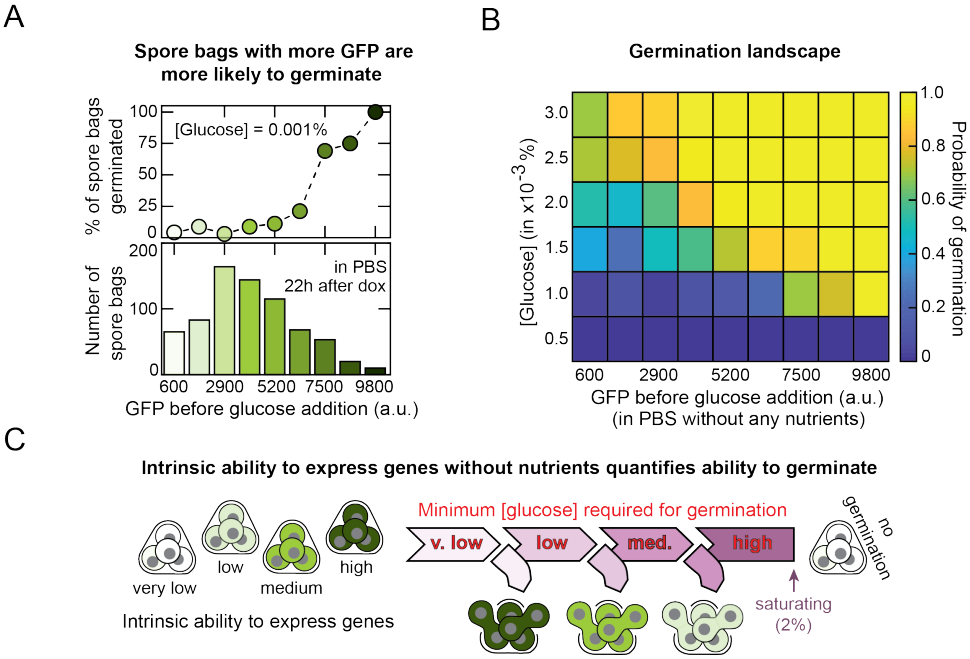


Figure 2.6: GFP expression predicts the ability to germinate (A) Top: Percentage of spore bags with the same GFP level ("GFP inducibility") from (B) that germinated after receiving a 0.001%-glucose. Percentages are averaged over all spore bags with the same binned GFP level (in the histogram below). Representative data shown (n = 145 spore bags in a population). (F) Germination landscape: colors represent the probability that a spore bag with a particular steady-state GFP level ("GFP inducibility") germinates for each glucose concentration (i.e., data in top panel of (E) represents a single row of this heat map). To measure each pixel, as in the experiment described in (D), we incubated spore bags in PBS with $100\mu g/ml$ of doxycycline for 22 hours before adding glucose ([glucose] as indicated along the rows). Columns indicate GFP inducibility of a spore bag, measured at 22 hours after adding the doxycycline. Each pixel is an average over 3 replicate populations (n = 3). (C) Given a spore bag, its GFP inducibility is a read-out of the probability to germinate ("germination ability") for each glucose concentration and the minimal glucose concentration required to guarantee germination. Spore bags with lesser gene-expressing abilities without nutrients require more glucose to germinate.

2.5. Ability to transcribe is key from spores to remain alive

2.5.1. Transcribing within a day during dormancy is crucial for spores to remain alive

GFP has no role in germination. So, it is puzzling why GFP inducibility so precisely predicts the germination ability for each glucose concentration. To make sense of this, we reasoned that the GFP inducibility represents the dormant spore's ability to express genes in general, not just GFP, when they are forced to do so without any nutrients (e.g., inducers such as doxycycline force an activation of GFP expression).

This is because there is nothing special about GFP. Notwithstanding the conventional view that gene expression has nearly ceased in dormant yeast spores, we then reasoned that GFP inducibility is a proxy for gene expressions that are actually occurring in dormant spores and which are required for germination. According to this reasoning, inhibiting global (genome-wide) transcription and translation during dormancy would decrease the percentages of spore bags that germinate (i.e., decrease germination ability) for each glucose concentration. To test this idea, we incubated spores in PBS for one day with one of three drugs - thiolutin, cycloheximide, and antimycin A (Fig. 4A). Each drug inhibits some key part of gene expression. Thiolutin globally inhibits transcription (Grigull et al. 2004; Parker et al. 1991; Guan et al. 2006; Lauinger et al. 2017; Pelechano et al. 2008). Cycloheximide globally inhibits translation (Belle et al. 2006; Buchanan et al. 2016). Antimycin A inhibits oxidative phosphorylation and, in turn, synthesis of ATP - a molecule that is required for expressing GFP and other genes (Ocampo et al. 2012). After the day-long drug treatment, we washed away the drug and then added a 2%-glucose with minimal medium to check how many spores could still germinate (i.e., were still alive) (Fig.2.7A).

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

We verified that all three drugs indeed inhibited GFP expression in yeast spores (Appendix Fig. S21). Nearly 100% of the spores were still alive after we inhibited either ATP synthesis with antimycin A or global translation with cycloheximide for 24 hours (Fig.2.7B). In contrast, inhibiting global transcription with thiolutin for 24 hours killed almost all spores: less than 5% of the spore bags were still able to germinate with a 2%-glucose after the thiolutin treatment (Fig.2.7B). These results establish that transcription - and potentially RNA metabolism in general - is the major process that keeps dormant spores alive.

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

To further explore how transcribing during dormancy is connected to germination ability - and thus to survival - for a given glucose concentration, we first devel-

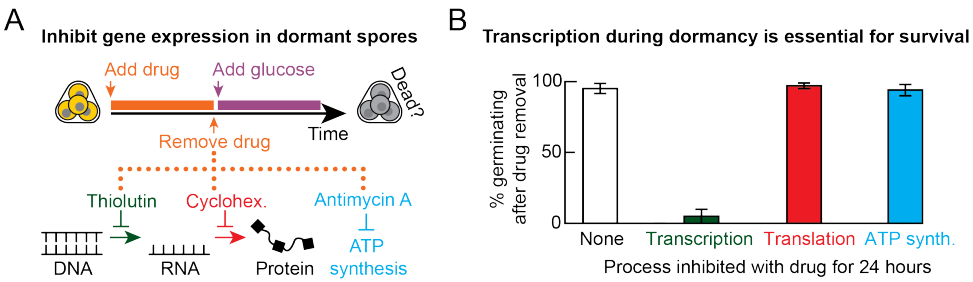


Figure 2.7: Transcription is necessary for short survival during dormancy (A) Protocol for (B). See "Protocol for Fig. 4B" in Methods. Thiolutin inhibits transcription. Cycloheximide inhibits translation. Antimycin A inhibits ATP synthesis by inhibiting oxidative phosphorylation. (B) For experiment in (A), percentage of spore bags that germinated. n=3; error bar are s.e.m.

oped a method to measure all the RNA that is actively being made in dormant yeast spores with a single-cell resolution. We modified an existing method for mammalian cells in which the cells uptake 5-Ethynyl Uridine (5-EU) and incorporates it into RNA that is being synthesized, after which the cells are fixed and fluorophores (e.g., Alexa 488) enter the fixed cells to bind the 5-EU-labeled RNAs (Jao et al. 2008) (Fig.2.8A). Since 5-EU would only be found in RNAs that are synthesized while the extracellular 5-EU was present, this method lets us determine the total amount of RNAs that are made during a specified time window (i.e., only while the 5-EU was present). The method therefore distinguishes these freshly made RNAs from the RNAs that were present before we added the 5-EU to the cell culture medium. By adapting this method for yeast, we could visually verify, by fluorescence, that yeast spores actively transcribe during dormancy and determine the amounts of RNA produced by the dormant spores, relative to one another, during a 24-hour incubation in 5-EU (Fig.2.8B). To our knowledge, this is one of the first demonstrations of active transcription in dormant yeast spores with a single-cell resolution, as previous studies used either indirect or bulk assays that required lyses of many spores (Brengues et al. 2002). We used several methods to verify that our method worked in yeast spores (Appendix Figs S22-S23).

To apply the adapted method to the GFP-inducible spores, we incubated the spores in PBS with both doxycycline and 5-EU for 24 hours. Hence, the total RNA level accumulated in a day is a proxy for the total transcription rate of a dormant spore. We found a statistically significant but weak, positive correlation ($R = 0.24$) between the spores' total transcription rate (i.e., accumulated RNA level) and their GFP inducibility (Fig.2.8C). This, in turn, means that the total transcription rate poorly correlates with the spore's chance of germinating. Thus, the total transcription rate cannot explain why the GFP inducibility so precisely determines the germination ability for each glucose concentration. Yet, the fact that a 24-hour

exposure to thiolutin kills nearly all spores (Fig.2.7B) suggests that some aspect of transcription during dormancy - other than the total amount of transcription - should strongly correlate with the GFP inducibility and, in turn, with the germination ability for each glucose concentration. Since RNA polymerases I, II, and III are required and central machineries for transcription, we next sought to address whether the amount of each RNA polymerase in a dormant spore strongly correlates with its GFP inducibility.

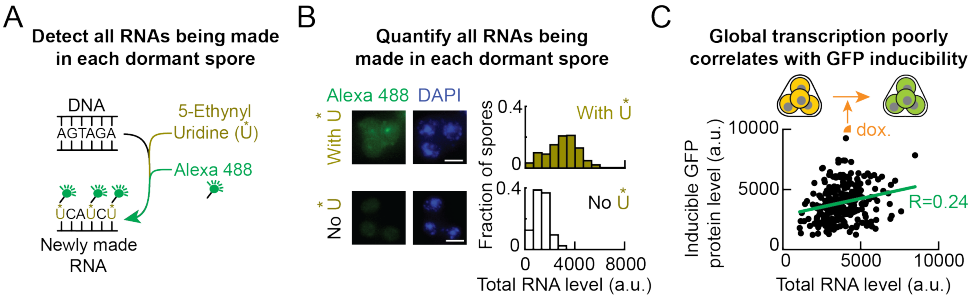


Figure 2.8: (A) Method to detect all RNAs being made in yeast spores with a single-cell resolution. Spores were incubated for 24 hours in PBS with 1 mM of 5-Ethynyl Uridine (5-EU; denoted U*) that incorporates into freshly made RNAs. We fix the spores afterwards and let fluorophore (Alexa 488) enter the spores and bind U* (see Methods). "Total RNA level" of a spore bag is the total fluorescence from all the 5-EU labeled RNAs. (B) Microscope images show a spore bag (from strain "TS3") after either incorporating 5-EU as described in (A) (top two images) or, as a control, following the protocol in (A) but without the 5-EU (bottom two images) (also see Appendix Fig S23A). Scale bar: $2\mu m$. Top histogram: total RNA level per spore bag with 5-EU (i.e., Alexa 488 fluorescence; n = 103 spore bags). Bottom histogram: fluorescence per spore bag in control population (i.e., without 5-EU; n = 95 spore bags). Also see Appendix Fig. S22A. (C) Each dot is from a single spore bag ("TS3" strain) with GFP-inducing circuit (Fig. 3A). For each spore bag, we measured its GFP protein level ("GFP inducibility") and total RNA level (5-EU fluorescence) after incubating the spores for 24 hours in PBS with both $100\mu g/ml$ doxycycline and 1 mM of 5-EU (see (C)). n = 245 spore bags. Alexa 594 fluorophore attached to 5-EU (see Methods). Green line: linear regression with R=0.24 and Pearson p-value = 0.00018.

2.5.3. Amount of RNA polymerases I-III are strong predictors of GFP inducibility and germination ability

To measure the amounts of RNA polymerase (RNAP) I, II, and III in individual spores through live time-lapse microscopy, we genetically engineered two yeast strains. One strain produced Rpb3 - a subunit of RNAP II - as a protein fused to the fluorescent protein, mCherry (Fig. 2.9A). The other strain produced Rpc40 - a subunit of both RNAP I and RNAP III - as a protein fused to mCherry (Fig. 2.9B). Both strains also have the GFP-inducing synthetic circuit (Fig. 3A). For these strains, the mCherry fluorescence was entirely confined to the nuclei of both spores and vegetative yeasts (Appendix Fig S24). Hence, the mCherry levels accurately reflected the amounts of each RNAPs in spores. After sporulating these two strains, we incubated the resulting spores in PBS with doxycycline for 24 hours to induce their

GFP. We then used a microscope to measure both the GFP and mCherry (RNAP) levels in individual spore bags. In one of the strains, mCherry level represents the level of RNAP II, which transcribes all the coding genes and also produces some non-coding RNAs. In the other strain, mCherry level represents the amount of RNAP I and RNAP III combined, both of which produce only non-coding RNAs such as ribosomal RNAs. Strikingly, the amount of RNAP II strongly (R=0.64) and positively correlated with the GFP inducibility (Fig. 2.9A). The combined amount of RNAPs I and III also strongly (R=0.63) and positively correlated with GFP inducibility (Fig. 2.9B). Hence the amounts of both classes of RNA polymerases - one for transcribing mostly coding genes (RNAP II) and one for transcribing non-coding genes (RNAPs I and III) - are strong predictors of GFP inducibility. We also used single-molecule RNA FISH (Youk et al, 2010; Raj et al, 2008) to quantify the amount of 18S ribosomal RNA (18S rRNA) - a ribosomal subunit - in dormant spores that we fixed after the 24 hours of doxycycline. We then determined how the amount of 18S rRNA in spores correlated with the GFP inducibility (Fig. 2.9C). We found that the amount of 18S rRNA in spores barely correlated with their GFP inducibility (R=0.19). Since the amount of 18S rRNA is a proxy for the amount of ribosomes, this result suggests that the amounts of ribosomes in a dormant yeast spore is virtually uncorrelated - and at most weakly correlated - with GFP inducibility and thus with germination ability. This is consistent with transcription, rather than translation, being the dominant process for surviving dormancy (Fig.2.7B).

Since GFP inducibility strongly and positively correlates with the amounts of all three RNAPs and germination ability strongly and positively correlates with GFP inducibility, we would expect that the amount of RNAPs in a dormant spore should also strongly and positively correlate with the spore's germination ability as well. To verify this for RNAP II - we will focus on RNAP II from now on instead of RNAPs I and III because RNAP II transcribes all coding genes such as GFP as well as some non-coding genes - we took the spores with Rpb3 fused to mCherry and then incubated them in a low concentration of glucose (0.0015%). Then, using the same method that we used for measuring the germination landscape (Fig. 2.3A), we determined how likely it is that a spore bag with a given amount of RNAP II germinates after receiving the 0.0015%-glucose. We found that spore bags with more RNAP II indeed were more likely to germinate (Fig. 2.9D).

Taken together, the results so far establish a triangular relationship in which a strong, positive correlation exists between any two of the following three quantities in dormant yeast spores (Fig. 2.9E): GFP inducibility without nutrients, amount of RNAPs (RNAP II level or level of RNAPs I and III combined), and the germination ability for each glucose concentration.

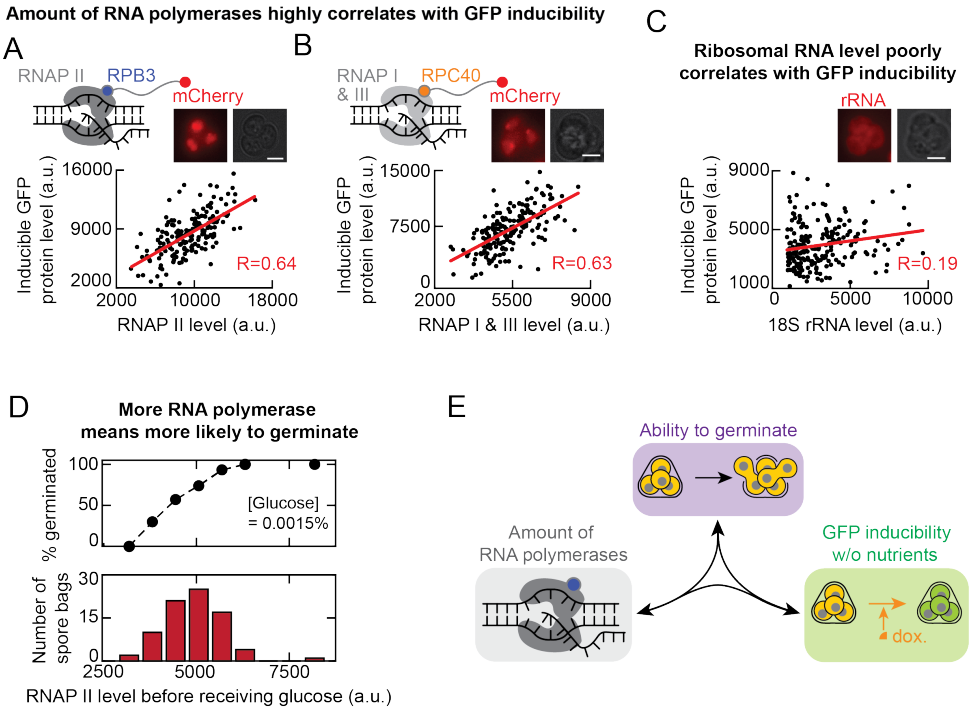


Figure 2.9: RNA-polymerase amount predict the ability to express genes (A) See "Protocol for Fig. 4F" in Methods. GFP inducibility per spore bag (each dot, "TS8" strain) measured as in (Fig.2.8C) but now with live time-lapse without the 5-EU. "RNAP II level" is the mCherry fluorescence per spore bag due to the mCherry protein fused to Rpb3, a subunit of RNA polymerase II. $n = 182$ spore bags; Red line: linear regression with $R = 0.64$ and Pearson p-value= $3.02x10^{-22}$. Scale bar = $2\mu m$. (B) Same protocol as in (A) but with "TS9" spores. "RNAP I and III level" is the mCherry fluorescence per spore bag due to the mCherry protein fused to Rpc40, a subunit of both RNAP I and RNAP III. $n = 185$ spore bags; Red line: linear regression with R=0.63 and Pearson p-value = $6.6x10^{-22}$. Scale bar = $2\mu m$. (C) Same protocol as in (A) but with "TT14" spores fixed after 24 hours of incubation in PBS with $100\mu g/ml$ doxycycline. 18S rRNA level is from CAL Fluor Red 610 fluorescence emitted by single-molecule FISH probes bound to 18S rRNAs (see Methods). $n = 213$ spore bags; Red line: linear regression with $R = 0.19$ and Pearson p-value = 0.005. Scale bar = $2\mu m$. (D) Bottom: RNAP II levels of spore bags ("TS8" strain) in a population, measured as in (A). Top: As a function of the RNAP II level (binned in the histogram), percentage of spore bags that germinated after receiving a 0.0015%-glucose, averaged over all spore bags with the same binned RNAP II level. $n = 80$ spore bags. (E) Triangular relationship. Any pair of the following three are positively correlated: Germination ability for each glucose concentration (purple), GFP inducibility (green), and amounts of RNA polymerases I-III.

2.6. Conclusion

Our study began by posing one broad question : how an organism whose life has almost ceased - including dormant organisms - remain alive, given that it has faint signatures of intracellular dynamics that are difficult to discern. We addressed key aspects of this question in the case of dormant yeast spores while also raising new

questions that invite future investigations. A conceptual difficulty in addressing these questions lies in the phrase, "almost ceased". Prior to our work, how slowly intracellular processes such as gene expression occur in dormant yeast spores has been unclear as firm numbers were missing. With single-cell level measurements, we identified key quantities that are relevant for studying the ability to survive during dormancy : probability of germinating (which we called "germination ability"), the ability to express genes during dormancy when forced to so (proxied by the "GFP inducibility") and the amount of RNAP polymerases inside the spores (Figs. 1-4).

We rigorously quantified these quantities and showed that there was strong positive correlation between them. Concretely, spores with a either GFP inducibility or a higher amount of RNAP polymerases had a higher probability of germinating. Beyond correlations, we also found that basal level of transcription but not translation was necessary for spores to maintain their germination ability. While in this study we did not investigate the molecular mechanisms underlying these dramatic loss of viability upon inhibition of transcription, our result together point towards a possible candidate. Indeed, one class of molecules, which we did not directly identify and measure here, are key transcripts that we argue are likely important for maintaining gene-expressing ability in yeast spores. While RNA polymerases are, as expected, required and important for maintaining gene-expression ability during dormancy, we have shown that ample RNA polymerases and ribosomes themselves are insufficient for yeast spores to maintain gene-expressing ability during dormancy. In fact, we found that after several hours of transcription inhibition, yeast spores with ample RNAP II and ribosomes cannot express coding genes both during dormancy and after receiving a saturating concentration of glucose - these spores died. By comparing the timescale of protein degradations in spores with the timescale of deaths that occur during the transcription inhibitions, we argue that certain transcripts - perhaps non-coding transcripts - that degrade on a timescale of at most hours are required for maintaining gene-expression ability and, in turn, for keeping spores alive. We expect that future studies will investigate this interpretation. Such future studies may benefit from the method that we have, for the first time, adapted for yeast spores here: 5-Ethynyl Uridine (5-EU) labeling of new RNAs being made during dormancy.

On a related topic, our work motivates an avenue of research that is underexplored: understanding the central dogma of molecular biology in eukaryotic spores by using dormant yeast spore as a model system. With a synthetic gene-circuit, we showed that inducing transcription and translation of a generic gene is possible in dormant yeast spores without any external energy source. We provided, to our knowledge, one of the first evidences of active gene expression inside intact, dormant yeast spores at a single-cell resolution. This approach - building synthetic

circuits in dormant yeast spores - may enable us to reveal fundamental principles of eukaryotic transcription and translation in dormancy. As a starting point, our work raises a fundamental question: how does a dormant yeast spore globally repress gene expression given that its total transcription rate appears to be nearly uncorrelated with the amount of RNA polymerases that it has? We believe that a good starting point for addressing this question is our finding that GFP expression stops after a day because the production - either transcription or translation - stops despite the inducer (doxycycline) still being present. Moreover, we may gain additional insights into how the central dogma of molecular biology functions in dormant eukaryotic spores by further investigating the glucose-triggered gene expressions in the primed, dormant yeast spores.

We hope that our study motivates future studies to find and probe system-level quantities that concretely link the ability to "wake-up" with the internal state/ activity during dormancy. Such studies will not only deepen our global understanding of dormancy, but also contributes find news ways artificially stop and resume cellular life on demand.

2.7. Supplementary materials

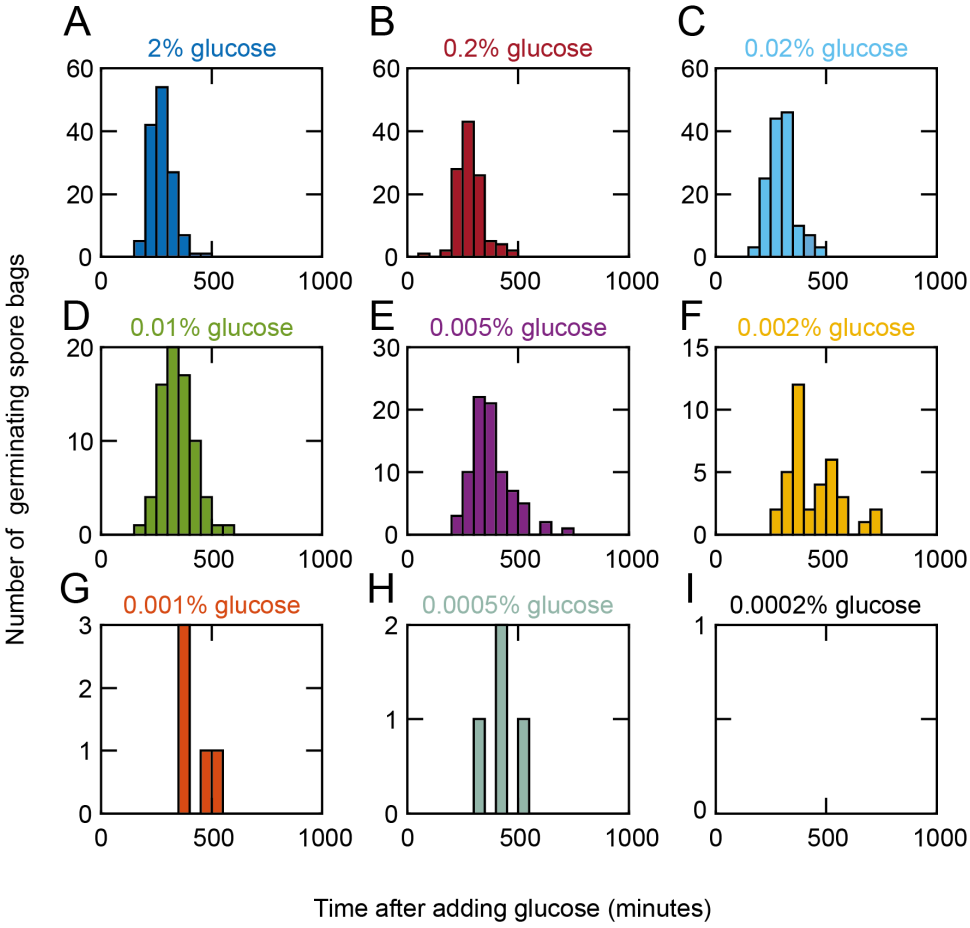


Figure 2.10: Supp.Fig - Representative histograms showing how many wild-type spore bags typically germinated for each glucose concentration (complements Figs. 2.1B-C). We incubated the spores in minimal media (which contains all essential amino acids and nitrogenous bases) that we supplemented with a 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).

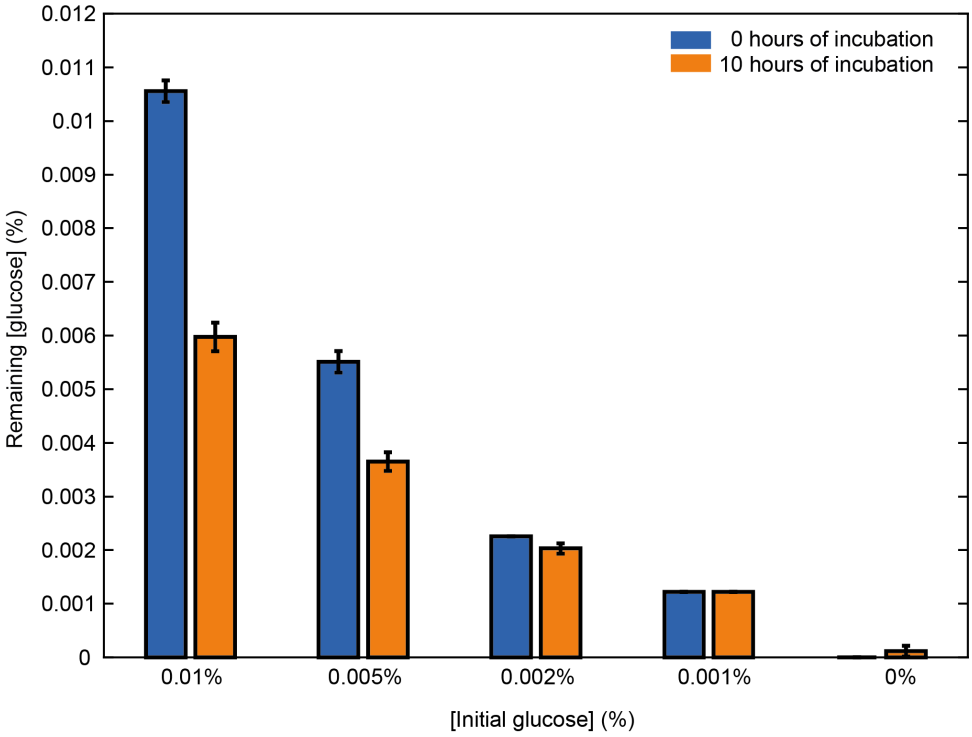


Figure 2.11: Appendix Figure S2 - Running out of glucose is not the reason for only a fraction of spore bags germinating for non-saturating glucose-concentrations (complements Figs. 1C-D). Before incubating the wild-type spores in a minimal medium with glucose (experiment shown in Fig. 1C), we measured the initial glucose-concentration in the medium (blue bars - "0 hours of incubation") with a hexokinase-based assay (see 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.

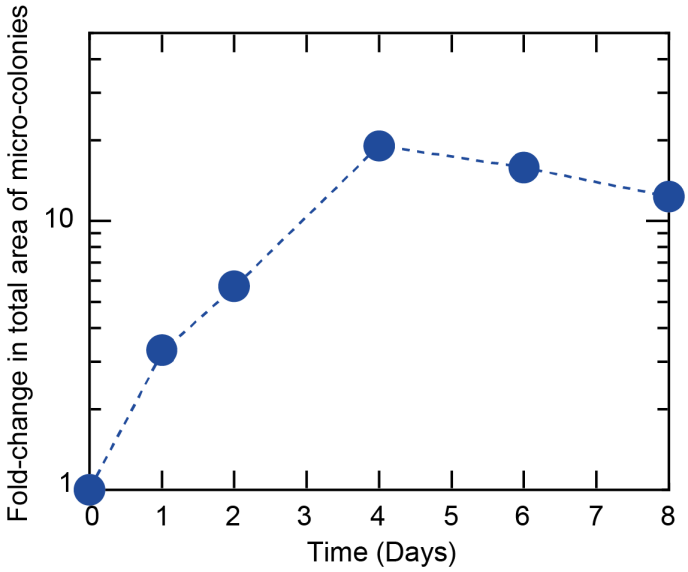


Figure 2.12: Appendix Figure S3 - A vegetative yeast cell can divide multiple times even with the lowest glucose-concentration (0.0002%) that we used, which could not germinate any spores (complements Figs. 1C-D). We sought to test if the lowest concentration of glucose that we used in Figs. 1C-D, which 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.

[!h]

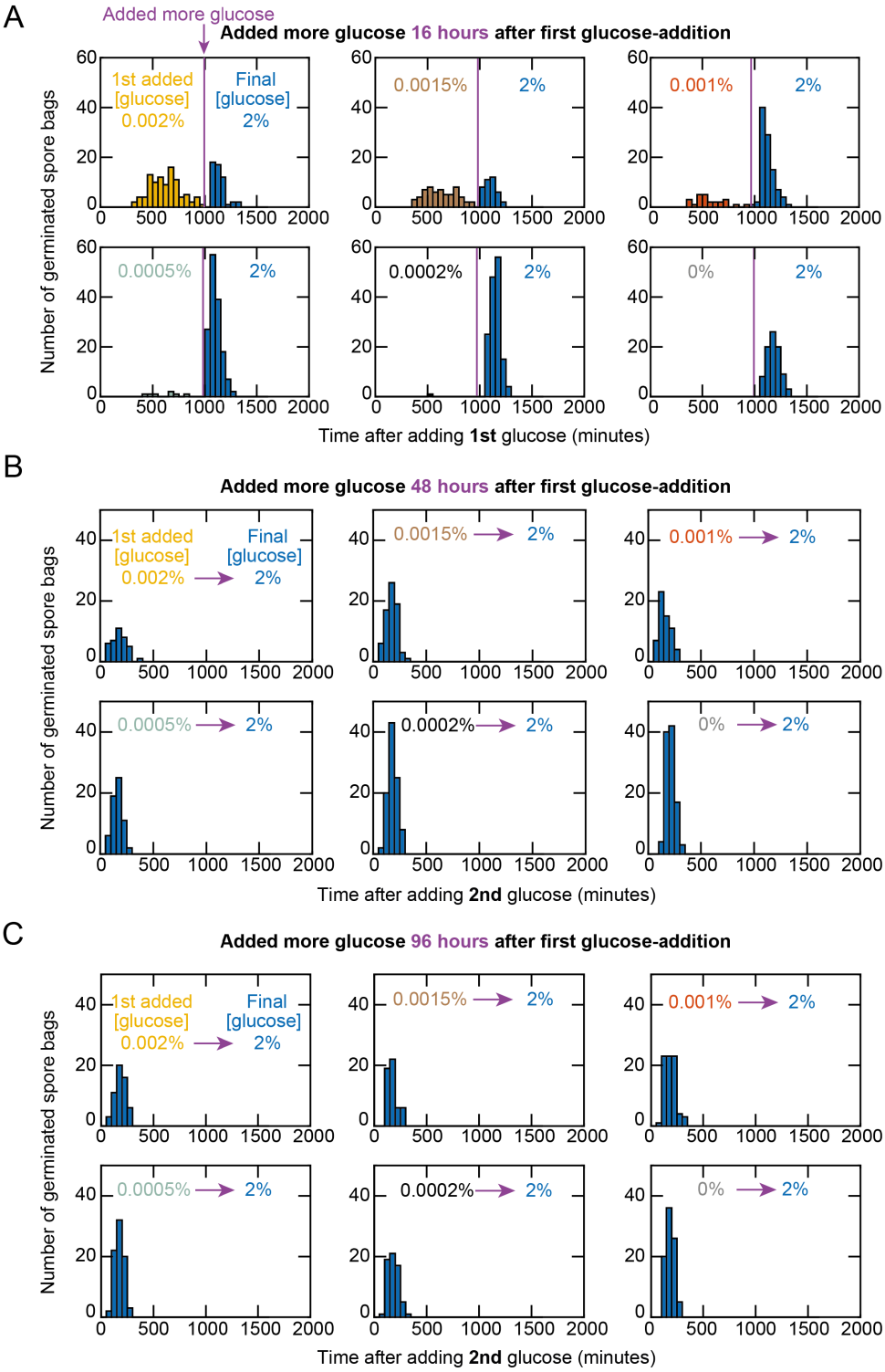

A
Added more glucose
Added more glucose 16 hours after first glucose-addition
1st added [glucose] 0.002%
Final [glucose] 2%
0.0015% 2%
0.001% 2%
0.0005% 2%
0.0002% 2%
0% 2%
Number of germinated spore bags
Time after adding 1st glucose (minutes)
B
Added more glucose 48 hours after first glucose-addition
1st added [glucose] 0.002% → 2%
Final [glucose] 2%
0.0015% → 2%
0.001% → 2%
0.0005% → 2%
0.0002% → 2%
0% → 2%
Number of germinated spore bags
Time after adding 2nd glucose (minutes)
C
Added more glucose 96 hours after first glucose-addition
1st added [glucose] 0.002% → 2%
Final [glucose] 2%
0.0015% → 2%
0.001% → 2%
0.0005% → 2%
0.0002% → 2%
0% → 2%
Number of germinated spore bags
Time after adding 2nd glucose (minutes)

Figure 2.13 ***(preceding page)*****:** Appendix Figure S4 - 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).

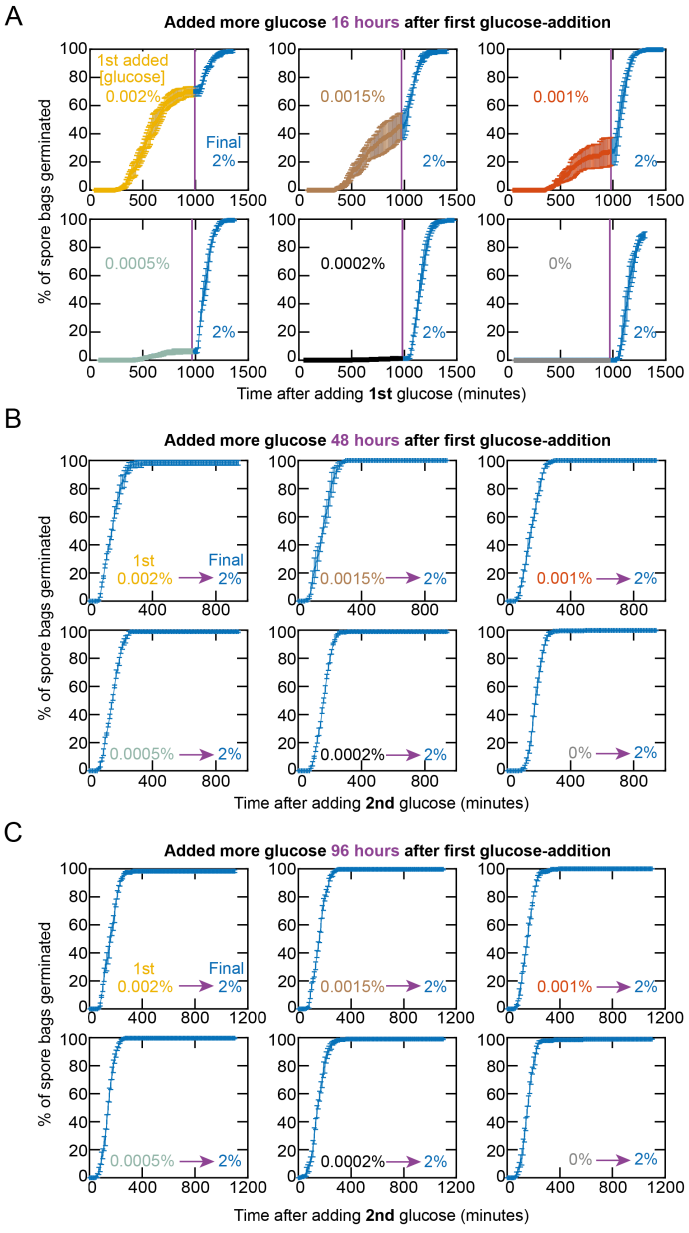

A
Added more glucose 16 hours after first glucose-addition
1st added [glucose] 0.002%
Final 2%
0.0015%
0.001%
0.0005%
0.0002%
0%
% of spore bags germinated
Time after adding 1st glucose (minutes)
B
Added more glucose 48 hours after first glucose-addition
1st 0.002%
Final 2%
0.0015%
0.001%
0.0005%
0.0002%
0%
% of spore bags germinated
Time after adding 2nd glucose (minutes)
C
Added more glucose 96 hours after first glucose-addition
1st 0.002%
Final 2%
0.0015%
0.001%
0.0005%
0.0002%
0%
% of spore bags germinated
Time after adding 2nd glucose (minutes)

Figure 2.14 ***(preceding page)*****:** Appendix Figure S5 - 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 Appendix Fig. S4 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 Appendix Fig. S4A 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 Appendix 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 Appendix 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.

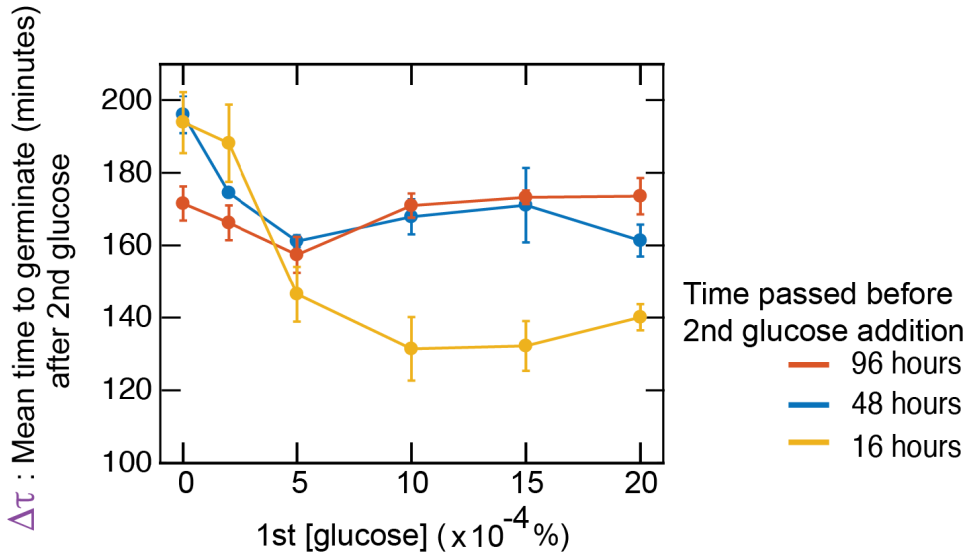


Figure 2.15: Appendix Figure S6 - Average time taken by wild-type spore-bags to germinate, as a function of the first, low glucose-concentration that primes the spores, in experiments in which we add glucose in two steps (shown in Fig. 2A) (complements Fig. 2F). We gave glucose in two steps to wild-type 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 (τ) 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 (τ) is now in minutes whereas Fig. 2F shows, for each color, the τ 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.

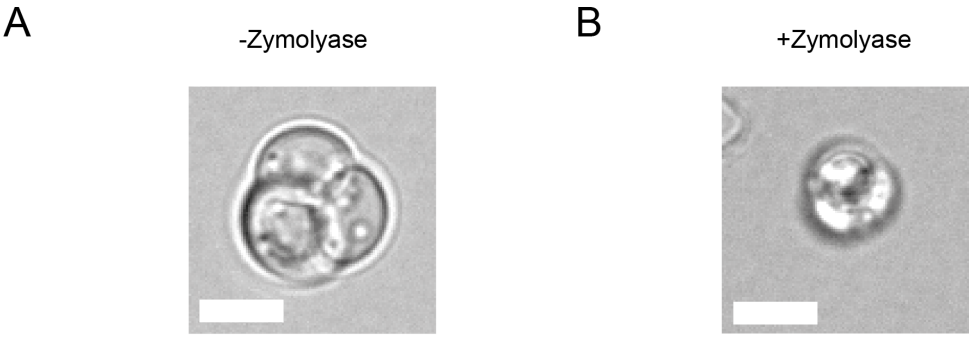
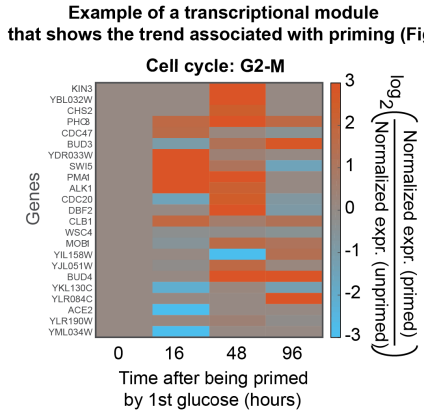
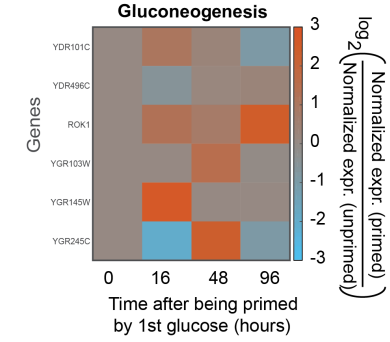


Figure 2.16: Appendix Figure S7 - Zymolyase leaves un-germinated spore-bags intact while lysing vegetative yeasts that result from germinated spores (complements Fig. 2G). In a mixture of spore-bags and vegetative yeasts, one typically isolates the spore bags by using zymolyase, which lyses vegetative 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, 5μm). (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, 5μm). 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 Appendix Fig. S8).

A


Example of a transcriptional module
that shows the trend associated with priming (Fig. 2F)
Cell cycle: G2-M
Genes
KIN3
YBL032W
CHS2
PHO8
CDC47
BUD3
YDR033W
SWI5
PMA1
ALK1
CDC20
DBF2
CLB1
WSC4
MOB1
YIL158W
YJL051W
BUD4
YKL130C
YLR084C
ACE2
YLR190W
YML034W
0 16 48 96
Time after being primed
by 1st glucose (hours)
3 2 1 0 -1 -2 -3
log2(Normalized expr. (primed) / Normalized expr. (unprimed))

B


Example of a transcriptional module
that shows no trend
Gluconeogenesis
Genes
YDR101C
YDR496C
ROK1
YGR103W
YGR145W
YGR245C
0 16 48 96
Time after being primed
by 1st glucose (hours)
3 2 1 0 -1 -2 -3
log2(Normalized expr. (primed) / Normalized expr. (unprimed))

C

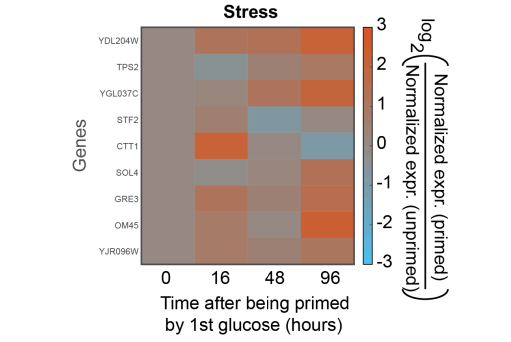

Example of a transcriptional module
that shows a trend other than one associated with priming
Stress
Genes
YDL204W
TPS2
YGL037C
STF2
CTT1
SOL4
GRE3
OM45
YJR096W
0 16 48 96
Time after being primed
by 1st glucose (hours)
3 2 1 0 -1 -2 -3
log2(Normalized expr. (primed) / Normalized expr. (unprimed))

Figure 2.17 ***(preceding page)*****:** Appendix Figure S8 - 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 Appendix Table S1). 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 Appendix Table S1). 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 Appendix Table S1). 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 Appendix Table S1). 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).

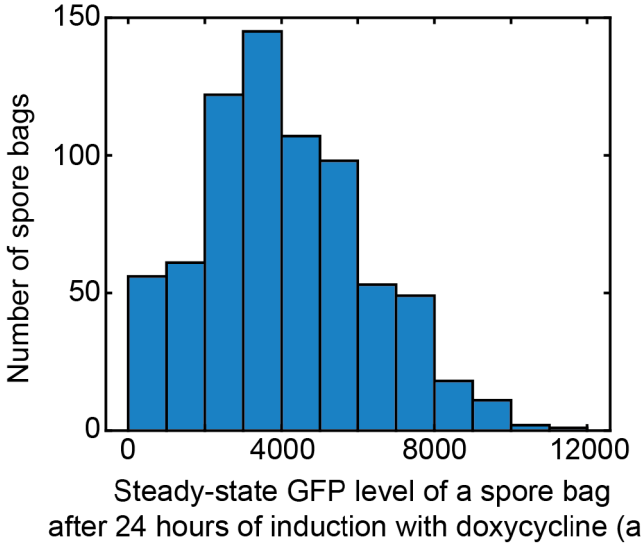


Figure 2.18: Appendix Figure S9 - GFP expression by dormant spores without any nutrients (in PBS) (complements Fig. 3C). We incubated a population of GFP-inducible spores (Fig. 3A) in a saline solution (PBS) with $100\mu g/ml$ of doxycycline for 24 hours. By the end of the 24-hour incubation, spore bags' 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).

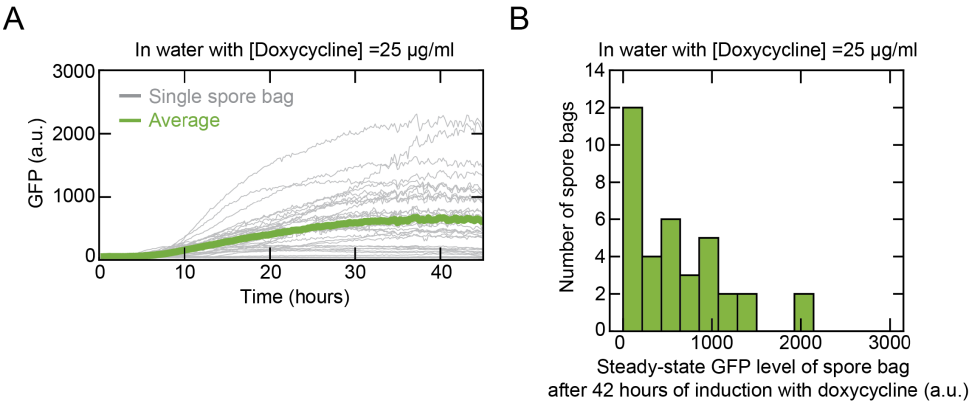


Figure 2.19: Appendix Figure S10 - Induction of GFP expression is possible for spores that are in water without any nutrients (no amino acids and no glucose) (complements Fig. 3B). (A) GFP level of individual spore bags (grey curves) over time during 42 hours of incubation in Milli-Q water (ddH2O) with 25$\mu g/ml$ 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 Appendix Fig. S12C). (B) Steady-state GFP-levels of individual spore bags after 42 hours of incubation in Milli-Q water with 25$\mu g/ml$ 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 Appendix Fig. S11C). Interestingly, while spore bags in PBS reach half of their saturating GFP levels after 10 hours of induction (see Fig. 3C or Appendix Fig. S12), we see here that the spore bags in water take $\sim$ 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 $\sim$ 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.

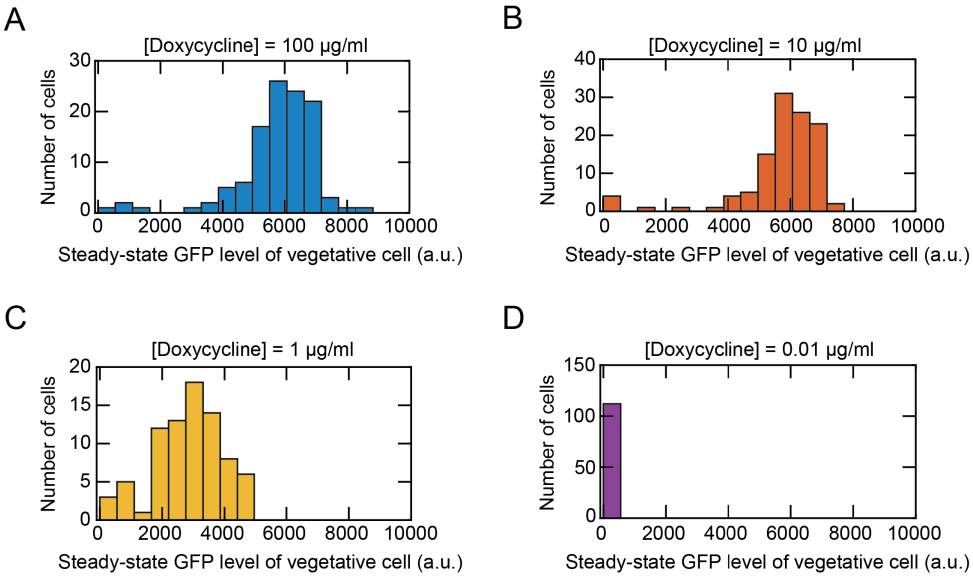


Figure 2.20: Appendix Figure S11 - Steady-state GFP levels of vegetative (replicating) cells that have the same synthetic gene-circuit as the GFP-inducible spores (Fig. 3A) (for comparison with the steady-state GFP levels of spore bags) (complements Fig. 3C). We sought to compare how the GFP levels of 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) $100\mu g/ml$ of doxycycline (n = 112 cells), or (B) $10\mu g/ml$ of doxycycline (n = 113 cells), or (C) $1\mu g/ml$ of doxycycline (n = 80 cells), or (D) $0.01\mu g/ml$ of doxycycline (n = 108 cells). Data obtained by using wide-field epifluorescence microscopy as in Appendix Fig. S9. By comparing these GFP levels of diploid, replicating cells with the GFP levels of spores (Appendix Fig. S9), 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 ($\sim$ 24 hours) to reach steady-state GFP levels where as the vegetative cells require only $\sim$ 8 hours to reach steady-state GFP levels and that (2) spores need more doxycycline ($100\mu g/ml$) than the vegetative cells (1 μg/ml) to reach similar GFP levels.

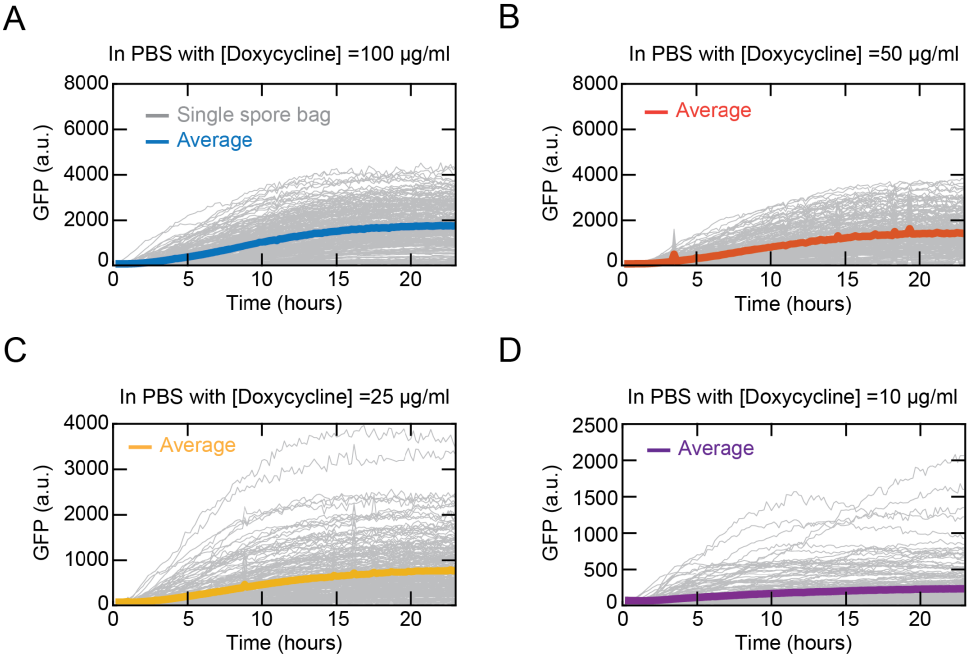


Figure 2.21: Appendix Figure S12 - GFP expression without nutrients as function of time for different doxycycline concentrations (complements Fig. 3C). GFP level of a single spore bag (grey curve) and population-level average (solid colored curves) over time while incubated in PBS (i.e., without any 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) [Doxycycline]=100$\mu g/ml$,n = 233 spore bags; (B) [Doxycycline] = 50$\mu g/ml$, n = 194 spore bags; (C) [Doxycycline] = 25$\mu g/ml$, n = 217 spore bags; (D) [Doxycycline] = 10$\mu g/ml$, 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 Appendix Fig. S9, 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 (Appendix Fig. S16) rather than in a stationary liquid medium inside a microscope well for 22 hours as shown here, due to the difference in culturing conditions.

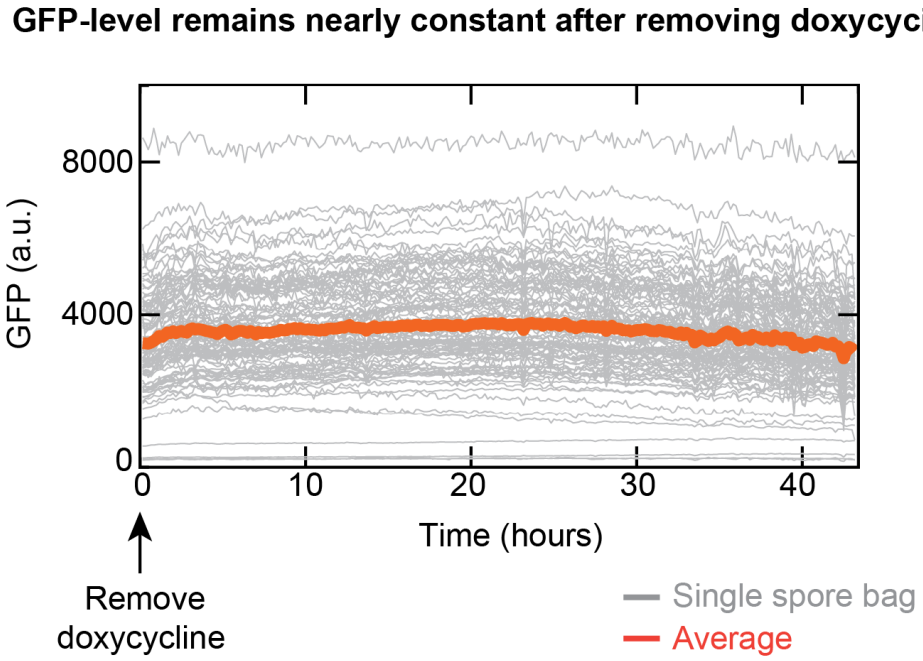


Figure 2.22: Appendix Figure S13 - GFP level of a spore bag remains nearly constant after removing doxycycline (complements Fig. 3C). GFP-levels of individual spore bags (grey curves) during 42 hours in PBS without doxycycline (n = 101 spore bags). The red curve shows the average GFP-level for these spore bags over time. We first induced GFP expression in these spores for 24 hours with $100\mu g/ml$ of doxycycline. At the end of the 24-hour incubation, the GFP levels reached their steady-state values (Fig. 3C and Appendix Fig. S12). Then, we removed the doxycycline and washed away any residual doxycycline with PBS several times. We then incubated these spores in PBS at 30 OC (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 doxycycline. 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.

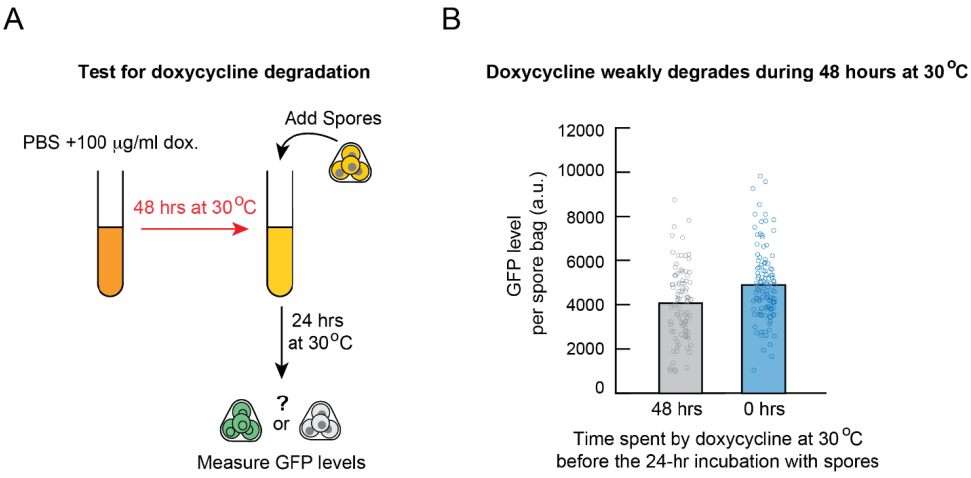


Figure 2.23: Appendix Figure S14 - Doxycycline barely degrades during two days at 30C (complements Fig. 3C). When we incubated spores in PBS with doxycycline, we always observed the GFP levels of individual spore bags plateauing after 15-to-20 hours (e.g., Appendix Fig S12). To test whether this is was due to large amounts of doxycycline degrading during after 15-to-20 hours, we first incubated PBS with 100$\mu g/ml$ of doxycycline for 48 hours in a rotating tube at 30 OC. Then, we incubated spores in this medium to induce GFP expression. After 24 hours of incubation, we measured the GFP levels in individual spore bags (grey data points, n = 96 spore bags). As a control, we repeated the above steps but without first incubating the PBS containing the 100$\mu g/ml$ of doxycycline for two days (just as we did in all experiments involving GFP induction (Figs. 3-6)) (blue data points, n = 116 spore bags). We found that, on average, incubating doxycycline for 48 hours caused the spores to express 20% less GFP (i.e., 4000 versus 5000 a.u.). Thus, doxycycline only weakly degrades after 48 hours. Hence, doxycycline degradation is not why the GFP levels plateau after 15-to-20 hours in Fig. 3C and any of the other GFP inductions in our work.

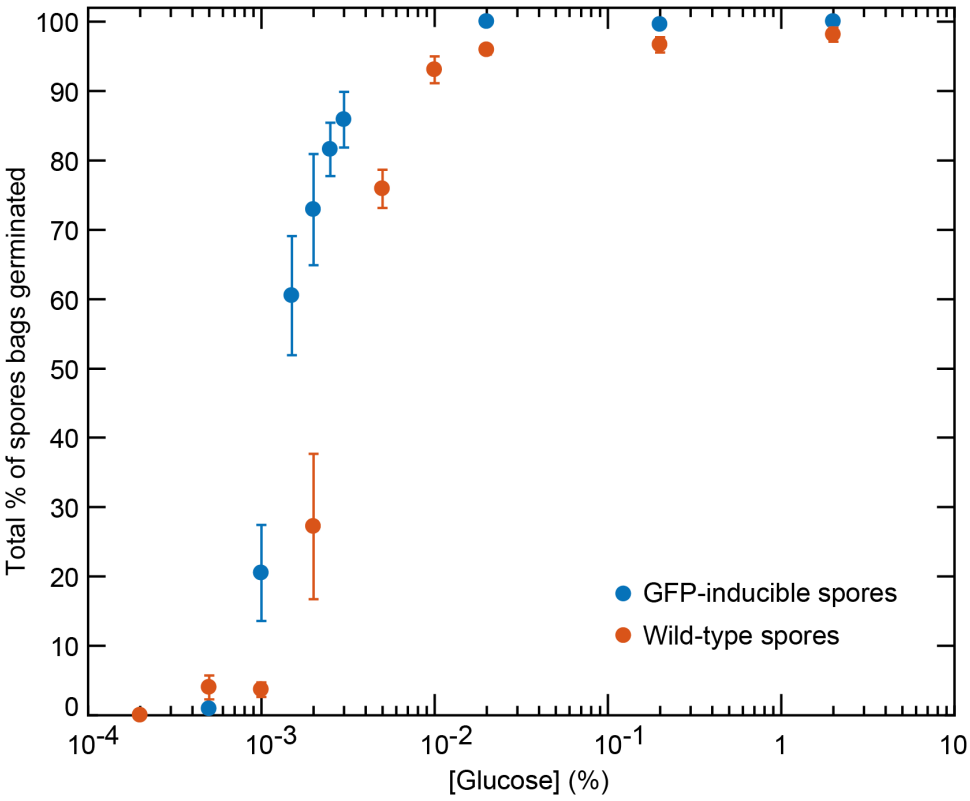


Figure 2.24: Appendix Figure S15 - Comparing efficiency of germination for wild-type spores with that of GFP-inducible spores (complements Fig. 3D). The GFP-inducible spores (Fig. 3A), aside from GFP, has additional genes (selection markers such as amino-acid biosynthesis genes) that we inserted during 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.

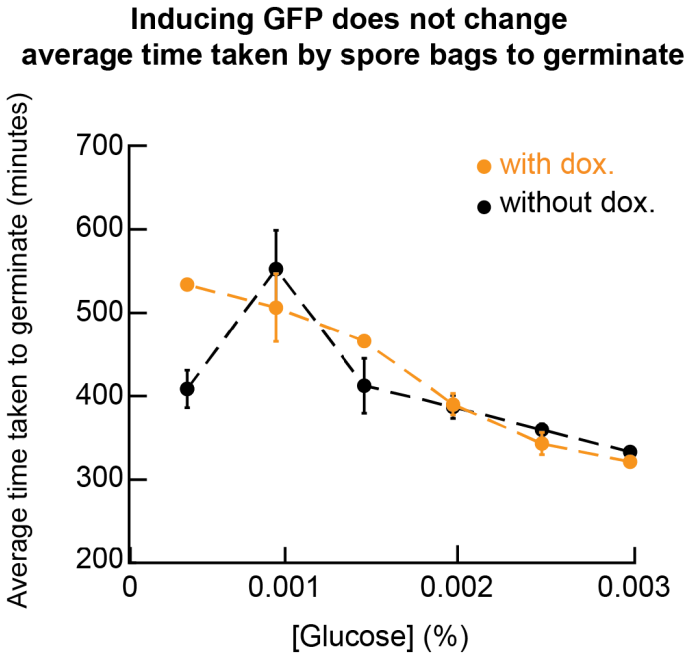


Figure 2.25: Appendix Figure S16 - Inducing GFP production does not appreciably alter the average time taken by spore bags to germinate (complements Fig. 3D). Same experiments and data as in Fig. 3D but now showing the average time taken by the GFP-inducible spore bags to germinate for low glucose-concentrations. 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 doxycycline (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. Appendix Fig S1H)). 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.

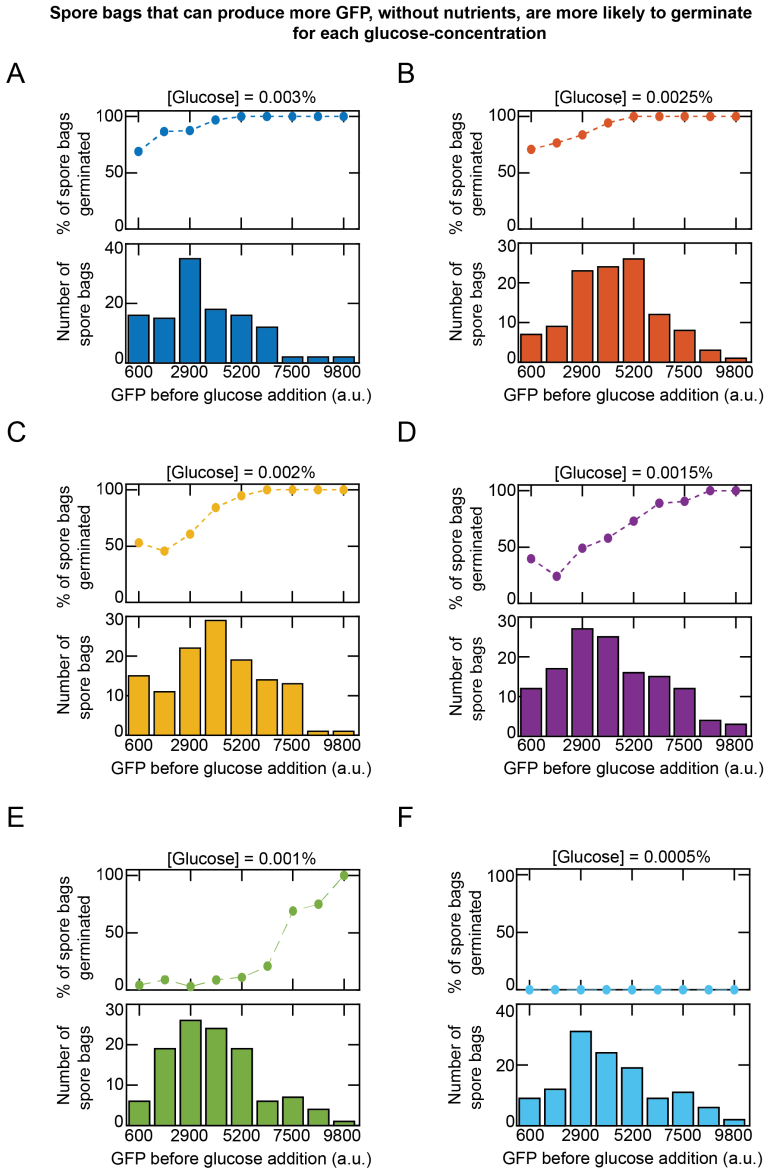

Spore bags that can produce more GFP, without nutrients, are more likely to germinate for each glucose-concentration
A
[Glucose] = 0.003%
B
[Glucose] = 0.0025%
C
[Glucose] = 0.002%
D
[Glucose] = 0.0015%
E
[Glucose] = 0.001%
F
[Glucose] = 0.0005%
% of spore bags germinated
Number of spore bags
GFP before glucose addition (a.u.)
600 2900 5200 7500 9800

Figure 2.26 ***(preceding page)*****:** Appendix Figure S17 - Spore bags that can express more GFP without any nutrients (higher GFP inducibility) 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 $100\mu g/ml$ 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 (Appendix Fig S12), 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 Appendix Figs S10, S12, and S13 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.

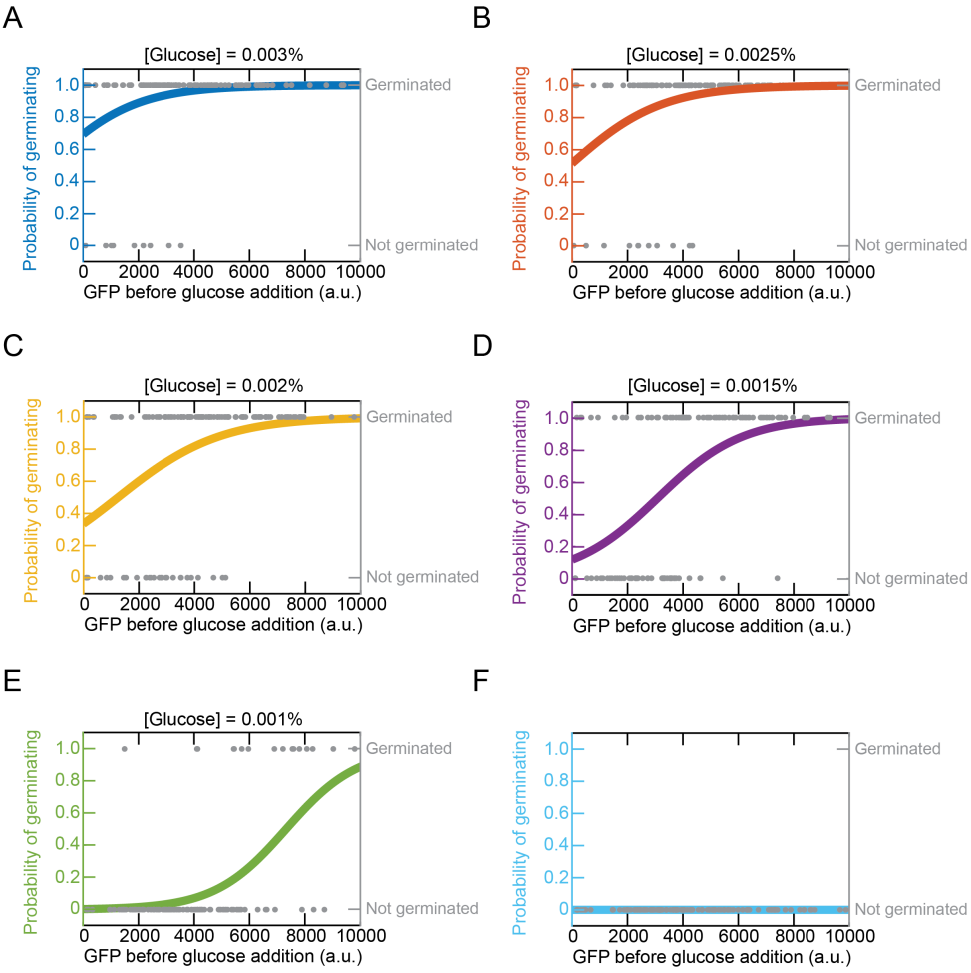

A
[Glucose] = 0.003%
Probability of germinating
1.0
0.8
0.6
0.4
0.2
0
Germinated
Not germinated
0 2000 4000 6000 8000 10000
GFP before glucose addition (a.u.)
B
[Glucose] = 0.0025%
Probability of germinating
Germinated
Not germinated
GFP before glucose addition (a.u.)
C
[Glucose] = 0.002%
Probability of germinating
Germinated
Not germinated
GFP before glucose addition (a.u.)
D
[Glucose] = 0.0015%
Probability of germinating
Germinated
Not germinated
GFP before glucose addition (a.u.)
E
[Glucose] = 0.001%
Probability of germinating
Germinated
Not germinated
GFP before glucose addition (a.u.)
F
Probability of germinating
Germinated
Not germinated
GFP before glucose addition (a.u.)

Figure 2.27 ***(preceding page)*****:** Appendix Figure S18 - Statistical test with a logistic regression-fit establishes that spore bags that can express more GFP without nutrients (higher GFP inducibility) are more likely to germinate for each glucose-concentration (complements Appendix Fig S17 and the germination landscape in Fig. 3F). Same experiments as in Appendix Fig S17 (i.e., GFP-inducible spores incubated for $\sim$ 22 hours in PBS with $100\mu g/ml$ 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 Appendix Fig S17 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, $p(x) = 1/(1 + e^{-(\alpha_0+\beta_1 x)})$, 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 β_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 = $3x10-5$; $\beta_1 = -0.00054 \pm 0.00026$, $n = 125$ grey data points (77% germinated); (D) for a 0.0015%-glucose, p-value = $4x10^{-7}$; $\beta_1 = -0.00066 \pm 0.00025$,$n = 131$ grey data points (61% germinated); (E) for a 0.001%-glucose, p-value = $2x10^{-5}$; $\beta_1 = -0.00077 \pm 0.00035$, $n = 118$ 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.

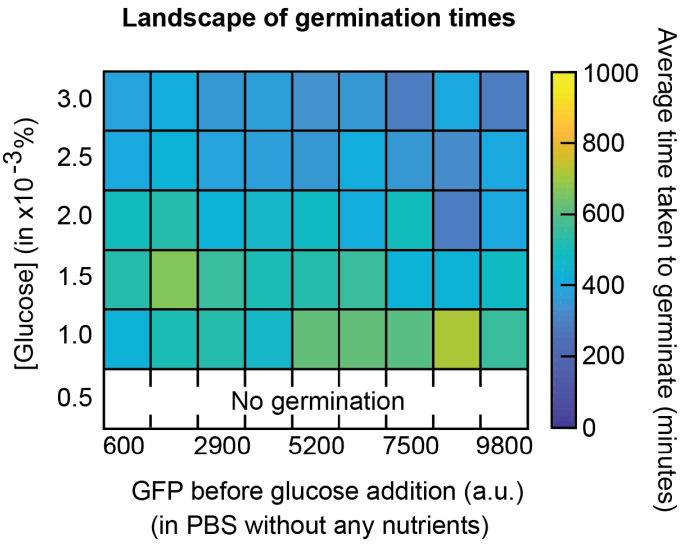


Figure 2.28: Appendix Figure S19 - Average time taken to germinate depends weakly on GFP inducibility (complements Fig. 3F). Each color represents the average time taken by a spore bag to germinate as a function of the glucose-concentration that it encounters and its steady-state GFP-level before it receives any glucose (result of GFP-induction with $100\mu g/ml$ 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).

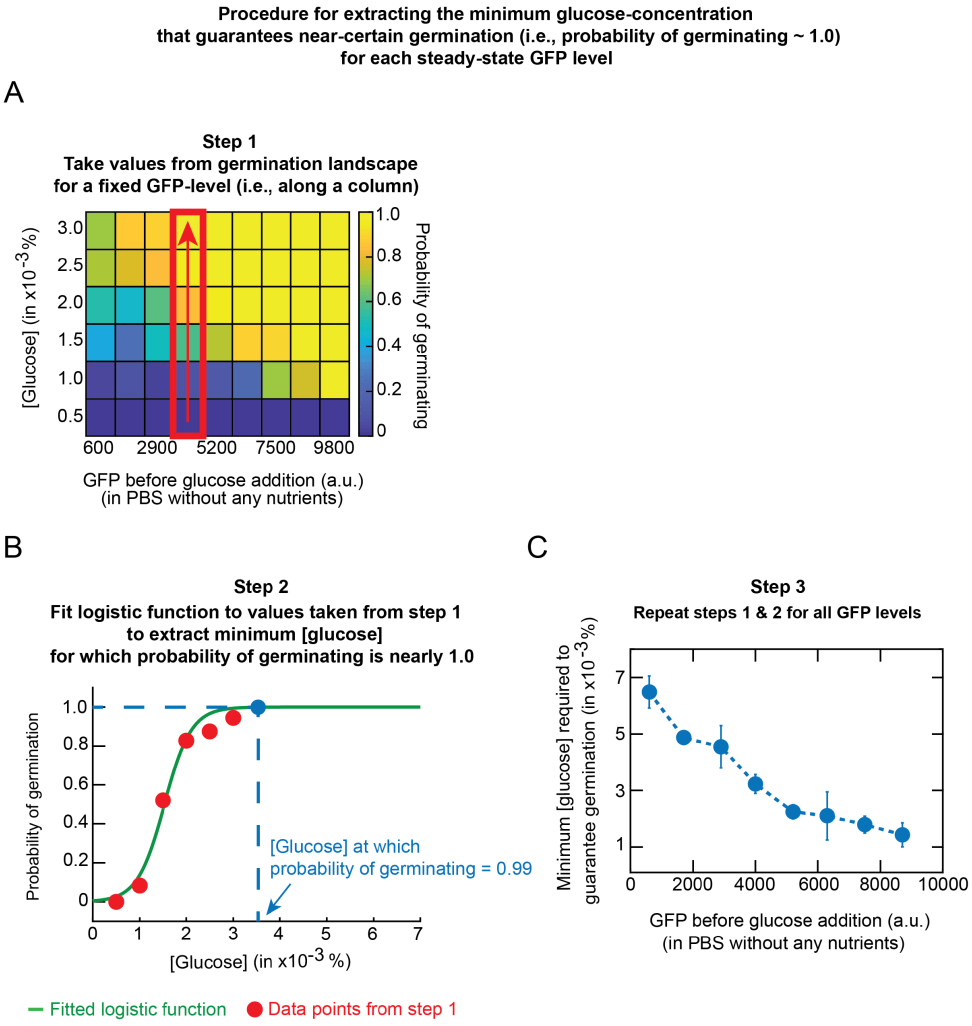


Figure 2.29: Appendix Figure S20 - Three-step procedure shown here establishes that the minimum glucose concentration that is required for guaranteeing that a spore bag will germinate (i.e., probability of germinating ~ 0.99) decreases as the spore bag's GFP inducibility increases (complements Fig. 3F). (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 Appendix Fig S18 but now with a different meaning: $p(x) = 1/(1 + e^{-(\beta_0+\beta_1 x)})$. 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 Appendix Fig S18 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 $p(x) \sim 0.99$ (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.

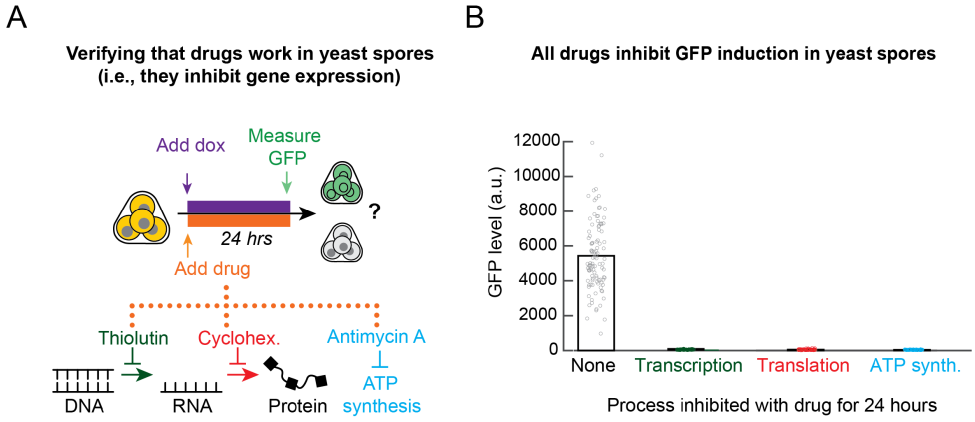


Figure 2.30: Appendix Figure S21 - Thiolutin, cycloheximide, and antimycin A all function in yeast spores as shown by their complete inhibition of GFP induction (complements Figs. 4A-B). (A) To verify that all three drugs that we used in Figs. 4A-B - thiolutin, cycloheximide, and antimycin A - actually functioned in yeast spores, we sought to test if all three drugs inhibited GFP expression in spores with the GFP-inducing synthetic circuit (Fig. 3A). We incubated spores for 24 hours in PBS with $100\mu g/ml$ of doxycycline (to induce maximum possible GFP expression) and one of the three drugs - $10\mu g/ml$ of thiolutin (inhibiting transcription) or $200\mu g/ml$ of cycloheximide (inhibiting translation) or $100\mu M$ of antimycin A (inhibiting ATP production). As a control, we also left out a drug in one case. (B) After the 24 hours of doxycycline with a drug, we measured the GFP levels of individual spore bags. Dots denote individual spores (n = 62 for thiolutin, n = 58 for cycloheximide, n = 63 for antimycin A, n = 75 for "None" (no drug)). The bars show the GFP-level averaged over all spores. The spores incubated in either thiolutin, cycloheximide, or antimycin A were not able to express any GFP. All three drugs thus inhibit gene expression in yeast spores.

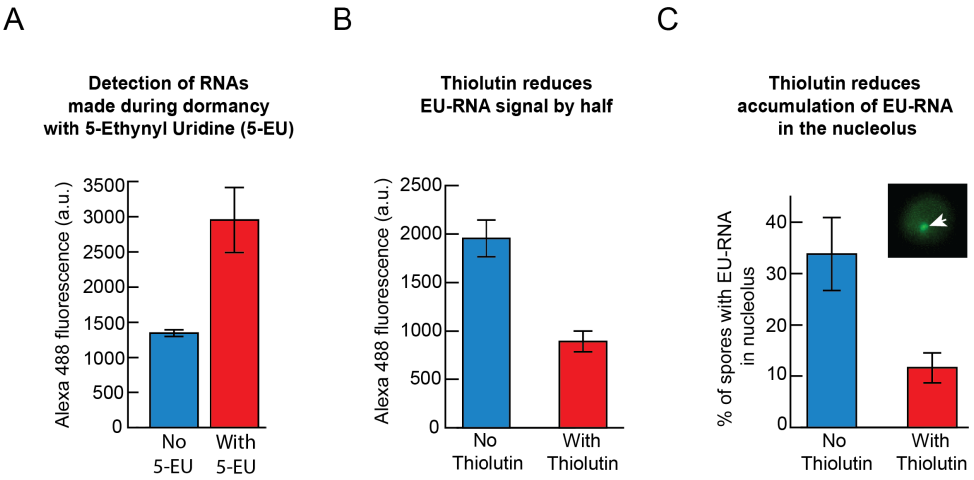
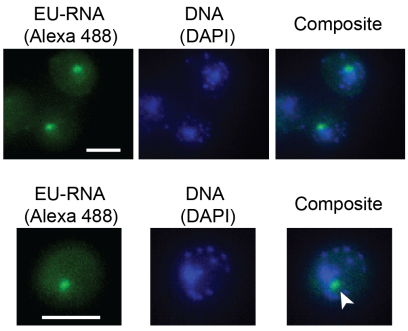


Figure 2.31: Appendix Figure S22 - Detection of freshly made during dormancy with 5-Ethynyl Uridine (5-EU) (complements Figs. 4C-E). (A) Alexa 488 fluorescence, on average, per spore bag after 24 hours of incubation in PBS without the 5-EU (blue) and with 1 mM of 5-EU (red). Hence, blue bar shows the background fluorescence which is ~ 2-fold lower than when 5-EU is present, indicating that the spores made RNAs during the 24-hour incubation. n = 3 populations (one of the populations is shown as histograms in Fig. 4D). (B) In order to further verify that the increase in fluorescence seen in (A) is from RNAs being synthesized during dormancy, we incubated spores for 24 hours in PBS with either 1 mM of 5-EU (blue) or 1 mM of 5-EU and 10$\mu g/ml$ of thiolutin (red). Thiolutin inhibits transcription. Thiolutin caused the Alexa 488 fluorescence per spore bag, on average, to be halved compared to the case without thiolutin. This is another validation that the 5-EU method for detecting freshly made RNAs is working in yeast spores. n = 3 populations. Errors bars are s.e.m. (C) (Blue) In ~ 35% of spores, we found RNAs being produced in the nucleoli of yeast spores that were incubated in PBS with 1 mM of 5-EU for 24 hours (blue). This number decreased to ~ 10% if the spores were in 10$\mu g/ml$ thiolutin with 5-EU for 24 hours (red). Inset picture shows a spore with the white arrow indicating the nucleolus where the RNA has accumulated during the 24 hours of incubation (images in Appendix Fig S23 actually prove that the spots like the one in the inset figure are RNAs in nucleoli). This indicates that thiolutin likely inhibits synthesis of non-coding RNAs as well. n = 3 replicates. Errors bars are s.e.m.

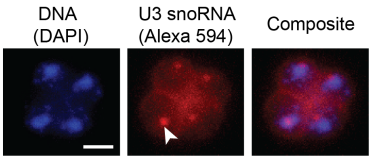
A

Accumulation of EU-RNA at the nuclear periphery



B

Localization of nucleolar RNA in dormant spores



C

Co-localization of nucleolar RNA and EU-RNA

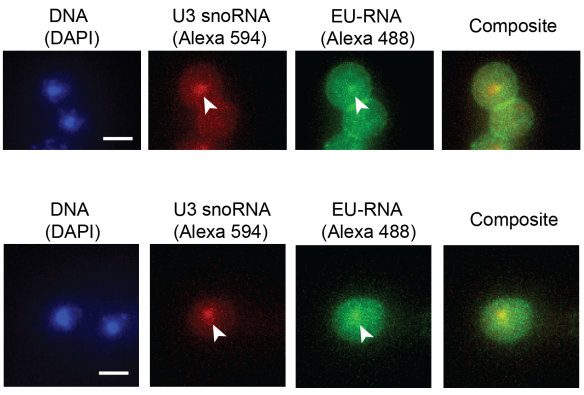


Figure 2.32 ***(preceding page)*****:** Appendix Figure S23 - Synthesis of non-coding RNAs in spores' nucleoli (e.g., U3 snoRNA) detected with 5-Ethynyl Uridine (5-EU) (complements Figs. 4C-E). (A) Images of fixed spores after 24 hours of incubation in PBS with 1 mM of 5-EU RNAs. During the fixation, we permeabilized the spores to let Alexa 488 fluorophores enter the spores and bind to the 5-EU-bound RNAs. Composite images show the merging of the Alexa 488 image (showing 5-EU bound RNAs) and DAPI image (showing DNAs) of the same spores. Top row shows three spores and the bottom row shows one spore. For both rows, scale bar is $2\mu m$. White arrow indicates the single bright green fluorescence spot localized at the nuclear periphery. In vegetative yeasts, an image like the ones shown here represent RNAs accumulated in the nucleolus. (B) Shows four spores in a spore bag. Scale bar represents $2\mu m$. To confirm that the bright spots of Alexa 488 mark the nucleoli of spores, we used single-molecule RNA FISH to make U3 snoRNAs fluorescently visible (via Alexa 594 fluorophores). U3 snoRNAs localize in the nucleolus (Narayanan et al. J. Cell Sci. 2003). As in Narayanan et al., We used an anti-sense deoxyoligonucleotide probe against the yeast U3 snoRNA for single-molecule FISH whose sequence is ATTCAGTGGCTCTTTTGAAGAGTCAAAGAGTGACGATTCCTATAGAAATGA (purchased from IDT DNA). We used Alexa-594 fluorophore fused at the 3' end of the probe. We observed bright red fluorescence spots at the nuclear periphery (indicated by the white arrow), indicating the yeast spore's nucleolus. This result, with (A), indicate that the spores transcribe RNAs during dormancy. (C) To further confirm that dormant spores are making nucleolar RNAs during the 24-hours of incubation in 5-EU, we performed a sequential staining experiment in which spores were first incubated for 24 hours in PBS with 1mM of 5-EU. We then fixed the spores and performed single-molecule RNA FISH with the probe for the U3 snoRNA, using Alexa 594 as the fluorophore bound to the probe (as in (B)). Afterwards, we added the Alexa 488 fluorophores that would bind to the 5-EU labeled RNAs (as in (A)). Images here show the resulting spores. These images show clear red fluorescence spots (U3 snoRNAs in the nucleoli) and green spots. The green spots are relatively dim compared to the FISH spots likely due to the Alexa 488 fluorophores having less spaces to bind due to the Alexa 594 probes binding first to the same target RNAs. But even with the relatively dim 5-EU spots, we can observe that U3 snoRNA, indicating nucleolar RNA (red), co-localize with the 5-EU focal point in a spore. This result confirms that yeast spores produce the non-coding, nucleolar RNAs during dormancy. Scale bars represent 2μ m.

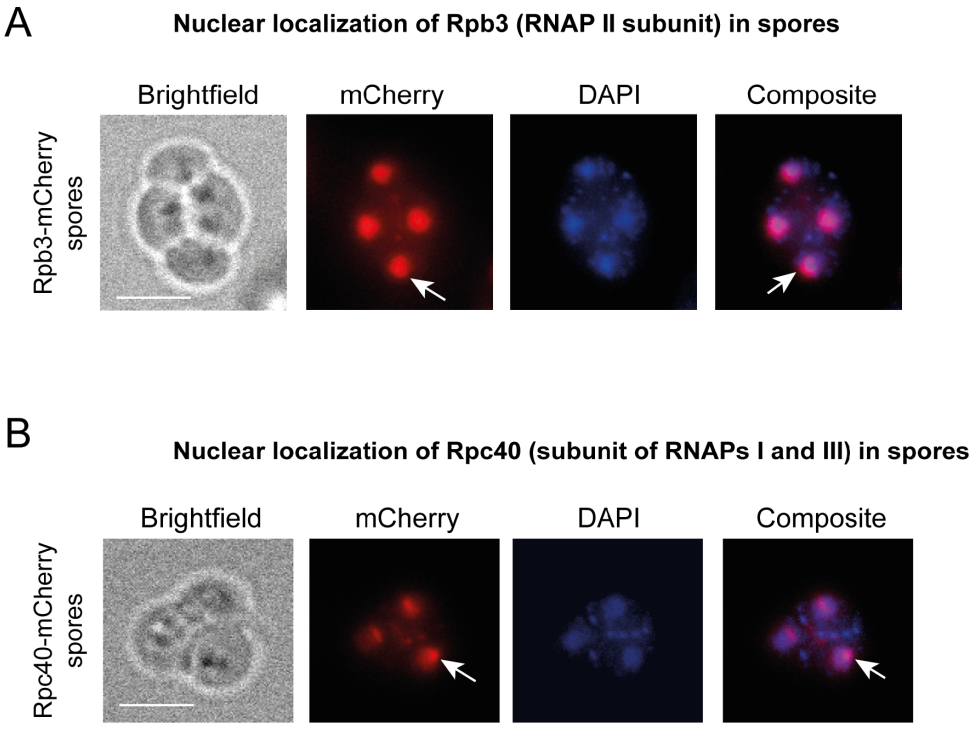
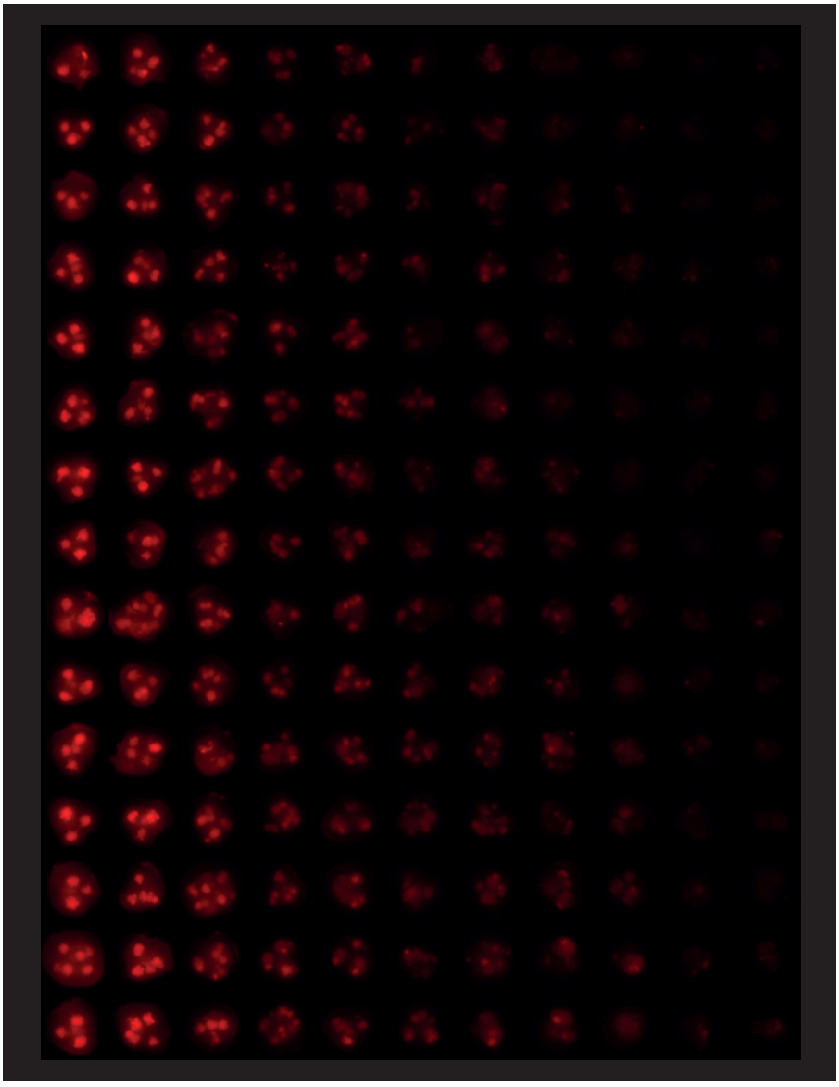


Figure 2.33: Appendix Figure S24 - Rpb3-mCherry and Rpc40-mCherry fluorescence are entirely confined to the nuclei of spores (complements Figs. 4F-G). (A) Representative image of spores whose Rpb3 (subunit of RNAP II) is fused to mCherry ("TS8" strain). As expected, mCherry fluorescence is almost entirely localized inside the nucleus of each spore. Nucleus is indicated by one uniform blue disk (DAPI) in each spore. White arrows show nuclear localized mCherry fluorescence. Scale bar represents $2\mu m$. (B) Representative image of spores with Rpc40 (subunit of both RNAPs I and III) fused to mCherry ("TS9" strain). White arrows show mCherry fluorescence localized at the nuclear periphery (nucleus indicated by DAPI), as expected of RNAPs I and III since they mostly make nucleolar transcripts. Scale bar represents $2\mu m$.

3

From dormant to dead



Contents

3.1. Introduction

Dormancy is colloquially defined as extending lifespan by being still. Upon starvation, numerous micro-organisms drastically reduce their internal activity but still remain viable for days to years (Lennon et al. 2021). Unlike replicating state, dormant state is characterized by a significant isolation from the environment, which confers protection external stresses but also imposes to function only with a limited amount of internal resources. Additionally, many dormant unicellular eukaryotes (for instance yeast spores or Giardia's cysts) are known to maintain a non-zero level of internal activity (gene expression or metabolism) during dormancy. This activity is thought to rely of the consumption of internal resources, which in the case of dormancy do not get renewed over time. Therefore a common rationale is that during dormancy, organisms progressively consume internal resources to sustain maintenance activity (necessary to remain viable) up to a point of exhaustion where the organism turn dead (Lennon et al. 2011). In parallel, reducing internal activities during dormancy is thought to minimize energetic expenditure and therefore, by postponing the exhaustion of internal resources, extend lifespan in the dormant state (Lennon et al. 2021).

While these rationales about dormancy are physically sensible, very little empirical evidence exists to support it. Concretely, several studies have uncovered the lifespan during dormancy (Brengues et al. 2002), but to our knowledge no study made the link with another quantity (ressource, amount of proteins,) being lost as organisms lose the ability to wake up. Here we propose to leverage on previous work on dormant *Saccharomyces cerevisiae* yeast spores (Chapter 2) to quantitatively address the following question :

How do dormant yeast spores die?

Previous studies showed that in the absence of external nutrients (kept in water at 30C), dormant yeast spores have a half-life of approximately 3 weeks (Brengues et al. 2002,Fig.1.7). This means that after 3 weeks of starvation, half of spores are still able to germinate upon addition of saturating glucose in minimal medium. In our previous work (Chapter 2), we found that inhibiting transcription but not translation drastically reduces the lifespan of dormant spores from 21 days to 12 hours. Moreover we uncovered that the ability to germinate is positively correlated to the ability to express genes (proxied by GFP inducibility) and to the amount of RNA polymerases. Based on these results, we set out to investigate how dormant spore die by monitoring these different quantities over the time-scale of dormancy lifespan (i.e. months), with or without inhibition of gene expression.

By combining these measurement with mathematical modeling, we could reveal what dynamic process underpin the gradual loss of germination ability upon long-

term incubation without nutrients. Finally we propose a conceptual model to explain how various aspects of gene expression dynamics during dormancy collectively set the lifespan of dormant spores.

3.2. Gene expression ability is lost as spore are ageing

3.2.1. Dormant spores gradually lose GFP inducibility as they age

To age the dormant spores, we incubated the wild-type and GFP-inducible spores in either water or minimal medium without any glucose for days or weeks at 30C. In this "ageing experiment", we found that the number of dormant (i.e., alive) spores - the ones that we could germinate when we gave them a 2%-glucose - decreased by similar rates over several weeks regardless of the strain type and regardless of whether we kept the spores in water or minimal medium (Fig.3.1A). Specifically, about half of the spores in the population died after 30 days without glucose and almost everyone died after 60 days without glucose (Fig.3.1A). Furthermore, we observed that spores needed more time to germinate if we kept them longer without nutrients (Appendix Fig S25), suggesting that germinating is becoming more difficult as the dormant spores age.

To measure the GFP inducibility in these ageing spores, we took some of the spores from the ageing population, incubated them with doxycycline in PBS for 24 hours (Appendix Fig S26), measured the resulting GFP levels in these spore bags with a microscope (Fig.3.1B and Appendix Fig S27), and thus determined how the GFP inducibility changed over the 80 days in PBS without nutrients (Appendix Fig S27). Since we also observed dead spores in the ageing population, we could also measure the dead spores' GFP inducibility with doxycycline on different days. The GFP inducibility of dead spores remained at near zero (near background fluorescence value) regardless of when the spores had died (Fig. 5B - grey points). The GFP inducibility of dormant spores, when averaged over every dormant spore bag, gradually decreased and approached the near-zero GFP inducibility of the dead spores that were in the same ageing population over tens of days (Fig.3.1B - blue points). The dormant spores' GFP inducibility eventually reached a barely detectable level - matching the dead spores' GFP inducibility - after 80 days. By this time, nearly all spores had died (Appendix Fig S27). Together, these results show that dormant spores indeed lose their GFP inducibility over time as they age and approach their deaths. Crucially, in accordance with our revised hypothesis, we did not find any dead spores with a detectable GFP inducibility. Hence dormant yeast spores lose their GFP inducibility before dying.

Moreover, the fact that the oldest dormant spores have barely detectable GFP in-

ducibility, just like the dead spores, suggest that GFP inducibility is nearly all lost moments before death.

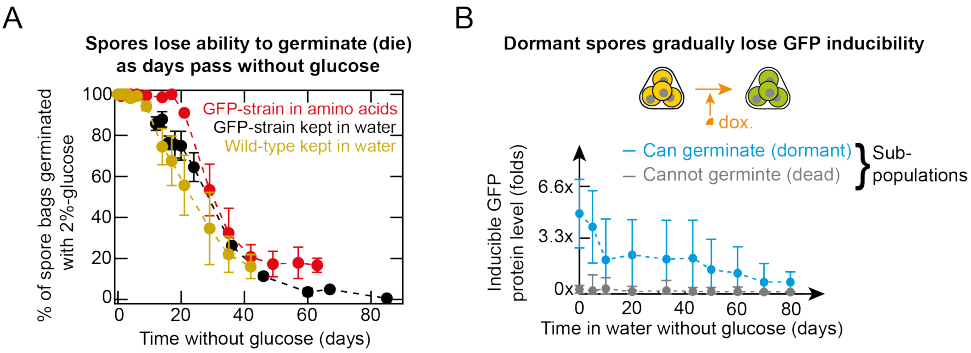


Figure 3.1: Dormant spores lose the ability to express genes as they age (A) Percentage of wild-type and GFP-inducible spore-bags that germinate due to a saturating glucose concentration (2%) that they encounter after ageing for 0 - 85 days in water (grey and black) or minimal medium with essential amino acids (red) at 30 OC. $n = 3$; error bars are s.e.m. (B) Mean GFP inducibility of dormant (blue) and dead (grey) spore bags that were incubated in water without nutrients at 30 OC over ~ 80 days (also see Appendix Figs S27-S28). Blue data points are the average GFP-levels from the histograms shown in Appendix Fig S27. Grey data points are averages of the dead spore bags' histograms shown in Appendix Fig S27. n=3, error bars show the average standard deviation from three biological replicates. n =3.

3.2.2. Dormant spores gradually have less copies of RNA polymerase II as they age

To further validate that dormant spores lose nearly all GFP inducibility just before dying, we sought to measure the RNAP II level in ageing spores since GFP inducibility strongly and positively correlates with the RNAP II level in spores. We repeated the ageing experiment but now with the spores that had the fluorescent RNAP II. By taking out aliquots of these spores from an ageing population and then using a microscope to measure the RNAP II levels in each of these aged spore bags, we found that spores' RNAP II level decreased as they aged. Moreover, after ~ 50 days of ageing, almost all the spores had barely detectable RNAP II levels which were near the background fluorescence value (Fig.3.2A and Appendix Figs S28-S29).

After a month of ageing, more than ~ 50% of the spores were dead. This was the case, for example, on day 39 of ageing in water. We aliquoted a subset of the aged spores on day 39. Then, we used a microscope to measure the RNAP II levels in each of the aliquoted spore bags. Afterwards, we incubated these spores in minimal medium with a 2%-glucose and then, with time-lapse microscopy, determined which spore bags germinated (i.e., were alive) and which ones did not germinate (i.e., were dead). Spores that had more RNAP II had a higher chance of being alive than spores with less RNAP II (Fig.3.2B). In particular, we found that nearly 50%

or more spores with the lowest observed RNAP II levels were dead. These spore bags had near background fluorescence level of ~ 2000 – 3000 fluorescence units (Fig.3.2B). Since GFP inducibility positively correlates with the RNAP II level, this finding complements the observation that spores lose almost completely their GFP inducibility just before dying, as also expected from the triangular relationship (Fig.2.9E).

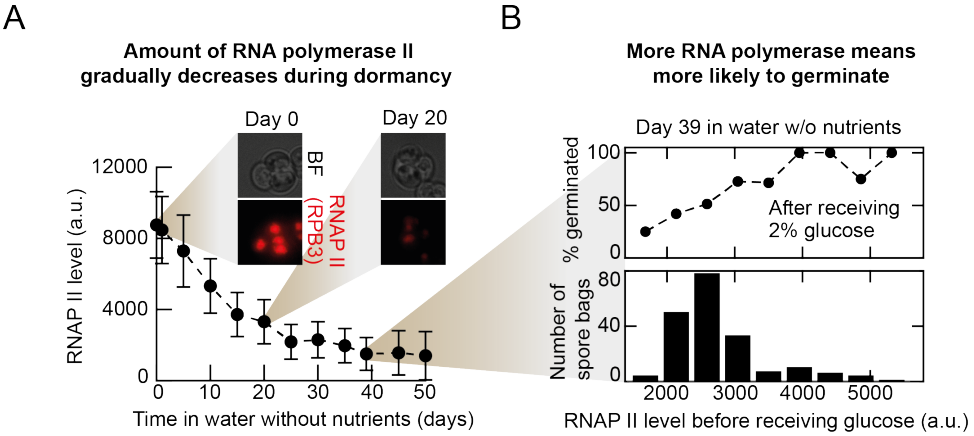


Figure 3.2: Dormant spores gradually have less copies of RNA polymerase II as they age (A) Average RNAP II level of spore bags incubated in water for 50 days at 30C. $n = 3$, error bars represent the average standard deviation in a population, each population having at least 100 spore bags (see Appendix Figs S28-S29). (B) Top: Percentage of spore bags, having the same RNAP II level on 39th day of ageing in water, germinating after receiving a 2%-glucose. Percentages are averaged over all spore bags with the same binned RNAP II level (corresponding histogram shown below). $n = 193$ spore bags.

3.2.3. Gene expression at the edge of dormancy

Aged spores at the edge of dormancy before dying have nearly depleted RNAP II and have strained gene-expressing ability On the 39th day of ageing, many spores with barely detectable RNAP II levels (2000-3000 fluorescence units in Fig. 5D) were dead but many others also with the same barely detectable RNAP II levels were alive, albeit with less than a 50% chance (Fig.3.2A). This 39-days-old population is thus ideal for understanding the last moments of dormancy - dormancy just before death. With this population, we sought to understand what actually distinguishes the dead from dormant spores when both have comparable, barely detectable RNAP II levels (i.e., 2000-3000 fluorescence units in Fig.3.2A). We examined two histograms of RNAP II levels: one for dead spores (Fig.3.3A - lower, grey histogram) and the other for dormant spores (Fig.3.3A - upper, blue histogram). The two histograms show many alive and dead spores having the nearly identical, low range of values for RNAP II level (Fig.3.3A). After measuring the RNAP II lev-

els in individual spore bags, we incubated both the dead and dormant spore bags together in a minimal medium with a 2%-glucose and then tracked the RNAP II levels in individual spore bags with time-lapse microscopy. We found that, after receiving the 2%-glucose, the dead spores did not make any detectable levels of new RNAP II (Fig.3.3B - grey traces and points) whereas the dormant spores did make observable amounts of new RNAP II until they germinated (Fig.3.3B - blue traces and points). Moreover, these dormant spores often started to make new RNAP II shortly before buds appeared (i.e., before they germinated) (Appendix movies 1-4, Fig.3.3B). They also, on average, took longer times to germinate (Appendix Fig S30). Strikingly, these aged dormant spores, near death, did not produce any detectable levels of new RNAP II for the first 5 to 10 hours after receiving the 2%-glucose (Appendix movies 1-4, Appendix Fig S30). After this "lag time", they began producing new RNAP II and then germinated shortly afterwards.

These results paint a picture in which dormant spores with barely detectable amounts of RNAP II are struggling through gene-expression and germination after receiving a 2%-glucose, as expected intuitively from dormant spores that are near death. This picture also makes sense given that RNA polymerases, including RNAP II, are required to make more RNAPs I-III and that the aged, nearly dead spores have barely detectable amounts of RNAP II. Crucially, these results establish that dead spores, which have virtually undetectable GFP inducibilities during dormancy (Fig.2.3B), also have virtually undetectable gene-expressing abilities after they receive a saturating amount (2%) of glucose. This is supported by the fact that RNAP II - required for expressing all coding and some non-coding genes - is at barely detectable levels in the aged spores that are near death (Fig.3.3A) and that a 2%-glucose does not induce gene expressions that lead to the making of a new RNAP II (Fig.3.3B).

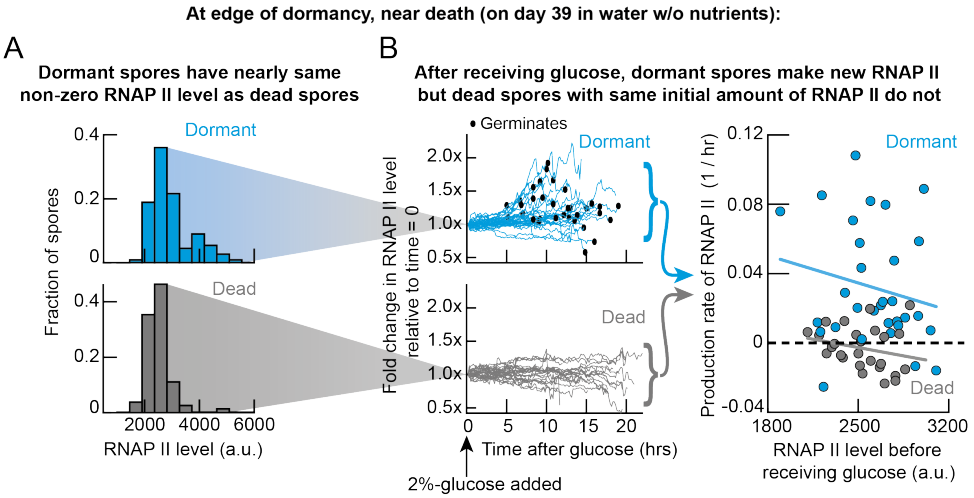


Figure 3.3: Gene expression at the edge of dormancy (A) RNAP II levels of dormant (alive) spore bags in blue and of dead spores in grey; both from the same population on 39th day of ageing in water before any glucose added. n = 111 spore bags (blue); $n = 82$ spore bags (grey). (B) Corresponds to histograms in (A). Blue is for dormant and grey is for dead spore bags. Line traces show, after receiving a 2%-glucose, the fold change in each spore bag's RNAP II level relative to the spore bag's initial RNAP II level (i.e., value just before we added the 2%-glucose). Black dots represent moments of germination. n = 38 for blue and n = 32 for grey. Data points in the rightmost plot show the RNAP II production rate in each spore bag profiled in the line traces. For each spore bag, we fitted an exponential function to its RNAP II level over time (one of the line traces). Average production rate of RNAP II in dormant spore bags (average over all blue points) is a positive value, 0.031 / hour and in dead spore bag (average over all grey points) is a negative value, -0.0030 / hour. For a t-test on the difference between the two values: p-value = $1.4x10^{-5}$. Linear regression line for dormant (blue) is $R = -0.19$ and for dead (grey) is $R = -0.23$.

3.3. Inhibition of transcription and ability to express genes

3.3.1. Transiently inhibiting transcription during dormancy permanently decreases GFP inducibility

We found that ageing spores gradually lose the GFP inducibility. In other words, older dormant spores, when forced by doxycycline to express GFP without nutrients, yield less steady-state GFP levels. Although dormant spores have less RNAP II as they age (Fig.3.2A), it is not obvious that less RNAP II should lead to less steady-state GFP level after 24 hours of doxycycline. This is all the more since we showed above that the GFP level reaches a steady-state value because the spores stop producing GFP, despite the doxycycline not degrading and still being present at the saturating concentration at the end of the 24-hour incubation (Appendix Figs S13-S14). To understand why spores lose the GFP inducibility as they age, we

sought a clearer understanding of gene expression in general, which occurs during dormancy as we have shown with the 5-EU labeling of RNAs (Fig.2.8).

As a start, we incubated the GFP-inducible spores in water with thiolutin for various hours (between 0 and 24 hours). Afterwards, we washed away the thiolutin and then added doxycycline for 24 hours to induce their GFP expression (Fig.3.4A). At the end of the 24-hr incubation with doxycycline, we measured the GFP inducibility of each spore bag. After measuring the GFP-inducibility, we incubated the spores in a 2%-glucose to determine which ones were dormant (alive) and which were dead. In this experiment, we found that the GFP inducibility of dead spore bags always remained at the minimally observable values, as expected (Fig.3.4B - grey points). The GFP inducibility of dormant spore bags gradually decreased as the hours spent in thiolutin increased, until the GFP inducibility reached barely detectable levels after $\sim$ 15 hours in thiolutin (Fig.3.4B - blue points). Hence, removing thiolutin did not restore the spore's GFP inducibility back to the level that it had before it encountered thiolutin. In other words, transiently inhibiting global transcription for $\sim$ 12 hours or more has permanently (irreversibly) reduced the GFP inducibility. Along with this irreversible decrease in GFP inducibility, we observed that thiolutin accelerated deaths, resulting in almost all spores being dead after $\sim$ 24 hours with thiolutin as opposed to the $\sim$ 2 months in the previous ageing experiment without thiolutin (Fig.3.1A and Appendix Fig S31A). These results establish that if dormant spores do not produce transcripts - and potentially proteins from these transcripts - during a day, then they irreversibly and completely lose GFP inducibility and die within a day. Thus transcribing - and potentially translating - during dormancy during a day is required for surviving and directly determines how much GFP the spore can make when it is forced to do so.

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

To better understand how the transcription inhibition by thiolutin causes death, we focused on spores that encountered thiolutin for 12 hours since many of them were either dead or nearly dead, thereby affording us enough dead and nearly dead spores to compare. During the 12-hours of thiolutin, the spores' production of RNAP II itself would be stopped. Indeed, these spores had $\sim$ 30% less RNAP II after the thiolutin treatment than they had before the treatment. They also had $\sim$ 30% less RNAP II after the thiolutin treatment than the spores that we kept in water for 12 hours without thiolutin (Appendix Fig S31). But these thiolutin-treated spores still had abundant RNAP IIs, nearly $2-3$ times more than 39-day-old spores that we kept in water without thiolutin (Fig.3.4C). Surprisingly, however, $\sim$ 50% of the spores were dead after the 12-hours of thiolutin, which we

discovered by incubating the spores in a 2%-glucose after washing away the thiolutin (Appendix Fig. S31A and Fig.3.4D). In comparison, without thiolutin, we would need to wait ~ 39 days for ~ 50% of the ageing spores to be dead (Appendix Fig. S32).

To test whether the RNAP II-abundant spores that died during the 12 hours of thiolutin could express genes after receiving a 2%-glucose, we used time-lapse microscopy to track the RNAP II levels in both dead and dormant spores after washing away the thiolutin and then incubating the spores in minimal medium with a 2%-glucose (Fig.3.4E - left half). As with the spores that aged for 39 days without thiolutin (Fig.3.2A), the thiolutin-treated dormant spores made new RNAP II after receiving the 2%-glucose, typically taking ~ 5 – 10 hours before producing detectable amounts of new RNAP II (Fig.3.4E - blue curves and points) just like the 39-day-old spores with little RNAP II. As for the spores that died from the thiolutin treatment, we did not observe any of them producing noticeable amounts of new RNAP II during the ~ 20 hours of incubation in the 2%-glucose, despite all of them having abundant RNAP IIs (Fig.3.4E - grey curves and data).

Taken together, the thiolutin experiments (Fig.3.4B-E) establish that dead spores can have many copies of RNAP II and yet a 2%-glucose may not induce gene expression in them, including those necessary for producing RNAP II. These results support the idea that spores need to produce some transcripts, within several hours (~ 12 hours as a conservative estimate), in order to maintain GFP inducibility - and thus for a 2%-glucose to be able to induce gene expression in them - and hence for the spores to survive. Moreover, the fact that transiently inhibiting transcription permanently reduces GFP inducibility (Fig.3.4B) strongly suggests that during the transcription inhibition, spores lost copies of key molecules that are required for gene expression and that the spores could not fully replenish these molecules after transcription became permissible again. In other words, the experiments suggest that the production rates for these molecules is lower than their degradation rates during dormancy. This is also consistent with the fact that dormant yeast spores eventually die if left they are left alone in water without any drugs (Fig.3.1A) - there is a net loss of the molecules because, during dormancy, the production rates for the molecules are lower than the degradation rates for the molecules.

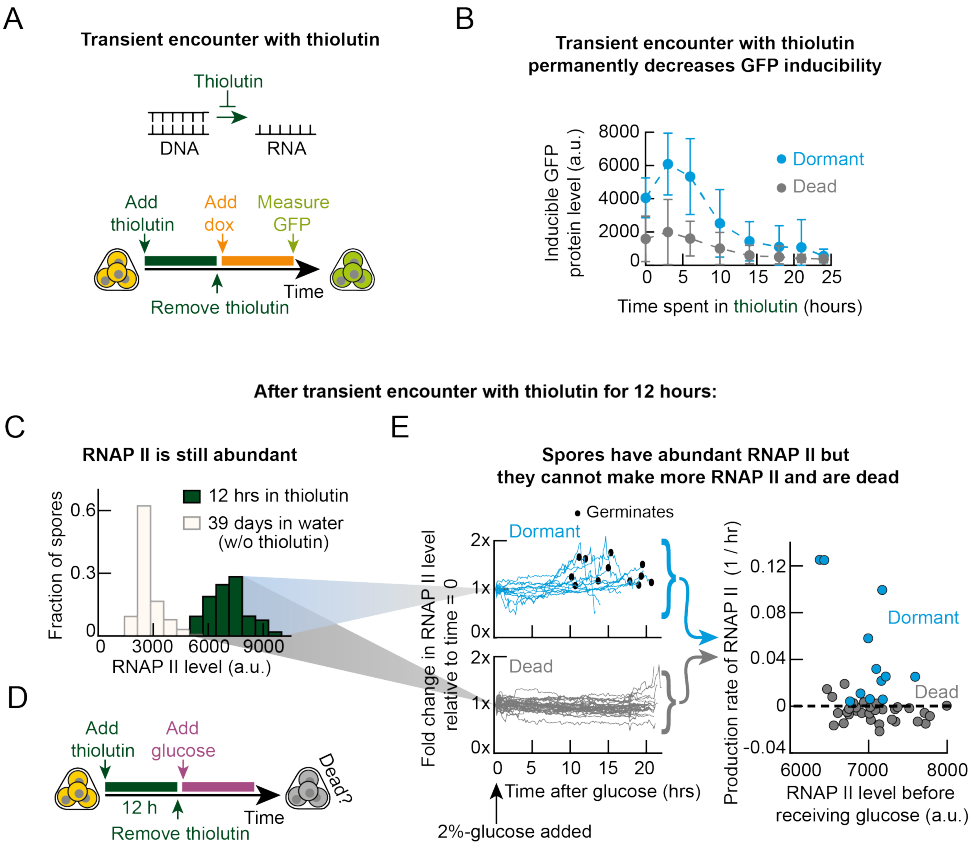


Figure 3.4: Stopping transcription for a day causes dormant spores with abundant RNA polymerases to lose all gene-expressing ability and thus die. (A) Protocol for (B-C) (See "Protocol for Figs. 6(A-C)" in Methods). (B) For protocol in (A), mean GFP inducibility of dormant (blue) and dead (grey) spore bags as a function of amount of time spent in thiolutin. n=3, error bars represent the standard deviation among spore bags in a population, averaged over three populations. (C) RNAP II level of spore bags after 12 hours of thiolutin (green; protocol in (A)) or after 39 days of ageing in water without drugs (light yellow; data from Fig. 5C). (D) Protocol for (E) (See "Protocol for Fig. 6E" in Methods). (E) Corresponds to green histogram in (C). Blue is for dormant and grey is for dead spore bags. Line traces show, after receiving a 2%-glucose, the fold change in each spore bag's RNAP II level relative to the spore bag's initial RNAP II level (i.e., value just before we added the 2%-glucose). Black dots represent moments of germination. n = 12 for blue and n = 42 for grey. Data points in the rightmost plot show the RNAP II production rate in each spore bag profiled in the line traces. For each spore bag, we fitted an exponential function to its RNAP II level over time (one of the line traces). Average RNAP II production rate in dormant spore bag (average over all blue points) is a positive value, 0.044 / hour and in dead spore bag (average over all grey points) is a negative value, -0.0045 / hour. For a t-test on the difference between the two values: p-value = $6.5x10^{-3}$.

3.4. Gradual net-loss of molecules required for gene expression causes spore to die

3.4.1. Globally inhibiting gene expression shortens spores' lifespans

To end our study, we tested the idea proposed above by estimating the production and degradation rates of key molecules that are necessary for gene expression during dormancy. To achieve this at a systems-level - without having to identify all such molecules and then measure their production and degradation rates - we continuously inhibited transcription and translation while ageing the spores that have the fluorescent RNAP II for ∼ 2 months in water with either a saturating concentration of cycloheximide or thiolutin. On various days, we took out an aliquot of spores from the ageing population, washed away the drug from the aliquoted population, measured the RNAP II level in these spores, and then incubated them in minimal medium with a 2%-glucose to check how many were still alive (i.e., dormant) (Fig.3.5A). From these measurements, we determined the rate at which a spore's ability to express genes during dormancy decreases - due to the net loss of key molecules required for gene expression - by measuring the net loss rate of RNAP II as a proxy (Fig.3.5B-C). The RNAP II level averaged over all dormant spores decreased by half after ∼ 8.4 days of ageing in cycloheximide (translation inhibitor) (Fig.3.5B and Fig.3.5C - red bar). As a comparison, the RNAP II level averaged over all dormant spores decreased by half after ∼ 14 days if the spores were left to age in water without any drugs (Fig.3.5C - white bar). Hence, inhibiting translation accelerates the net loss of RNAP II by ∼ 1.5 times compared to natural (drug-free) dormancy. Moreover, with cycloheximide, 50% of the spore population were dead after ∼ 19 days (Fig.3.5D and Fig.3.5E - red bar) whereas, without any drugs, half of the spore population were dead after ∼ 38 days (Fig.3.5E - white bar). The approximately 2-fold difference caused by cycloheximide in both the rate of RNAP II loss and the half-life of dormancy supports the idea that dormant spores age and eventually die because they gradually lose enough of the molecules that are required for gene expression, including RNAP II , and that spores cannot replenish these after receiving a 2%-glucose.

The fact that cycloheximide accelerates the net loss of RNAP II proves that RNAP II production, which requires transcription and translation, occurs during dormancy. Cycloheximide prevents spores from producing RNAP II and thus accelerates the RNAP II loss. By how much it is accelerated tells us the production rate of RNAP II during dormancy. We found that dormant spores in water without any drugs produced RNAP II at an extremely slow pace, having a characteristic production time of ∼ 26−30.3 days (Appendix Fig S33). In comparison, after receiving a 2%-glucose

but before germinating, spores produced new RNAP II 150-50 760 times faster than they did during dormancy (i.e., before receiving the 2%-glucose) (Fig.3.1A and Appendix Fig S33). The extremely slow production of RNAP II during dormancy supports the idea that spores do not replenish the molecules required for production, such as RNAP II, fast enough for preventing their net losses. As RNAP II is certainly required for expressing coding genes and thus for germination (i.e., for building a new cell after receiving a 2%-glucose), the net loss of RNAP II ensures that dormant spores will eventually die if left alone. Our measurements on RNAP II also strongly suggest that the molecules required for gene expression, which are lost on the order of at most several hours during the thiolutin treatment (Fig.3.4B), require transcription for them to be made and are relatively unstable compared to macromolecular machineries such as RNAP II that are also required for gene expression. In fact, since the RNAP II fluorescence arises from a single protein, Rpb3, fused to mCherry, the slow degradation of RNAP II also means that smaller proteins likely also degrade over days, not hours, during dormancy. Taken together, these results suggest that key transcripts, which are required for gene expression, are lost on the order of at most several hours during the transcription inhibition. In accordance with this view, spores retained more than half of their original RNAP II level after a day thiolutin (Fig.3.5C) yet the number of surviving spores were halved after just ~ 12 hours (Fig.3.5E).

3.4.2. Model for dormancy-to-death transition

Taken together, our experiments suggest a model of how dormant spores gradually approach their deaths (Fig.3.6). In this model, when yeast spores enter dormancy sometime after sporulation (Brengues et al. 2002; Thacker et al. 2011), they initially possess high amounts of key molecules that are necessary for gene expression which include, but are not limited to, RNA polymerases. Spore bags would vary in the initial amounts of these key molecules - leading to different germination abilities among them for each glucose - but all of them initially have sufficiently high amounts of the molecules to let them germinate to a 2%-glucose. During dormancy, retaining the ability to express genes is crucial for being able to germinate and thus for survival because a spore must build a new cell after glucose appears (and thus express genes that code for the building blocks of the new cell). The molecules required for gene expression become less abundant as time passes without any nutrients because spores cannot replenish these molecules faster than the rate at which they degrade. The resulting net loss of these molecules over time leads to the spores gradually losing the ability to express genes such as those involved in making RNAP II. The spores also become less able to germinate as days pass by, requiring more glucose to guarantee that they germinate as they age (Fig.2.3G). An aged spore that has lost the gene-expressing ability can neither build

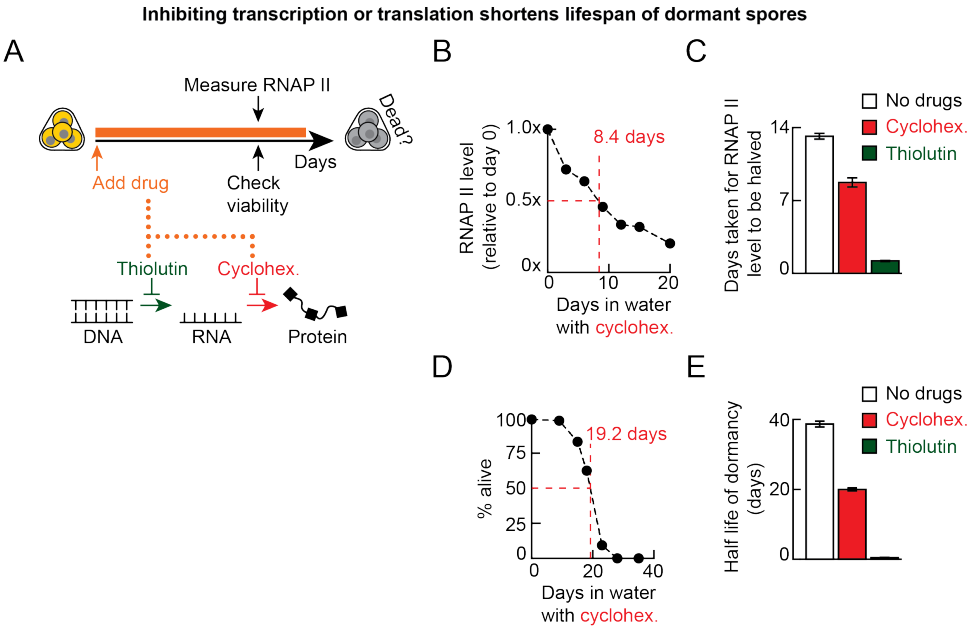


Figure 3.5: Gradual loss of molecules that are required for gene expression underlies ageing and eventual death of dormant yeast spores. (A) Protocol for (B-E). See "Protocol for Fig. 7(B-E) in Methods". Two drugs: Thiolutin inhibits transcription and cycloheximide inhibits translation. (B) Experiment in (A) with cycloheximide. For one representative population with cycloheximide (other replicates and data for thiolutin are in Appendix Fig S32). RNAP II level relative to its day 0 value, averaged over all dormant spore bags in a population. We used a linear interpolation determine the time taken for the halving of RNAP II level (8.4 days). (C) Experiment in (A). Time taken for the RNAP II level to decrease by half in dormant spores without drugs (white), with cycloheximide (red), and with thiolutin (green). For spores with thiolutin, we extrapolated this value from the measurements because, even when every spore had died after 2 days of thiolutin, average RNAP II level was still more than half its initial level (Appendix Fig S31). n=3; error bar are s.e.m. (D) Experiment in (A). For one representative population with cycloheximide (other replicates and data for thiolutin are in Appendix Fig S32). Time taken for half of the spore population to die ("half-life of dormancy"), estimated by linear interpolation (19.2 days). (E) Experiment in (A). Time taken for half of the spore population to die for populations without drugs (white), with cycloheximide (red), and with thiolutin (green). n=3; error bars are s.e.m.

a new cell when a 2%-glucose appears - so it cannot germinate - nor can it make the lost molecules that are crucial for regaining the gene-expression ability when a 2%-glucose appears. Accordingly, dead spores cannot express genes including GFP (Fig.3.1B) and those involved in the making of RNAP II when a 2%-glucose appears (Fig.3.3B). Once the amounts of the molecules required for gene expression decrease below certain threshold values - note that different molecules can have different threshold values - the spore cannot germinate even with a saturating amount of glucose and is thus considered dead. Our results suggest that among the many molecules that are required for gene expression, the key molecules - the ones that degrade the fastest during dormancy - are RNA polymerases and, likely,

certain crucial transcripts which may be non-coding transcripts. Further studies, however, are required to definitively show that such transcripts play a role and identify them.

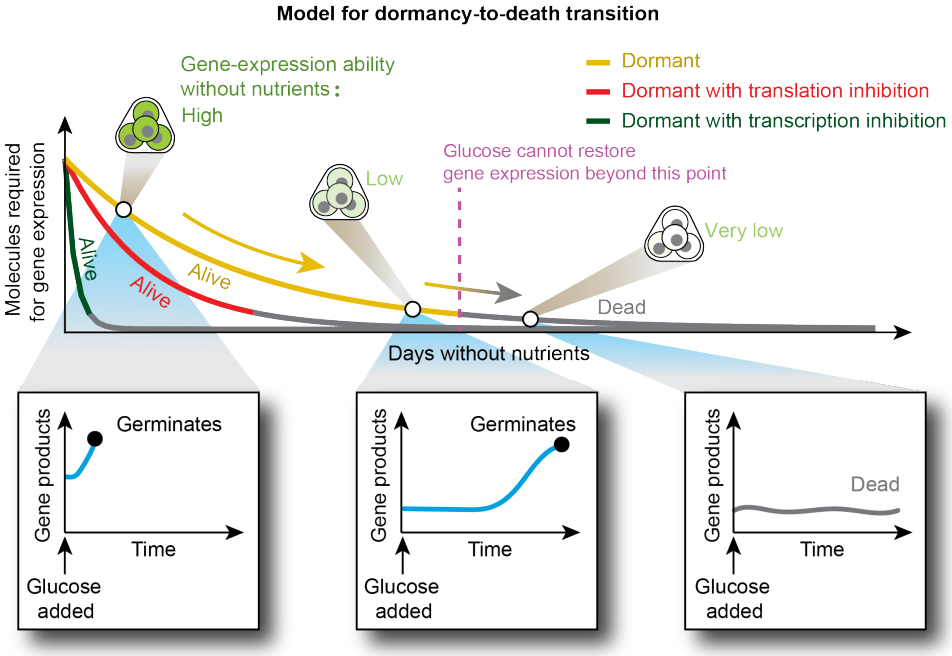


Figure 3.6: Conceptual model of how dormant spores die. Putting all our data together leads to a conceptual model of how spores age during dormancy and eventually die: key molecules required for gene expression, including RNA polymerases, are gradually lost during dormancy because spores cannot produce them faster than they degrade. These molecules eventually reach below some threshold amounts after which the spore has permanently lost the ability to express genes, meaning that even ample glucose cannot restore the gene-expressing ability (inset at bottom right). Since, after receiving glucose, spores must express genes in order to build a new cell that will bud off the spore (i.e., germinate), the permanent loss of gene expressing ability at the final moments of dormancy marks the spore's death (grey parts of the curves in the top plot).

3.5. Conclusion

In this chapter we started by posing a simple yet open question : *how do dormant yeast spores die?*. Leveraging on previous discoveries about mechanisms underlying the ability of germinate (see Chapter 2), we sought to uncover system-level principle explaining why dormant yeast spores have a half-life of ~ 3 weeks. With single-cell level measurements, we showed that key quantities such as the ability to express genes during dormancy when forced to so (proxied by the "GFP inducibility"), and the amount of RNAP polymerases inside the spores, decays over time as spore gradually lose their ability to germinate. We found that the RNAP II production and degradation take days whereas they take minutes in vegetative yeasts. We measured the lifespan of dormancy and found that one can shorten it from months - for spores kept in water without any drugs at 30C - to either hours (by inhibiting transcription during dormancy) or days (by inhibiting translation during dormancy). We revealed, with a single-cell resolution, how these quantities decayed over time up to the last moments of dormancy (i.e., just before the yeast spores die), and then determined when a yeast spore is no longer dormant but dead. Crucially, we found that these quantities are linked to one another and therefore we can study the dormancy-to-death transition by monitoring several different quantities. By linking the different quantities and measuring the extremely slow production rates - compared to degradation rates - of gene expression machineries such as RNAP II, we showed that a complete loss of gene expressing ability is inevitable for a yeast spore. These measurements led us to a new conceptual model for how yeast spores gradually lose dormancy (Fig.3.3). One way to monitor the gradual loss of dormancy is by measuring the gradually decreasing ability of the yeast spore to express a generic gene when forced to do so without nutrients (e.g., GFP inducibility). For this reason, we propose here a concept of "amount of dormancy". The amount of dormancy gradually decreases in a yeast spore and once it goes below a certain amount, the spore dies because it cannot express genes even when glucose appears.

While much is known about bacterial spores, particularly B. subtilis spores (Süel et al. 2007; Segev et al. 2012; Ablett et al. 1999; **Parry 2014**; Sinai et al. 2015; Dworkin et al. 2010), the dormant state of yeast spores - a model for eukaryotic spores - has remained comparatively underexplored. Our discoveries on yeast spores may not apply to bacterial spores such as B. subtilis spores and determining the similarities and differences between dormant state of yeast spores with dormant states of bacterial spores is challenging because dormancy of both remain incompletely understood. To bridge the gap, one may adapt the approach that we introduced here: building synthetic gene circuits in bacterial spores (e.g., B. subtilis spores) and then studying their dormancy-to-death transition. Since eukaryotic gene regula-

tions have many features that are distinct from those of prokaryotes, we expect that studies of gene regulation in dormant bacterial spores - another underexplored topic - along with further studies of gene regulation in dormant yeast spores will deepen our understanding of both forms of dormancy and help us elucidate the similarities and difference between the two.

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

3.6. Supplementary materials

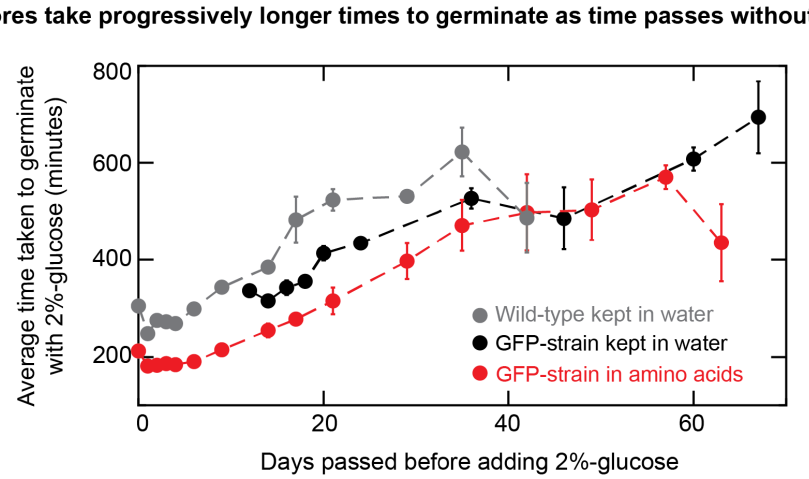


Figure 3.7: Appendix Figure S25 - Dormant spores gradually take longer times to germinate as days pass by without any glucose (complements Fig. 5A). Same experiments as in Fig. 5A but now plotting the average time taken by the GFP-inducible (black and red) and the wild-type (grey) spore bags to germinate 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. 5A), 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.

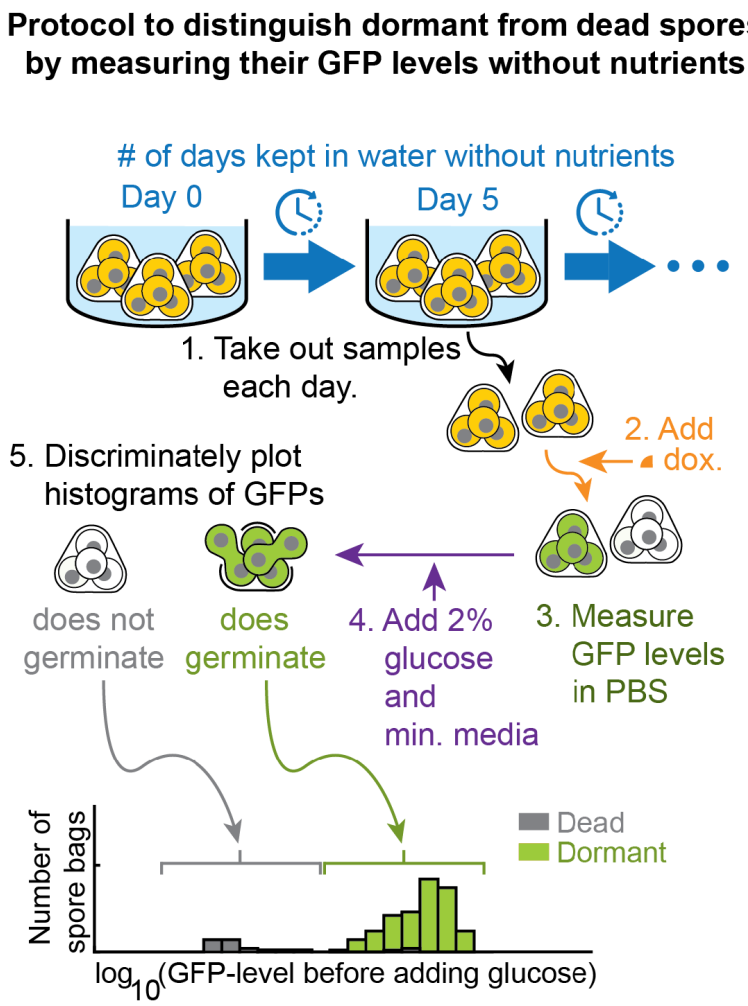


Figure 3.8: Appendix Figure S26 - Protocol for Appendix Fig S27, which distinguishes dormant from dead spores by measuring their GFP levels without nutrients on different days of incubation in water without any nutrients at 30 OC (complements Fig. 5B). Protocol for measuring how the ability to express 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. 5B), 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. 5A). 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 Appendix Fig S27 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 Appendix Fig S27 and discriminate them from the GFP levels of dead spore bags.

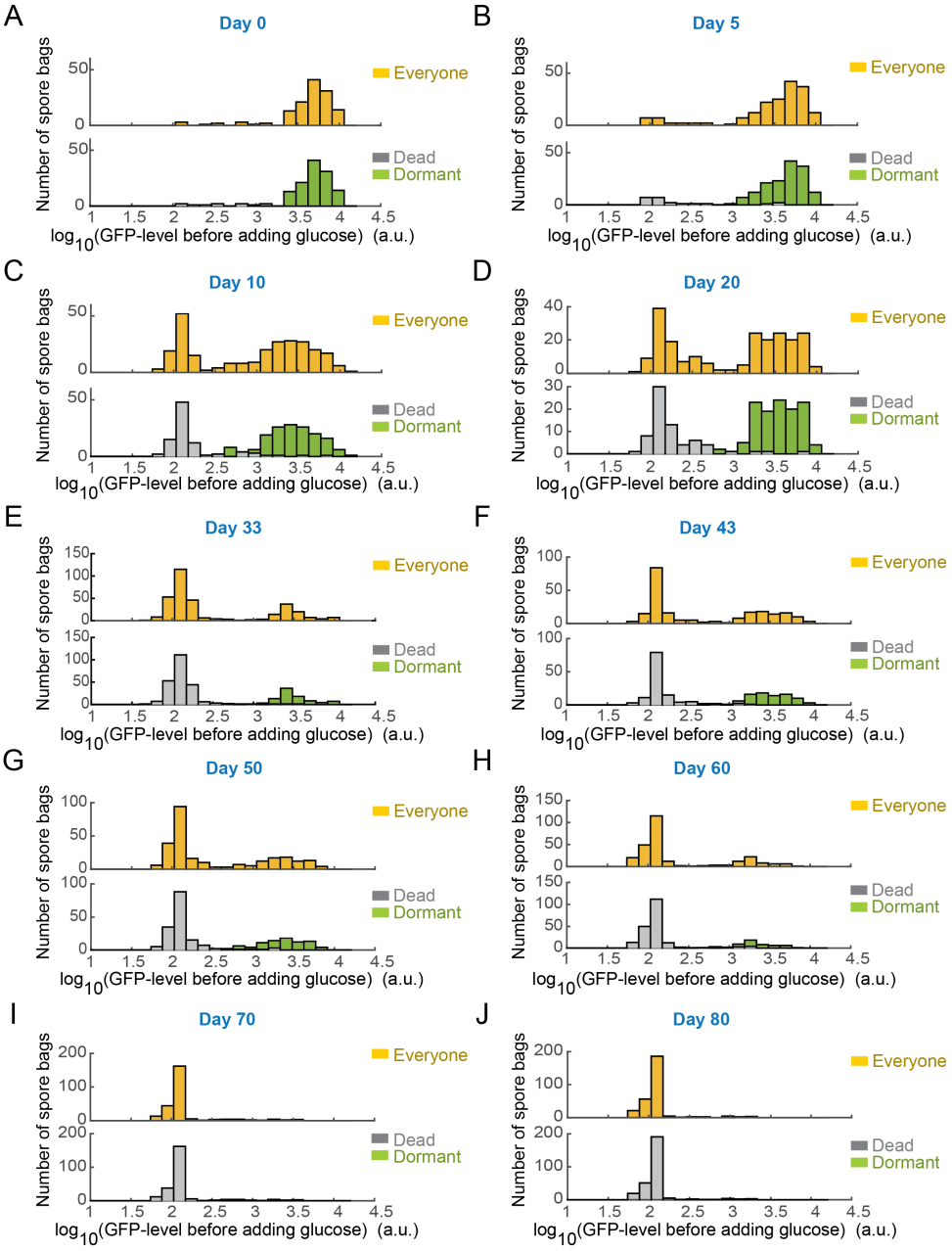

A
Day 0
B
Day 5
C
Day 10
D
Day 20
E
Day 33
F
Day 43
G
Day 50
H
Day 60
I
Day 70
J
Day 80
Number of spore bags
Everyone
Dead
Dormant
log10(GFP-level before adding glucose) (a.u.)

Figure 3.9 ***(preceding page)*****:** Appendix Figure S27 | Dormant spores lose their gene-expressing ability before dying (complements Fig. 5B). With the procedure in Appendix Fig S26, 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 OC). 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).

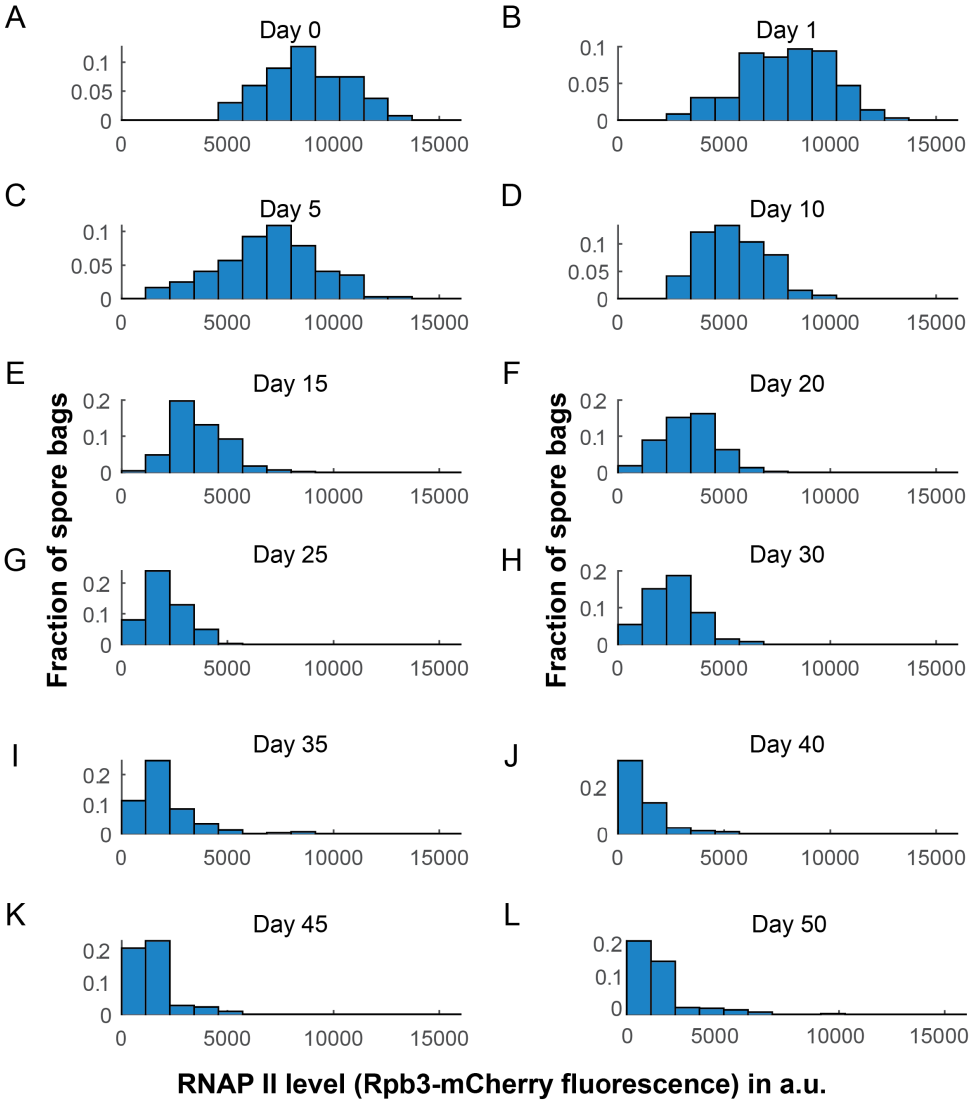


Figure 3.10: Appendix Figure S28 - Histograms showing RNAP II levels of ageing spores on different days (complements Fig. 5C). For each day of ageing shown in Fig. 5C, we show here a representative histogram (one population). Histograms show the RNAP II level of each spore bag in a population (i.e., fluorescence value from mCherry fused to Rpb3). Population averages of these histograms (3 histograms per day) are plotted in Fig. 5C. (A) Day 0. n = 134 spore bags. (B) Day 1. n = 181 spore bags. (C) Day 5. n = 184 spore bags. (D) Day 10. n = 169 spore bags. (E) Day 15. n = 228 spore bags. (F) Day 20. n = 191 spore bags. (G) Day 25. n = 194 spore bags. (H) Day 30. n = 139 spore bags. (I) Day 35. n = 160 spore bags. (J) Day 39. n = 117 spore bags. (K) Day 45. n = 109 spore bags. (L) Day 50. n = 196 spore bags.

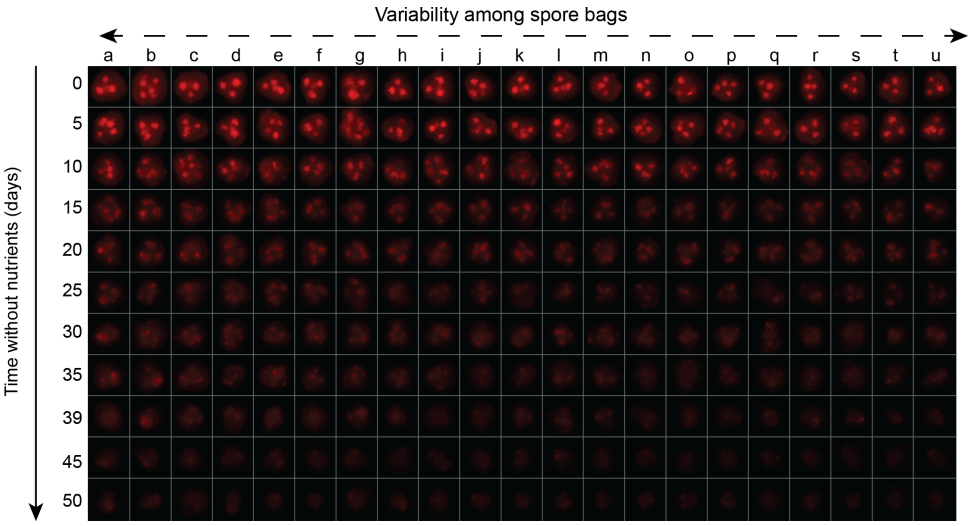


Figure 3.11: Appendix Figure S29 - Montage of spore bags showing RNAP II level (Rbp3-mCherry fluorescence) on different days of ageing (rows), with multiple spore bags per row to show variability in RNAP II level among spore bags on the same day (complements Fig. 5C). Each square contains a single spore bag and is $5\mu m$ x $5\mu m$. Red color shows the mCherry fluorescence (Rpb3 fused to mCherry) and its intensity represents the amount of RNAP II in each spore bag. In each row, the spore bag becomes dimmer as one traverses towards the right end of each row (towards "u"). Each row displays 21 representative spore bags that were sampled on a given day, from a single population that was ageing in water for 2 months at 30 OC (the same rotating tube for 2 months at 30 OC) (data shown in Fig. 5C and Appendix Fig S28). Rows show different days of ageing (from 0 to 50 days). Since the RNAP II level is sorted in each row, from highest (a) to lowest (u), we can see the variability in RNAP II level among the spore bags on each day. This representation allows us to directly visualize the main phenomenon described in Fig. 5C: RNAP II level gradually decreases over days during dormancy (i.e., the pictures become dimmer within a column as we traverse from top to bottom).

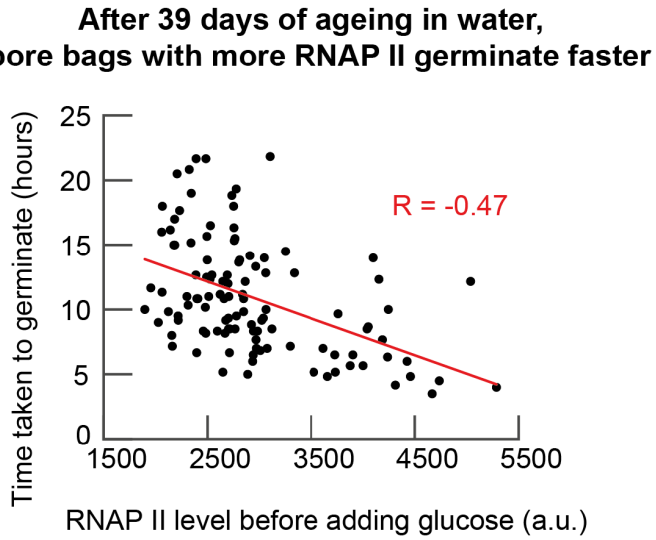


Figure 3.12: Appendix Figure S30 - Having more RNAP II means germinating faster (complements Fig. 5F). Time taken to germinate as a function of the RNAP II level (Rpb3-mCherry fluorescence level) in a spore bag. Each point represents a single spore bag from the same population. The population had been ageing in water for 39 days at 30 OC (same data as Fig. 5F). Red line is a linear regression fit: R = -0.47, Pearson p-value = 1.67 x 10-7. n = 111 spore bags. Note that some germinate after more than 10 hours after receiving the 2%-glucose.

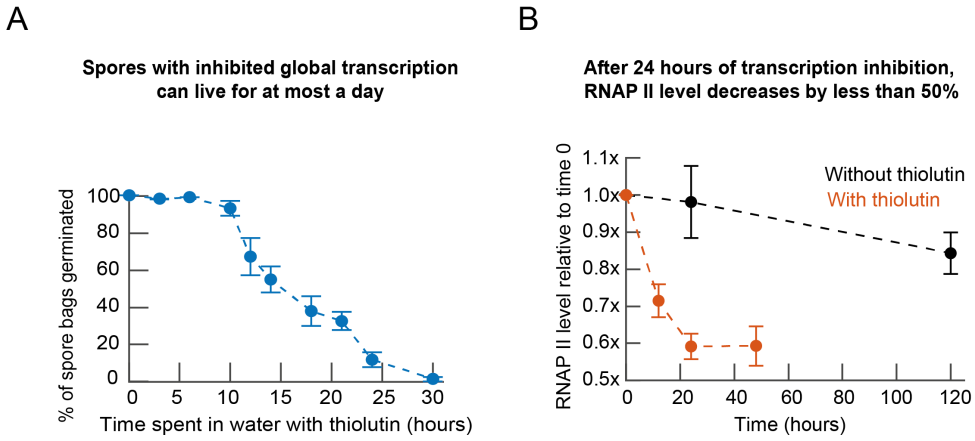


Figure 3.13: Appendix Figure S31 - Spores with inhibited global transcription (with thiolutin) can live for at most a day while their RNAP II levels stay relatively high throughout (at most 40% reduction in the RNAP II level after one day of thiolutin) (complements Figs. 7A-E). (A) Experimental protocol shown in Fig. 7A. Percentage of spore bags germinated for different durations of incubation in water with $10\mu g/ml$ of thiolutin. n = 3 populations. Errors bars are s.e.m. (B) RNAP II level relative to its value at time zero (i.e., value at the start of incubation in water with thiolutin (red) or without thiolutin (black) for different amounts of time. n = 3 populations. Errors bars are s.e.m. After 24 hours of thiolutin, almost all spores are dead (see (A)) while the RNAP II level in these dead spores is, on average, 60% of the level at time zero. Moreover, RNAP II level did not continue to decrease (or decrease at the same fast rate) between 24 hours and 48 hours of thiolutin as seen by comparing the last two points.

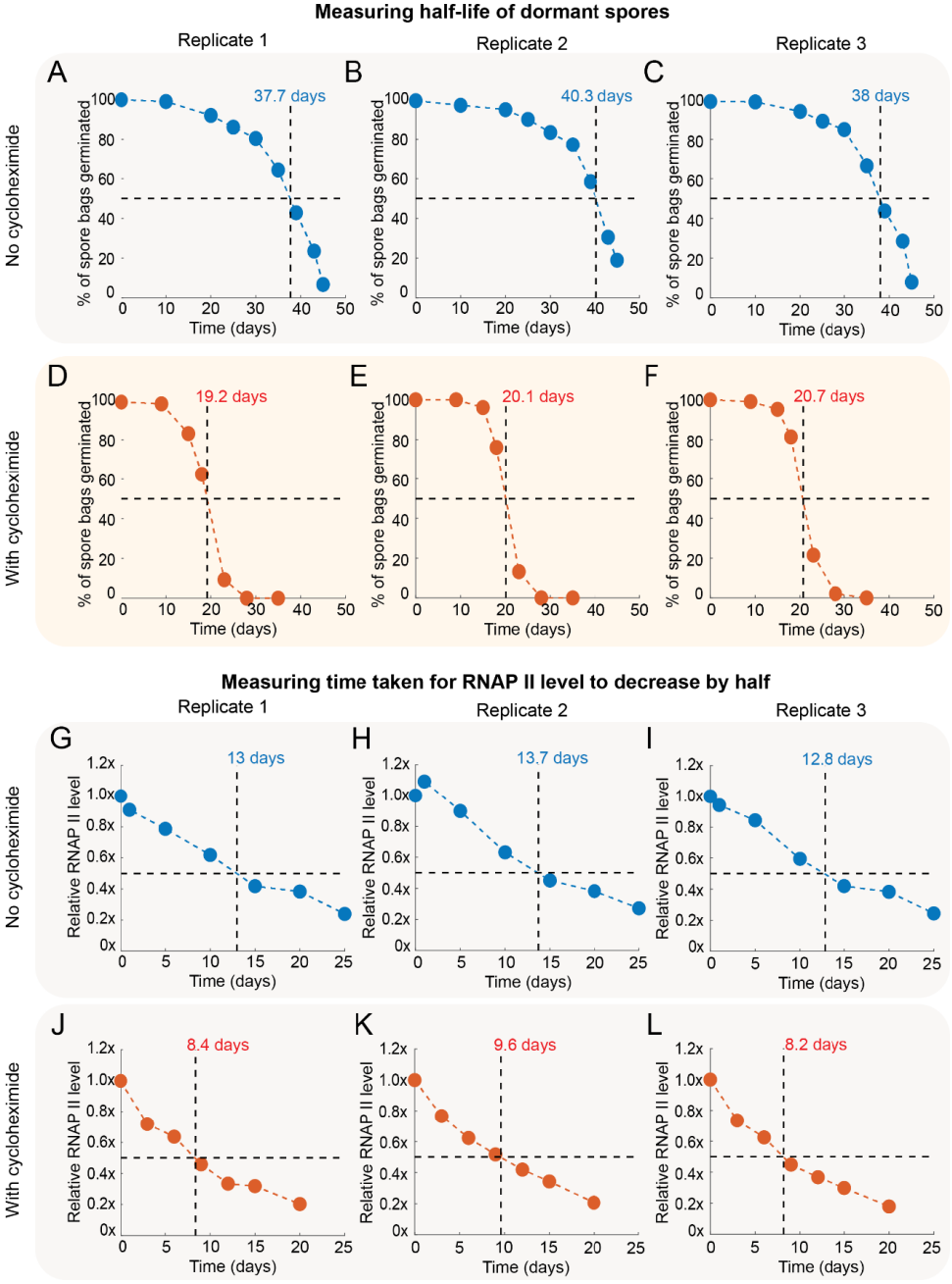
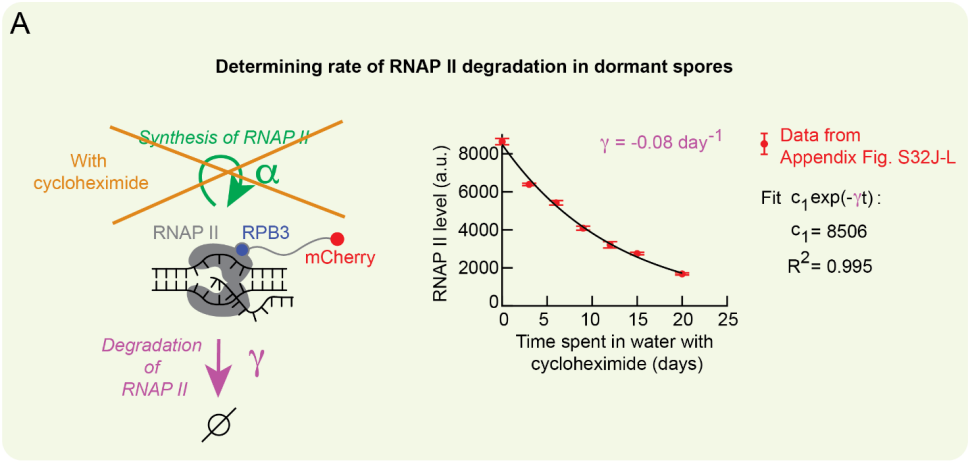

Measuring half-life of dormant spores
Replicate 1
Replicate 2
Replicate 3
No cycloheximide
With cycloheximide
A
B
C
D
E
F
37.7 days
40.3 days
38 days
19.2 days
20.1 days
20.7 days
% of spore bags germinated
Time (days)
Measuring time taken for RNAP II level to decrease by half
G
H
I
J
K
L
13 days
13.7 days
12.8 days
8.4 days
9.6 days
8.2 days
Relative RNAP II level

Figure 3.14 ***(preceding page)*****:** Appendix Figure S32 - Estimating half-life of dormancy and RNAP II (complements Figs. 7A-E). (A-F) Experimental protocol in Fig. 7A. Percentage of spore bags germinating after receiving a 2%-glucose after ageing in water without cycloheximide (blue points, A-C) or with 200$\mu g/ml$ of cycloheximide (red points, (D-F) for the designed days (values on horizontal axis). Each panel shows the half-life of dormancy (either in blue or red text) which we determined by a linear interpolation from finding the time at which 50% of the population had died (indicated by dashed black lines). (G-L) Average RNAP II level of dormant spore bags (Rpb3-mCherry fluorescence averaged over all dormant spore bags) relative to the level at the beginning of the ageing experiment in Fig. 7A. Blue points (G-I) are for spores aged in water without cycloheximide. Red points (J-L) are for spores aged in water with 200$\mu g/ml$ of cycloheximide. Each panel shows the time taken for the RNAP II level to decrease by 50% during ageing, which we determined by a linear interpolation, as indicated by the dashed black lines.



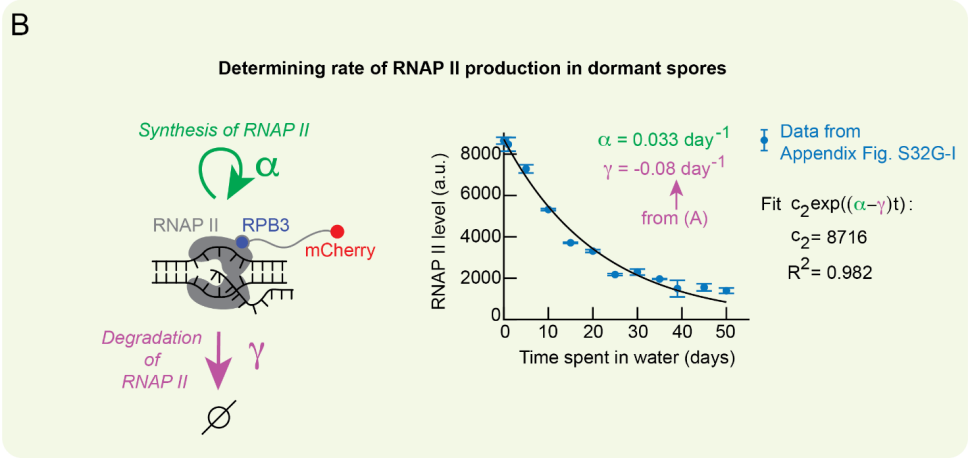
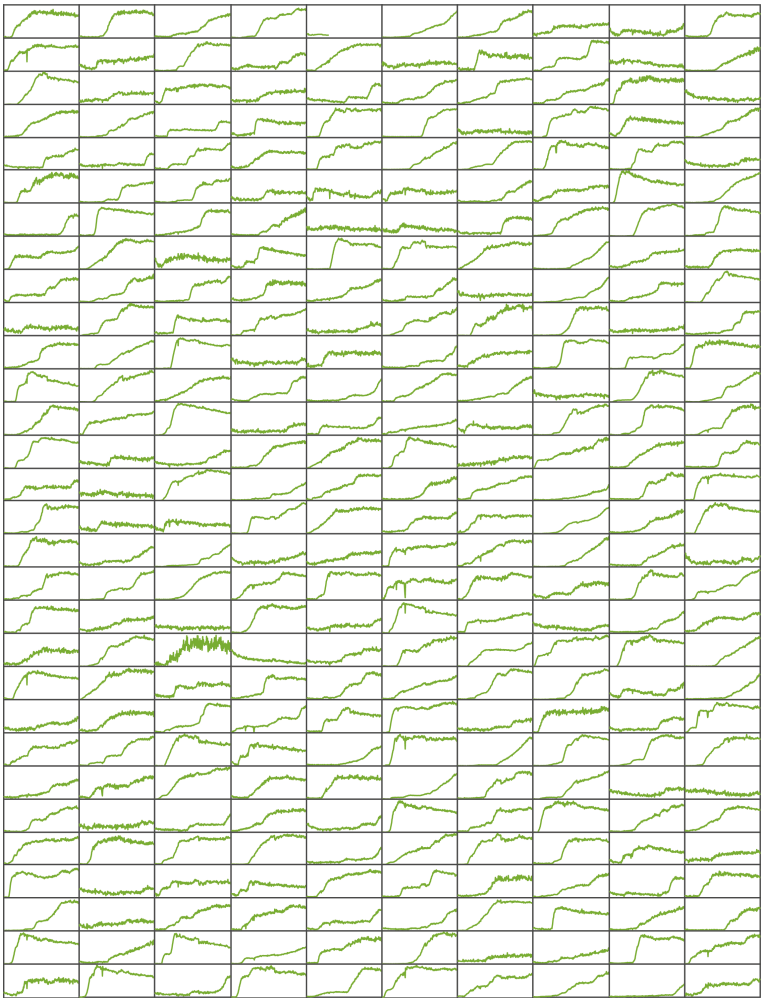


Figure 3.15 ***(preceding page)*****:** Appendix Figure S33 - Estimating production rate of RNAP II during dormancy (complements Fig. 7F). (A) We determined the degradation rate of RNAP II in dormant spores by using Rpb3-mCherry. For this, we assumed that when spores age in water with cycloheximide (translation inhibitor), their RNAP II level decreases purely due to the Rpb3 degrading, with each RNAP II molecule degrading with a probability per unit time of (see cartoon). Hence, we have: $(\frac{d[RNAPII])}{dt} = -\gamma.[RNAPII]$. Note that γ is the degradation rate constant. This equation yields an exponentially decaying solution, $[RNAPII](t) = c_1.exp(-\gamma.t)$, where c_1 is initial RNAP II level. We fit the solution (black curve in the graph) to the data (red points in the graph) for the ageing experiment done with cycloheximide. Red data points are from averaging the three replicate populations shown in Appendix Fig. S32J-L; $n = 3$, errors bars are s.e.m. From the fit, we obtained a degradation rate constant, $\gamma = -0.08$ with the fit agreeing well with the data ($R^2 = 0.997$). (B) Having determined the degradation rate, we now turn to determining the production rate of RNAP II during dormancy by using Rpb3-mCherry. For this, we assume that when spores age in water without any drugs, then the observed decrease in the RNAP II level during ageing occurs at a rate - a net loss rate - that is the difference between the production rate and degradation rate of RNAP II (with the degradation rate being higher than the production rate) (see cartoon). Then we have, $(\frac{d[RNAPII])}{dt} = (\alpha - \gamma).$[RNAP II], with γ being the degradation rate determined in (A) and α being the production rate. We thus have as the solution, [RNAP II](t)=$c_2 * exp((\alpha - \gamma).t)$ where c_2 is the initial RNAP II level at the start of the ageing. We fit the solution (black curve in the graph) to the data (blue points in the graph) for the ageing experiment without any drugs. Blue data points are from averaging the three replicate populations shown in Appendix Fig S32 G-I;$n = 3$, errors bars are s.e.m. Equivalently, we can fit the solution to the data in Fig. 5C. For the fit, we fix $\gamma = -0.08$ as determined in (A). Hence α and c_2 are the only unknown parameters to fit. The fit yielded, $\alpha = -0.033$, which agreed well with the data ($R^2 = 0.982$). If we ignore degradation by setting, then we would get $[RNAPII](t) = exp(\frac{t}{\tau})$, with $\tau = \frac{1}{\alpha} = 30.3$ "days" , being the "characteristic time" for RNAP II production in a dormant spore in water without any drugs. As a comparison, this is about $15-to-76$-fold smaller than the characteristic time of 0.4-to-2 days measured in spores that have received a 2%-glucose and are on their way to germinating (Fig. 5E - blue points). These results establish that RNAP II production occurs during dormancy and that gene expression is occurring, albeit extremely slowly, during dormancy (e.g., expression of genes coding for RNAP II).

4

Gene expression dynamics during dormancy



Contents

4.1. Introduction : quantifying gene expression in dormant spores

Confronted to growth-inhibiting conditions, many organisms undergo a programmed isolation from the environment, usually called "dormancy", whereby they both shield from the outside stress and survive without external nutrient (Chapter1,Fig.1.4). This specific state is thought to rely on a "nearly ceased" metabolism, allowing survival with minimal energetic expenditure, and thus providing long lifespan under growth prohibiting conditions (Lennon et al. 2011,**Ritterhaus2013**,Lennon et al. 2021). Indeed, several studies have established that during dormancy, energetic metabolism or gene expression are reduced to a few percent of their non-dormant, replicating phenotype (**Pagget1993**, Brengues et al. 2002,Segev et al. 2012). Furthermore, several studies showed that inhibiting that "nearly ceased" activity drastically shortens the lifespan during dormancy (**Gengenbacher2010**, Maire et al. 2020). Thus, while being of weak intensity, the biochemical reactions taking place during dormancy are necessary for survival. Therefore, identifying and quantifying these biochemical reactions in greater details is a critical step towards understanding how organisms remain alive during dormancy. Specifically quantifying the rates of the central dogma of molecular biology, i.e. transcription, translation and degradation of mRNA and proteins (Fig.4.1A), would be an important step towards deciphering the gene expression dynamic supporting survival during dormancy. Historically, this has been challenging, simply because one needs methods that allow sufficient resolution to distinguish the weak signal of the nearly ceased activity from the instrument's noise.

Here, based on insights from previous work (Maire et al. 2020), we propose to overcome that limitation by adopting a different strategy. Instead of trying to measure the endogenous "nearly ceased" gene expression, we leverage on the expression of a single inducible reporter gene (exogenous) during dormancy. Indeed, our previous work showed that it was actually straightforward to induce GFP expression during dormancy of yeast spores (Maire et al. 2020). The main advantage of using a synthetic inducible gene is that we can easily and unambiguously control and measure its expression at the single-spore level, allowing to gain critical insight on the expression dynamic (Rosenfeld et al. 2005). The main inconvenient, however, is that we measure the expression of an induced synthetic gene, which can sensibly differ from the expression of an endogenous gene. In this study, we aim to learn something new about the endogenous gene expression that is important for survival during dormancy. But *what do we already know about gene expression of endogenous genes during dormancy of yeast spores?*

Biochemical studies using radioactive labelling showed that in the absence of exter-

nal nutrients, *S.cerevisiae* yeast spores survive for weeks, transcribe and translate at a reduced rate, estimated to be 5% of vegetative cells (Brengues et al. 2002). Moreover, in our previous work we showed that inhibiting transcription during dormancy killed spores in 24 hours, whereas inhibiting translation only divide the lifespan by two (Maire et al. 2020,Fig.4.1B). These results indicated to us that transcription, despite being greatly reduced, plays a critical role in survival of dormant spores. Therefore, we sought to focus on quantifying transcription dynamics during dormancy of yeast spores.

As a starting point, we examine methods to directly look at transcription dynamic of endogenous genes during dormancy of yeast spores. Then we leverage on the versatility of the synthetic inducible circuit to perform quantitative single spore experiments. In parallel, we use these results to build a minimal mathematical model of gene expression dynamic during dormancy. Finally, we show preliminary results to probe the relevance of this model on endogenous genes expression dynamics.

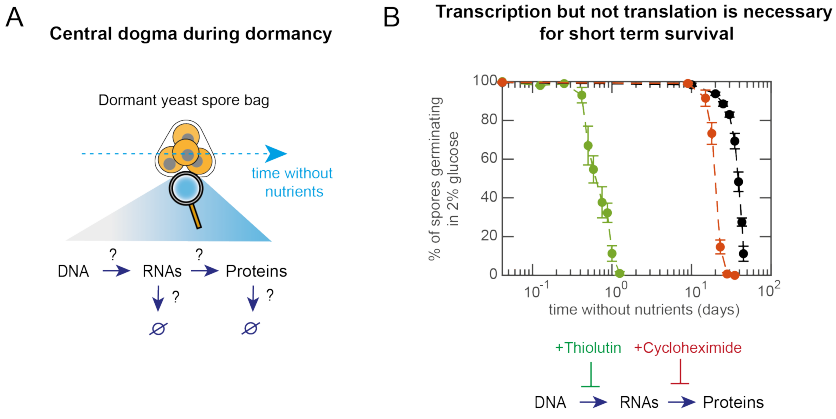


Figure 4.1: Investigating dynamic of gene expression in dormant yeast spores. A, While gene expression of *S.cerevisiae* replicating cells has been quantitatively investigated in numerous studies, basic quantification of production and degradation of mRNA and proteins are unknown in dormant yeast spores as they survive without nutrients,**B**. Spores were incubated for days in either PBS (black dots), PBS+100$\mu g/ml$ Cycloheximide (red dots) or PBS+10$\mu g/ml$ Thiolution (green dots) and the fraction of spores able in to germinate in minimal medium (SC) with 2% glucose was measured at several time point with time-lapse microscopy. error bar are s.e.m $n = 3$ biological replicates.

4.2. Probing transcription of endogenous mRNA during dormancy

Knowing that transcription was necessary for short term survival of dormant spores, we first sought to directly measure transcript level during dormancy. Here we present our tentative to do so by combining a genome wide approach (4tU-seq) to measure the relative average transcript level of many genes, with single molecule RNA FISH to measure the absolute transcript number at single spore level for a few target genes.

4.2.1. Towards 4tU-seq to measure transcription during dormancy

Based on previous work, we know that it is possible to perform RNA-seq of dormant spores RNA (Maire et al. 2020,Fig.2.3B). However, on itself, this assay does not allow distinguishing between the RNA made during dormancy and the one made during sporulation or inherited from the diploid cell before sporulation.

In order to sequence only the RNAs made during dormancy, we used 4tU-seq, a technique based on metabolic labelling that physically select for the newly synthesized RNA (Baptista et al. 2018,Barrass et al. 2015). We incubated dormant spores with a modified pre-cursor of uridine, 4-thiouracil, which incorporates in newly made transcripts in place of uridine. Then after total RNA extraction, we physically separated the newly made RNA (containing modified uridine) from already present RNA (containing non-modified uridine) (Fig.4.2A). As a proof of principle, we performed a "pulse" experiment by sequencing 4tU-labelled RNA 0, 5 and 15 minutes after addition of 4tU in the medium (Fig.4.2B). Using a known a amount of 4tU-labelled S.pombe RNA (see methods) as a reference spike in we could accurately normalize the RNA content for each time point. Looking at the total transcript level per time point, we found a small increase between 0 and 5 minutes but an important increase between 5 and 15 minutes (more than 10 fold). This confirmed that transcription was happening during dormancy and indicated that it might be happening relatively fast (in tens of minutes), similar to replicating yeasts cells (Barrass et al. 2015).

Looking at a few specific *S.cerevisiae* genes known to be highly expressed in replicating cells (like ENO1, RPS3 etc), we found that these were also highly produced (relatively to other genes) during dormancy (Fig.4.2C). Additionally, comparing the level of newly made transcripts after a short pulse of 4tU with total transcript (newly made + already existing transcripts) revealed that positive correlation (Fig.4.2D , Supp.Fig.4.11). This last result may indicate that the distribution of mRNA in dormant spores results mostly from the transcription during dormancy.

Importantly, these results should be interpreted with caution, since we have only

one biological replicate and 3 time points (other replicates and time points two were "lost" during sequencing). Therefore, we can only say that 4tU-seq is a promising technique to explore genome-wide transcription at the population level during dormancy.

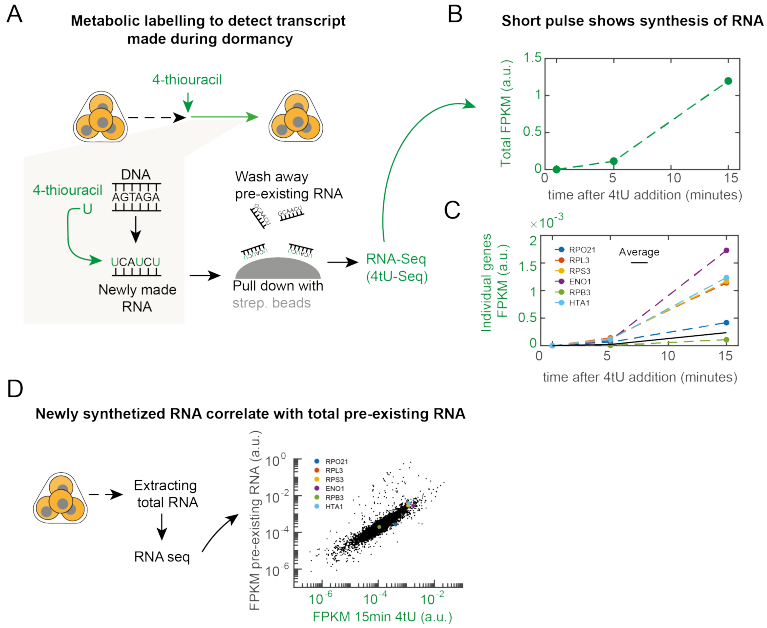


Figure 4.2: Metabolic labelling of RNA during dormancy pinpoints rapid transcription in dormant spores. A 4thio-uracil linked to biotin, an analog of uracil incorporate in newly made RNA (after natural transformation into 4tU uridine) if transcription is happening in dormant spores. After incubation and total RNA extraction, 4tU-biotin bind streptavidin beads, allowing to selectively enrich for newly made transcripts, which with sequence and relative abundance are probed by RNA-seq. **B** Total FPKM, i.e. the sum of FPKM for all transcripts detected, as function of time after addition of $[\mu g/ml\ ]$ 4thio-uracil to TT14 spores preincubated for 4 days in PBS at 30C. All FPKM are normalized by a reference S.pombe spike-in. (see Methods). Data from only 1 biological replicate. 2 other replicates were prepared and sequenced but a technical issue during the library preparation prevented further analysis. Average over n=5058 transcripts. **C** Same as B, but for 6 individual coding genes **D** (Left)Total RNA not (not distinguishing newly made from already present) of TT14 spores were extracted after 4 days of incubation in PBS at 30C, then these RNA were sequenced with RNA-seq. (Right) Correlation between the FPKM of pre-existing transcripts and FPKM of newly made transcripts after 15minutes incubation with 4tU. Each black dot represent a single ORF, $n =$. Colored dots are the genes used in C.

4.2.2. RNA FISH shows low and variable amounts of RNA per spore

While transcriptome-wide technique such as RNA-seq or 4tU-seq allows could be powerful to estimate relative abundance and production rate of RNA, they can not be used to estimate the absolute number of RNA molecules present in individual spores during dormancy.

Using single molecule RNA FISH ((Raj et al. 2006,**Youk2010**)), we counted the number of individual mRNA molecules of a specific gene within fixed dormant spores (Fig.4.3A,Supp.Fig.4.16), as well as in diploids replicating cells. For RPL3, we found on average ~ 100 copies per diploid cells, but in spores we found only ~ 2.5 copies. (Fig.4.3B,C). We found similar results for RPO21 and RPS3 (Fig.4.3D).

In order to check that this low number of mRNA was not due to an artifact of RNA FISH on spores, we counted mRNA of ENO1, which is known to be extremely abundant in vegetative yeast cells (not countable by RNA FISH) (Supp.Fig.4.16A). There we found individual spores with up to ~ 60 mRNAs copies. Moreover, by looking at other genes (7 in total), we found that in spores, while the average number of mRNA is low (around 1 copy per spore), a large fraction do not have any mRNA (around 50%) and a small fraction have around 10 copies or more (Supp.Fig.4.16B). Therefore, these results seem to indicate that rather than a measurement artifact, the low average number of mRNA is due to an important phenotypic variability, with most of the spores having close to zero mRNA and some rare spores having up to 5-50 mRNAs.

In conclusion, we confirmed that transcription is happening during dormancy in spores, and revealed that the total number of RNA transcribed is on average very low and highly variable between individual spores.

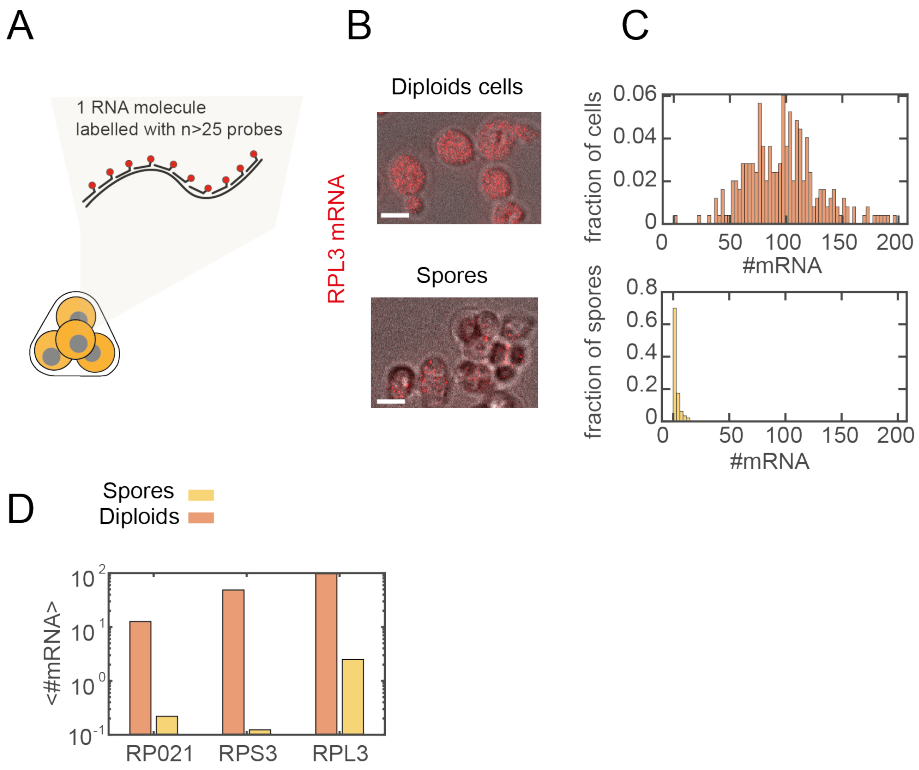


Figure 4.3: Single molecule FISH on endogenous mRNA during dormancy A. Multiple probes ($20 > n > 45$) each labelled with the same fluorophore are hybridized to a specific mRNA. Therefore single molecule RNA FISH allow to detect a specific single mRNA as a diffraction limited dot, and then count the number of mRNA of a given genes per individual spore (using FISHquant Mueller et al. 2013). **B** Composite image of RPL3 FISH in replicating cells in log phase in SC+2% (top) and dormant spores after 4 days in PBS (bottom). Brightfield and red represent the fluorescence of Qasar570 fluorophore. Scale bar is $5\mu m$. **C** Histograms RPL3 mRNA copy number of $n = 247$ diploids (top) cells and $n = 444$ spores.(bottom) **D** Average mRNA copy number for 3 different genes, RBP1, RPS3 and RPL3 for both diploids replicating cells (red bars) and dormant spores (yellow bars). error bar are s.e.m. over $n = 3$ replicates.

4.3. Dynamic of induced transcription

What is the underlying dynamic of transcription during dormancy?

Accordingly, previous results are not sufficient to conclude about transcription dynamics during dormancy. Beyond technical difficulties, one major challenge in quantifying transcription dynamics is to distinguish the mRNA pre-existing in spores from the mRNA that are newly transcribed after spores formed (i.e. during dormancy).

Here, we propose to overcome that ambiguity by quantifying the transcription of

a single exogenous reporter gene controlled by an inducible promotor. In previous work we demonstrated that upon induction with doxycycline, genetically engineered spores can slowly accumulate GFP proteins, even in the absence of external nutrients (Fig.2.4A-C). If we can detect accumulation of GFP proteins (by fluorescence), this means GFP mRNA were produced as well. Now, we sought to probe the synthesis of GFP mRNA upon induction with doxycycline during dormancy.

4.3.1. Time course mRNA FISH

Using single molecules RNA FISH, we measured the number of GFP mRNA molecule per individual spore after induction in PBS with $100\mu/ml$ doxycycline (Fig.4.4A). After 3 hours of induction we could already observe some individual spores had more than 40 mRNAs while others had close to 0 mRNA (Fig.4.4B).

In order to assess the transcriptional dynamic upon induction, we measured the abundance of mRNA over a time course from 2 minutes to 120 hours. We found that average number of mRNA per spore regularly increased over the first hours before starting to decrease irregularly from 24h to 120h (Fig.4.4C). First, let us notice this early dynamic is consistent with our previous genome-wide pulse experiment, in which we could detect newly made transcript after 15 min (Fig.4.2B). However, within a population and for a given time point, the amount of mRNA varied greatly, with a small fraction of spores (less than 5%) having between 10 and 100 mRNAs (Fig.4.4B,supp.Fig.4.17). Specifically, we found this "long-tail" distribution to remain qualitatively the same throughout the time-course.(supp.Fig.4.17). Interestingly, this distribution of mRNA copy number from the induced gene qualitatively matches the distribution of the most abundant endogenous gene we could measure (ENO1, Supp.Fig.4.16).

Knowing that for a given time point, the abundance of mRNA varied greatly between individual spores, with most spore (80%) without any detectable RNA, we looked separately to the fraction of spores having 1 or more RNA (Fig.4.4D) and the average number of mRNA in spores having 1 or more RNA (Fig.4.4F). This distinction revealed that the average number of mRNA in spores having 1 or more RNA actually increased regularly before reaching a steady state, while the fraction of spores having 1 or more RNA decreased irregularly after 24 hours.

Then we repeated the same induction experiment but with lower Doxycycline concentrations, which is equivalent to vary the promotor strength, (see Fig.2.4A), and look at the fraction and average number of mRNA after 24 hours of induction. This showed that the average number of mRNA increased regularly with Doxycyline concentration (Fig.4.4E) (as expected if you increase the strength of transcription) while the fraction of spores only slightly changed, remaining between 0.1 and 0.2

across all tested Doxycyline concentrations (Fig.4.4G).

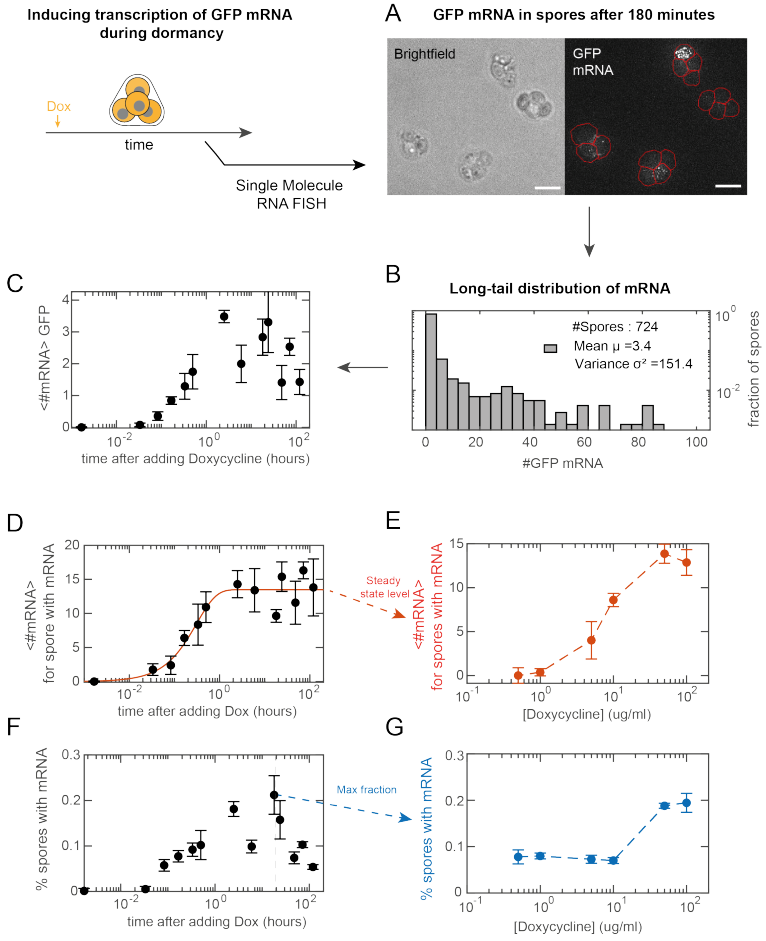


Figure 4.4: Transcription of GFP mRNA in inducible dormant spores. **A**. TT14 spores (GFP inducible) after 180min in PBS+100$\mu g/ml$ Doxycycline, fixed and GFP mRNA hybridized with smFISH probes (Calfluor 610) (see methods) (Left) Brightfield image, (right) Maximum z-projection projection of 40 slices of 0.2μm. Red lines indicate segmented masks, within which the number of dots (i.e. number of GFP mRNA) is counted. Scale bar is 5μm.**B**, Histogram of GFP mRNA, n=724 spores.**C** Average number of GFP mRNA after different times in PBS+100$\mu m/ml$ Doxycycline.error bar are s.e.m n=3.**D** Average number of GFP mRNA for spores having at least one GFP mRNA.error bar are s.e.m n=3. Red line is a fit from equ.4.6 :$y = \frac{\nu}{\delta}(1 - e^{-\delta t})$; fitted $\nu = 43.2 mRNA.hours$; $\delta = 3.2 hours^{-1}$; $R^2 = 0.89$. **E** Steady state average number of GFP mRNA for spores having at least 1 GFP mRNA, as function of Doxycycline concentrations (0.10.551050100$\mu g/ml$).**F**. Fraction of spores having at least 1 mRNA. error bar are s.e.m $n = 3$. **G**. Maximum fraction of spores having at least 1 mRNA as function of doxycycline concentrations. error bar are s.e.m $n = 3$.

4.3.2. Random telegraph model of transcription

With these first insights on induced transcription, we sought to build a simple model. Indeed, while mRNA FISH assay does not directly probe the transcription dynamic of single spores over time, previous observations (long-tail distribution and effect of inducer on the fraction) in other systems such as mammalian cells have been interpreted to be the consequence of the random activation and inactivation of transcription (Raj et al. 2006,Raj et al. 2008,Munsky et al. 2012). Specifically, researchers have been building upon the "two-state random-telegraph model", initially defined and studied in (Peccoud et al. 1995). We will now briefly describe that model before using it to infer the features of transcription dynamic in our experiments in dormant spores.

In its simplest version, the model assumes transcription randomly and spontaneously switches between an active state "A", in which mRNA is transcribed at a high rate ($\nu > 0$), and an inactive state "I", in which mRNA is not transcribed ($\nu = 0$) (Peccoud et al. 1995). The transition dynamic between the two state is fully determined by both the activation rate λ and the inactivation rate μ. When transcription is in the active state "A", mRNA molecules "M" are then produced at a constant rate ν. Moreover, mRNA molecules "M" are always degrading at rate δ :

$$I \xrightarrow{\lambda} A \tag{4.1a}$$

$$A \xrightarrow{\mu} I \tag{4.1b}$$

$$A \xrightarrow{\nu} A + M \tag{4.1c}$$

$$M \xrightarrow{\delta} \emptyset \tag{4.1d}$$

This model has been studied extensively (Peccoud et al. 1995 Munsky et al. 2012), so we will only summarize important results and notation we will use afterwards. First, assuming the system is at steady-state, we have that the probability to be in the active state p_{ON} (or inactive state p_{OFF}) and that the average time spent in the active (rsp. inactive state), T_{ON} (rsp. T_{OFF}) :

$$p_{ON} = \frac{\lambda}{\lambda + \mu} \tag{4.2a}$$

$$p_{OFF} = \frac{\mu}{\lambda + \mu} \tag{4.2b}$$

$$T_{ON} = \frac{1}{\mu} \tag{4.2c}$$

$$T_{OFF} = \frac{1}{\lambda} \tag{4.2d}$$

We also introduce α_M the steady state number of mRNA and τ_M the half-life of mRNA "M".

$$\alpha = p_{ON} \frac{\nu}{\delta} \tag{4.3a}$$

$$\tau_M = \frac{log(2)}{\delta} \tag{4.3b}$$

Depending on the ratios between these two last parameters, the model yields qualitatively different regimes (Munsky et al. 2012). Of interest for us here is the "bursting" regime ($\frac{\tau_M}{T_{ON}} << 1$ and $\frac{\tau_M}{T_{OFF}} >> 1$), which yields a long-tail distribution of mRNA (Munsky et al. 2012), similar to such as the one we observed by measuring GFP mRNA upon induction with doxycycline (Fig.4.4B).

Importantly, this type of model with random activation and deactivation of transcription is the simplest to reproduce the distribution of mRNA we observed (Fig.4.4B). Indeed, a "simple" constitutive production of mRNA, (i.e. assuming in our model 4.1 that the gene is always in the active state) always yield a Poisson distribution of mRNA at steady state (Raj et al. 2008), which can not capture the variance we observe (Fig.4.4B). Additionnaly looking at the distribution of mRNA over time supports the assumption that the transcription dynamic quickly reaches a steady state (Supp.Fig.4.17).

Assuming that the transcription follows the random activation de-activation model (4.1), it is possible to directly extract of the rates of de-activation and activation (λ and μ) from the steady state distribution of mRNA Peccoud et al. 1995). Applying this method, the GFP mRNA distribution yielded : (4.2):

$$p_{ON} = 0.03 \tag{4.4a}$$
$$T_{ON} = 4.6 minutes \tag{4.4b}$$
$$T_{OFF} = 2.6 hours \tag{4.4c}$$

These values are given within a constant, and estimating their values in units of time (minutes, hours) requires scaling by the degradation rate of mRNA (Peccoud et al. 1995). Here we used a degradation rate of mRNA-based on a half-life $\tau_M = 10 minutes$, which corresponds to a conservative estimate based on the timescale of GFP mRNA accumulation upon induction (Fig.4.4C-D) and the known mRNA decay kinetics in replicating yeasts cells (Chan et al. 2018) (see Box.4.3.2). However, several other measurements seem to indicate a significantly longer half-life (potentially days) of mRNA during dormancy (see Box.4.3.2).

This discrepancy points out our current lack of knowledge about the specificity of mRNA decay kinetics during dormancy compared to replicating cells. Therefore, for future work we should not exclude the possibility that some (or all) mRNAs in dormant are particularly stable because active degradation mechanism (with nucleases) are shut down during dormancy. Here for the rest of this chapter (all simulations) we will use the assumption that mRNAs are degrading relatively fast in spores (half-life ~ 10 minutes), so that our work should be read as :

Assuming that in dormant spores mRNA are degrading fast (half-life of ~ 10 minutes), what is the most likely model of gene expression to explain our experimental data?

What is the half-life of GFP mRNA in dormant spores?

1. The most direct way to measure the degradation rate of GFP mRNA is by inhibiting transcription (with Thiolutin) after induction with doxycycline and following the decay of GFP mRNA by smFISH or qPCR. These experiments showed virtually no decay of GFP mRNA even after several days of incubation (not shown here in this thesis).
2. An alternative way to estimate mRNA degradation rate is to infer it from the dynamic of mRNA accumulation upon induction. Specifically, assuming a simple model of production / degradation of mRNA, the typical time it takes for the average copy number of mRNA to reach a steady state is directly linked to the degradation rate of mRNA. Indeed, in its simplest version (with constitutive expression), the evolution of the average number of mRNA $< M >$ is given by (mean-field approximation) :

$$\frac{d < M >}{dt} = \nu - \delta \cdot < M > \tag{4.5}$$

which yields the solution :

$$< M > (t) = \frac{\nu}{\delta}(1 - e^{-\delta t}) \tag{4.6}$$

This simplified model fitted accurately our measurement of the average number of mRNA *in spore having mRNA* (Fig.4.4D). This yielded a half-life of GFP mRNA around 11 minutes :

$$\tau_M = \frac{log(2)}{\delta} = 10.7 minutes \tag{4.7}$$

3. In dormant yeast spores, previous studies estimated with Northern Blot that PGK1 mRNA gradually decayed over 2 days, with a half life of 10 hours. (Brengues et al. 2002). However, we know that mRNAs are being produced in during dormancy (this work and Brengues et al. 2002) and in this assay transcription was not inhibited. Therefore, the measured half-life of 10 hours is the result of both production or degradation. As a consequence, the half-life when mRNAs are only degrading could be at most 10 hours, but potentially much less.
4. In replicating vegetative *S.cerevisiae* cells, non-pertubative methods (based on extensive 4tU-seq) indicate the average half life of mRNAs at 30C is around 5 minutes, with long-lived mRNA around 20-30 minutes (Chan et al. 2018).

With that assumption on the degradation rate of mRNA and with the other parameters estimated from the distribution of mRNA (4.4), we ran simulation of our model 4.1(with the Gillespie algorithm) to visualize predicted mRNA dynamic within single spores (Fig.4.5B).

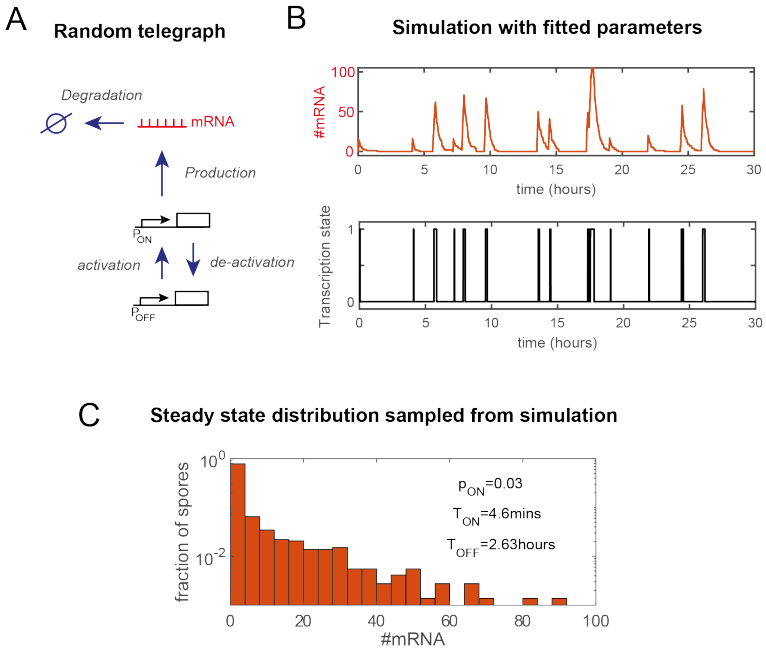


Figure 4.5: Simulation of mRNA dynamic with the random telegraph model.A.Random telegraph model for transcription (see also 4.1). 2 variables : "Transcription state" (can be 1 active or 0 inactive) and "#mRNA" (number of mRNA molecules, can be any positive integers). 4 reactions are modeled : (1) "Transcription state" goes from active to inactive (deactivation) (2) "Transcription state" goes from inactive to active (activation) (3) "#mRNA" is increased by 1 (production of mRNA) (4) "#mRNA" is decreased by 1 (degradation mRNA). **B** Simulation of model in (**A**) as a continuous time Markov jump process with the Gillespie algorithm. Simulation was stopped when the reaction time was 30 hours. Reaction rates were set according to normalized parameters : (fraction of time transcription is active) $p_{ON} = 0.03$; (average time transcription is active) $T_{ON} = 4.6 minutes$; (half-life of mRNA) $\tau_M = 10.7 minutes$; (steady number of mRNA) $\alpha = 3.4 mRNAs$ **C**. Histograms of mRNA at steady state obtained by taking the number of mRNA ("$\#mRNA$") after 40 hours, after $n = 1000$ independent simulations.

As expected from the shape of the distribution of mRNA (**Musnsky2012**), this model predicts that at the single spore level, transcription occurs in discrete "bursts". Concretely, over time we distinguish two dynamics : either the promotor is in the "active" state and GFP mRNA are quickly produced, accumulating up to $\sim 80-100$ mRNAs in a $5-10$ minutes, or the promotor is in the "inactive" state and mRNA only degrades before staying close to zero for several hours

. We also sampled the distribution at steady and verified it reproduced the distri-

bution of GFP mRNA we measured with RNA FISH (Fig.4.5C).

Importantly, even if that "burst-like" dynamic looks similar to the burst of transcription observed in many systems such as yeasts and mammalian cells (Raj et al. 2006,Raj et al. 2008), the timescale of deactivation is here predicted to be much longer (1-10 hours instead of a few minutes).

Overall, our model predicts that at in single spores, transcription randomly stops for hours. This mechanism is sufficient to explain why when we measure the mRNA distribution at a given time, most spores have close to zero mRNA, while some rare spore have up to 50 mRNAs. We will now confront this prediction to the dynamic of protein synthesis at single spore level.

4.4. Dynamic of induced protein synthesis

In our previous work, we already measured GFP protein expression upon induction (Fig.2.4) and found two interesting features : protein synthesis was very slow (increasing for 24 hours) and highly variable between spores. Crucially, these measurements were based on averaging GFP fluorescence over "spore bag" (containing 2-4 spores) and not on single spores within a spore bag. However, by measuring GFP transcript level we found that within spore bag, sometime one spore had plenty of mRNAs while the 3 other spores had zero (see image Fig.4.4A). With that insight, we sought to measure GFP protein expression at the single spore level.

4.4.1. Single-spore tracking reveals discontinuous GFP expression

We segmented and tracked GFP fluorescence of individual spore after induction in PBS+30$\mu g/ml$ Doxycycline over 42 hours (Fig.4.6A). Strikingly, by looking at several individual traces, we found that many spores displayed a "stair-like", discontinuous expression with 1 to 5 distinct "steps" (Fig.4.6B). In other words, for many spores, GFP expression alternated between periods of slow but regular increase and periods where fluorescence level remained constant (or slightly decreased) for hours (up to 30 hours). Importantly, we verified these kinds of curves were reflecting the behavior of single spores and not the sum of the signal of different spores (especially when in the same spore bag), as shown in Fig.4.6 where only one spore within the spore bag expresses GFP over 42 hours, and still display 2 distinct "steps".

4.4.2. Steps are caused by an arrest of protein synthesis

What could cause the discontinuous expression in single expression protein expression?

We first hypothesized that periods during which GFP fluorescence did not increase occurred because protein synthesis stopped. Indeed, as reported in a previous chapter, GFP proteins seem to be very stable in spores, such that their apparent half-life exceeded the duration of our time-lapse (Supp.Fig.2.28). Therefore, if protein synthesis stops, we expect GFP protein level to virtually remain constant over tens of hours. In order to test that hypothesis, we performed an experiment in which we inhibited protein synthesis with Cycloheximide only 12 hours after inducing GFP expression. As expected, addition of cycloheximide caused the average fluorescence level to remain constant, whereas it continued to increase in the control without cycloheximide (Fig.4.7A). Additionally, tracking the same spores before and after inhibition of protein synthesis revealed that we could artificially create a step in fluorescence level, similar to the ones observed previously (Fig.4.6B). Overall, these experiments showed that randomly stopping protein synthesis is a

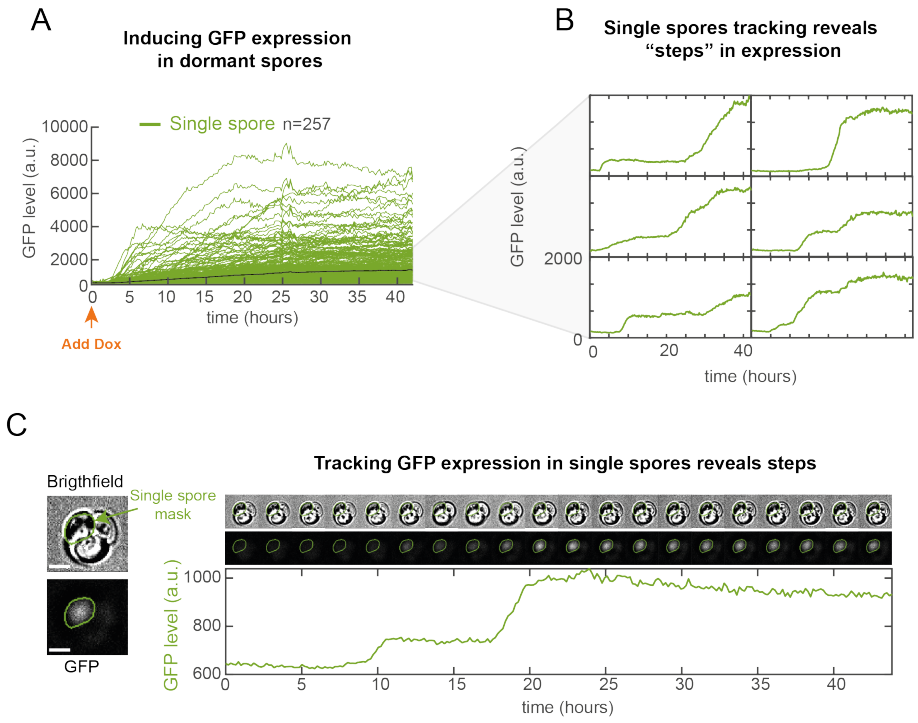


Figure 4.6: Discontinuous GFP protein synthesis in dormant spores A TT14 spores were incubated in PBS+30ug/ml Doxycycline for 42h. Each curve represents the GFP fluorescence of a single spore (as shown in **C**, i.e. not a single spore bag) over time. n = 254 individual spores. **B**. Example of 6 individual spore traces from **A** with distinct discrete steps in expression. **C** Example of single spore tracking and expression. Microscopy time-strip with images sampled uniformly over the 42h, of both brightfield and GFP fluorescence channel. Mask is indicated in green on the images. GFP Fluorescence of the single spores was computed by averaging GFP fluorescence of pixels within the segmentation mask (green line on images). Tracking of single spores over 42h (green curve) reveals two expression events at ~ 10 hours and ~ 17 hours that not due to independent increase of fluorescence from neighboring spore within the spore bag. scale bar are $2\mu m$. See methods for details on the procedure used to segment and track spore.

sufficient mechanism to explain why at single spore level GFP fluorescence profiles are discontinuous, with discrete "steps". In other words, a discontinuity in protein synthesis is sufficient to observe the discontinuity in GFP accumulation.

One simple explanation for the fact GFP protein synthesis stops could be that GFP mRNA are absent from spores for extended periods of time (despite doxycycline present in the medium). Looking at the mRNA copy number together with protein level (fluorescence) in the same spores indeed showed many spore with high GFP fluorescence level and no GFP mRNA (Fig.4.7C white arrow). More generally, mRNA and protein level were only very weakly correlated ($\rho = 0.24$ Fig.4.7.D,Supp.Fig.4.19). These observations are then consistent with the hypoth-

esis that protein synthesis stops because mRNA are no longer present in spores.

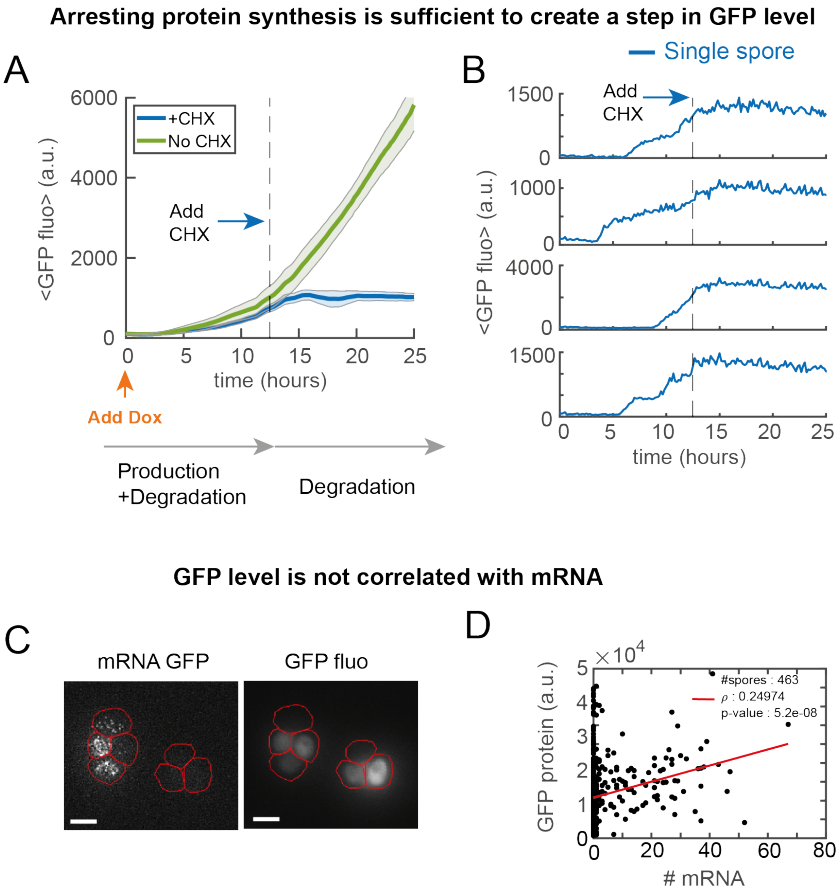


Figure 4.7: Stopping protein synthesis reproduced steps in expression. **A**. TT14 in PBS+30ug/ml Doxycyline, at t=12.6h after addition of Doxycyline, 100ug/ml Cycloheximide (CHX) was added (blue curve) or not (green curve). Shaded areas represent the standard error of the mean over 3 biological replicates, each containing more than 100 individual spores. **B**. Example 6 individual spores (blue curves), tracked as in Fig.4.6C, corresponding to data of **A** blue curve (with Cycloheximide added at 12.6h, as indicated by the dashed black line). **C** Microscopy images of TT14 spores after 18 hours incubation in $PBS + 30\mu g/ml$ Doxycycline, red line indicates the mask used to extract the number of GFP mRNA molecules measured by FISH dots (CalFluor610 same as in Fig.4.4) (Left image) and the average GFP protein fluorescence (Right image). Scale bar is $2\mu m$. **D** Scatter plot of the correlation between the number of GFP mRNA molecule (measured as shown in **B**) left and the average GFP protein fluorescence (measured as shown in **B** right) for $n = 463$ individual spores (black dots). Red line indicates the linear regression fit $y = \rho * x + b$, $\rho = 0.25$. and Pearson correlation test p-value = $5.2 * 10^{(}-8)$.

4.4.3. Modeling with protein synthesis

How is that hypothesis consistent with our observation of transcription dynamics?

We now incorporate protein synthesis dynamic in our previous model4.1. We added a first order translation reaction, together with first order protein degradation, as follows :

$$I \xrightarrow{\lambda} A \tag{4.8a}$$
$$A \xrightarrow{\mu} I \tag{4.8b}$$
$$A \xrightarrow{\nu_M} A + M \tag{4.8c}$$
$$M \xrightarrow{\delta_M} \emptyset \tag{4.8d}$$
$$M \xrightarrow{\nu_P} P + M \tag{4.8e}$$
$$P \xrightarrow{\delta_P} \emptyset \tag{4.8f}$$

This model naturally yielded a discontinuous protein expression profile with discrete steps, similar to what we observed experimentally, on the condition that we set the half-life of protein to be very high (typically more than 40h, i.e. what we estimated experimentally in Supp.Fig.2.28). (Fig.4.8).

Aside from producing discrete steps, this model also explain why we observed that upon induction, the GFP protein level is generally not correlated with the GFP mRNA copy number (Fig.4.7C-D,Supp.Fig.4.19). Indeed, in this model we assume the half life of proteins to be in days whereas the half life of mRNA is assumed to be in minutes. This order of magnitudes difference causes the mRNA level to be completely uncorrelated from protein level. However, one consequence is that the mRNA level become strongly correlated to the *derivative* of the protein level (Fig.4.8). Indeed, in the model 4.8e is described deterministically by :

$$\frac{d < P >}{dt} = \nu_P \cdot < M > - \delta_p \cdot < P > \tag{4.9}$$

Here we see that with a very slow degradation compared to production ($\delta_p \cdot < P > << \nu_P \cdot < M >$), then the derivative of protein level is mostly driven by :

d<P>$_{\overline{dt \sim <M>}}$
(4.10)

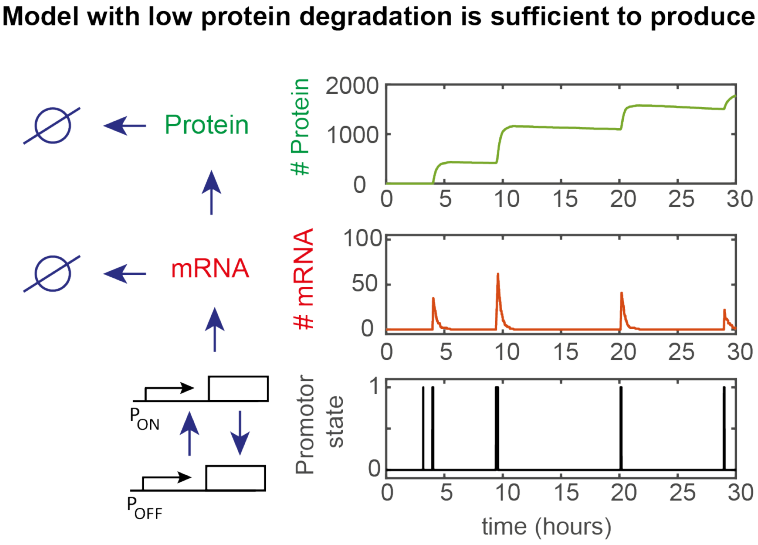


Figure 4.8: Simulation of protein synthesis with the random telegraph model. Representative simulation with the parameters used in Fig.4.5 and a half life of proteins of 40h. Simulation with the Gillespie algorithm of model 4.8. Compared to Fig.4.5, 1 variable was added : "#Proteins" (can be any positive integers). 2 reactions were added : (5) Production of proteins ("#Proteins" is increased by 1); (6) Degradation of proteins ("#Proteins" is decreased by 1). Simulation was stopped when the reaction time was 30 hours. Reaction rates were set according to normalized parameters : (fraction of time transcription is active) $p_{ON} = 0.03$; (average time transcription is active) $T_{ON} = 4.6 minutes$; (half-life of mRNA) $\tau_M = 10.7 minutes$; (steady number of mRNA) $\alpha = 3.4 mRNAs$; (half-life of proteins) $\tau_P = log(2)/\delta_P = 40 hours$; (protein production rate) $\nu_p = 0.1$ is arbitrary since in experiments we measure GFP fluorescence arbitrary units, no protein number.

This makes two predictions : (1) within a single spore, mRNA level should be correlation to derivative of protein level. (2) at the population level, mRNA level should be distributed similarly to the derivative of protein level. We could only test the second prediction by comparing the distribution of #mRNA from FISH data, with the distribution of dGFP/dt (our proxy for dP/dt) in single spore fluorescence data. Interestingly, we did find that similarly to the distribution of #mRNA, the distribution of dGFP/dt looked "long-tailed" (Supp.Fig.4.23). This result further supports the qualitative validity of our model to explain both distribution of mRNA and discontinuous protein expression.

In conclusion, our model predicts that a unique mechanism of random activation-deactivation of transcription is sufficient to qualitatively explain both single spore mRNA FISH and fluorescent protein time-lapse data. We next aimed at testing whether that prediction was valid quantitatively.

4.5. Quantitative modelling reveals a random switch of transcription

4.5.1. Automated analysis of GFP expression steps

Using a basic filtering method, we automatically detected steps in single spore GFP expression profile(Supp.4.20). Then we extracted several features such as the duration between two steps (Fig.4.9B), the duration of a step (Fig.4.9C), the maximum slope of step (Fig.4.9D). We then extracted these features for hundreds of individual spores induced with different doxycycline concentrations and computed their average value (Fig4.9A-D).

First we found the GFP expression rate (i.e. the maximum slope of a step, Fig4.9D) increased linearly as a function of Doxycycline. This is consistent with the fact that increasing doxycycline effectively increases the amount of mRNA produced (Fig4.4E). Indeed, having more GFP mRNA could mean having more GFP proteins translated simultaneously and therefore a higher GFP expression rate. Then we also found that the fraction of spores expressing GFP (i.e. spore having at least one expression step) increased as a sigmoid function from 0 to 1 with 0.5 reached for $15\mu g/ml$ (Fig4.9A).

Strikingly, we found that both the average time interval between steps and the duration of a step did not change as a function of doxycycline, staying a constant value of 10h and 5h respectively (Fig4.9B-C). Additionally, when looking at the variability within a population for a given doxycycline concentration (Supp.Fig4.22), we found that there was no preferential time of expression over the 42 hours, meaning that there was a similar probability for step to occur at any time between 5h and 40h after induction (Supp.Fig4.22E).

In conclusion, that quantitative analysis revealed that the process generating discontinuous GFP expression (stair-like with discrete steps) can be decomposed into two distinct components : one about the timing of expression, which is independent of doxycycline and one about the intensity of expression which strongly depends on the amount doxycycline.

Moreover, we also found that for a step of expression in a single spore (or between tow consecutive steps), there was no correlation between any of the quantities (duration, max slope, time of start etc.) (Supp.Fig.4.24).

Finally, we noticed that this behavior is merely similar to what we observe with FISH for the distribution of mRNA at a given time after induction. Indeed, there we observed the average amount of mRNA produced but not the fraction of spores

having mRNA depended on the amount of doxycycline (Fig.4.4E and G).

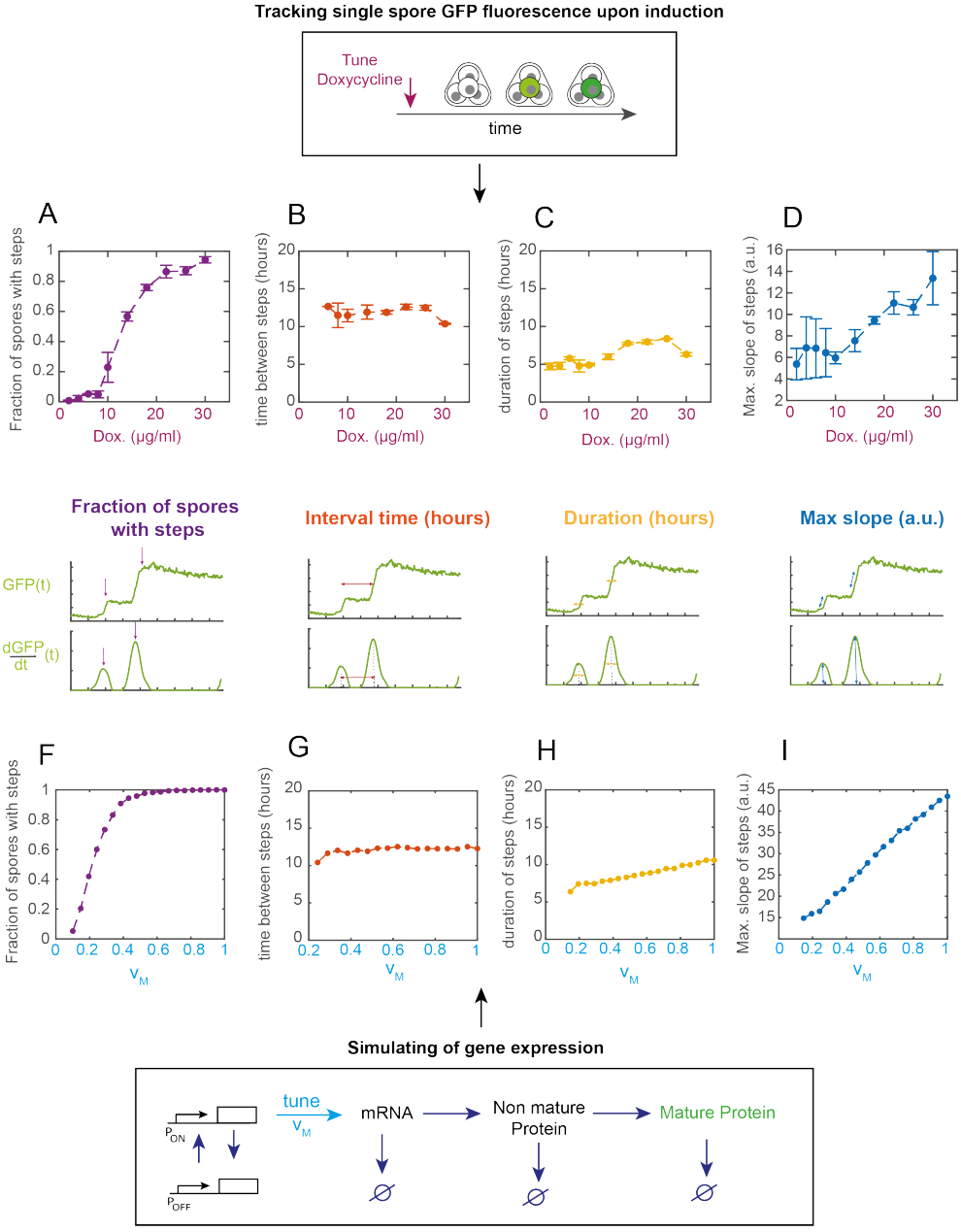
Now we will turn into modeling to investigate whether these two independent and quantitative observations can be jointly explained by our hypothesis : random activation-deactivation of transcription.

4.5.2. Model with limiting maturation of protein

As we saw previously, simply having a low degradation rate is sufficient for our gene expression model (4.8) to generate a discontinuous "stairs-like" protein profile, qualitatively similar to what we observe experimentally (Fig.4.8). However, by looking carefully at single spore GFP fluorescence profile, we found that, in some spore, expression is nearly continuous over 40 hours upon induction (Supp.Fig.4.20A). Importantly, this behavior could not be explained by our model with "simple" first order protein production. Indeed, in this model, protein production is directly driven by mRNA level, which is always discontinuous. Therefore, in this model, increasing the average amount of mRNA produced only cause "more steps" in protein expression, but not a continuous profile (Supp.Fig.4.20B).

What do we need to add in our model to yield both continuous and discontinuous protein expression?

According to our model (4.8), a continuous protein expression can only be explained by a continuous presence of mRNA (4.4.3). Specifically, a constant protein production rate means a constant amount of mRNA copy number. In other words, the systems behaves as if there was a memory of the presence of mRNA that already degraded, allowing GFP fluorescence to continue to accumulate after mRNA were gone. We reason that a biologically sensible candidate would be that right after translation from mRNA, proteins are not maturated enough to be visible by GFP fluorescence (for instance by being incompletely folded), and that an extra "maturation" steps would be necessary for the non-mature protein to become visible by fluorescence microscopy. We then implemented that idea in the model by adding a species P_1, representing non-mature proteins, intermediate between mRNA M and mature protein P_2 that we measure with GFP fluorescence, as follows :


Tracking single spore GFP fluorescence upon induction
Tune
Doxycycline
time
A
B
C
D
Fraction of spores with steps
time between steps (hours)
duration of steps (hours)
Max. slope of steps (a.u.)
Dox. (μg/ml)
Fraction of spores with steps
Interval time (hours)
Duration (hours)
Max slope (a.u.)
GFP(t)
dGFP/dt (t)
F
G
H
I
V_M
Simulating of gene expression
tune
mRNA
Non mature Protein
Mature Protein
P_ON
P_OFF

$$I \xrightarrow{\lambda} A \tag{4.11a}$$

$$A \xrightarrow{\mu} I \tag{4.11b}$$

$$A \xrightarrow{\nu_M} A + M \tag{4.11c}$$

$$M \xrightarrow{\delta_M} \emptyset \tag{4.11d}$$

$$M \xrightarrow{\nu_{P1}} M + P_1 \tag{4.11e}$$

$$P_1 \xrightarrow{g(P_1)} P_2 \tag{4.11f}$$

$$P_1 \xrightarrow{\delta_{P_1}} \emptyset \tag{4.11g}$$

$$P_2 \xrightarrow{\delta_{P_2}} \emptyset \tag{4.11h}$$

Moreover we assumed a non linear sigmoid function $g(P_1)$, α

$$\alpha(P_1) = \nu_{P_2} \cdot \frac{[P_1]^n}{[P_1]^n + K_P^n} \tag{4.12}$$

Assuming this model was sufficient to produce both discontinuous (Fig.4.10C) and continuous (Supp.Fig.4.20C)protein expression, with the same underlying mRNA dynamics (burst of production). Beside reproducing these two qualitative behaviors, this model could quantitatively reproduce the effect of tuning doxycycline concentration on steps' features (Fig.4.9A-D) by simply tuning the transcription rate ν_M.

We will now justify why we use ν_M (transcription rate) as the control parameter to mimic the effect of changing doxycycline concentration, i.e. changing how much we induce transcription.

In our model, tuning ν_M concretely only modifies the theoretical average number of mRNA $\alpha_P = p_{ON}\frac{\nu}{\delta}$. Moreover, we found that tuning doxycycline directly in-

Figure 4.9 *(preceding page)*: Comparison of GFP protein synthesis statistics between experiments and simulations Average statistics of single spore protein expression steps for experiments (A-D) and model (F-I). The fraction of spores having at least one steps (A and F), the time between two steps (B and G, red), the duration of a step (C and H, yellow) and the maximum slope of step (D and I,blue) were automatically extracted as in Supp.4.21 and Supp.4.22. For experiments (A-D), TT14 spores were incubated for 42 hours in PBS with different doxycycline concentration ($2, 4, 6, 8, 10, 14, 18, 22, 26 and 30 \mu g/ml$) error bar are s.e.m on n=3 replicates. For simulation (F-G), model 4.11 Average over 1000 simulations.

creased the measured average number of GFP copy number (Fig.4.4E). Therefore, changing ν_M is experimentally consistent with changing the doxycycline concentration.

Now we will briefly explain what features of our model (4.11) concretely enables to reconcile experimental data of both mRNA and protein. The crucial element here was to implement the fact that maturation of proteins saturated above a certain amount of non-mature proteins. In other words, a spore is limited by the amount of non-mature protein it can maturate per unit of time. As a direct consequence of that assumption, we naturally get that for high doxycycline (high average mRNA), the production rate of mature protein becomes constant, because the amount of non-mature proteins always exceed the saturation limit (Supp.Fig.4.20C).

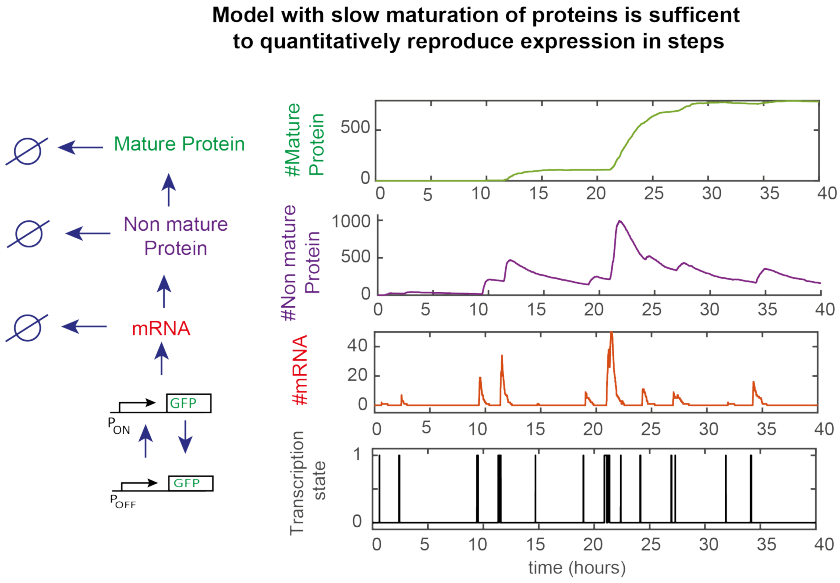


Figure 4.10: Simulation of gene expression with random telegraph transcription and limiting maturation of proteins Simulation with the Gillespie algorithm of model 4.11. Compared to Fig.4.8, 1 variable was added : "#Non mature Proteins" (can be any positive integers). 2 reactions were added : (7) Production of mature proteins ("#Mature Proteins" is increased by 1); (8) Degradation of mature proteins ("#Mature Proteins" is decreased by 1). Simulation was stopped when the reaction time was 40 hours. Reaction rates were set according to normalized parameters : (fraction of time transcription is active) $p_{ON} = 0.03$; (average time transcription is active) $T_{ON} = 4.6 minutes$; (half-life of mRNA) $\tau_M = 10.7 minutes$; (steady number of mRNA) $\alpha = 3.4 mRNAs$; (half-life of proteins) $\tau_P = log(2)/\delta_P = 40 hours$; (protein production rate) $\nu_p = 0.1$ is arbitrary since in experiments we measure GFP fluorescence arbitrary units, no protein number.

In conclusion, we showed that adding a reaction of saturating protein maturation to our model is sufficient to resolve the contradiction between the mRNA FISH and GFP time-lapse data. We next reason that if such reaction of saturating protein maturation takes place in dormant spores, we should observe that after stopping

protein GFP, GFP fluorescence should continue to accumulate for some time. Indeed, by stopping protein synthesis we would stop the production of non-mature proteins P_1, but we would not stop their subsequent maturation. In our model, the time protein will continue to maturate after protein synthesis is stopped should scale with the average duration of GFP steps. We verified that prediction experimentally by simply looking at the results of Fig.4.7A where we inhibited protein synthesis after 12 hours of induction in PBS with Doxycycline. There we found that on average it took 2.5 hours for GFP fluorescence to completely stop accumulating after inhibiting protein synthesis, which matches the average duration of a step (5 hours, Fig.4.9C). This result supports our assumption that saturating maturation of protein is likely to take place in dormant spores.

Overall, the combination of mRNA FISH data, single spore protein fluorescence time-lapse and quantitative modeling supports the idea that in dormant spore, transcription randomly alternates between short periods of activity (few minutes) with long periods of inactivity (hours to tens of hours). Crucially, varying the amount of doxycycline indicates that the random switch of activation to deactivation is likely independent of how much transcription is induced. We next wonder what mechanism could underpin random activation-deactivation of transcription.

4.6. Random switch of transcription controls two inducible genes

Up to this point, all the experiments we carried over a single inducible gene (ptet-GFP). Quantitative experiments and modeling pointed out towards a random switch of transcription, which properties were independent on how much the gene was induced. Since the inducer itself did not seem to influence the switch, we hypothesized that the underlying mechanism controlling it might not be specific to the single gene we induced. Specifically, the random switch may influence more than one gene simultaneously. In this part, we aim at testing this hypothesis.

4.6.1. Two-color dynamic of induced expression

We constructed spores in which we could induce simultaneously both GFP and mCherry expression(Fig.4.11A). Similarly to what we did for one inducible gene (Fig.4.6), we induced and then tracked expression of GFP and mCherry proteins at single spore level (Fig.4.11B). Strikingly, we found that in many spores, both GFP and mcherry had discontinuous expression profile (stairs-like), with some steps occurring at the same time and some other not (Fig.4.11B).

In order to know whether discontinuous expression of both gene was correlated, we looked at the number of GFP steps against the number of mcherry steps within the same spores (Fig.4.11C). There we found a clear positive correlation, meaning that a single spore with 3 steps of GFP expression was very likely to also have 3 steps of mCherry expression.

Next, we sought to estimate quantitatively whether GFP and mcherry steps started at the same time. To that aim, we computed the probability to find a mcherry steps at time $\tau_{mcherry}$ knowing there was a GFP step a time τ_{GFP} (Fig.4.11D). Intuitively, this corresponds to ask *How likely is it for GFP and mcherry to be expressed simultaneously?*. We found that given any τ_{GFP}, the probabilty to find a mcherry expression steps within 3 hours before or after was very high (i.e. the probability density is concentrated on the diagonal) compared to shuffled data (Fig.4.11E-F;Supp.Fig.4.25). Moreover, we found that unlike the timing of expression,

the intensity of expression was only weakly correlated (Supp.Fig.4.27).

According to our model (Fig.4.11E), discontinuous protein expression is caused by random activation and deactivation of transcription. Therefore, a correlation in protein expression between two genes should stem from a correlation in mRNA expression. Indeed, we found that mRNA copy number of GFP and mCherry were positively correlated as well (Supp.Fig.4.26).

Overall, these correlations in protein and mRNA expression seem to all agree that

the random activation deactivation of transcription controlled both gene (GFP and mCherry) at the same time. Next, we turn into modelling to ask whether such co-activation/deactivation was sufficient to explain these correlations.

In order to make sense of the experimentally observed positive correlation between the two switches, we formulated two limit models. In the first "null" model (4.13) we assumed the two switches controlling GPF and mcherry to be fully independent (Fig.4.12A). In the second model (4.14) we assumed only one global switch controlling both GFP and mcherry expression (Fig.4.12B). Apart from parameters controlling activation and deactivation of transcription, all the other reaction and associated parameters are assumed to be identical for the two genes.

Concretely, we have two different genes that can switch between

A_1, I_1 and A_2, I_2 respectively. For the first null model of "Independent control" we have the following set of reactions :

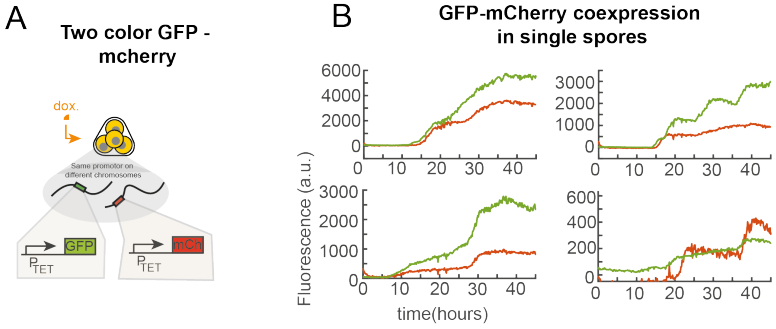
$$I_1 \xrightarrow{\lambda} A_1 \tag{4.13a}$$
$$A_1 \xrightarrow{\mu} I_1 \tag{4.13b}$$
$$I_2 \xrightarrow{\lambda} A_2 \tag{4.13c}$$
$$A_2 \xrightarrow{\mu} I_2 \tag{4.13d}$$

And for the second "global control" model we have :

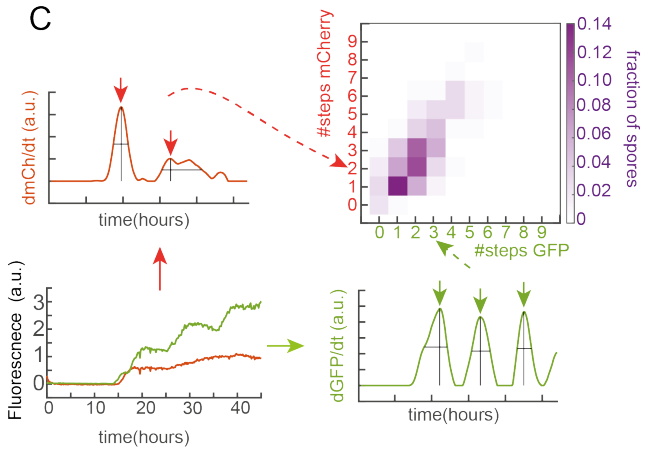
$$I_1, I_2 \xrightarrow{\lambda} A_1, A_2 \tag{4.14a}$$
$$A_1, A_2 \xrightarrow{\mu} I_1, I_2 \tag{4.14b}$$

Importantly in that model with a unique common random switch, even when transcription is in ON state, both production of GFP or mcherry mRNA are still governed by independent stochastic processes, and therefore do not have to be identical.

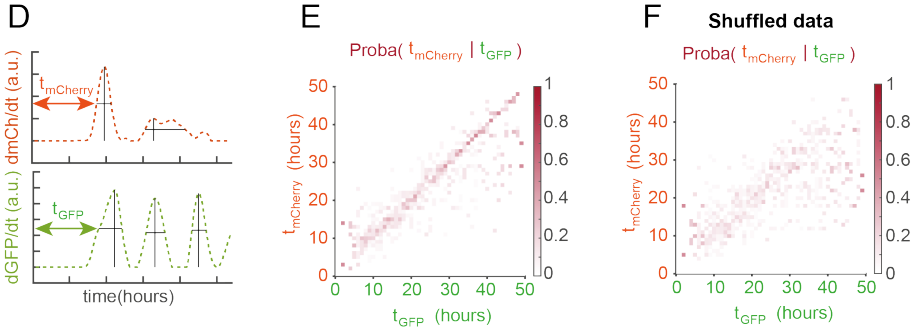
We simulated the two different models and computed the same probability heatmap as for experiments (Fig.4.11E) (Fig.4.12E-F). We found that only the second model with a unique switch could reproduce the experimental data (Fig.4.11E and Fig.4.12F), whereas the null model yielded a flat probability map, similar to what we found



Number of steps is positively correlated



Timing of expression is positively correlated



with shuffled experimental data ((Fig.4.11F and Fig.4.12E). As shown in example simulation, the global control model could accurately reproduce single spore expression of GFP and mcherry that we observed with experiments (Fig.4.12D).

In conclusion, experiment and modelling with two-color synthetic induction confirmed our hypothesis that the random transcription switch controls more than one gene. Importantly, since both gene were located on different chromosomes, the putative random switch control can not correspond to local compaction-decompaction of chromatin.

Motivated by this discovery, we will now close up this part (Chapter 4) by examining an extreme hypothesis :

What if the random switch we uncovered for inducible GFP actually controls transcription of all endogenous genes in spores?

Figure 4.11 ***(preceding page)*****: Two color experiments reveals that mRNA and proteins are globally co-expressed. A** Two inducible synthetic circuit (ptet-mcherry and ptet-GFP) were integrated in yeast in two different chromosomes (VI and II) and then homozygous spores were prepared from that strain (TM3 see methods). **B** 4 GFP and mcherry traces of 4 single "TM3" spores incubated in PBS+25$\mu g/ml$ for 45 hours. Fluorescence was extracted as in Fig.4.6C. **C** For each individual spore, number expression steps of both GFP and mcherry traces were automatically extracted as in Supp.4.21, as indicated by the red or green arrows on the derivative plots (top left and bottom right). (Top left) Normalized heatmap indicating in purple the fraction of spores with a (x,y) doublet of steps, with given "x") number of GFP steps (x-axis in green and "y" number of mCherry steps (y-axis in red). n = 457 spores, same condition as in **B**. **D** For each individual spore and for both GFP and mCherry, the time of start of single expression steps was extracted, as indicated by the green and red arrows. **E** Heatmap showing the conditional probability (in dark red) of having an mCherry expression step at "$t_{mCherry}$" (y-axis), given that there has been a GFP expression step at "t_{GFP}". Results were obtained by binning the data for t_{GFP} and $t_{mCherry}$,t per hour (1 bin = 1 hour) and normalizing over a total of n=457 spores. For spores with multiple mCherry steps, only the closest (if exists) GFP step was taken into account. **F** Heatmap of conditional probability with same data as **E** but with shuffled data.

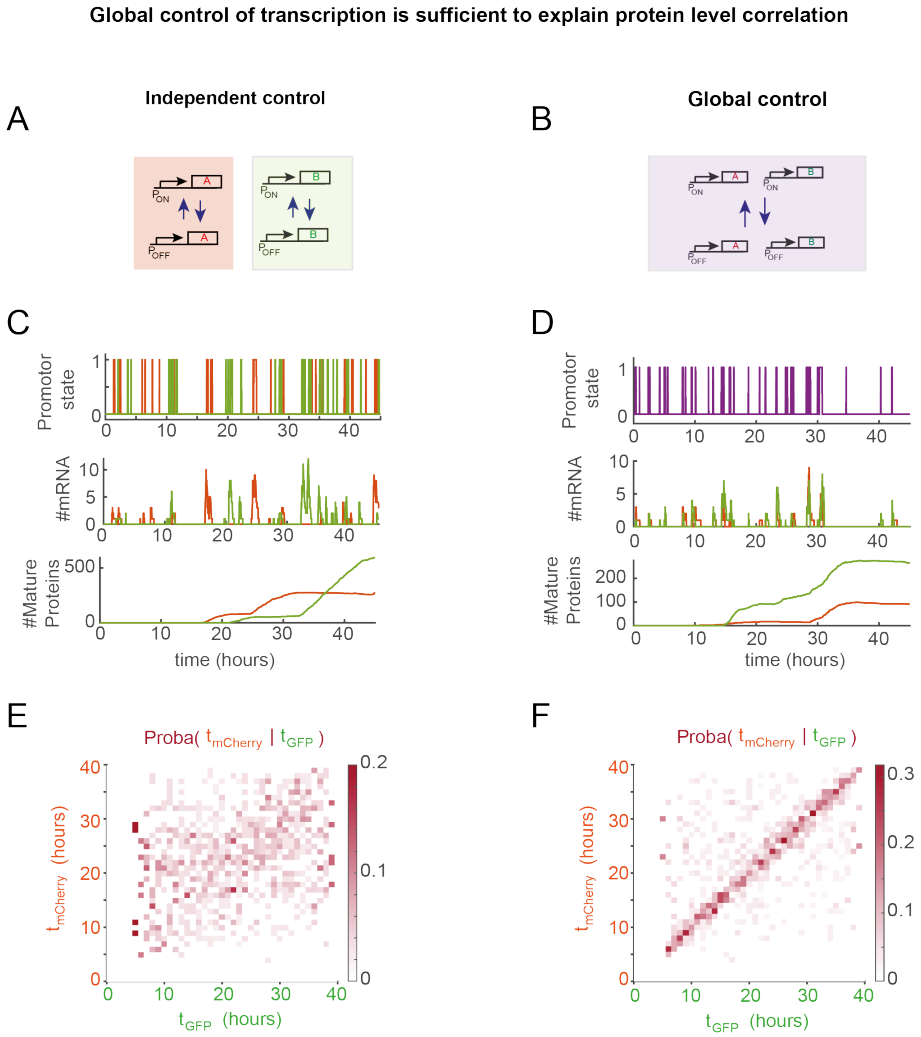


Figure 4.12: Simulation with global control of random telegraph A In the first model "independent control", the two promotors controlling the expression of gene A (in red, representing mCherry) and the expression of gene B (in green, representing GFP) switch between active and inactive states with the same rates (λ and μ) but independently, i.e. that the state of one promotor at time t has no influence over the switching probabilities of the other promotor between t and $t + dt$. **B** In the "global control model", both promotor activate or de-activate at the same time, as indicated in 4.13. **C** Example simulation with the independent control model. For both GFP and mCherry, parameters are same as in Fig.4.11. **D** Example simulation with the global control model. For both GFP and mCherry, parameters are same as in Fig.4.11 and for the global activation de-activation parameters are the same as each gene of **C**. **E** Heatmap of the conditional probability of having a step for mCherry at time $t_{mCherry}$ given that there is a step for GFP at time t_{GFP}, computed the same way as in Fig.4.11E. Data from simulation of n=1000 spores, with same model and parameters as **C**. **F** Heatmap of the conditional probability same as **E** but for the global control model.

4.7. Probing random switch of transcription on endogenous genes

We started out that chapter by showing that during dormancy, transcription was happening genome-wide, but that the amount of mRNA copies was low and highly variable. This made us wonder what transcription dynamic was taking place in a single spore to generate such distribution of mRNA. To address that question, we used synthetic inducible circuits, that we could control and measure accurately. In parallel to experiments, we constructed a stochastic model of gene expression that could reproduce our single cell data with minimal assumptions. In its final version, this model stipulates that transcription of two genes randomly co-activate and co-deactivate, with hour-long periods of deactivation, causing both mRNA level to potentially stay close to zero for hours. We now close off the loop by testing this model on endogenous gene expression.

4.7.1. Method to probe the model from RNA FISH data

Unlike synthetic inducible circuit, the transcriptional activity of endogenous genes can not be so easily controlled and monitored. However, we reasoned that if a similar random switch is controlling the transcriptional activities of multiple endogenous genes, this could leave a specific signature in the joint distribution of mRNA copies. Specifically, this signature should be similar for any pair of genes, whether they are controlled by a native promotor or a synthetic inducible promotor. Using RNA FISH, we therefore measured the joint distribution of 8 different pairs of genes, using various combination of inducible (such as ptet-GFP) and endogenous (such as ENO1, RPL3 etc.) genes. (Fig.4.13A). If an inducible gene was present in the pair, we induce it with Doxycycline for 24 hours before measuring mRNA distributions (Histograms in Fig.4.13B and Supp.Fig.4.28)

Having measured the joint distribution in mRNA level of several pairs of genes, we then asked how to probe the existence of a random switch for each of the pairs. Naively, we can first say that if two genes are controlled by the same random switch, then the mRNA copy number should be positively correlated. Indeed, in our previous double induction experiments, we did find a strong positive correlation in the number of mRNA of both GFP and mcherry (Supp.Fig.4.26). While we did find a significant positive linear correlation for all the pairs we used (Supp.Fig.4.29). However, we find that evidence insufficient, because a linear correlation can be caused by a wide class of mechanisms and therefore can not probe our model of gene expression.

In order to probe our model on joint distributions of mRNA, we sought to generalize it to more realistic situations where two genes are not completely indepen-

dent (such as in Fig.4.12A-C-E) and not completely globally controlled (such as in Fig.4.12B-D-F)). To do so, we defined a "hybrid model" (see 4.9 for details) which has one global control parameter $'R'$ to continuously interpolate between the two extreme cases (independent vs global control). In that model, $R = 0$ means that the two genes are controlled by two independent random switches, whereas $R = 1$ means they are both controlled by a unique random switch. A value of R such that $0 < R < 1$ means that the two random switches controlling the transcription of the two genes are influencing each other. Concretely, for a higher value of R, if one gene is activated, the activation rate of the other gene is higher.

The idea is then to find, for each pair of genes, which value of R is best at reproducing the experimental joint mRNA distribution measured by FISH. To do so, we computed the mutual information between the two distributions of mRNA of given a pair of genes, either from experimental data, or from simulation. Then we looked at the value of R that made the simulated mutual information match the experimental mutual information (Fig.4.13C, Supp.Fig.4.30).

In total, for all simulations we only varied :

1. The average number of mRNA for each genes, that we measured directly from the FISH data (see Supp.Fig.4.28 for details)
2. The value of R, which we varied to find which one was best at reproducing experimental data.

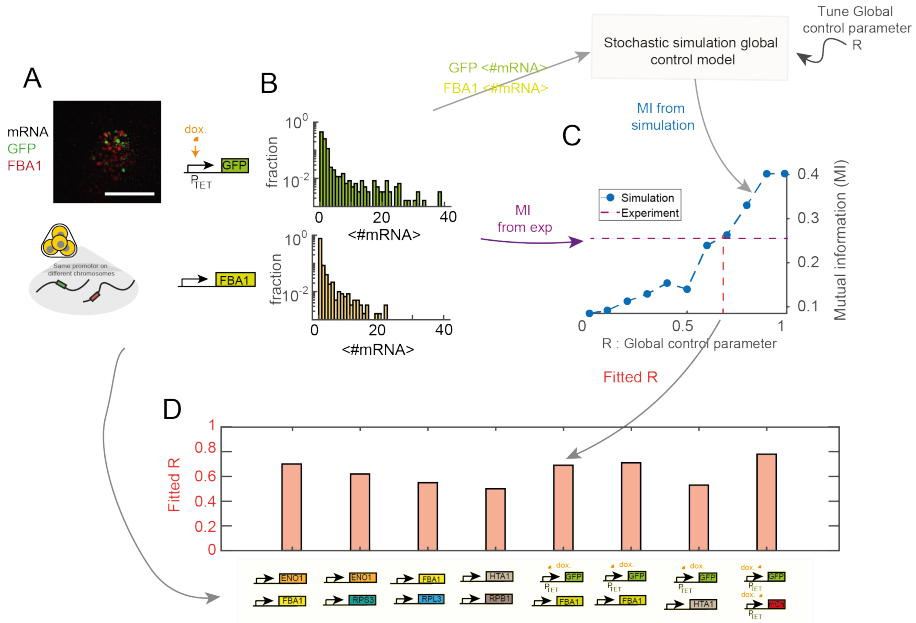
Importantly, all the other parameters are the same we estimated and used to simulate GFP transcription dynamic upon induction with doxyycline (Fig.4.5, 4.4).

4.7.2. Results and interpretation

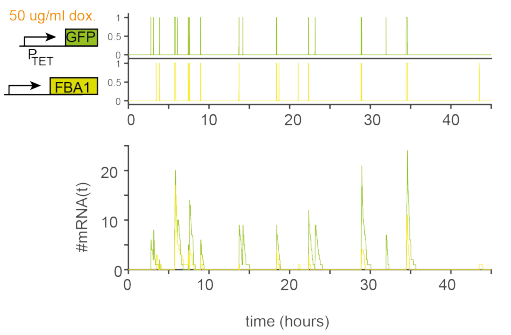
Applying our strategy for each of the 8 pairs, we found that the best global control parameter $"R"$ to reproduce the joint distribution of mRNA was always between 0.5 and 0.8, with an average value of 0.65 (Fig.4.13D). The fact these values are always much above 0 signifies that a model in which the transcription of both genes are controlled independently ($R = 0$) is clearly not sufficient. On the other hand, the fact R is always much below 1 seems to indicate that a model in which all genes are simultaneously activated and deactivated is not the most likely. Rather, this analysis seems to indicate that in the most likely scenario, transcription of several genes is "often but not always" activating and deactivating simultaneously. As a concrete reference, the pair of inducible genes, ptet-GFP and ptet-mCherry yielded an "R" of 0.8. Therefore, any pair of genes that yielded an "R" close to 0.8 should activate and deactivate simultaneously, as much as we directly observed for ptet-GFP and ptet-mCherry (Fig.4.11).

Of particular interest are the pairs with inducible GFP and an endogenous gene

Estimating the level of global transcripitonal control for different multiple pairs of genes



Simulation with estimated global control parameter



(ptet-GFP with HTA1 or FBA1). These pairs constitute a "bridge" between the world of synthetic inducible genes (ptet-GFP and ptet-mcherry), for which we could uncover the single-spore dynamic and the world of endogenous genes (ENO1, HTA1, RPL3), for which we only have access to statistical distribution of mRNA. Indeed, the transcription factor inducing the expression of GFP (rtTA bound to doxycycline) has no reason to directly influence the transcription of endogenous yeast genes. However, our experiments and model suggests that both type of genes are co-regulated by the same random switch of transcription. Specifically, we found that quantitatively, these pairs are as co-regulated as much as the pair of inducible genes (ptet-GFP and ptet-mCherry). This means that according to our model, an endogenous gene is as much co-regulated with ptet-GFP as ptet-mcherry is. Similarly, when our method estimates an R of 0.7 for a pair of endogenous genes such as ENO1 and FNA1, this means their transcription should start and stop with the same time delay we observed for ptet-GFP and ptet-mcherry. In order to visualize what this model exactly predicts about endogenous genes, we simulated pairs of mRNA trajectories based on the value of R that best reproduced experimental data (Fig.4.13E,Supp.Fig.4.31).

Figure 4.13 ***(preceding page)*****: Pair-gene distributions of mRNA support a model of global control of transcription A** Composite image of mRNA FISH dots of a single spore bag for after induction of TT14 spores (GFP inducible) in PBS+50$\mu g/ml$ Doxycycline for 24 hours .GFP mRNA (Qasar570) are colored in green and FBA1 mRNA (Qasar670)in red. Scale bar is $3\mu m$. **B** Pair of Histograms of the number of molecules of GFP mRNA (top, green bars) and FBA1 mRNA (bottom, yellow bars) for spores incubated and images as in **A**. n=346 individual spores. **C** Plot of the mutual information between mRNA distribution of the pair of genes (ptet-GFP,FBA1), from simulated data. (blue dots). Pairs of histograms were simulated by computing the steady state distributions (see methods simulation) with the hybrid model (part 4.6.3). All parameters are fixed and the same as Fig.4.12, except for "R", which is varied between 0 and 1, and α_A and α_B (theoretical steady state level of mRNA), which are taken equal the *measured* average number of mRNA from FISH (i.e average of GFP mRNA and average of FBA1 mRNA in that example). Purple dashed line indicates the mutual information between the *measured* mRNA joint distribution of mRNA (here between GFP and FBA1). The red dashed line indicates the extrapolated value of "R" that best matches the experiments (measured mutual information). **D** Bar graph of the "fitted" value of the global control parameter "R" for 8 different pairs of genes. Simulated and measured mutual information for individual pairs of genes are in Supp.4.30. **E** Simulation with Gillespie algorithm of transcription of one example pair of genes (ptet-GFP and FBA1) with fitted global control $'R = 0.67'$. Average GFP mRNA number is set to 2.8
and average FBA1 mRNA number is set to 1 (from FISH histograms see Supp.Fig.4.28). All other parameters are similar to our initial model of transcription (4.1, Fig.4.5) : (fraction of time transcription is in the active state)$p_{ON} = 0.03$; (average time transcription is active) $T_{ON} = 4.6 minutes$; (half-life of mRNA) $\tau_M = 10.7 minutes$

4.8. Conclusions

Motivated by previous experiments showing that inhibiting transcription killed dormant yeast spores in less than a day, we sought to uncover transcription dynamic during dormancy. We started out by observing that with bulk genome-wide assay, the de novo production of endogenous RNA was detectable in a few minutes. However, at the single spore level snapshot, mature mRNA were in low amount and highly heterogeneous. In order to uncover what was the underlying transcription dynamics, we turned into measuring and controlling the transcription of an inducible gene. Combining RNA FISH and single-spore tracking of protein and mathematical analysis of that inducible gene, we constructed a minimal model that explained our observations. This model stated that for a single dormant spore, transcription randomly alternate between short periods of transcription where RNA are produced within minutes and hour-long periods of inactivity where RNA are not produced and degrade quickly. This model is akin to random activation deactivation models of transcription (also called "bursting transcription") in replicating yeast and mammalian cells (Raj et al. 2006,Raj et al. 2008,**Tineke2016**). In these systems, variation in transcriptional activity is thought to be caused by local effect, underpinned by variation in chromatin compaction or binding of transcription factors. The main experiment supporting this theory is that two genes' transcription is activated and deactivated simultaneously only if the two genes are physically close (i.e. regulation in cis). In dormant yeast spores, we showed evidence that transcription activate and deactivate simultaneously even if the two inducible genes are physically distant (not on the same chromosome). Finally, by comparing the distribution of endogenous mRNA with the distribution of induced mRNA at the single spore level, we found a random switch of transcription operating genome-wide, i.e. activating and deactivating the transcription of all genes simultaneously (with some level of noise), was the most likely model (Fig.4.14).

We constructed this model by monitoring expression of synthetic inducible reporter genes during dormancy. One key assumption we made along the way was that during dormancy, the degradation of GFP mRNA was fast, so that mRNA half-life around 10 minutes. This value was "conservative", since it is similar to the mRNA decay kinetics of replicating cells (Chan et al. 2018). However, one should not exclude the possibility that in dormant spores, mRNA decay kinetics qualitatively differ from replicating cells. Importantly, since mRNA are transcribed during dormancy, methods to probe the degradation of mRNA should explicitly decouple the balance between production and degradation.

In our opinion, the most accurate (and feasible) way to decipher the balance between production and degradation of mRNA in spores would be to extensively

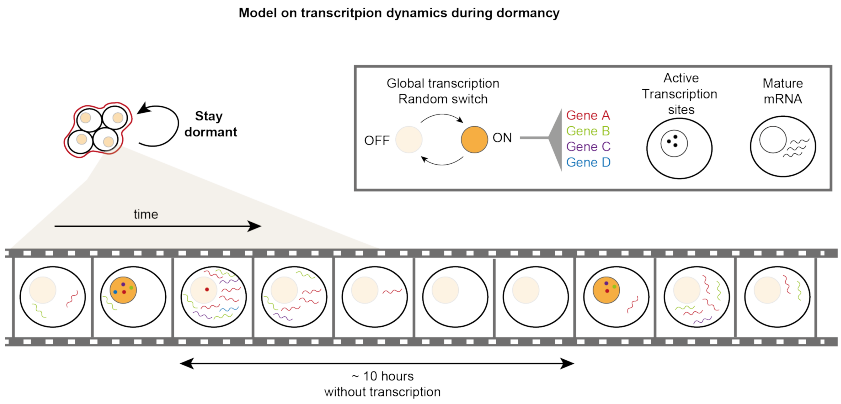


Figure 4.14: A model for transcription dynamics during dormancy of yeast spores

perform metabolic labelling with 4tU-sequencing during dormancy. Indeed, we presented preliminary results showing that 4tU-seq can be used to detect mRNA production during dormancy of spores (Fig.4.2). With sufficient time point and qPCR controls, this method can be used to accurately infer the production and degradation rates of mRNA genome-wide (Chan et al. 2018).

One different way to probe mRNA production and degradation in spores could be to measure live transcription activity of one (or a few) gene(s)

with single spore imaging. Indeed, recent studies showed

that methods using PP7-MS2 tagging can be used to directly probe the live accumulation of mRNA at the transcription site and to lesser extent the live-abundance of mature mRNA in the cytoplasm (Lenstra et al. 2016). Therefore, applying these live-assays to inducible gene expression in dormant spores would be well-suited to probe GFP mRNA dynamic upon induction with doxycycline. Specifically, it could be used to test our model of random activation and deactivation of transcription during dormancy.

If confirmed to be true, this model would raise several questions about gene expression during dormancy of yeast spores. First, what molecular mechanism could simultaneously activate or deactivate transcription of several genes that are not physically close? One possible other direction could be to look at the biophysical properties of the nucleus during dormancy. Indeed, several studies have found that under nutrient deprivation conditions, cells including yeast spores undergo a transition to a glassy-state, whereby molecular diffusion becomes greatly reduced (**Munder2018**). One possible way to test this would be following the activity and or distribution of RNA polymerases over time in dormant spores.

In conclusion, using inducible gene expression allowed us to open a new window

on the "nearly ceased" activity allowing dormant spores to survive. Accordingly, we found more questions than definitive answers. Specifically, our results suggest two things : (1) production and/or degradation of mRNA during dormancy have kinetics that strongly differ from replicating cells (2) mRNA content and/or mRNA dynamic is highly variable between individual dormant spores. Therefore, developing assay to systematically estimate mRNA production and degradation genome-wide or at single spore level, would likely yield exciting discovery in the future.

4.9. Supplementary materials

An hybrid model for interaction between two random switch

In order to get closer to experimental data, we would like to define a general model with one continuous "global control parameter" ($r \in [01]$) that recovers both previous model as limit cases. This means that for $r- > 0$ we want to recover the model with two independent switches and for $r- > 1$ the model with one unique switch controlling both genes. To that aim we started from the model with a unique switch and extended it to allow transitions to intermediate states A_1, I_1 and I_1, A_2, so that the full chain G_h can jump between 4 states : $(A_1, A_2), (I_1, A_2), (A_1, I_2), (I_1, I_2)$. For simplicity, let us rename these 4 states X_1, X_2, X_3, X_4 respectively, so that we write transition rate matrix :

$$P = (a_{ij})_{i,j \in (1,2,3,4)^2}$$

with a_{ij} the transition rate from X_i to X_j. With these notations, a_{23} is the transition rate from X_2 to X_3, i.e. the transition rate from (I_1, A_2) to (A_1, I_2).

Now if we set the transition matrix P to $P = P_1$

$$P_1 = \begin{pmatrix} 0 & 0 & 0 & \lambda \\ \infty & 0 & 0 & \infty \\ \infty & 0 & 0 & \infty \\ \mu & 0 & 0 & 0 \end{pmatrix}$$

we remark that our chain G_h is equivalent to our model with one unique switch controlling both genes :

Similarly, if we set the transition matrix P to $P = P_0$

$$P_0 = \begin{pmatrix} 0 & \mu & \mu & 0 \\ \lambda & 0 & 0 & \mu \\ \lambda & 0 & 0 & \mu \\ 0 & \lambda & \lambda & 0 \end{pmatrix}$$

we remark that our chain G_h is equivalent to the system :

$$A_1, I_2 \xrightarrow{\lambda} A_1, A_2 \tag{4.15a}$$
$$A_1, A_2 \xrightarrow{\mu} A_1, I_2 \tag{4.15b}$$
$$I_1, A_2 \xrightarrow{\lambda} A_1, A_2 \tag{4.15c}$$
$$A_1, A_2 \xrightarrow{\mu} I_1, A_2 \tag{4.15d}$$
$$I_1, I_2 \xrightarrow{\lambda} A_1, I_2 \tag{4.15e}$$
$$A_1, I_2 \xrightarrow{\mu} I_1, I_2 \tag{4.15f}$$
$$I_1, I_2 \xrightarrow{\lambda} I_1, A_2 \tag{4.15g}$$
$$I_1, A_2 \xrightarrow{\mu} I_1, I_2 \tag{4.15h}$$
$$\tag{4.15i}$$

with is itself equivalent to :

i.e. our model with two independent random switches.

Our goal now is to define a transition matrix $P(r)$ such that

$$\lim_{r\to 0} P(r) = P_0$$
$$\lim_{r\to 1} P(r) = P_1$$

While an infinity of matrix could satisfy that condition, we chose a simple interpolation with :

$$P(r) = \begin{pmatrix} 0 & \mu(1-r) & \mu(1-r) & \lambda r \\ \lambda\frac{1}{1-r} & 0 & 0 & \mu\frac{1}{1-r} \\ \lambda\frac{1}{1-r} & 0 & 0 & \mu\frac{1}{1-r} \\ \mu r & \lambda(1-r) & \lambda(1-r) & 0 \end{pmatrix}$$

With that transition matrix $P(r)$ we obtain a model that can be continuously tuned between our two previous models.

Supplementary figures

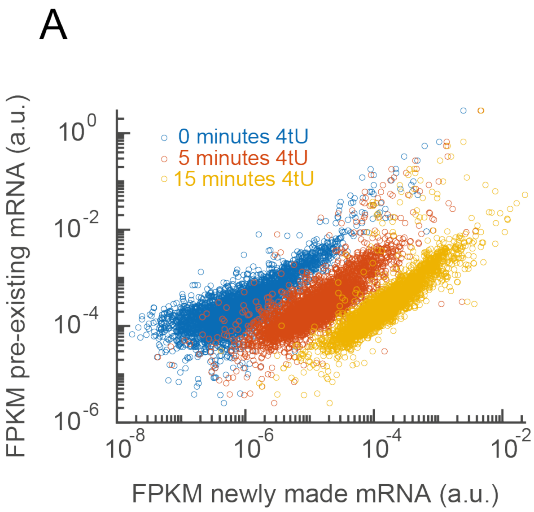


Figure 4.15: Genome wide correlation between pre-existing mRNA and newly made RNA. Correlation between the FPKM of newly made RNA (measured with 4tU seq, see methods []) and the FPKM of pre-existing mRNA (measured with total RNA-seq, see methods []). Individual dots are single genes (like ENO1,RPB3 etc) and different colors indicates different incubation times with 4tU (blue 0 minute, red 5 minutes and yellow 15 minutes.). n= genes. We see that longer incubation time with 4tU causes a slightly higher correlation, suggesting that the mRNA sequenced from 4tU-labeled RNA (x-axis) are gradually approaching the distribution of steady state mRNA (y-axis). The cloud of point for t=0 minute after incubation with 4tU indicates the experimental noise there is in purifying 4tU-labelled RNA. Indeed this process is not perfect and we should expect a residual amount of mRNA that are not labelled with 4tU (mRNA already present in spores and mature before we add 4tU) to leak into the purification process (see methods). Overall the evolution of the shape of the cloud of point makes sense but is not sufficient to conclude anything. However it suggests that 4tU sequencing is a promising method to detect newly made RNA during dormancy of *S.cerevisiae* spores.

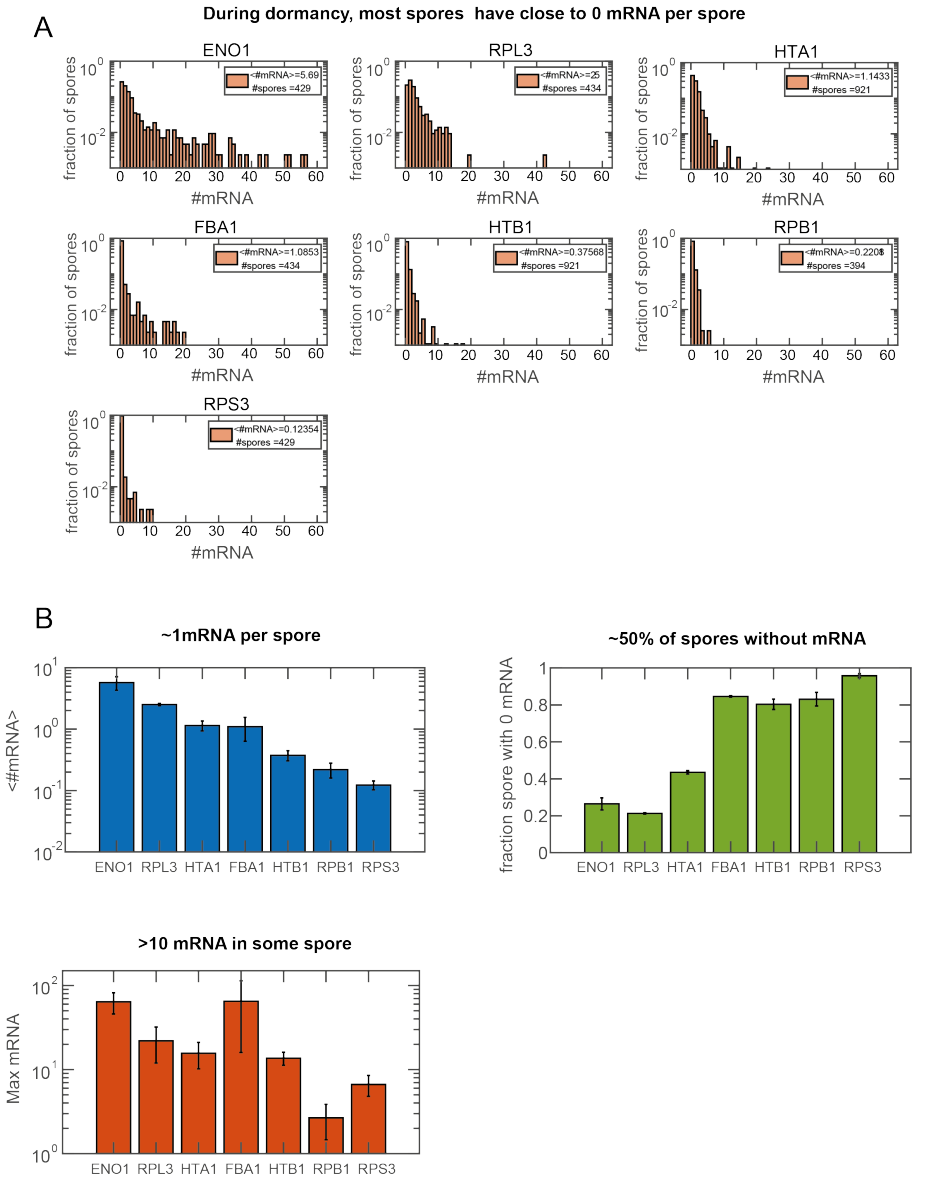


Figure 4.16 ***(preceding page)*****: mRNA abundance of endogenous genes in dormant spores**. Supporting data and supplementary for Fig.4.3. **A** Histograms of mRNA copy numbers (measured by single molecule FISH) of 7 different genes ENO1 (Qasar570), RPL3(Qasar570), HTA1(Qasar670), FBA1(Qasar670), HTB1(Qasar670), RPB1(Qasar570) and RPS3(Qasar670),from TT14 spores after 4 days incubation in PBS at 30C. See methods [] for list of probes and sequences. **B** Bar graph showing for each 7 genes, either the average number of mRNA per spore (arithmetic mean) (in red), the fraction of spores with more than 0 mRNA (1 and more) in green and maximum number of mRNA per spore in red. error bar are s.e.m on n=3 replicates.

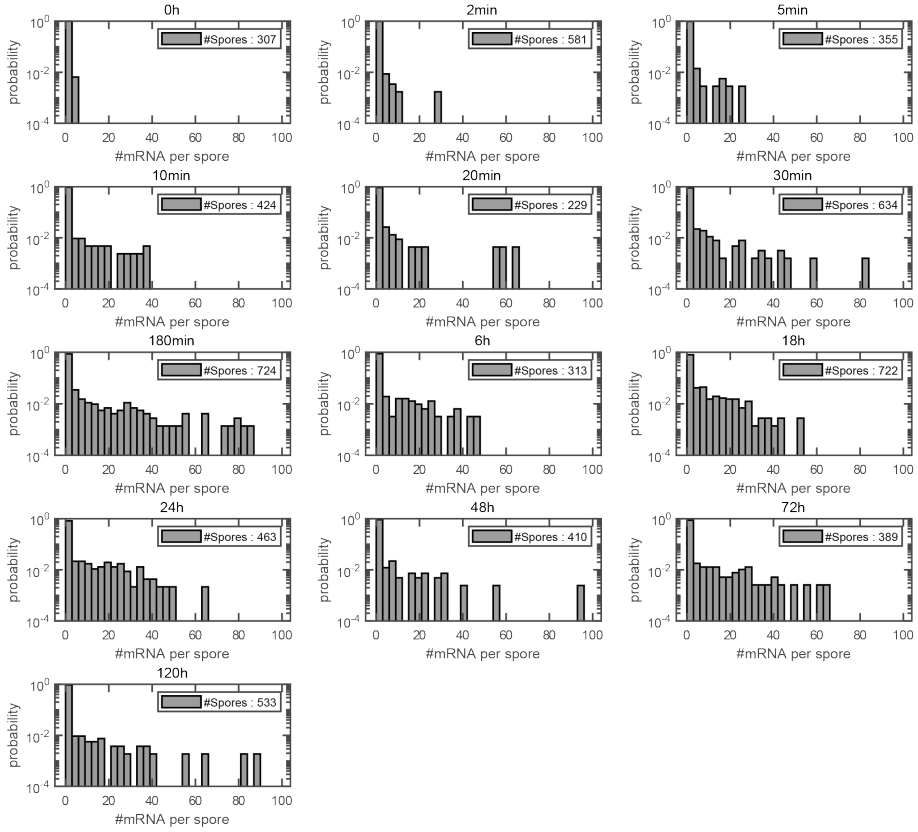


Figure 4.17 ***(preceding page)*****: Histograms of GFP mRNA copy number after induction with Doxycycline** Supporting data of Fig.4.4 Histograms of copy number GFP mRNA in TT14 spores incubated in PBS+100$\mu g ml$ after different times logarithmically spaced from 0 minutes to 120h.

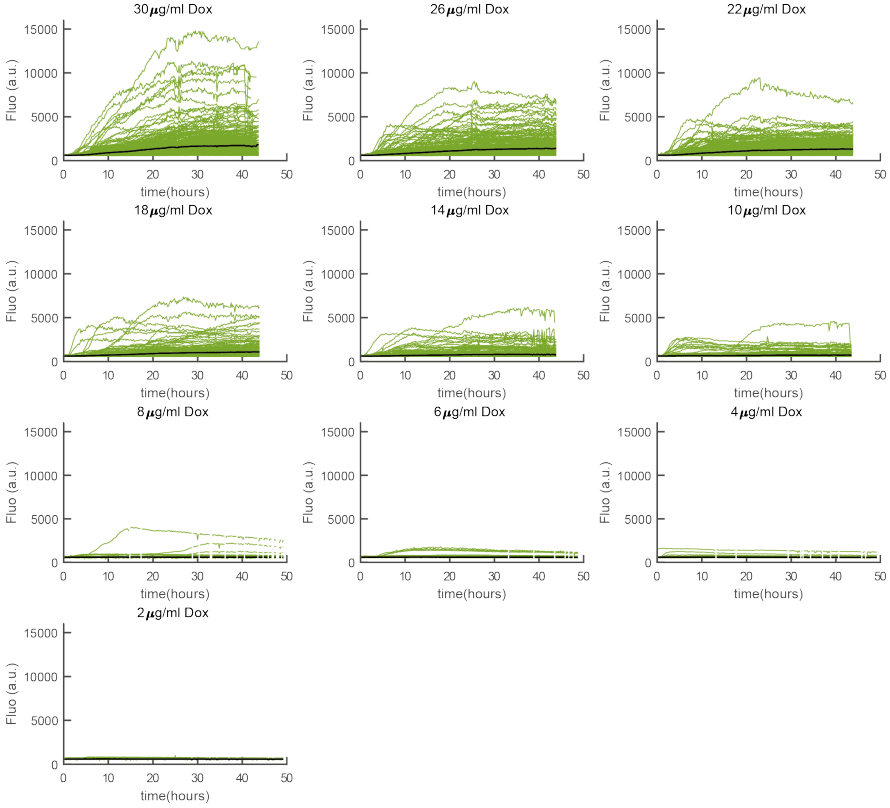


Figure 4.18 ***(preceding page)*****: Supp. GFP proteins expression for different doxycycline concentrations** Supporting data for Fig.4.6. Population of single spore traces of GFP protein fluorescence of TT14 spores (extracted as in Fig.4.6) during incubation in PBS with different Doxycycline concentrations for more than 40h.

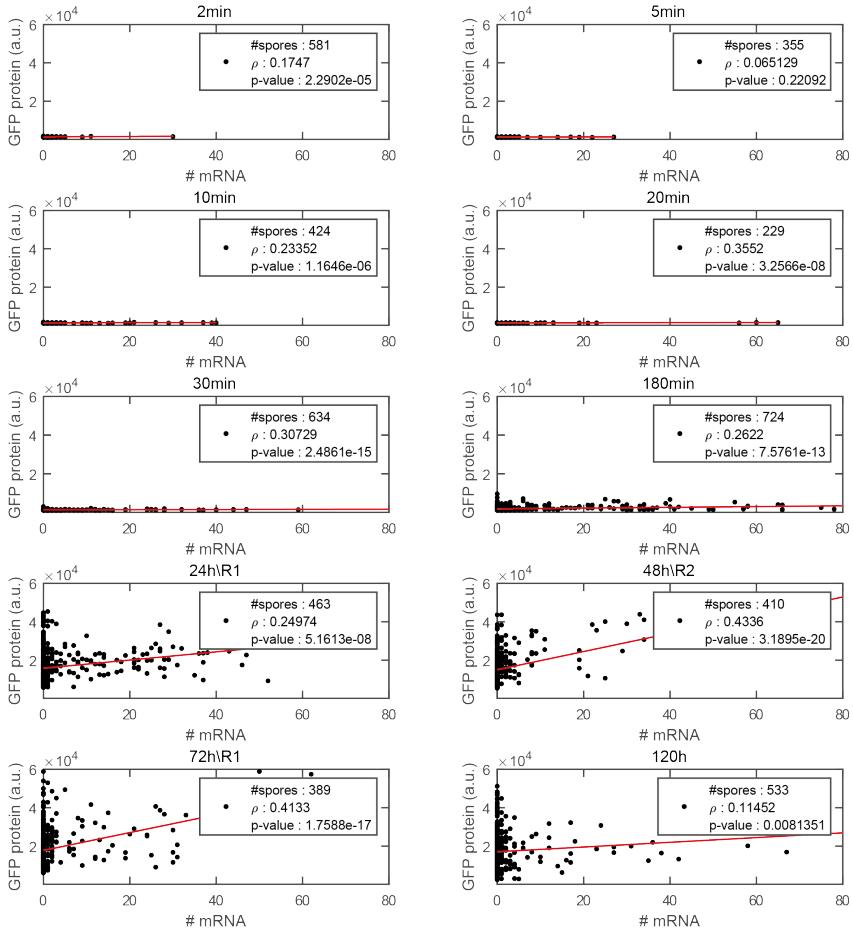


Figure 4.19 ***(preceding page)*****: Supp. Correlation between GFP mRNA and proteins after induction** Supporting data for Fig.4.7. Correlation between the number of GFP mRNA molecules (measured by smFISH) and the average GFP protein fluorescence for single TT14 spores incubated in PBS+100$\mu g/ml$ of Doxycycline for different times (same as data as in Supp.Fig.4.17). Each black dots is a single spore and red line indicate the linear regression fit. While for some time point there is a weak but significant positive correlation, overall GFP protein and GFP mRNA level seems to be un-correlated at the single spore level. This likely indicates that the two processes happens at very different time-scales. Other results suggests that mRNA production is fast and randomly stops for hours whereas GFP proteins level slowly accumulate and most importantly do not degrade of the time-lapse observation time-scale (a few days).

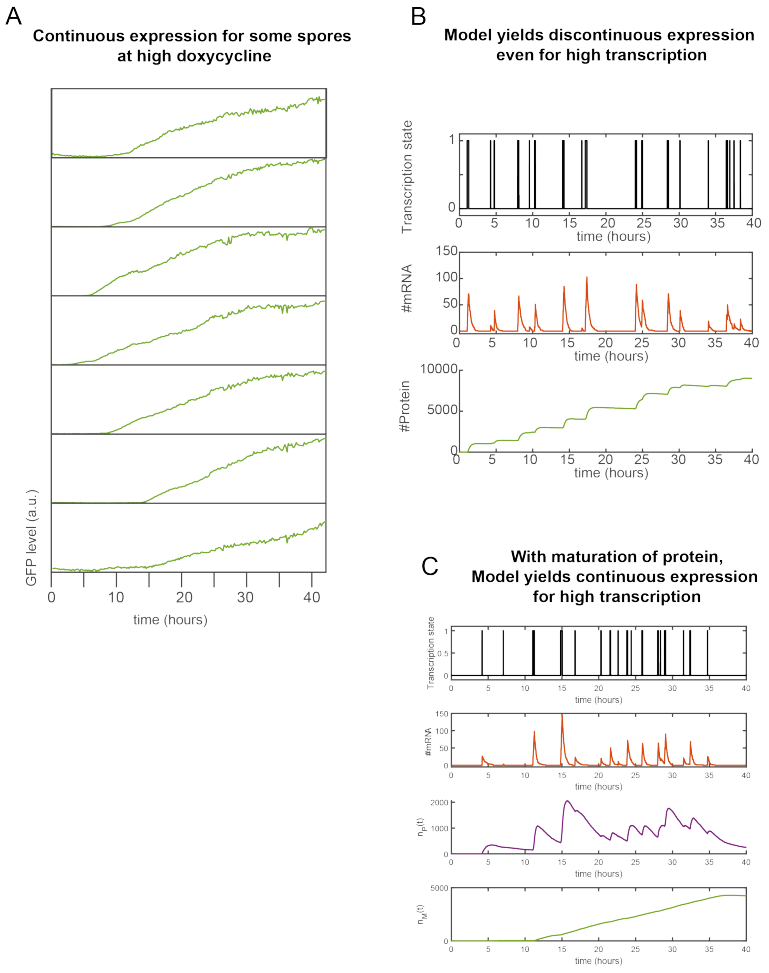

A
Continuous expression for some spores
at high doxycycline
GFP level (a.u.)
time (hours)
B
Model yields discontinuous expression
even for high transcription
Transcription state
#mRNA
#Protein
time (hours)
C
With maturation of protein,
Model yields continuous expression
for high transcription

Figure 4.20 ***(preceding page)*****: Supp. Continuous GFP expression - Model and experiments** Supporting data and experiments for Fig.4.9 and Fig.4.10. For high doxycycline concentration (typically higher than $30\mu g/ml$), we observed than some spores (but not all) do not have the typical "steps" profile we usually observed for lower Doxycycline concentrations (Fig.4.6B-C). As showed in 7 individual examples in **A**, these spores display nearly continuous expression profile. Moreover we see that the rate of expression is low and not constant. This behavior could not captured by simulation of the model with "simple" protein production as a first order reaction, i.e. with translation rate directly proportional to the number of mRNA as in Fig.4.8, reaction 4.8e. From previous experiments (**??**E), we saw that varying the Doxycycline concentrations only increased the averaged number of GFP mRNA molecules per spores. Thus in our model increasing the doxycycline concentration should be implemented by increasing $\mu_M = p_{ON} * \frac{\nu}{\gamma}$ (average number of mRNA at steady state in the random telegraph model 4.1) without changing p_{ON}, so we have increase ν. Strikingly, increasing ν (translation rate) still yielded discontinuous profile but with a higher number of steps (**B**). On the other hand, adding a additional non linear reaction that can interpreted as protein maturation (model 4.11), was sufficient to produce continuous profile as we increase ν (translation rate) only. Indeed in panel **C**, which is a simulation of the model 4.11 with high value of ν (equivalent to $50 to 100 \mu g/ml$ Doxycycline), we see that the number of mature proteins increase nearly continuously (green curve). This happens because the intermediate species non mature protein (in purple) acts as a transient memory of the past mRNA spikes (in red).

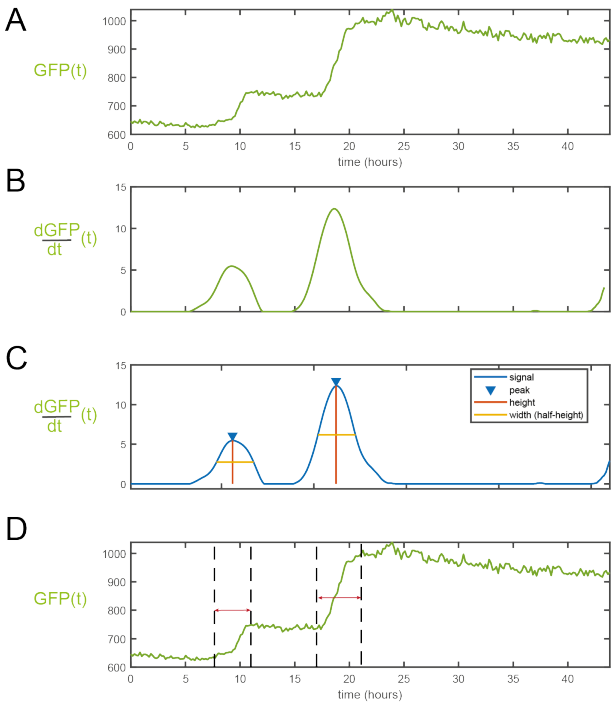


Figure 4.21 ***(preceding page)*****: Method to extract GFP expression events from raw single spore fluorescence** Supporting Fig.4.9. Here we demonstrate the automated extraction on the example of the spore used in Fig.4.6, replotted here in **A**. **B** First we compute the smooth the raw curve, compute the derivative and smooth again the derivative to obtain a clean filtered derivative of the fluorescence over time ("dGFP/dt"). **C** Then we use the function "findpeaks" from Matlab that is detecting peaks (blue arrows). Then it computes the height of peaks (in red), which corresponds to the maximum expression rate of raw profile **A** ; the width of peak (width at half of height) in yellow, which corresponds to the duration of the expression event.

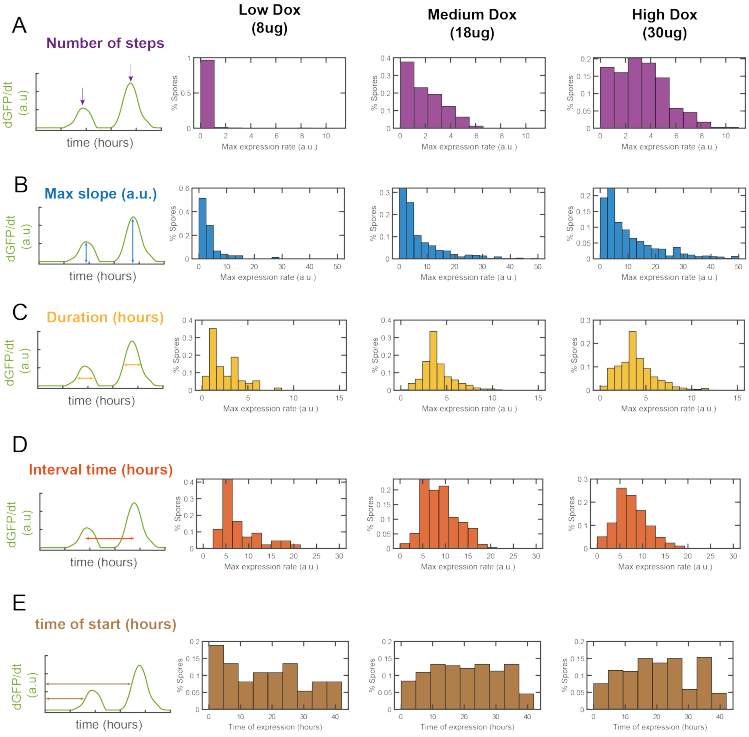


Figure 4.22 ***(preceding page)*****: Supp. Histograms of statistics of GFP expression steps** Supporting Fig.4.9 (same dataset). Using the automated method described in Supp.Fig.4.21 on the filtered derivate of single spore GFP traces, we compute **A** the number of events (in purple) as the number of peaks detected (purple arrow) ; **B** the maximum slope as the height of the peak (blue vertical line) ; **C** the duration of the event as the width of the peak (horizontal yellow line) ; **D** the interval between two events as the distance between the two peaks (time where the maximum slope is reached, indicated by purple arrows above) ; **A** and the time of start of an expression event (brown lines) going from t=0 to the time at which the curve reaches half the height of the peak (beginning of the yellow line in). Then we show the histograms of these quantities for 3 representative Doxycycline concentrations (8,18 and 30$\mu g/ml$).

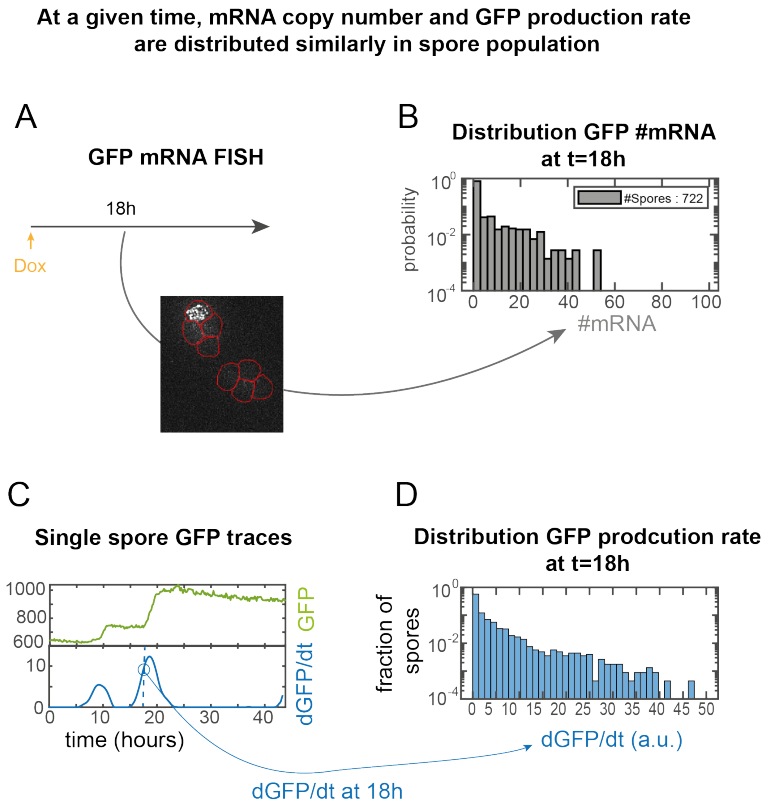


Figure 4.23 ***(preceding page)*****: Supp. Comparison between mRNAs and translation rate distributions**. In our work we look at GFP expression upon induction during dormancy in distinct perspectives : (1) From the perspective of mRNA copy number distribution at a given time (through single molecule FISH), (2) From the perspective of the GFP protein fluorescence dynamic at the single spore level. Since we do not directly measure the dynamic of mRNA production at the single spore level, we assumed a model (4.11) that is sufficient to reproduce both the mRNA distribution (Fig.4.5C) and the statistics of single spore GFP protein profiles (Fig.4.9 and Fig.4.10). We also see the problem in the opposite direction, and "assuming the model is true, what should be see in the data?". In that line of thoughts, here we compare the distribution of the number of GFP mRNA copy number at a given time (t= 18h **A** FISH image and **B** histogram) to the distribution of the protein synthesis rate at the same time (t=18h) (**C** and corresponding histogram in **D**.) Indeed we already that these two are not positively correlated (Supp.Fig.4.19) but if, as our model assume, the GFP steps are caused by discrete spikes of mRNA production (Fig.4.10), then the distribution of mRNA copy number and GFP production rate should be similar. Indeed in that model, a step in GFP production only occur when sufficient mRNA are produced during a spike. On the other hand, when no mRNA are present for a long time, the GFP production rate is zero, causing the GFP fluorescence profile to be flat. Interestingly we do observe that the two distributions (mRNA grey histogram in **B** and protein production rate blue in **D**) are qualitatively similar, strengthening the validity of our model.

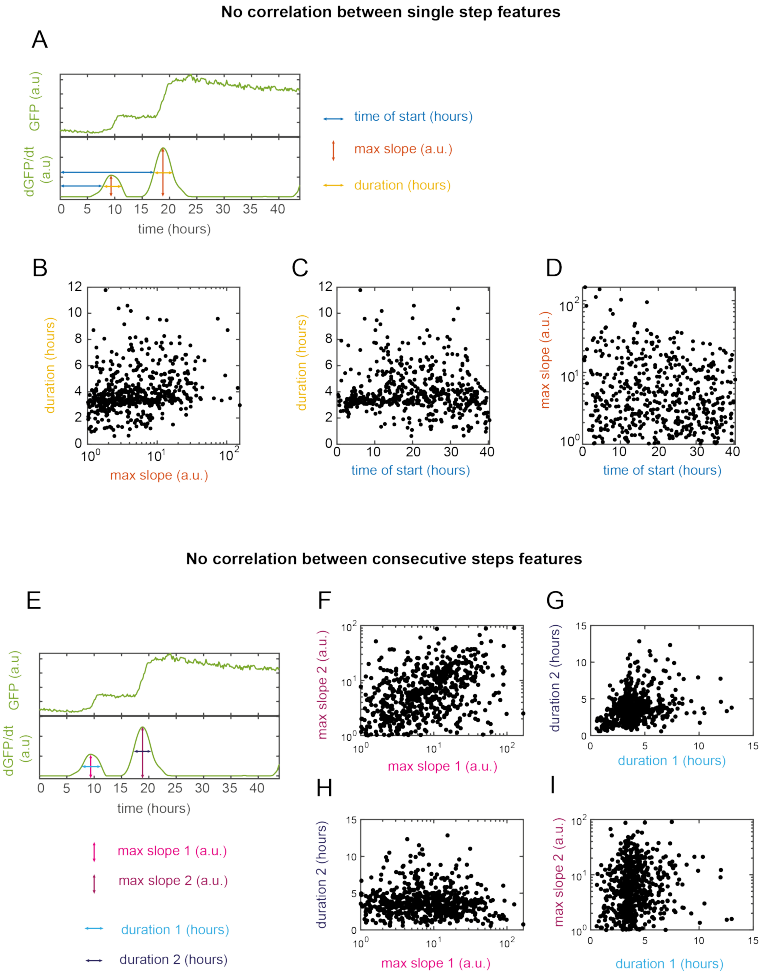


Figure 4.24 ***(preceding page)*****: Supp. Correlation between statistics of GFP expression steps** Supporting Fig.4.10. **A-D** For a given step in expression, we look at the correlation between the different quantitative features (duration, max slope and time of start) and found no striking positive correlation. In the case of more than one step per single spore, we looked at correlation by the different step features (max slope, duration etc) and also did not find any striking positive correlation.

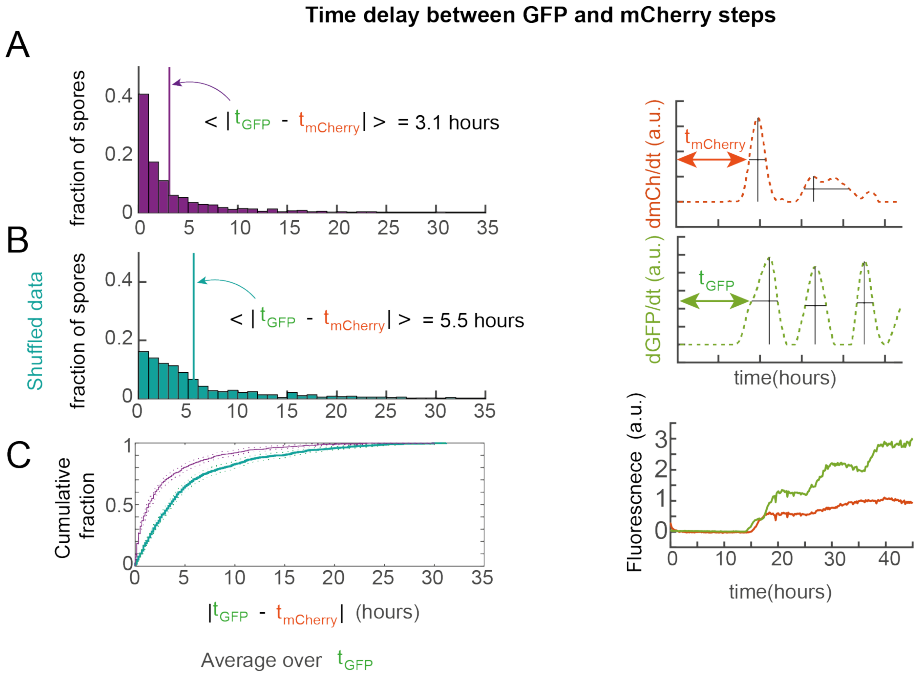


Figure 4.25 ***(preceding page)*****: Supp. Time delay between GFP and mCherry expression in experiments** Supporting analysis of data in Fig.4.11. **A** Histogram of the time between t_{GFP} (as computed in plot on the right, green curve) and $t_{mCherry}$ (as computed in plot on the right, red curve)). Vertical purple line indicates the average over n=349 spores. **B** Same as **A** but with shuffled data. **C** Cumulative density plot with data from **A** and **B**.

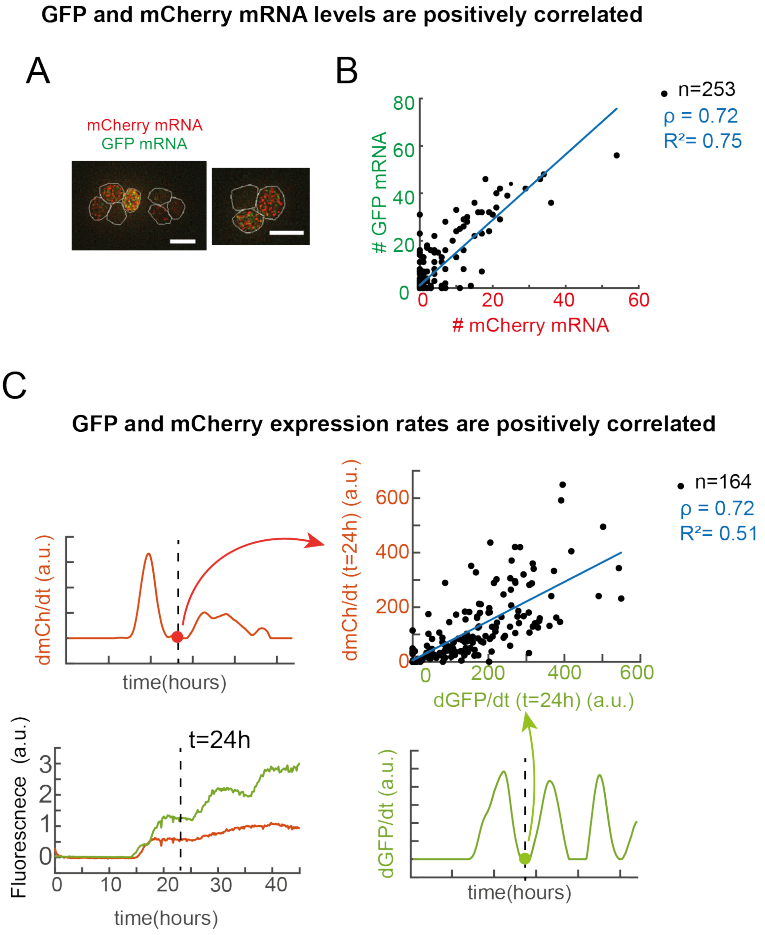

GFP and mCherry mRNA levels are positively correlated
A
mCherry mRNA
GFP mRNA
B
n=253
ρ = 0.72
R²= 0.75
# GFP mRNA
# mCherry mRNA
C
GFP and mCherry expression rates are positively correlated
dmCh/dt (a.u.)
time(hours)
dmCh/dt (t=24h) (a.u.)
dGFP/dt (t=24h) (a.u.)
n=164
ρ = 0.72
R²= 0.51
t=24h
Fluorescnece (a.u.)
time(hours)
dGFP/dt (a.u.)
time(hours)

Figure 4.26 ***(preceding page)*****: Supp. Correlation between mRNA level or translation rate in two color experiments** Supporting data and analysis of Fig.4.11. **A** Composite microscopy image of TM3 spores (GFP+mCherry inducible) after 24 hours incubation in PBS+25μ/ml Doxycycline. Red dots represent mCherry mRNA (Qasar670) and green dots represent GFP mRNA (Qasar570). White line indicated the segmentation mask. Number of mRNA molecules was counted within each mask with FISHquant (see Methods). Scale bar are 3 μm. **B** Scatter plot of single spore GFP and mCherry copy number, measured and segmented as in **A**. Black dots indicates single spores, blue line indicates the linear regression fit. n=253. **C** Scatter plot of single spores GFP and mCherry protein fluorescence production rate (dGFP/dt and dmCherry/dt), after 24hours of incubation in PBS+25μgml Doxycycline (i.e. same as **A-B**). Production rates were extracted from the filtered derivative of fluorescence over time (as Supp.Fig.4.20). Black dots are single spores and blue line is the linear regression fit.
We see that (1) mRNA copy number are positively correlated ($\rho = 0.72$) and that the protein production rate are positively correlated as well ($\rho = 0.72$). Comparing the two quantities makes sense with the reasoning explained in Supp.Fig.4.23. The idea being that if our model is correct, the mRNA copy number and the protein production rate should behave similarly. Here we look at the correlation between two genes (GFP and mCherry), and we indeed observe that both the mRNA copy numbers and the protein production rates display a similar positive correlation. Therefore this results support our claim that transcriptions of GFP and mCherry are activated and de-activated simultaneously. We observe a strong correlation in protein synthesis of both genes (Fig.4.11-4.21) because both transcription of both genes is globally controlled by the same random switch (Model,4.14,Fig.4.12B).

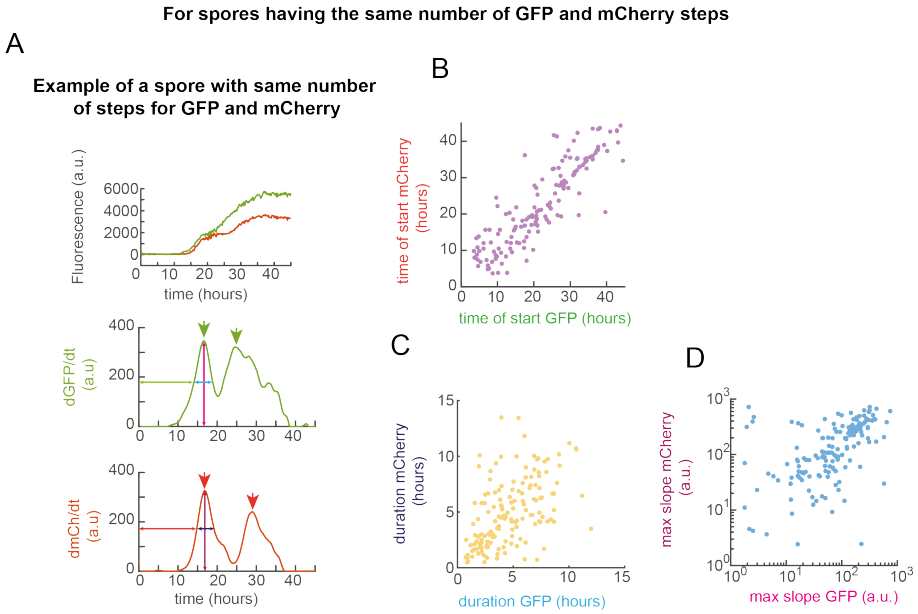


Figure 4.27 ***(preceding page)*****: Supp. Correlation between steps features in two-color experiments** Supplementary analysis of data presented in Fig.4.11) Here we look at alternatives to probe the correlation between GFP and mCherry protein synthesis at single spore level. **A** We consider here the special case where both GFP and mCherry fluorescence profile have the same number of steps, i.e. the same number of peaks on the derivative (here two green arrows for GFP and two red arrows for mCherry). There we can map un-ambigously the peaks of GFP and mCherry by respecting the temporal order (first GFP peak with first mCherry peak,second GFP peak with second mCherry peak etc). **B** Scatter plot of the times of start of a step (as defined in Supp.Fig.4.22) between GFP and mCherry. Each purple dot corresponds to a single expression step (or peak in derivative). **C** Similar to **B** but the duration of a step (width of peak in derivative). **D** Similar to **B** but for the maximum slope of step (height of peak in derivative)

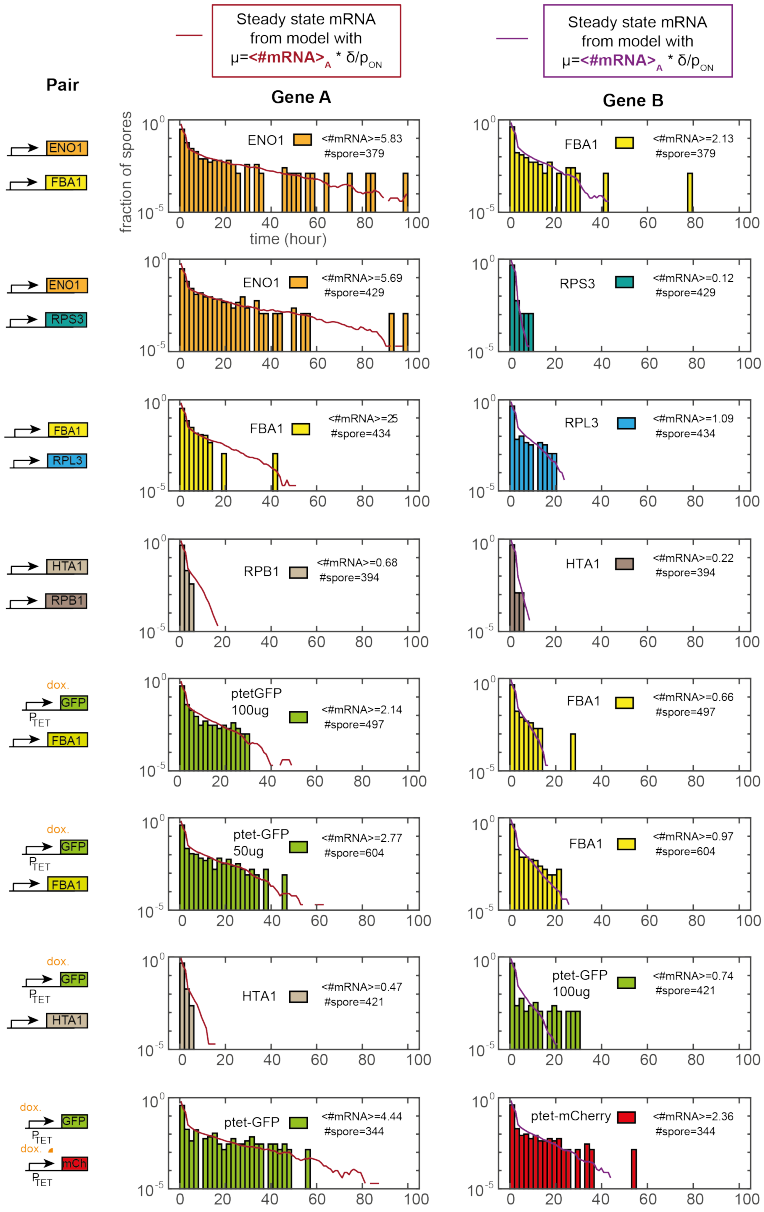

Steady state mRNA from model with μ=<#mRNA>_A * δ/p_ON
Steady state mRNA from model with μ=<#mRNA>_A * δ/p_ON
Pair
Gene A
Gene B
fraction of spores
time (hour)
ENO1
FBA1
ENO1 <#mRNA>=5.83 #spore=379
FBA1 <#mRNA>=2.13 #spore=379
ENO1
RPS3
ENO1 <#mRNA>=5.69 #spore=429
RPS3 <#mRNA>=0.12 #spore=429
FBA1
RPL3
FBA1 <#mRNA>=25 #spore=434
RPL3 <#mRNA>=1.09 #spore=434
HTA1
RPB1
RPB1 <#mRNA>=0.68 #spore=394
HTA1 <#mRNA>=0.22 #spore=394
dox
GFP
P_TET
FBA1
ptetGFP 100ug <#mRNA>=2.14 #spore=497
FBA1 <#mRNA>=0.66 #spore=497
ptet-GFP 50ug <#mRNA>=2.77 #spore=604
FBA1 <#mRNA>=0.97 #spore=604
HTA1
HTA1 <#mRNA>=0.47 #spore=421
ptet-GFP 100ug <#mRNA>=0.74 #spore=421
ptet-GFP <#mRNA>=4.44 #spore=344
ptet-mCherry <#mRNA>=2.36 #spore=344

Figure 4.28 ***(preceding page)*****: Supp. Histogram of mRNA from model and experiments** Supplementary analysis of data presented in Fig.4.13. Here we show the mRNA copy number distribution for pairs of gene used in Fig.4.13 to compute the mutual information and fit the "R" of the global control model. Histograms obtained from measured FISH data on TT14 spores incubated in PBS or PBS+Doxycycline for pair where there is a ptetGFP gene. From these histograms we extracted the average number of mRNA per single spore for each pair of gene (A,B), $< \#mRNA >_A$ and $< \#mRNA >_B$. We then used these values to tune the parameter ν in the model (4.11), which represents the translation rate, so that for gene A $\nu_A =< \#mRNA >_A * \frac{\delta}{p_{ON}}$ and for gene B : $\nu_B =< \#mRNA >_B * \frac{\delta}{p_{ON}}$. In all simulation δ the degradation rate of mRNA and p_{ON}, the fraction of time promotor spend in the activated state, were kept the same, to the value we obtained from GFP induction only (Fig.4.4-Fig.4.5). With these parameters and the value of R fitted (Supp.Fig.4.30) hybrid control model, we sampled the steady state distribution of both mRNA (gene A and B) from this model by running 10^4 times the same simulation and taking the number of mRNA (for gene A and B) after 40 hours (until the Gillespie algorithm reached 40 h). The sampled steady state distribution are shown as a plain line on top of the histograms (red for gene A and purple for gene B). Importantly for all these pairs, it is not given that a model such as the random telegraph could reproduce accurately the distribution of mRNA (for instance for all endogeneous genes such as EN01, FBA1 etc), simply by changing the value of ν_A and ν_B (translation rates), but by keeping the transcription regulation the same for all pairs.

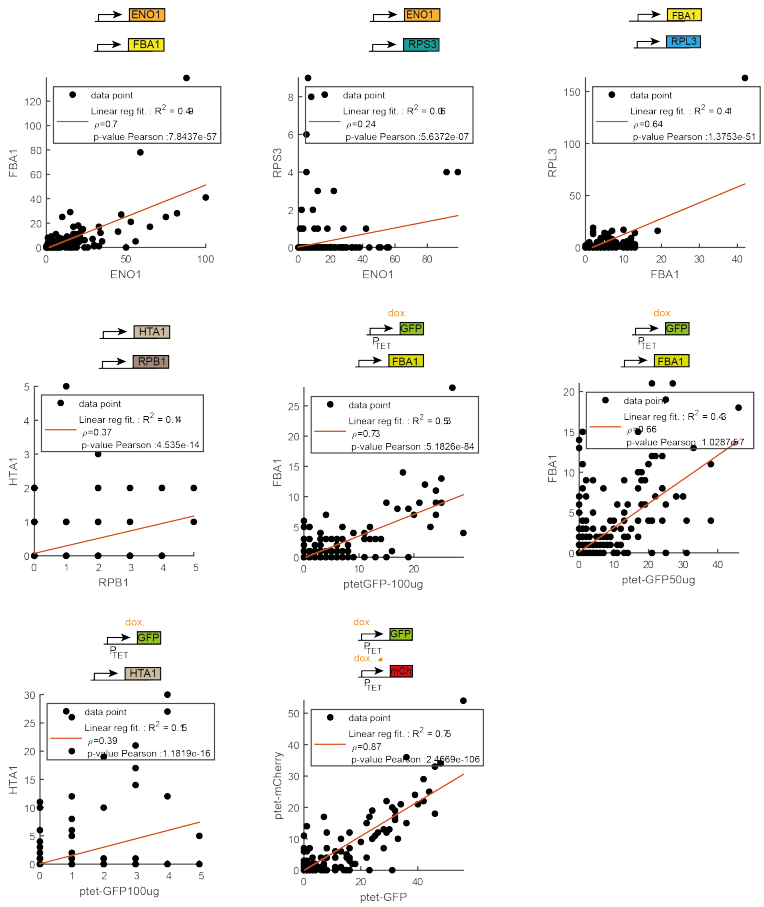


Figure 4.29 ***(preceding page)*****: Correlation mRNA distribution experiments for all pair of genes** Supplementary analysis of data presented in Fig.4.13. For each 7 pairs of gene, we show a scatter plot with in x-axis the mRNA copy number of gene A and in y-axis the mRNA copy number of gene B. (each black dot is a single spore measured by smFISH). Red line represents the linear regression fit.

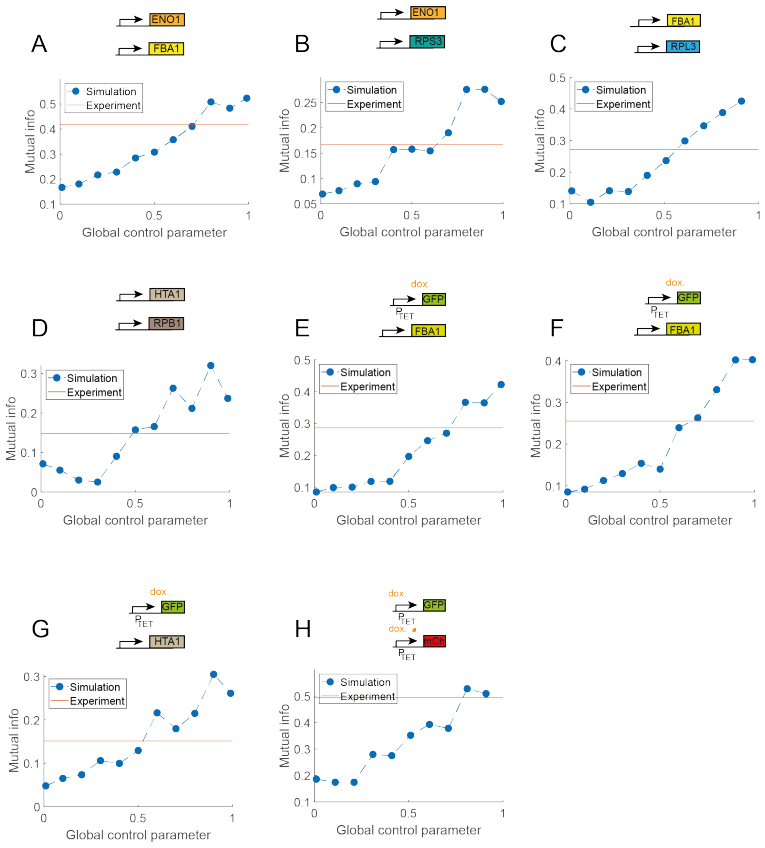


Figure 4.30 ***(preceding page)*****: Comparison experiment-model Mutual information for all pair of genes** Supplementary analysis of data presented in Fig.4.13. As depicted in Fig.4.13C, for each pair of gene we ran simulations by (1) Tuning "R" the global control parameter defined in 4.1 between 0 and 1 (2) Tuning the translation rate ν_A and ν_B according to the mRNA FISH data (see Supp.Fig.4.28), (3) All the other parameters are kept the same for all simulations (same as 4.10). For each simulated pair of histograms we then compute the mutual information using the "Normalized mutual information" ("nmi") from the Information Theory toolbox. `https://fr.mathworks.com/matlabcentral/fileexchange/35625-information-theory-toolbox`. Resulting value of mutual information are shown as blue dots. We observe that as expected, increasing the value of the global control parameter "R" increases the value of the mutual information. This is expected because higher value of "R" means that both genes are more likely to activate and de-activate transcription at the same time. In turn having both mRNA produced at the same time naturally makes the distribution mRNA "more correlated". In the of probability and information theory language, Being "more correlated" signifies to have a higher mutual information.
Having computed this "standard curve" to map the value of the global control parameter "R" to the mutual information between the pair-distribution of mRNA, we similarly compute the mutual information from the mRNA distribution measured by FISH (red line) and infer the corresponding global parameter value by extrapolating from the simulated mutual information curve. Accordingly this approach is an heuristic. It was done this way to estimate, given our model with hybrid, what is the "amount" of correlation between the two genes. Simply measuring the mutual information from mRNA FISH histograms and computing the linear coefficient of correlation is not sufficient because we can not directly interpret such values. Therefore this procedure allows to convert these numbers into a tangible feature we can interpret in our model (i.e. the global control parameter)

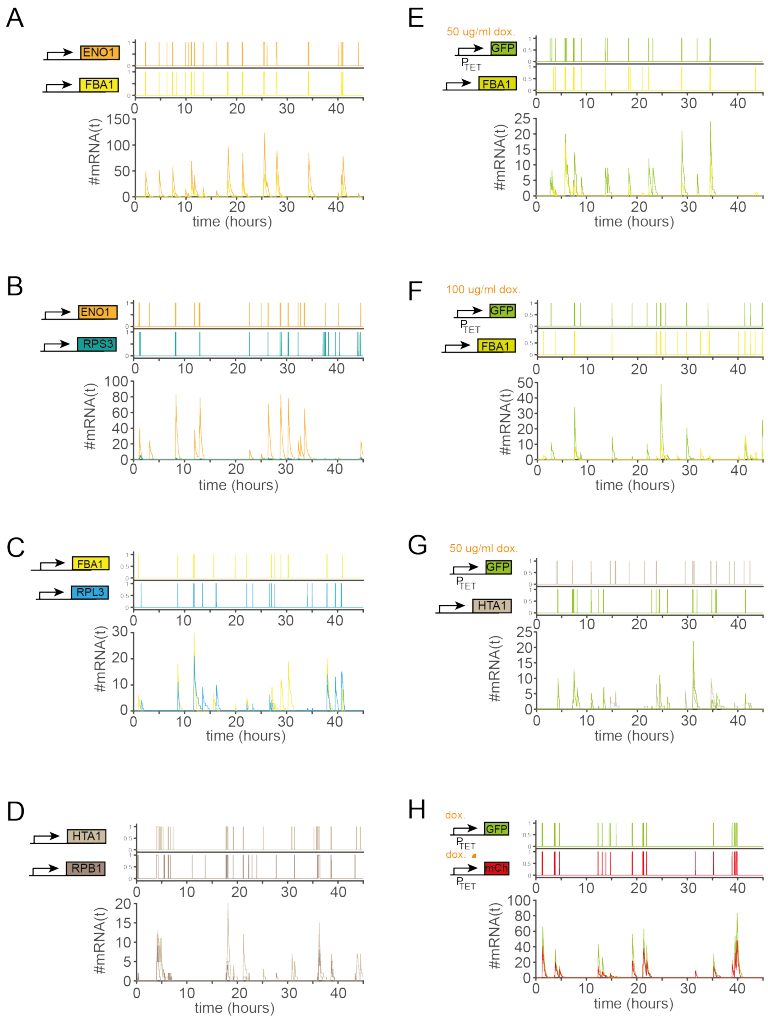


Figure 4.31 ***(preceding page)*****: Supp. Simulation RNA dynamics for pair of genes** Supporting simulation to Fig.4.13. Here we show a representative simulation for each pair of genes, with the fitted "R" from Supp.Fig.4.30.

5

Conclusion

Being dormant is surviving with drastically reduced internal activity.

We started out this thesis by interrogating the mechanism underlying this phenomenon, namely "how to survive by being still?". To do so, we investigated how reduced gene expression in dormant yeast spores was linked to their ability to survive. We used single cell experiments and modelling to address three different questions, based on three different fates of a dormant spore :

- *What determines whether a dormant spore germinates?* (Chapter 2)
- *What determines whether a dormant spore dies?* (Chapter 3)
- *What is a dormant spore doing before it germinates or dies?* (Chapter 4)

Throughout these three parts we covered different approaches to capture the link between gene expression and survival during dormancy of yeast spores, with synthetic circuit, single molecules FISH, fluorescence time-lapse and genome wide assays, perturbation with drugs etc. We tried our best to bind these different assays through simple mathematical modeling. Specifically, when direct single spore live-measurement was not possible, we tried to estimate what was the most likely single-spore dynamical process consistent with most of our experimental data. Therefore, our model of how gene expression dynamics set timescale of survival during dormancy (Fig.5.1) should be interpreted as a general indicative map to guide future more targeted studies. Indeed, quantifying "nearly ceased" processes going on in cells during dormancy is still an emerging research field. The work presented in this thesis indirectly revealed the extent of our ignorance concerning the biological and physical processes underpinning the term "nearly ceased".

We will now briefly discuss more in details three points regarding our emerging conceptual model about the link between gene expression and survival during dormancy :

- what unifying model emerges from our experiments on gene expression during dormancy?

- what are its limitation and the experiments that are required to test it?
- what perspectives it opens on studying gene expression during dormancy?

5.1. Model of dynamics and functions of gene expression during dormancy

Overall, a common theme emerged : transcription is fast and crucial for short term survival, whereas translation is slow and determines long-term survival.

Transcription In our model, single gene are transcribed and degraded fast ($\sim 10-30$ minutes), but transcription is randomly deactivated for hours. Together, this yields relatively short "spikes" of RNA production ($\sim 30min-1hour$), during which RNA are quickly produced and degraded. These spikes are then spaced by random 5–20hours long periods during which RNA level is close to zero. Finally, in our model, the random hour-long deactivation of transcription is acting genome-wide, so that several if not all genes are not transcribed simultaneously. On the other hand, functional results suggest that transcription is necessary for short term survival, so that inhibiting transcription kills half of dormant spores in less than $10-12$ hours. Given this similarity between these two time-scales (functional and dynamic), we could speculate that there are causally linked.

Protein synthesis In our model, mature protein production is limited and degradation is very slow (half-life higher than 2 days). On the long-term, proteins are more degraded than produced, yielded a slow but inevitable net-decay over months.

On the other hand, protein synthesis is only necessary for long term-survival, so that inhibiting translation reduces the lifespan of spore by 2 (from 40 to 20 days). Similarly to transcription, it is striking that the two time-scales (functional and dynamic) closely match. This may indicate that the long-term survival of dormant spores is actually set by the timescale of protein's dynamic decay.

One simple model to bind everything would be the following : proteins necessary for transcription (such as RNA polymerases) are slowly decaying until a critical point is reached, after which transcription is prohibited and spores die quickly. In that model, both RNA and protein dynamic metabolism jointly determine the timescale of dormant spores' survival.

5.2. Current limitation of our model

Accordingly, this work leaves more door opened than closed. Several new avenues would require specific work to be investigated. We will stress two crucial points

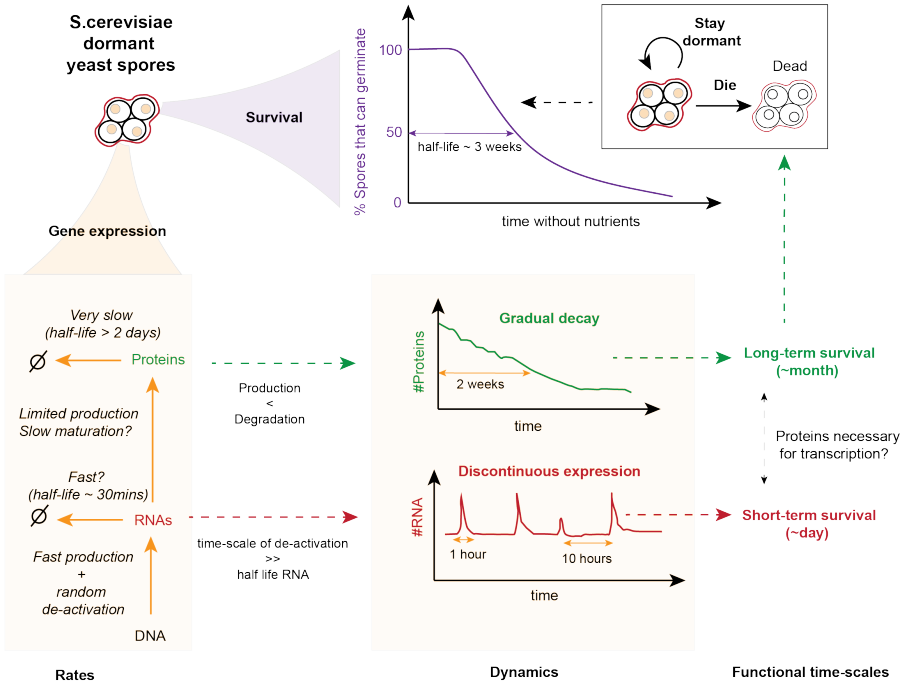


Figure 5.1: Gene expression and survival during dormancy of yeast spores. Summarizing view on the links between the dynamic of gene expression and the timescale of survival during dormancy of yeast spores. In this thesis, we gained insights about some of the fundamental rates of gene expression during dormancy (orange shades) : mRNA (in red) production seems to be fast but discontinuous (transcription randomly stopped over tens of hours) and degradation relatively fast, yielded a train of spikes in mRNA level. On the other hand, protein are degraded slowly but produced even more slowly, causing a net-loss and long-term (in weeks) gradual decay of proteins. These two distinct dynamics are consistent with the effect of drugs inhibiting gene expression : translation is necessary for long term-survival (months) while transcription is necessary for short term survival (day). Our model speculates that a process to explain the loss of spores viability (in purple) is the decay of proteins necessary to maintain gene expression.

about transcription dynamic and function during dormancy that would require special attention for future work.

What is the genome wide stability of RNA in dormant spores? Throughout this thesis we accumulated evidence that exogenous (e.g. GFP) as well as endogenous proteins (e.g. rpb3, subunit of RNA polymerase II) are stable for several days in dormant spores. On the other hand, for RNA we got conflicting results, either supporting stability similar to replicating cells (half-life of $\sim$ 30 minutes) or extremely long stability (half-life in days, similar to what we see for proteins) (see Chapter 4, Box 4.3.2). We built our model based on the parsimonious assumption that RNA are not particularly stable in dormant spores, but this point should be clarified by future studies. We suggest two possible to way to directly probe degradation of RNA in dormant spores. Either by tracking live transcription of an inducible gene (such as GFP) at the single spore level with PP7-MS2 fluorescent tagging transcript in live cells (**Tineke**); or by inferring the stability of RNA genome wide with metabolic labelling technique (such as 4tU-seq).

Why does inhibiting transcription kills dormant spores within a day? One of the key unexpected result we presented in this thesis was that inhibiting transcription with Thiolutin drastically reduces the lifespan of dormant spores from 3 weeks to half a day. While several other evidence that dormant are transcribing, we did not uncover the mechanism explaining why stopping transcription impairs survival during dormancy. Two classes of mechanisms could be investigated. In the first one, one could speculate that stopping transcription has a global impact on the biophysics of transcription in the nucleus of dormant spores, for instance on aggregation of polymerases, chromatin compaction state or nucleolus composition. Once disrupted for long enough, a dormant spore might be unable to recover transcription and subsequently become unable to germinate in the presence of abundant nutrients. One evidence supporting this idea is that quickly after thiolutin is added to dormant spores, we observed that the protein RPC40, a subunit of RNAP I-III polymerases, stopped being aggregated in the nucleolus, as this is the case in replicating cells and non-inhibited dormant spores.

One other class of mechanism could be that the presence of some specific RNAs is necessary for spores to remain viable. For this mechanism to be valid, we would need these RNAs to 1) degrade within $\sim$ 12 hours if not produced, 2) be necessary for gene expression to function in spores. While RNA FISH revealed that non-coding RNA forming ribosomes are likely not a good candidate, we did not exclude the possibility that transcription of other unstable non-coding RNA (*snRNA*,*snoRNA*,*CUTs*,*SUTs* etc) might play a crucial role in survival during dormancy. Performing extensive sequencing of newly made RNA in dormant spores (through metabolic labeling) is a promising way to address that question.

5.3. New direction to study dormancy in other systems

Methods We found out that two types of general methods, both classically to study the biology of replicating cells, happened to be particularly useful to study gene expression during dormancy.

The first one is to inhibit various sub-process of gene expression (or other metabolic processes) during dormancy to probe their functional importance. Indeed, while transcription and translation are obviously fundamental processes supporting cellular growth and replication, the exact role they play in supporting survival during dormancy is far obvious in many systems. Crucially, in cases where

it remains unclear whether dormant state is metabolically active or not, measuring whether inhibiting transcription or translation affects lifespan during dormancy represents an easy preliminary test. If any drugs have an effect lifespan, it will indicate that dormant cells are metabolically active.

The second method is to induce an exogenous gene with a synthetic promotor. Crucially, if RNA and protein are being detected upon induction, it immediately demonstrates that gene expression machinery *can* function during dormancy. While this does not provide direct insight into the endogenous gene expression, such synthetic system already exist for replicating cells and could be easily test in the context of various dormant stage (spores, cysts, endospores etc).

Due to their simplicity and generality, we expect these two tools to be particularly helpful to quickly probe basic features of gene expression dynamic during dormancy of many different organisms.

Concepts Beyond concrete experimental methods, our work uncovered several useful concepts about dormancy that could potentially be applied to other systems. First, our work demonstrated that dormant spores (1) display an important phenotypic variability (the amount of certain proteins or the ability to express genes) and (2) this phenotypic variability underpin in the ability to exit dormancy (germinate for yeast spores) (3) This phenotypic variability is modified as spores decay to death (i.e. are ageing). Together, these results invite us to look at dormancy as a dynamic quantity that is varies greatly between individual, rather than a simple state of general inactivity. Generally, measuring the phenotypic variability during dormancy and looking for predictors of the ability to wake-up or of the lifespan, likely represents a fruitful strategy to start uncovering molecular mechanisms underpinning survival during dormancy.

Beyond phenotypic variability, our work uncovered that at the single-cell level, dormant spores seems to have a gene expression activity that varies greatly over time (see Chapter 4), alternating between long period of inactivity and short period

of strong activity. Given that dormancy is thought to be a state of "nearly-ceased activity", one would intuitively tend to pool together as many individual as possible in order to increase our chance to detect a good signal. Counter intuitively, we propose that the opposite strategy, consisting in focusing on tracking the activity of a single dormant individual over time, might actually be more fruitful to detect rare but measurable events of activity that would be hidden in bulk-approaches.

6

Material and methods

Protocols

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

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

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

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

Protocol for Fig. 7(B-E). We incubated a population of spores (“TS8” strain) in

water with either $10\mu g/ml$ of thiolutin or $200\mu g/ml$ of cycloheximide, or without any drugs. At various time points during the incubation, we aliquoted some of the spores from the population and then, with a microscope, measured the mCherry fluorescence of each spore bag (representing RNAP II level since these spores had mCherry protein fused to Rpb3, a subunit of RNAP II). The "RNAP II level" for each spore bag is the maximal mCherry fluorescence obtained from 36 z-stacks with a z-step $0.2\mu m$. After measuring the RNAP II level, we kept these spores under the microscope and washed away the drug four times with PBS. Then, while still keeping the spore bags under the microscope, we incubated them for 24 hours in minimal medium with 2%-glucose. We measured the percentage of spore bags that germinated during the 24 hours (these are counted as dormant (alive)).

Drugs used in Fig. 4A. We used thiolutin from Sigma Aldrich (CAS Number 87-11-6). For storage, we aliquoted thiolutin into DMSO at a final concentration of 1 mM. We used cycloheximide from Sigma Aldrich (CAS Number 66-81-9) which we maintained in DMSO at a final concentration of $100mg/mL$. We used antimycin A from Sigma Aldrich (CAS Number 1397-94-0) which we diluted into ethanol at $10mg/mL$.

Strains

The "wild-type", homozygous diploid yeast strain that we used is from Euroscarf with the official strain name "20000D" and genotype as follows: $MATa/MAT\alpha his3-11_15/his3-11_15;leu2-3_112/leu2-3_112;ura3-1/ura3-1;trp12/trp12;ade2-1/ade2-1;can1-100/can1-100$. This strain generated four genetically identical, haploid spores for each spore bag. For engineering the GFP-inducible spores, we started from the haploid versions of "20000D", which were also from Euroscarf. These haploid strains were "20000A" (isogenic to another standard laboratory strain called "W303" with mating-type "a") and "20000B" (isogenic to W303 with mating-type "alpha"). The 20000A's genotype is as follows:$MATahis3-11_15;leu2-3_112;ura3-1;trp12;ade2-1;can1-100$. The 20000B's genotype is exactly the same as 20000A's, except that it is of the opposite mating type (mating type "alpha"). The GFP-expressing diploid strain, called "TT14", is nearly identical to the wild-type's except for the addition of selection-marker genes that we introduced during the construction of the strain. TT14 has the following genotype: $(MATa/MAT\alpha his3-11_15/his3-11_15;leu2-3_112/leu2-3_112;ura3-1/ura3-1;ADE2/ADE2;can1-100/can1-100;HygB/HygB;trp12/TRP1;URA3/ura3-1;pADH1-rtTA/pADH1-rtTA;pTET07-GFP/pTET07-GFP)$. Here, pADH1 is the constitutive promoter for ADH1 gene in yeast (631 bases upstream of ADH1's ORF), rtTA is the reverse tetracycline-controlled transactivator whose transcription-activation domain is from the yeast's Msn2, and pTET07 is the promoter with 7 binding sites for rtTA. We sporulated TT14 to form the "GFP-inducible spores" (Fig. 3A). These constructs

are more fully described in a previous publication (**Youk2014**). For experiments involving the metabolic labelling of RNA with 5-Ethynyl-Uridine (5-EU) (Fig. 4C), we had to obtain spores that were able uptake the extracellular 5-EU for incorporation into intracellular RNA. To achieve this, we engineered a diploid yeast strain, "TS3", which is the same as TT14 - TS3 also has the same GFP-inducing synthetic circuit (Fig. 3A) as TT14 - but now with additional genotype modifications ($HIS3/HIS3;hENT1-ADH1/hENT1-ADH1;HSV-TKGDP/HSV-TKGDP$) for which we used a construct that was previously shown to let yeast import BrdU (Viggiani et al. 2006). hENT1 is the "human Equilibrative Nucleoside Transporter", under the control of the promotor for ADH1, HSV is the Herpes Simplex Virus thymidine kinase under control of the promotor for GPD1, and HIS3 is a selection marker gene used for the cloning. To obtain the spores whose subunits of RNA polymerases were fused to mCherry (Rpb3 for RNAP II and Rpc40 for RNAP I and III) (Figs. 4F and 4G), we engineered two diploid strains, "TS8" and "TS9", which were the same as "TS3" - TS8 and TS9 both have the GFP-inducing synthetic circuit and allow 5-EU uptake) - but now with additional genotype modifications: (RPB3-mCherry-NatR / RPB3-mCherry-NatR) or (RPC40-mCherry-NatR / RPC40-mCherry-NatR). Here "RPB3-mCherry-NatR" is the RPB3 gene on the yeast chromosome IX (YIL021W) fused at its 3'-end to the mCherry gene, using the protein-linker sequence from (Sheff et al. 2004). NatR is the Nourseothricin resistance marker inserted behind the stop codon of the RPB3-mCherry fusion gene. "RPC40-mCherry-NatR" is the same as the "RPB3-mCherry-NatR" but now with the RPC40 gene that is on the yeast chromosome XVI (YPR110C) instead of RPB3.

Strain construction.

For each yeast transformation, we integrated a single copy of an appropriate, linearized yeast-integrating plasmid at a desired genomic locus through a homologous recombination. We first introduced a promoter of ADH1 controlling rtTA expression pADH1-rtTA into 20000A (wild-type haploid, mating type "a") and 20000B (wild-type haploid, mating type "alpha") at the HO locus, by inserting a linearized, singly integrating, yeast-integrating plasmid with pADH1-rtTA and Hygromycin-resistance gene as a selection marker. This yielded two strains, "W303r1" (From 20000A) and "W304r1" (from 20000B). We next replaced the ade2-1 "ochre mutation" in W303r1 and W304r1 with a functional ADE2 gene by a homologous recombination of ADE2 that we obtained by PCR from the S288C reference genome (100-bp homology on both flanking sites of the PCR product). This yielded two strains, "TT2" (from W303r1) and "TT8" (from W304r1). We then inserted a pTET07-GFP at the LEU2 locus in TT2 and TT8 by linearizing a yeast-integration plasmid that contains pTET07-GFP. This yielded two strains, "TT7" (from TT2) and "TT9" (from TT8). We then introduced two constitutively expressed selection-marker genes: URA3 into TT7 to create "TT10" and TRP1 into TT9 marker to

create "TT13". These selection markers allowed us to select the diploid strain ("TT14") that resulted from mating TT10 with TT13 by using a double drop-out medium (lacking -ura and -trp). TT14 is homozygous for ADE2, pADH1-rtTA, and pTET07-GFP, thus all four haploids in a TT14 spore-bag has an inducible GFP. For the construction of TS3 strain (EU-incorporating diploids), we transformed two haploid strains, "TT10" and "TT13", as stated in (Viggiani et al. 2006) with a linearized fragment (digested with NheI) of the plasmid "p403-BrdU-Inc" (from (Viggiani and Aparicio, 2006) and obtained from Addgene). This plasmid contains hENT1-ADH1, HSV-TKGDP, and a constitutively expressed selection marker, HIS3. We linearized and then integrated the plasmid, as a single-copy, into the HIS3 locus in the yeast genome. This resulted in two strains, "TS1" (from TT10) and "TS2" (from TT13), of opposing mating types. We mated these two to obtain a diploid strain "TS3". To construct the strain, "TS8" (which has Rpb3 protein fused to mCherry), we transformed the haploids strains, TS1 and TS2, by integrating mCherry-NatR linker at the RPB3 locus in the yeast genome. We obtained the mCherry-NatR linker taking a plasmid "HypI" (used in (**LamanTrip 2020**) and derived from "pkt150" (Sheff and Thorn, 2004)) and then amplifying the mCherry-NatR linker through PCR. This resulted in two strains, "TS4" (from TS1) and "TS5" (from TS2), which had opposite mating types. We mated them to obtain the diploid strain, "TS8". For constructing the diploid strain, "TS9" (which has Rpc40 protein fused to mCherry), we transformed the haploids strains "TS1" and "TS2" to obtained two strains, "TS6" (from TS1) and "TS7" (from TS2), which had opposite mating types. We mated them to obtain the diploid strain, "TS9".

Yeast transformations and mating

We transformed yeasts with the standard, lithium-acetate-based method. In short, log-phase cells were resuspended in 0.1M of lithium acetate in 1x TE, together with the DNA to be inserted by homologous recombination at a desired location within the yeast genome and herring ssDNA. We then added PEG3350, 10X TE and 1 M of lithium acetate to the yeast culture so that we had final concentrations of 40% PEG 3350, 1X TE, and 0.1 M of LiOAc. We then incubated the culture at 42C for 30 minutes. We pelleted the resulting cells by centrifuging and then washed them with water. Afterwards, we plated the cells on agar plates with dropout media to select from transformed yeasts. We used a standard method of mating yeasts. Namely, we mated yeasts of opposite sexes by inoculating a 500 μL of YPD medium with two colonies - one from each sex. We then incubated the culture overnight in 30C on a benchtop shaker (Eppendorf Mixmate) that agitated the culture at 300 rpm. We then spread 100μL of the culture on double-dropout agar plates which selected for the diploids that resulted from successful mating.

Spore formation (sporulation). We used a standard protocol for sporulating yeasts.

In short, we first grew diploid yeasts (homozygous diploid wild-type or GFP-inducible strains) to saturation overnight. We then transferred these cells to a "pre-sporulation media" (i.e., YPAc: consists of Yeast Peptone media with a 2% potassium acetate) which we then incubated for 8 hours at 30°C. We subsequently transferred the diploid yeasts to a "sporulation medium" (i.e., 2% potassium acetate) and left them to sporulate for 5 days at 20C, while rotating as a liquid culture in a tube. Afterwards, we transferred the resulting spores to water and stored them at 4°C. Through measurements, we found that we could store these spores for several months without loss of viability (i.e., 2%-glucose still germinates ~ 100% of the spore bags).

Microscope sample preparation

We performed all microscope imaging (including single molecule RNA FISH) with 96-well glass-bottom plates (5242-20, Zell-Kontakt). Prior to each microscope imaging, we pre-treated the glass-bottom by incubating it for 20 minutes with 0.1 mM of concanavalin A (ConA, C2010, Sigma-Aldrich). We washed the ConA and then typically added 1 μL of spores in 200$\mu\mu L$ of minimal medium per well. The plates were then centrifuged at 1000 rpm for 1 minute to sediment and attach all spore bags to the glass-bottom. We then performed the microscope imaging.

Microscope data acquisition

We used Olympus IX81 inverted, epifluorescence, wide-field microscope. For each time-lapse movie, we collected images once every 10 minutes for every field of view. The temperature during microscope imaging was maintained by an incubator cage (OKO Lab) that enclosed the microscope. We acquired each image with an EM-CCD Luca R camera (Andor) and IQ3 software (Andor). We used a wide-spectrum lamp (AMH-600-F6S, Andor) for exciting fluorescent proteins.

Microscope data analysis

We processed the microscope images with ImageJ and MATLAB (Mathworks). To measure the times taken to germinate (and to count spore bags that germinated), we looked for the first haploid spore that formed a bud for each ascus (spore bag). We segmented the spore bags by using the Sobel filtering of the brightfield images to create a mask. We extracted fluorescence values inside the mask. We corrected for the background fluorescence, for each ascus one by one, by subtracting the average background in a 50-pixel area that surrounded each ascus. For GFP fluorescence (measuring GFP inducibility) and mCherry fluorescence (measuring RNAP I-III levels through Rpb3-mCherry or Rpc40-mCherry), we computed the fluorescence per spore by taking the maximal intensity from 36 z-stacks with a z-step size

of 0.2 μm per spore bag.

Hexokinase-based assay to measure glucose concentrations.

10 hours after adding a low concentration of glucose to germinate some of the spores in a 1-mL minimal medium in a 24-well microscopy plate (5242-20, Zell-Kontakt), we took 800 μL of the supernatant to measure the concentration of glucose in it. We determined the glucose concentration by using a hexokinase-based glucose assay kit (Glucose (HK) Assay, G3293, Sigma-Aldrich) that is based on converting glucose through hexokinase and NADP+ dependent glucose-6-phosphate-dehydrogenase.

RNA-seq on un-germinated spores.

We collected un-germinated spores by first collecting 1 mL samples of spores that were incubated in a 0.002%-glucose at 0, 16, 48, and 96 hours after the incubation began. To isolate the un-germinated spores, we treated the 1-mL samples with zymolyase (786-036, G-Biosciences). Zymolyase lysed vegetative cells that formed from germinated spores, thus ensuring that we only collected RNA from un-germinated spores for sequencing. We then extracted the RNA from the left-over, un-germinated spores RiboPure Yeast Kit (Ambion, Life Technologies) as described by its protocol. Next, we prepared the cDNA library with the 3' mRNA-Seq library preparation kit (Quant-Seq, Lexogen) as described by its protocol. Afterwards, we loaded the cDNA library on an Illumina MiSeq with the MiSeq Reagent Kit c2 (Illumina) as described by its protocol. We analyzed the resulting RNA-Seq data as previously described (Trapnell et al, 2012): We performed the read alignment with TopHat, read assembly with Cufflinks, and analyses of differential gene-expressions with Cuffdiff. We used the reference genome for S. cerevisiae from ensembl. We used the transcriptional modules listed in Appendix Table S1 for grouping the relevant genes into transcriptional modules.

4tU seq on dormant spores.

We prepared large cultures (400ml) of wild-type spores (from W305 diploids), pre-incubated it for 4 days in PBS at 30C and added 4-thiouracil (4tU) media at a final 5 mM concentration. Samples were subsequently collected after desired amounts of time (0, 5 and 15 minutes). We discarded the supernatant and re-suspended the pellet in 1 mL RNAlater (Cat. No. AM7021, Thermo Fischer Scientific). As a spike-in of 4tU labelled RNA we used a fixed amount of cells from Schizosaccharomyces pombe (YFS110) analogously to previous work (Baptista et al. 2018). We then spun down our samples in a pre-cooled centrifuge, removed the RNAlater,

and proceeded with RNA extraction as described in the paragraph "preparing cells and RNA extraction". After RNA extraction, we proceeded with biotinylation and purification of the 4tU labelled RNA following existing protocols with minor modifications. After sequencing, we processed all sequencing data with the Salmon tool to quantify relative transcript abundance. Finally, we converted the transcript levels for S. cerevisiae to gene expression levels (Transcripts Per Million, TPM), merged all samples using the package tximport from Bioconductor.

Fixing and permeabilizing spores.

Spores were fixed and made permeable - to let fluorophores into the spores for 5-EU labeling of RNAs and single-molecule RNA FISH - according to the standard protocols for vegetative S. cerevisiae. An important modification to these protocols is that to digest the spore wall, we extended the typical incubation in zymolyase to 2 hours during which the spores were constantly agitated at 300 rpm in 30 OC. We used this fixation and permeabilization treatment for performing single-molecule RNA FISH (Fig. 4H) and 5-EU labeling of RNAs (Fig. 4C-D).

Single-molecule RNA FISH.

FISH probes. We designed single-molecule FISH probes for detecting mRNA or rRNA with the Stellaris FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA): www.biosearchtech.com/stellarisdesigner. The probes were coupled to CAL Fluor Red 610, Qasar 570 or Qasar 670 (Biosearch Technologies, Inc.).

Probe set name	Gene name	Number of probes	Fluorophore
rRNA_25S_TM	RDN25	48	CAL Fluor Red 610
18S_CAL610	RDN18	48	CAL Fluor Red 610
mRNA_yeGFP_Q670	yeGFP	28	Quasar 670
mRNA_yeGFP_Q570	yeGFP	28	Quasar 570
mRNA_yeGFP_TM	yeGFP	28	CAL Fluor Red 610
mCh_Q670	mCherry	27	Quasar670
mCh_Q570	mCherry	27	Quasar670
RPB1_Q670	RPO21	48	Quasar670
RPB3_Q570	RPB3	39	Quasar570
HTB1_Q670	HTB1	25	Quasar670
HTA1_Q570	HTA1	25	Quasar570
ENO1_Q570	ENO1	48	Quasar570
FBA1_Q670	FBA1	48	Quasar670
RPL3_Q570	RPL3	48	Quasar570
RPS3_Q670	RPS3	30	Qusar670

Table 6.1: List of Single molecule RNA FISH probes with fluorophores

Sample preparation We used the standard protocol for single-molecule RNA FISH in yeast, detailed in "Protocol for S.cerevisiae from Stellaris RNA FISH" (Biosearch Technologies, Inc., Petaluma, CA) and also in (**Youk2010**; Raj et al. 2008). We always used a minimum of 90 minutes incubation with Zymolyase and overnight hybridization with 20% formamide.

Imaging We used Olympus IX81 inverted, epifluorescence, wide-field microscope. For each RNA FISH experiments (single replicate for a given condition), we imaged around 10 fields of view (corresponds to around 500 spores), 40 z-stacks spaced by $0.2\mu m$ for FISH fluorophores (CAL Fluor Red 610, Qasar 570 or Qasar 670) and one single stack for other channel like fluorescence proteins (GFP) or brightfield. For CAL Fluor Red 610 we used a mcherry filter cube, for Qasar 570 we used a Cy3 filter cube and for Qasar 670 a Cy5 filter cube. We used maximum exposure time to still avoid significant bleaching, which we found to be 300ms for all three fluorophores.

Analysis Microscope raw images were saved as TIFF. First we manually segmented individual spores within spores bags using brightfield channel and a custom made matlab interface. Then we used the software (implemented in Matlab as well) FISHQuant (Mueller et al. 2013) in order to automatically count the number of FISH dots per individual spores. For each probes, we marginally adjusted the software settings so that the dot count matched what we could observe in z-projection images.

Metabolic (5-Ethynyl Uridine) labelling of freshly synthesized RNA with click reaction.

We used click chemistry to bind fluorophores to the 5-Ethynyl Uridine (5-EU) labeled RNAs in spores. To do so, we followed the protocol for mammalian cells, from "Click-iT Plus Alexa Fluor picolyl azide toolkits" (Thermo Fisher) but with the following modifications so that the protocol would work in yeast spores: (1) all reactions and washing steps were done in a 1.5-mL Eppendorf tube with a centrifugation speed of 800 x G; (2) spore fixation and permeabilization were performed as mentioned above; (3) total volume for the click reaction was $50\mu L$; (3) for the click reaction, fixed spores were at OD $\sim$ 0.02; (4) the click-reaction cocktail was incubated for 1 hour and 30 minutes. All components (Ethynyl-Uridine, Fluorophore-Azide, and reagents for the click reaction) were from the "Click-iT Plus Alexa Fluor picolyl azide toolkits" (Thermo Fisher). For simultaneously imaging 5-EU bound RNAs with GFP proteins inside spores (Fig. 4E), we used Picolyl Azide Alexa 594 from JenaBioscience. Here, the ratio of CuSO4 to the copper protectant was set to 1:20 in order to simultaneously detect the fluorescence from both GFP and Alexa

594.

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List of Publications

1. **Maire, T.**, Allertz, T., Betjes, M., Youk, H., "Dormancy-to-death transition in yeast spores occurs due to gradual loss of gene-expressing ability", *Molecular Systems Biology* (2020).
2. Laman Trip, D., **Maire, T.**,Youk, H., "Fundamental limits to progression of cellular life in frigid environments", *bioarxiv* (2022) (manuscript in revision).
3. **Maire, T.**, Youk, H., "Molecular-level tuning of cellular autonomy controls the collective behaviors of cell populations", *Cell Systems* (2015).

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