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REVIEW

Wake-up alarm: virtual time-lapse gene expression landscape illuminates mechanisms underlying dormancy breaking of germinating spores

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Abstract

Dormancy breaking is a common physiological phenomenon that is shared by eukaryotes. Germination of spores in fungi is one of the most representative cases of dormancy breaking. Understanding the mechanisms of spore germination is therefore fundamental to basic studies on the control of cell proliferation and differentiation, as well as agricultural applications and medical investigation of fungal pathogenesis. In fission yeast, spores are generated as a consequence of sexual differentiation under nutrient starvation, remaining dormant until further nourishment, but little is known about how dormant spores germinate in response to environmental change. In a breakthrough, methods for single-cell-based gene expression profiling have recently been introduced. Several mRNA expression profiles were assembled from single spore cells during dormancy or germination. Single-cell RNA-seq profiles were aligned sequentially according to their similarities. The alignment of transcriptomes visualised how gene expression varies over time upon dormancy breaking. In this review, we revisit knowledge from previous studies on germination, select candidate genes that may be involved in germination, and query their expression from the temporal transcriptomic dataset so that studies on *S. pombe* germination can be extended further.

Keywords Germination · Dormancy · Spore · Fission yeast · Single-cell RNA-seq · Gene expression

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Introduction

Eukaryotic cells undergo repeated cell division during proliferation. Occasionally, however, cells escape from mitotic cycles and are arrested at the dormant stage called the G_0 phase in response to external signals from the surrounding environment. When environmental circumstances change again, cells re-enter the G_1 phase of the cell cycle. Such entry and exit from the dormant state are widely shared in fungal, plant, and animal cells.

In general, dormancy breaking is related to cell birth. In animals, dormancy breaking is seen in the early development of fertilised eggs (Schulz and Harrison 2019). Somatic cells are often dormant in the G_0 phase, but cancer cells restart the mitotic cell cycle in response to internal or external abnormal signals (Sun and Buttitta 2017). In plants, seed germination is a typical scenario for dormancy breaking (Née et al. 2017). In fungi, depletion of nutrition from the media causes cell-cycle arrest in G_0; alternatively, cells enter sexual differentiation depending on environmental conditions (Sherwood and Bennett 2009; Feofilova et al. 2012).

Fungal spores are generated as a result of asexual or sexual reproduction (mating, meiosis, and sporulation) (reviewed in Money 2016). In the fission yeast *Schizosaccharomyces pombe*, haploid cells of homothallic *h*90 strains undergo mating followed by meiotic divisions and sporulation (corresponding to gametogenesis) in the absence of nitrogen sources (reviewed in Yamamoto et al. 1997). *S. pombe* spores remain dormant under starvation conditions until they are exposed to a nutrient-rich environment.

Since spore germination may be an evolutionary origin of dormancy breaking in higher eukaryotes, understanding of *S. pombe* germination could provide a potential clue as to how dormancy is broken in other organisms. Recently, our group established a system to investigate the gene expression profiles in a single cell, as well as a single spore, of *S. pombe* (Tsuyuzaki et al. 2020). Poly(A) + RNAs were extracted from single spore cells, and the cDNAs were amplified to examine expression profiles in each cell through deep sequencing (single-cell RNA-seq; scRNA-seq). We then employed bioinformatics tools to compare gene expression profiles originating from each single cell, defining a virtual timeline where cells were arranged temporarily as their gene expression profiles changed.

In this review, we revisit previous genetic studies regarding spore germination in fission yeast and compare previous knowledge with the expression landscape revealed by our scRNA-seq profiling.

Overview of spore germination

The sequence of events involved in spore germination in *S. pombe* is shown in Fig. 1. The proliferating *S. pombe* cells in rich media are cylindrical. In response to nutritional starvation, heterothallic cells are arrested in the G_1 phase of the mitotic cell cycle and undergo sexual differentiation. After conjugation (mating), cells undergo division and sporulation (Shimoda and Nakamura 2004; Yamamoto et al. 1997). After a while, asci are broken, and the spores are released. Spores survive for a long time in the absence of

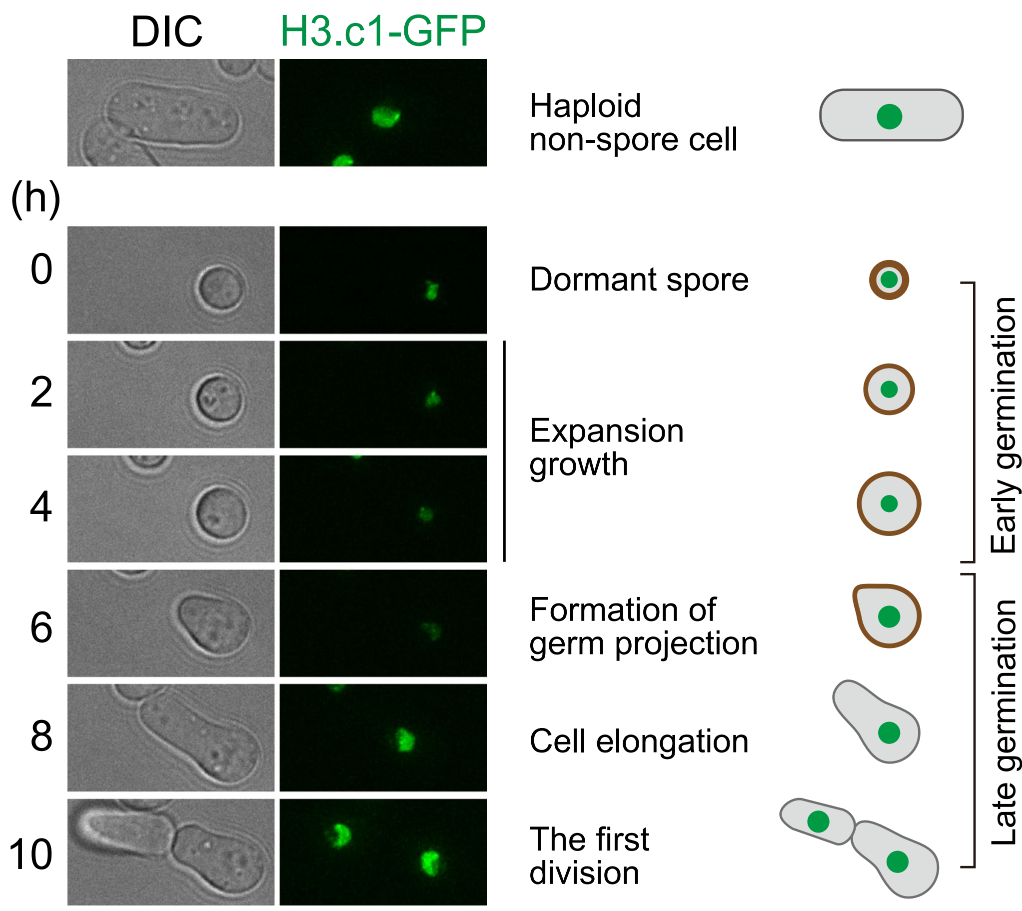

Fig. 1 Morphological change of germinating spores in DIC microscopy. DIC images of a spore cell germinating from dormancy (taken sequentially as time-lapse images) and a non-spore cell in vegetative cycles. Images for H3.c1-GFP (histone H3 copy 1 encoded by *hht1*) of the same cells (green) and schematics are also shown. Scale bar (bottom), 10 µm

nutrition; they are round, and spore walls are harder than the cell walls of vegetative cells (Yoo et al. 1973; Tahara et al. 2020). This is partly due to sporulation-specific wall components, including β-glucan and chitin synthesised by Bgs2 (1,3-β-glucan synthase) and Chs1 (chitin synthase), which are specifically expressed during meiosis (Liu et al. 2000; Martín et al. 2000). The physical solidity of the spore wall endures severe conditions in the surrounding environment.

Little is known regarding what happens inside dormant spores, which simply appear to keep “silent”, without any morphological changes until nourished. In phase contrast microscopy, dormant spores are observed as refractile cells (Hatanaka and Shimoda 2001). In differential interference contrast microscopy, dormant spores were visualised with a slightly darker contrast than vegetative cells (Fig. 1). When nutrients are supplied to the spores, germination is induced. Using phase contrast microscopy, the spores lose their "brightness" within 2 h of refeeding (Hatanaka and Shimoda 2001), which is not observable by DIC (Fig. 1). After 6 h, spores swell (expansion growth) and by 8 h extend a protrusion, called germ projection or germ tube (Fig. 1). The outgrowth of a germ projection is a landmark for "wake-up" of spores that can be easily detected under standard light microscopes, and this process (or processes leading up to it) is often called “germination”, in a narrow sense. The germ projection maintains outward elongation until the cell size reaches that of the cycling interphase cells. The first nuclear division takes place after 10 h of induction, followed by cytokinesis (septation) for 12 h. The entire process can also be called "germination" in a broad sense.

In this review, we analyse germination in 2 stages: “early germination” which includes all the events that occur prior to the formation of the germ projection, and “late germination”, composed of the events from germ projection, including those considered as part of the first mitotic cell cycle, such as replication, mitosis and cytokinesis. These late events could be regulated in a manner analogous to that in cycling cells. In contrast, early germination machinery operates in a distinct manner.

Preparation of fission yeast spores

Experiments for physiological analyses of *S. pombe* spore germination have been conducted since the 1970s. In contrast to the explosive growth of studies on proliferation cycles, as pioneered by the discovery of Cdc2/Cdk1, the molecular mechanisms underlying events during the initial stages of germination remained elusive. A major obstacle to more extensive research on germination may be technical difficulties. To reopen doors for investigating germination mechanisms, we revisited technical operations in the history of germination studies, based on how dormant and germinating spores have been prepared in previous studies.

To study the mechanisms of germination, it is essential to separate dormant spores from a mixed population of spore and non-spore cells after induction of sporulation under nitrogen-starvation conditions. In the 1970s, spores were selectively collected via sedimentation rate (zonal density) centrifuge procedures using a density gradient of sucrose, urografin, or Ficoll-hypaqe (Padilla et al. 1975; Nishi et al. 1978; Johnke and Padilla 1979). These methods to separate spore cells are based on the size-dependent fractionation of spore and non-spore cells. The spores were further fractionated after centrifugation into several groups by size and monitored as to when morphological outgrowth started. Larger spores tended to start outgrowth earlier after induction of germination in rich media (Padilla et al. 1975). This indicates that there is an individual difference in the timing of morphological outgrowth in each spore. The timing appears to be linked to the size (volume) of spores, although size is not the sole determinant, as individual differences still remain among spores of similar sizes.

Notably, if measured from the initiation of outgrowth, DNA synthesis started at nearly the same time in all germinating cells. This suggests that there is a size-dependent "pre-germination" lag to prepare the germination cue in response to environmental changes. Once the germination cue starts to exert its influence, the following two downstream phenomena are both tightly scheduled: morphological outgrowth and DNA replication. This suggests that events in late germination events are strictly controlled by underlying molecular mechanisms after germination cues.

In the 1990s, dormant *S. cerevisiae* spores were purified using a cell wall-lysing enzyme (zymolyase) and Triton X-100 to break unsporulated cells (Herman and Rine 1997). Germination was induced by the addition of rich media, and cells in the culture were collected sequentially during the course of the experiments. No specific methods have been used to synchronise the progression of germination.

Single-cell RNA-seq used for high-resolution gene expression analysis

In a recent study (Tsuyuzaki et al. 2020), we first attempted to profile the gene expression landscape of *S. pombe* germinating spores, as previously reported in other fungal species (Tisserant et al. 2012; Lanfranco and Young 2012; Geijer et al. 2012). We particularly focused on initial stages of early germination, as these may include "kick-off" genes for germination that dictate the initial fate of spores in reaction to environmental nutrients. For this purpose, we sought to determine temporal changes in the expression of each gene during germination. Practically, pure mRNA samples are needed for RNA-sequencing (RNA-seq); therefore, mRNAs should be extracted from a highly synchronised population of germinating cells in culture, as contaminating mRNAs

from cells in other stages may obscure the actual temporal kinetics of gene expression. As mentioned, the progression of early germination varies among individual cells, hampering the collection of cells with high synchronicity.

Therefore, we developed a single-cell-based RNA sequencing approach to avoid noise from other cells. mRNAs were extracted from each of the 66 individual dormant or germinating spores, and cDNA was amplified. As a quality control, we removed two unqualified cells that showed low cDNA amplification, possibly due to technical reasons. Then, using the monocle toolkit (Trapnell and Cacchiarelli 2014; Qiu et al. 2017a, b) for 64 qualified single-cell-based profiles, we estimated the temporal changes in global transcription and assembled a "virtual germination timeline", which is particularly useful when it is difficult to obtain a highly synchronised population of cells (Tsuyuzaki et al. 2020).

We used microneedles to select the single cells. This simple method does not require any expensive devices other than a light microscope; therefore, this method can be recommended to anyone, anytime, anywhere. In addition, every cell in the population can be visually monitored under a microscope to avoid selecting sick, dead, or morphologically abnormal cells. Using a cell sorter could be another option for users to monitor the conditions of cells. Thus, depending on the availability of equipment, users can choose methods for single-cell isolation, either machine-based cell sorting or an inexpensive method using a microneedle. This flexibility can appeal to a broad range of researchers.

RNA and protein synthesis are silent increase during early germination

The landscape of virtual time-lapse transcriptomes has been elaborated by newly introduced technologies, and this can be compared to previous physiological studies in the 1970s and 80 s. In the dawn of cell cycle studies, Nurse and Thuriaux (1977) discussed the relationship between cell size and the initiation of DNA replication. One of the key experiments that demonstrated cell-cycle regulatory systems measured the amounts of DNA and proteins during germination. They collected a population of spores using sedimentation rate centrifuge techniques to investigate these amounts. In their experiments, dormant spores were fed with rich media at 35 °C – note that the temperature was higher than our sampling for single-cell RNA-seq profiling, in which spores were inoculated at 30 °C.

The amount of proteins was low in dormant spores, but started to increase 1–1.5 h after feeding and kept increasing until they stopped sampling at 8 h (Nurse and Thuriaux 1977). The increase in protein levels correlates with an increase in cell size. The cell size then determines the timing of late germination events, such as germ projection outgrowth and DNA replication (Padilla et al. 1975; Nurse and Thuriaux 1977). These results indicate that global protein synthesis occurs at the start of late germination events.

The RNA levels in germinating spores were similarly estimated by Johnke and Padilla (1979). In their experiments, spores were collected using a Ficoll-Hypaqe gradient centrifuge, and a slight increase in total RNA was detected prior to morphological outgrowth, which was observed at ~ 5–10 h after exposure to minimal medium (EMM-2) supplemented with glucose (Johnke and Padilla 1979).

These studies demonstrate that global gene expression (both transcription and translation) is accelerated prior to, and in preparation for, late germination.

Temporal kinetics of RNA levels during germination

In the scRNA-seq methods used in a previous study (Tsuyuzaki et al. 2020), the total amount of RNA could not be estimated, as cDNAs were individually amplified from each cell lysate. RNA levels across genes may not be directly compared because of possible amplification bias, although we employed the bead-seq method to minimise this bias (Matsunaga et al. 2015). Virtual time-lapse analysis is a powerful tool for revealing temporal changes in the RNA level of each gene. As previously described, this revealed differential expression of three histone H3 genes during germination.

Figure 2 illustrates the overall landscape for temporal change of each *S. pombe* gene, for ~ 7000 genes (~ 5000 protein-coding genes and ~ 2000 non-coding transcripts) annotated in PomBase (https://www.pombase.org/). Quality control of samples was performed in two ways: cells with few sequence reads (2 out of 66 single cells), and genes frequently showing low expression in many cells, were excluded from the informatics operations to estimate the virtual timeline.

To validate our virtual timeline, we studied individual genes. Our study indicates that *hht1* mRNA fluctuates during the germination process, which was further confirmed using a fluorescently labelled H3.c1-GFP (Hht1-GFP) and following changes in GFP intensity via microscopy (see Figs. 1 and 3). Furthermore, *fbp1* (encoding fructose-1,6-bisphosphatase) expression is repressed in the presence of glucose (Vassarotti and Friesen 1985). Our expression analysis showed that indeed *fbp1* is present in dormant spores but is silenced after refeeding (Fig. 3). These results show that our estimated virtual timeline is valid for setting the temporal landscape of spore germination.

The temporal scRNA-seq landscape unexpectedly indicated that transcripts of many genes exist even in dormant spores, and that these decreased once spores were in the middle of germination. In the later stage, notably, many of these genes tended to regain transcript levels. Genes exhibiting such "high→low→high" temporal kinetics tend to be

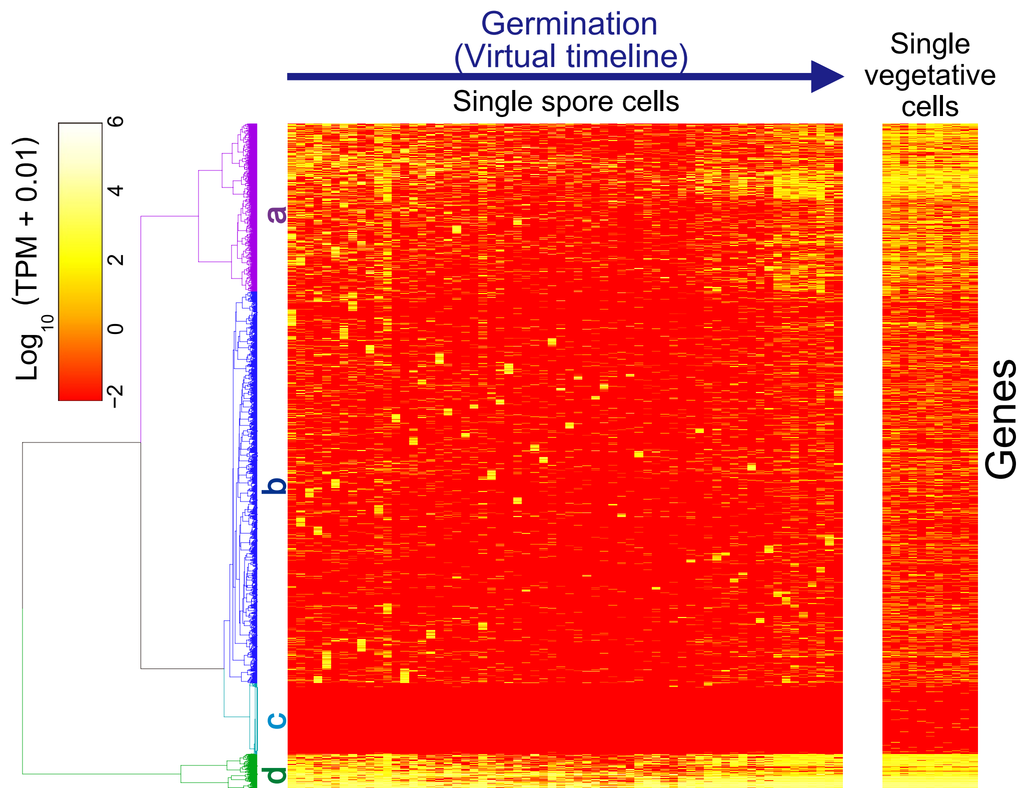

Fig. 2 Landscape of gene expression with temporal kinetics during spore germination. Heat-map diagrams indicating the mRNA levels of each gene in each scRNA-seq profile. Approximately 7,000 *S. pombe* genes annotated in PomBase were aligned in the order indicated by cluster analyses along the vertical axis. Sixty-four scRNA-seq datasets from dormant and germinating spores (left) and 11 scRNA-seq datasets from vegetative cells (right) were aligned along the horizontal axis. The genes are categorised as indicated: a, "high→low→high" kinetics; b, moderately expressed; c, silent or non-detectable transcripts; d, constitutively expressed genes. TPM (transcripts per million mapped reads) is defined as the number of sequence reads normalised by the number of total reads and the length of each gene

highly expressed during vegetative growth, as indicated by the heatmap (category a, Fig. 2). 'Category a' includes *hht1*-encoding histone H3.c1, one of three canonical H3 histones (Takayama and Takahashi 2007), which has been experimentally shown to promote germination (Tsuyuzaki et al. 2020). Many genes showed medium-level fluctuation in kinetics: genes expressed moderately during vegetative growth remained at similar levels, with minor fluctuations during germination (category b).

In general, genes with highly abundant transcripts in vegetative growth are also abundant in dormant and germinated spores (category d). In contrast, some genes remained silent in all states of dormancy, germination, and vegetative growth (category c). This may indicate that expression of these genes is under detectable levels; alternatively, this might be due to some technical reasons in preparation of cDNA library prior to scRNA-seq assays. Poly(A) + mRNAs were selected for cDNA amplification; therefore, ncRNAs without poly(A) cannot be detected. Of 747 genes in category c, 180 are ncRNA genes without poly(A) (tRNA, snoRNA, and rRNA), and 9 were pseudogenes. The protein-coding genes in category c are diverse. We did not specifically cut off genes with low or no expression for further analyses regarding expression patterns of individual genes, because it was possible that low- or no-expression genes may reflect the real transcriptomic state of dormant or germinating spores.

The restorative expression of genes in categories a and b in the virtually late stage may correspond to the increase in total RNA level in the middle of germination, as shown in a previous study (Nurse and Thuriaux 1977). In earlier stages of germination, it is possible that the total RNA level may be downregulated for a reset, so that cellular events in the later stage can be efficiently promoted.

Transmembrane transporters

Glucose is one of the most effective nutrients that induces spore germination (Johnke and Padilla 1979; Shimoda 1980). This indicates that the glucose-sensing pathway may be an upstream event that works in response to the induction

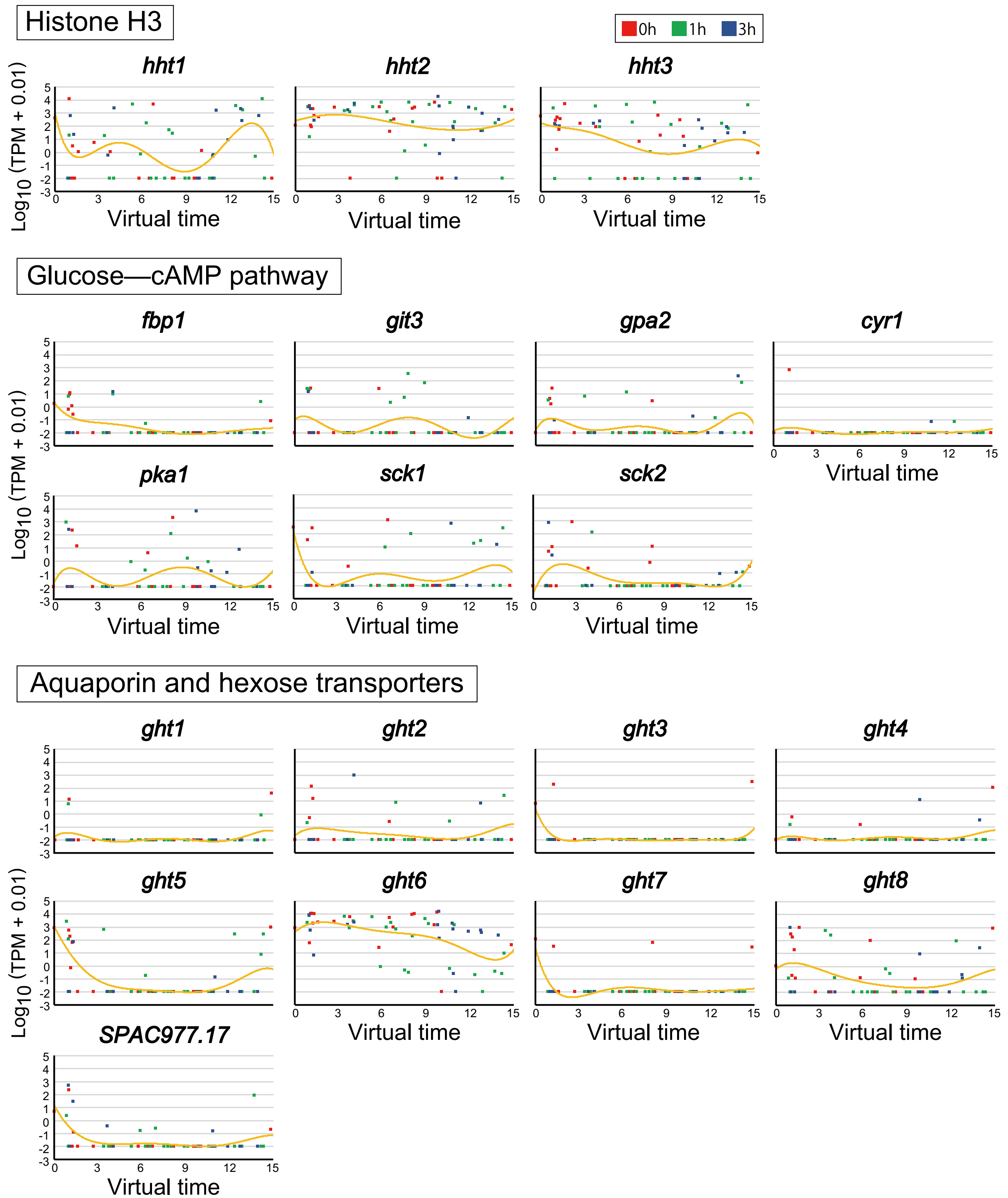

Fig. 3 Transcription kinetics of selected genes involved in nutrient sensing and reaction (1). Expression levels (vertical axis) of the indicated genes were extracted from each of the 64 scRNA-seq profiles and spotted along the virtual timeline during germination (horizontal axis, a.u.; note that the values 0–15 are not hours). Three histone H3 genes (*hht1*, *hht2*, and *hht3*) are shown as references published in a previous study (Tsuyuzaki et al. 2020). In total, 64 single spore cells taken after 0, 1, or 3 h of feeding (red, green, or blue, respectively) were subjected to profiling and plotted along the virtual timeline estimated by monocle operations

of germination. Since the late 1980s, molecular pathways for nutrient sensing, including those for glucose, have been identified. First, we focused on transmembrane hexose transporters that are essential for the transport of hexoses, including glucose. *S. pombe* has eight hexose transporter genes (*ght1*–*ght8*) (Matsuzawa et al. 2013; Saitoh et al. 2015). The *ght5* gene is the major hexose transporter in *S. pombe*; it is transcriptionally upregulated under low-glucose conditions to accelerate glucose uptake (Saitoh et al. 2015). Upon germination, the *ght5* transcript is decreased and shows "highlowhigh" kinetics similar to those of the *hht1* gene (Fig. 3). *ght8* shows a similar pattern along the virtual timeline, although its fluctuation is moderate. *ght6* is also known to increase under low-glucose conditions, but is constantly expressed throughout germination. Other transcripts were rarely detected, and they may be silent or technically difficult to detect.

The profiling of transcripts upon exit from quiescence (G_0 stage) has been previously examined (Shimanuki et al. 2007). As this is another type of *S. pombe* dormancy breaking in reaction to nutritional change (nitrogen replenishment), it would be interesting to compare it with single-cell RNA profiles upon germination. Upon exit from G_0 quiescence, some hexose transporter genes (*ght3*–*ght6*) showed a transient decline. The difference between these two profiles could be due to differences in biological mechanisms or experimental methodologies.

Transmembrane aquaporins (including aquaglyceroporins) transport water and glycerol across the cellular membrane. *S. pombe* has a single aquaporin gene (SPAC977.17) in the database. Although the gene product has not been characterised, it may be regulated during sporulation and germination. *AQY1*, a *S. cerevisiae* aquaporin gene, is expressed during spore formation but is degraded during dormancy and germination, possibly to control water outflow (Sidoux-Walter et al. 2004). SPAC977.17 transcription also remained low during germination, although it appears to be maintained at a certain level in dormant spores, unlike *S. cerevisiae AQY1* (Fig. 3).

Glucose–cAMP pathway

The glucose signal is first mediated by glucose receptor Git3 at the cell surface (Byrne and Hoffman 1993; Nocero et al. 1994; Welton and Hoffman 2000). Git3 then activates trimeric GTP-binding proteins Gpa2 (Gα), Git5 (Gβ), and Git11 (Gγ) (Isshiki et al. 1992; Nocero et al. 1994; Welton and Hoffman 2000; Landry and Hoffman 2001), which then activates adenylate cyclase Cyr1 (Yamawaki-Kataoka et al. 1989; Maeda et al. 1990; Kawamukai et al. 1991). The signal is then transduced to Pka1, a protein kinase A (PKA) (Maeda et al. 1994; Jin et al. 1995). In general, these factors in the recognition pathway of environmental glucose are essential for spore germination, as evidenced by genetic analyses; spores of *gpa2*Δ, *cyr1*Δ, and *pka1*Δ mutants show defects in germination (Maeda et al. 1994; Hatanaka and Shimoda 2001).

Two kinases, Sck1 and Sck2, play redundant functions with respect to Pka1. Single and double mutants of these two kinases do not show defects in spore germination, unlike *pka1*Δ. However, the *pka1*Δ *sck1*Δ and most likely *pka1*Δ *sck1*Δ *sck2*Δ mutants are defective in germination (Jin et al. 1995; Fujita and Yamamoto 1998). These mutant spores display defects in expansion growth as well as colony formation; the glucose–cAMP pathway is clearly required at the initial stage of early germination.

It has been circumstantially deduced that these factors reside underneath the cell surface during dormancy so that they immediately react in response to environmental glucose. It would therefore be interesting to refer to the virtual time-lapse scRNA-seq profiles, although scRNA-seq data do not provide any information regarding protein amounts.

In the scRNA-seq profiles, these factors showed distinct behaviour during germination (Fig. 3). *git3*, *gpa2*, *pka1*, and *sck1/2* genes show similar patterns in the virtual time-lapse scRNA-seq; these transcripts are detected in dormant cells, but they fluctuate during germination. The mRNAs of these genes (except for *sck2*) appear to rebound in the middle of germination, possibly because the glucose–cAMP pathway needs to repress retrograde entry into sexual differentiation. The spike of transcription does not seem to last long, and this may be due to the avoidance of hyper-activation of the pathway upon entry into vegetative cycles. The low level of *cyr1* transcripts in scRNA-seq profiles may reflect its low abundance after nitrogen starvation (~1.2 copies/cell), examined in a previous RNA-seq study (Marguerat et al. 2012).

Glucose–trehalose metabolism

Another essential pathway for the recognition of environmental glucose in eukaryotic cells is glycolysis, including trehalose metabolism. Glucose transported into cells is phosphorylated to glucose 6-P by hexokinase, and is further converted to trehalose 6-P by trehalose 6-P synthetase (Tps1 in *S. pombe*). Neutral trehalase (Ntp1 in *S. pombe*) is a gluconeogenic enzyme that catalyses the conversion of trehalose to glucose, thereby neutralising trehalose levels. Both enzymes promote germination, although there is a slight difference in their mutant phenotypes: *tps1*Δ is lethal with possible germination defects, while *ntp1*Δ shows a markedly reduced rate of germination (Blázquez et al. 1994; Beltran et al. 2000).

The scRNA-seq transcriptome indicates that the *tps1* gene is highly expressed during germination in many cells, if not all. It is possible that the metabolic pathway to produce

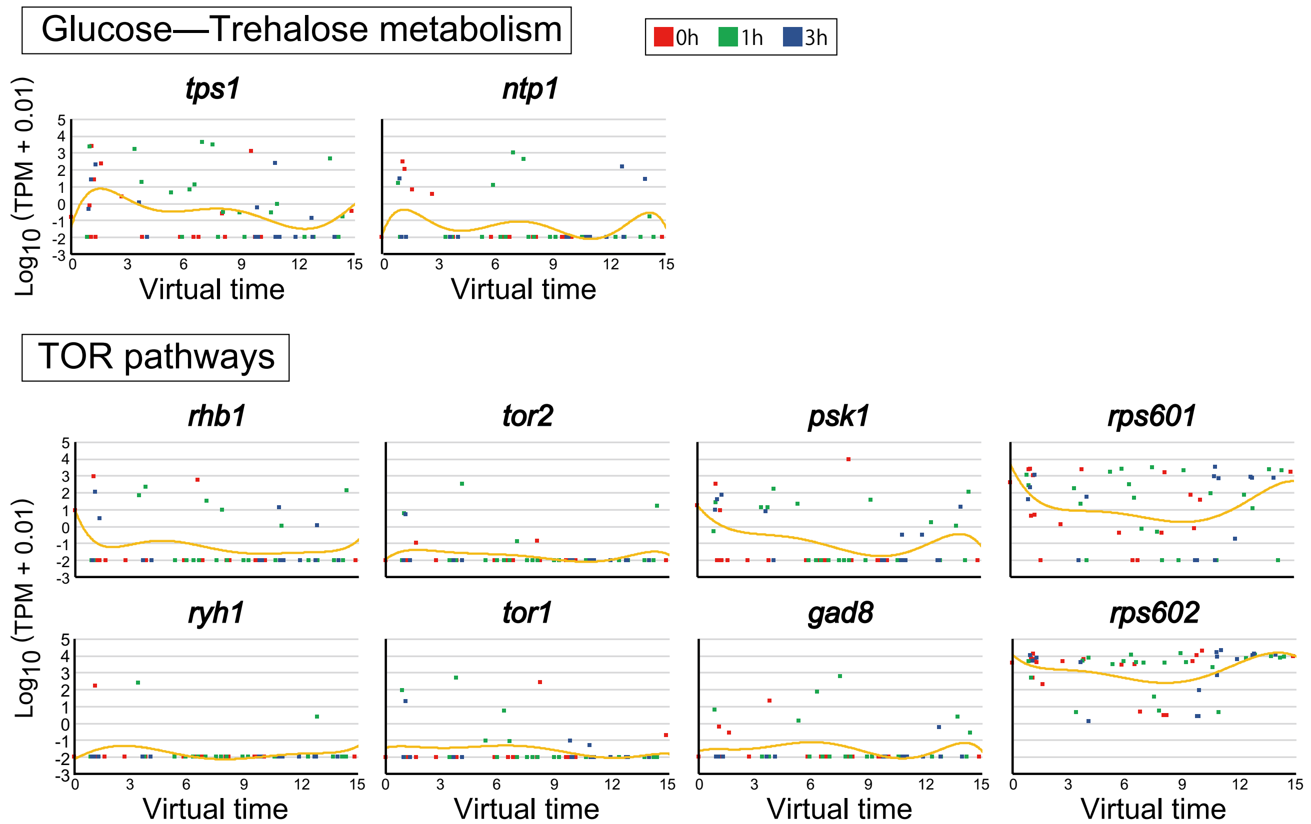

Fig. 4 Transcription kinetics of selected genes involved in nutrient sensing and reaction (2). Diagrams are prepared as in Fig. 3

trehalose remains almost constant (Fig. 4). In contrast, *ntp1* decreased upon germination, this is in line with the fact that the expression of the gluconeogenic enzyme is repressed in response to glucose addition.

TOR pathways

The target of rapamycin (TOR) is an evolutionarily conserved kinase, and two distinct complexes (TORC1 and TORC2) containing distinct TOR kinases have been reported for fission yeast (Weisman et al. 2007). These TOR complexes are known to modulate cell proliferation and growth in response to nutrient status. Although their involvement in spore germination remains to be elucidated, we refer to the time-lapse scRNA-seq profiles for some indication of fluctuation in expression.

TORC1 promotes growth and represses autophagy and differentiation, while TORC2 promotes differentiation, represses growth, and is required for stress survival. Since they play opposing roles, it is expected that transcriptional regulation would also be opposite for different complexes. The fission yeast TORC1, which contains Tor2 as its catalytic subunit, is activated in the presence of nitrogen and carbon sources, such as glucose through the Rheb GTPase Rhb1, and then targets the major S6 kinase Psk1. The TORC1-Psk1 pathway finally phosphorylates ribosomal protein S6 Rps6 (encoded by two paralogous genes *rps601* and *rps602*, both of which are thought to be targets) (Uritani et al. 2006; Matsuo et al. 2007; Nakashima et al. 2010, 2012).

For TORC1 factors, on the other hand, expression of *rhb1* and *psk1* was rather high throughout germination, although the *tor2* transcript was hardly detected (Fig. 4). In contrast, two Rps6 genes (*rps601* and *rps602*) were abundant. This can be interpreted as a general requirement for subunits of ribosomal proteins. Alternatively, this may be due to the dual specificity of Rps6, which can be targeted by TORC1-Psk1 and TORC2-Gad8 (see below) (Du et al. 2012; Nakashima et al. 2012).

The other complex, TORC2, which contains Tor1 as its catalytic subunit, is activated in response to glucose through the Rab GTPase Ryh1 (Hatano et al. 2015). TORC2 then activates the downstream AGC-family kinase Gad8 (Matsuo et al. 2003; Ikeda and Shiozaki 2008). For TORC2 factors, the scRNA-seq transcriptome indicated that expression of *ryh1* remained almost constant, and *tor1* and *gad8* were detected only slightly during germination (Fig. 4).

These profiles imply that the TORC1 pathway plays a positive role in germination, which is consistent with the generally accepted idea that TORC1 coordinates environmental nutrients to aid in cell proliferation (Shiozaki 2009).

Formation of germ projection followed by outgrowth

Formation of the germ projection and its outgrowth are actin-dependent landmark events in the middle-to-late stages of the whole germination process in yeast (Hatanaka and Shimoda 2001; Kono et al. 2005; Tahara et al. 2020). The mechanism of symmetry breaking in the round shape of a spore has recently been revealed. The germ projection is formed when the expansion growth of a spore doubles the volume. Cdc42, a Rho-type GTPase that regulates actin dynamics during polarised cell growth (Miller and Johnson 1994), is essential for germ tube formation. Before the projection is made, GTP-bound (active) Cdc42 localises to only a part of the plasma membrane of a round spore. The region harbouring active Cdc42 changes dynamically every moment. After the oscillatory movement, a zone showing persistent localisation of active Cdc42 is determined and promotes assembly of the actin cytoskeleton to synthesise the cell wall for expansion growth (Bonazzi et al. 2014).

In cells undergoing proliferation and sexual differentiation, Cdc42 is activated by its associating factors. The GTPase complex includes Scd1/Ral1 (a guanine nucleotide exchange-factor (GEF) for Cdc42), which is activated by the Ras protein Ras1 (Fukui and Yamamoto 1988; Chang et al. 1994) and Scd2/Ral3 (the scaffold subunit of the complex). Cdc42 is also activated by Gef1, another GEF for Cdc42 (Coll et al. 2003; Hirota et al. 2003).

In the scRNA-seq profiles (Fig. 5), the *cdc42* transcript fluctuated in a wavy "high→low→high" manner. The G-actin gene *act1* is abundant over time.

Among the activators for Cdc42, *ras1*, *scd1/ral1*, and *scd2/ral3* remained silent or undetected in most cells, although they were detected in some cells in the middle of the virtual timeline. *gef1*, the other GEF for Cdc42, was detected in many cells from the dormant state until the middle of germination, but was not evident in the late stage.

Cdc42 is inactivated by several GTPase-activating proteins (GAPs), including Rga3, Rga4, and Rga6 (Das et al. 2007, 2012; Tatebe et al. 2008; Dudin et al. 2015; Gallo Castro and Martin 2018). In the scRNA-seq profiles, *rga4*, and *rga6* remained low, whereas *rga3* was detected in many cells from dormancy until early germination. It is intriguing to test the possibility that fluctuating factors, such as Gef1 (GEF) and Rga3 (GAP), contribute to spore germination, particularly through regulation of the activity or polarised localisation of GTP-bound Cdc42 on the spore cortex.

Towards the first DNA replication

In the late germination stage, DNA replication and formation of germ projections occur. These phenomena are independent of each other (Shimoda and Nishi 1982). We then chose the following factors as transcriptional markers for the S phase and examined the scRNA-seq profiles:

[1] Factors showing low expression levels.

As shown in Fig. 5, the *pcn1* gene encoding proliferating cell nuclear antigen (PCNA) (Waseem et al. 1992) is rarely transcribed during germination.

Transcription of the following genes is induced by the MCB binding factor (MBF, the Cdc10-Res1-Res2-Rep2 transcription complex) in cycling cells: Mrc1 is a downstream factor in Rad3- and Tel1-dependent checkpoints (Zhao and Russell 2004), Cdt1 is a factor for replication licencing (Hofmann and Beach 1994), and Cdc18/CDC6 is an MCM loading factor for the initiation of DNA replication (Kelly et al. 1993). Unlike in the G_1–S phases of cycling cells, these genes are silent in most cells during germination, or they are undetected.

The scRNA-seq profiling suggests that these factors might be dispensable for starting the first DNA replication in late germination. Alternatively, since Cdc18 and Cdt1 are part of the replication machinery, they might remain attached to the DNA after meiosis and sporulation, ready to promote S phase when the germination signal is sensed.

[2] Factors upregulated during late germination.

Other target genes of Cdc10 include the *cdc22* gene, which encodes the large subunit of ribonucleoside reductase (Fernandez Sarabia et al. 1993) and the *ams2* gene, which encodes a transcriptional activator (see below). These show similar expression patterns along the virtual timeline: both are detected in dormant cells and in early germination (Fig. 5). The levels appeared considerably decreased in the middle of germination, whereas they were upregulated in late germination. The timing of upregulation coincided with the initiation of the first S phase. Ams2 is a transcriptional activator of histone genes (Takayama and Takahashi 2007), and it may boost the subsequent expression of histone genes during late germination.

S. pombe has three genes that encode canonical histone H3: *hht1* (copy 1 = H3.c1), *hht2* (H3.c2), and *hht3* (H3.c3). Although these three genes encode the same proteins comprising identical amino acid sequences, it remains unclear how they are utilised in *S. pombe* cells. During mitotic cell cycles, mRNA expression of these genes is differentially controlled (Takayama and Takahashi 2007), and transcription of *hht2* appears constant, whereas *hht1* and *hht3* are upregulated in G_1–S phases depending on the transcription

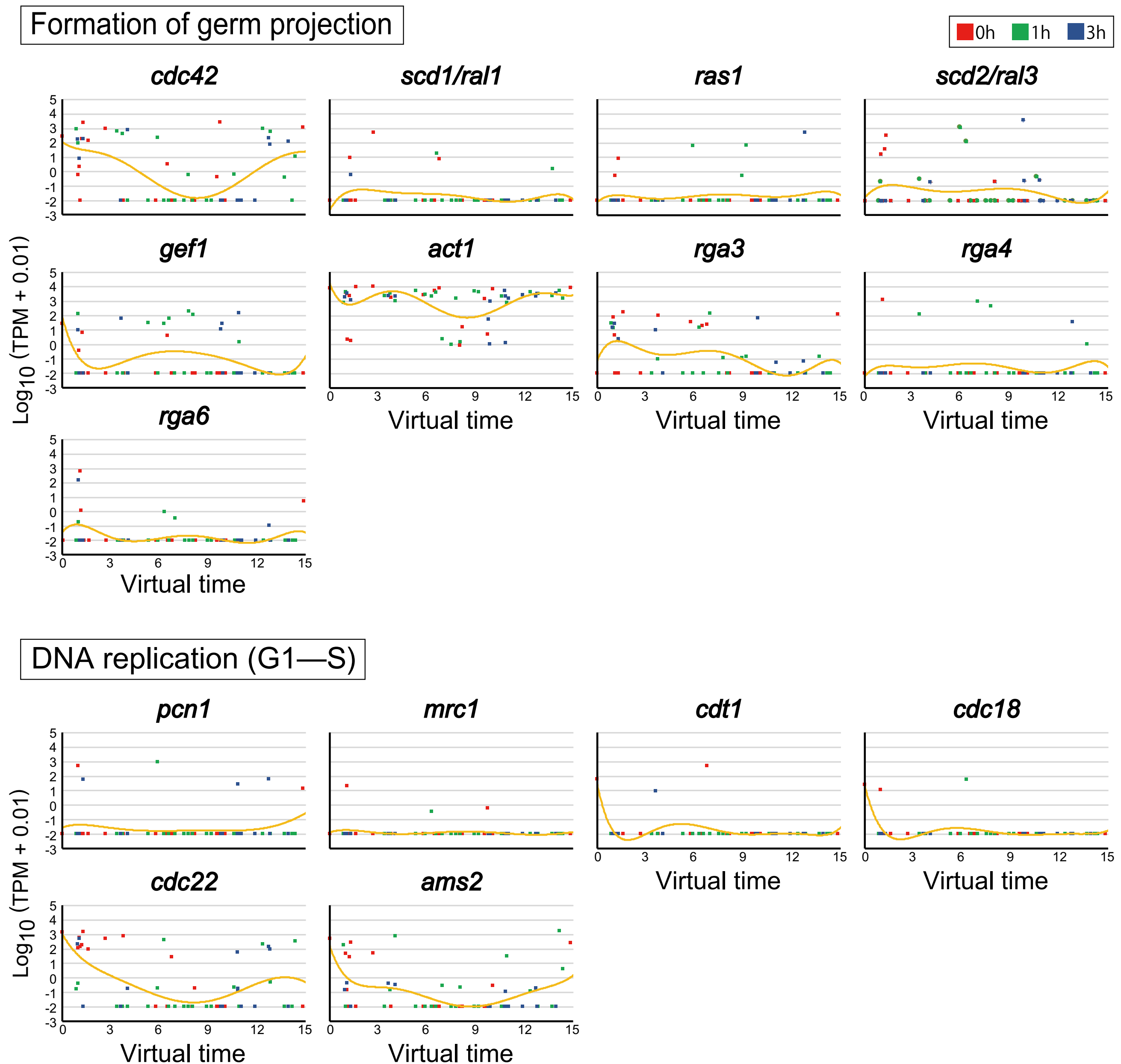

Fig. 5 Transcription kinetics of selected genes possibly involved in late germination events. Diagrams are prepared as in Fig. 3

activator Ams2. Thus, it is possible that upregulation of *hht1* mRNA in late germination depends on the preceding increase in *ams2* expression. The *hht3* mRNA pattern appears distinct in mitotic cycles and spore germination. As *hht3* is dispensable for both germination and proliferation, this gene may serve as a backup at any time.

In conclusion, there are two germination-specific regulations for histone expression: reduction of *hht1* transcripts during early germination and maintenance of *hht2* at a steady level. Although the DNA replication process is shared in two physiological phenomena—germination and proliferating cycles—the global transcription behind it may be differently regulated in these two stages.

Stepwise transition of gene expression status upon germination

Finally, we summarise a schematic showing how gene expression changes along the virtual timeline (Fig. 6). The morphological appearance of germinating spores is also

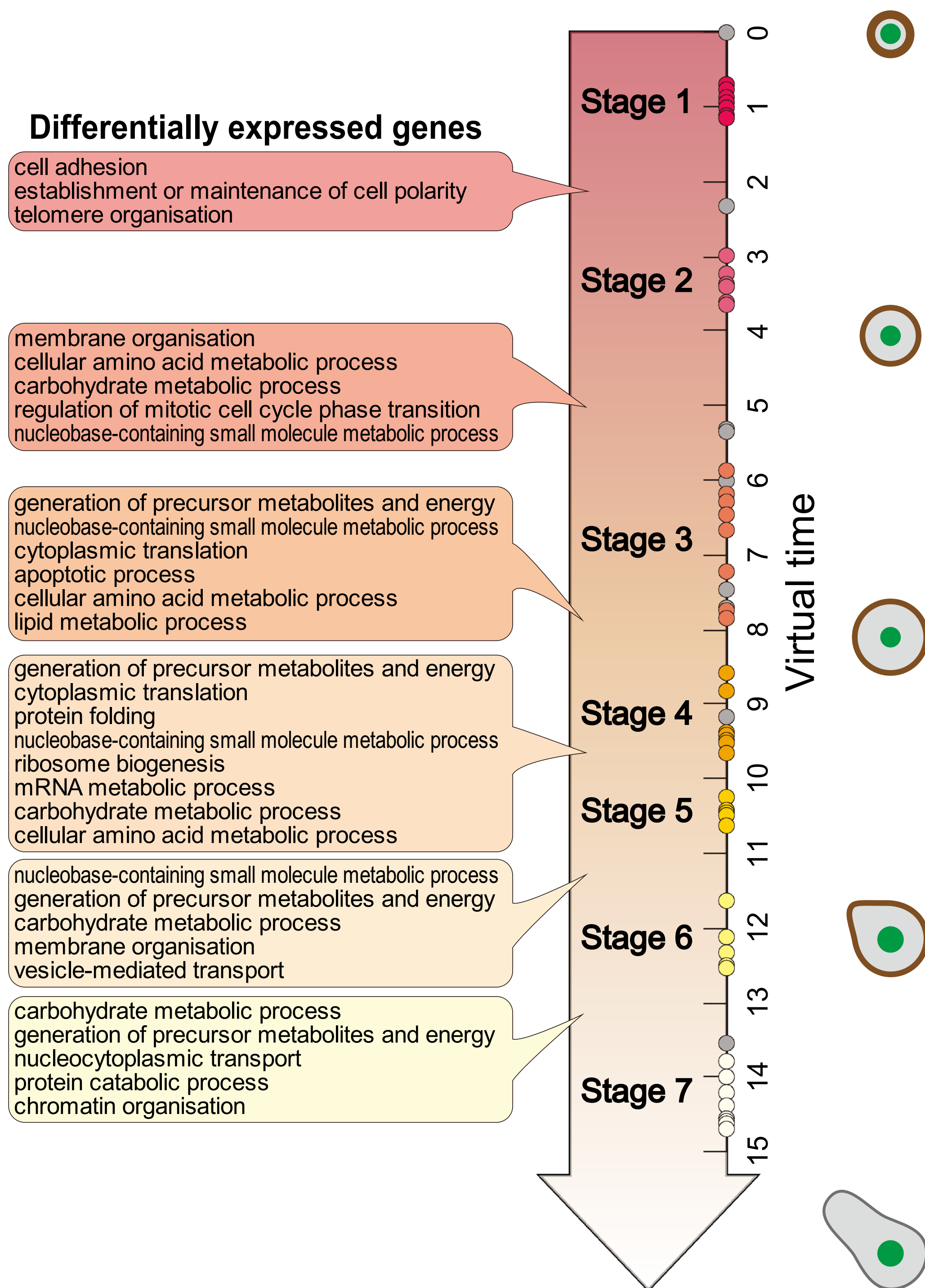

Fig. 6 Temporal change of transcripts during germination. The virtual timeline estimated using the monocle operation is shown vertically. Values 0–15 (virtual time) are shown with an arbitrary unit (not hours). The whole procedure was divided into seven putative stages, based on mapping of the 64 single-cell transcriptomes (small circles) along the virtual timeline. Gene cohorts (based on GO slim terms) listed on the left indicate the timing at which their RNA levels are significantly changed. Temporal changes in cell morphology are shown on the right. This was estimated by linking the temporal kinetics of *hht1* mRNAs along the virtual timeline with the kinetics of nuclear Hht1-GFP, observed together with cell morphology. Green, nucleus; brown, spore wall (thick) and cell wall (thin)

displayed along with the timeline. As analysed in a previous study, the transcriptional status of germinating cells can be divided into seven sequential stages (Tsuyuzaki et al. 2020). Genes were classified using GO slim terms, and gene cohorts were listed according to the stages in which their transcript levels significantly changed. Genes involved in metabolism are upregulated in the middle of germination, and chromatin-related genes are in the late stage. Notably, only a few groups were listed at the initial stage of germination. The initial stage is not occupied by genes of any specific category, but is instead composed of a mixture of genes from many GO slim categories.

Relation to other types of dormancy breaking in yeast

Next, we compared our scRNA-seq data for *S. pombe* spore germination with other profiles regarding dormancy breaking or similar cellular phenomena.

In budding yeast studies, comparison between germination and exit from the stationary phase has been made previously (Radonjic et al. 2005). In general, genes upregulated in these two types of dormancy breaking were similar to each other. However, there are some differences: expression of genes involved in ribosomal biosynthesis is particularly accelerated upon exit from the stationary phase compared to upon germination.

Interestingly, this tendency was also observed in *S. pombe* cells when spore germination and exit from the G_0 phase were compared (Shimanuki et al. 2007; Tsuyuzaki et al. 2020). Expression of ribosomal biogenesis genes, which quickly increases upon G_0 exit, does not immediately react upon germination. Instead, it changes in the late stage of the germination procedure (stages 4–5, Fig. 6).

S. cerevisiae has a faster cell cycle than *S. pombe*, and this is also the case with germination speed. *S. cerevisiae* spores start swelling after 2 h of induction and elongate after 3 h (Herman and Rine 1997). In contrast, *S. pombe* spores start to display morphological changes after 6–8 h (Hatanaka and Shimoda 2001) (Fig. 1), indicating that *S. pombe* requires a longer time for germination. Upon recovery from the stationary phase, some *S. cerevisiae* genes reacted quickly for 0.15 h. This is similar to the recovery from G_0 in *S. pombe* cells, in which genes for ribosome biogenesis are upregulated within 1 h (Shimanuki et al. 2007). We speculate that *S. pombe* spore germination specifically requires a longer lag phase at the initial step, likely for commitment in reaction to the nutritional change. After commitment, genes involved in ribosome biogenesis may start to upregulate to boost global gene expression.

It is intriguing to examine the difference between profiles of *S. pombe* germination and those of G_0 exit, to further screen for genes that specifically regulate spore germination. Ideally, a comparison should be made between the single-cell-based profiles.

Dormancy breaking in other organisms

Germination of plant seeds is a widely known example of breaking dormancy. Germination of *Arabidopsis thaliana* seeds can be initiated by water uptake. Germination progresses with a number of procedures, including imbibitional swelling, activation of metabolism, respiration, morphological changes, and gene expression (Weitbrecht et al. 2011). Dormancy is induced and maintained by the plant hormone abscisic acid (ABA), whereas germination is induced by the hormone gibberellin (GA). GA inhibits ABA and activates amylase synthesis to promote hydrolysis of starch stored in the seed, thereby internally producing glucose (Rodríguez-Gacio et al. 2009). Indeed, a transcriptional increase in genes involved in glycolysis, as well as aerobic and anaerobic respiratory pathways, contributes to energy synthesis (Weitbrecht et al. 2011). An artificial external supply of glucose to dormant seeds inhibits germination (Price et al. 2003), suggesting that the glucose uptake machinery is dispensable during germination.

Profiling of mRNAs in *Arabidopsis* seed germination indicated that genes involved in translation were highly detected (Nakabayashi et al. 2005), whereas they were not evident in the early stages of *S. pombe* spore germination (Fig. 6). This difference may be due to the use of maternal mRNA. Plant seeds autonomously germinate, initially relying on stored materials, such as the translation of maternal mRNAs and reserved energy sources. Germinating yeast spores incorporate an energy source from the environment, and they may largely rely on de novo transcription.

Chromatin factors play an essential role in seed germination. A member of the SWI/SNF subfamily, chromatin-remodeling factor PKL acts as a negative factor in ABA signalling to promote germination (Perruc et al. 2007). SNL, a histone deacetylase-binding factor, is downregulated upon seed imbibition, which allows the acetylation of histone H3 K9/K18. This induces the expression of auxin transporter AUX1, which promotes germination (Wang et al. 2016). Polycomb repressive complex 2 (PRC2) deposits histone H3 Lys27 trimethylation (H3K27me3), which is essential for germination of *Arabidopsis* seeds (Bouyer et al. 2011). Thus, chromatin remodelling is essential for altering global gene expression to promote seed germination.

In animals, dormancy breaking is observed in the early development of fertilised eggs. Fertilised eggs can be considered dormant cells because zygotic gene expression is silent, and instead maternal mRNAs exist. During the

early development of mammalian embryos, RNA synthesis begins, and zygotic gene activation (ZGA) is globally induced (Mintz 1964; Woodland and Graham 1969; Golbus et al. 1973; Wu et al. 2016; Jukam et al. 2017). Global gene activation is promoted by chromatin remodelling, particularly in gene regulatory elements, such as promoters and enhancers. These elements are structurally loosened to form accessible chromatin (Wu et al. 2016). In mouse embryos, chromatin reorganisation during ZGA correlates with the reprogramming of H3K4me3 marks. In the open chromatin regions, H3K4me3 modification preferentially occurs to loosen the chromatin, which is thought to promote transcription during ZGA (Wu et al. 2018). In later stages, H3K4me3 marks decrease, making the region inaccessible.

The fluctuating expression of histone H3 (*hht1*) during *S. pombe* germination may be correlated to the open-close remodelling of chromatin during mouse ZGA. In *S. pombe*, the level of histone H3 decreases upon germination, which may open the chromatin to promote transcription globally. Recapturing histone H3 expression during late germination may seal the chromatin to an adequate level upon entry into mitotic cycles. It would be interesting to test whether histone modification also contributes to dormancy breaking in *S. pombe*. For instance, genes classified as histone lysine methyltransferases and acetyltransferases are upregulated upon G_0 exit (Shimanuki et al. 2007), though transcriptional regulation of these groups is not evident in the early stage of germination (Fig. 6).

Another well-known aspect of dormancy breaking is observed in cancer cells. Cancer stem cells (or cancer-initiating cells) are generally quiescent and therefore resistant to anticancer agents, because these drugs mainly target dividing cancer cells (Clevers 2011; Phan and Croucher 2020). A possible strategy to overcome cancer is to prevent quiescence of cancer stem cells or to break their dormancy, thereby inducing entry into the proliferation stage (Takeishi et al. 2013). Investigation into mechanisms for dormancy breaking and genetic reprogramming by histones and chromatin remodelling factors through single-cell-based multi-omics will therefore assist drug-based cancer therapy in the future.

Conclusion

The scRNA-seq profiles arranged by bioinformatics operations visualised the temporal changes in global mRNA expression patterns in *S. pombe* spores. Methods built with brand-new technologies have been validated by specific marker genes, as shown here and in a previous report (Tsuyuzaki et al. 2020). The time-lapse mRNA landscape also revealed unexpected findings: a number of gene transcripts were reduced in the middle of germination. There may be some unknown mechanism that modulates the transcription state. For these genes, upregulated transcription during late germination may be a comprehensive preparation for entering the cell cycle.

Our scRNA-seq datasets have been recently deposited and equipped in Pombase, so that the raw sequencing data can be visually mapped to the *S. pombe* genome via the genome browser: https://www.pombase.org/reference/PMID:32152323.

Regarding global gene expression upon germination, reduction of mRNA and protein levels of histone H3 at the initial stage may be a trigger for global upregulation of genes as preparation for launching the first cell cycle. Reprogramming global gene expression is a key event that determines the fate of developing cells. Reprogramming based on chromatin remodelling is a common mechanism in a wide range of eukaryotic cells. Detailed mechanisms to orchestrate chromatin states may evolutionarily differ in each organism, possibly through modulation of histone levels during fission yeast germination, and through deposition of histone marks in higher eukaryotes.

As introduced herein, the virtual time-lapse scRNA-seq dataset upon spore germination could be a powerful tool for genetic screening of candidates involved in germination. In addition, the dataset may also illuminate the existence of universal systems that orchestrate the physiological states of cells. It is notable that scRNA-seq detects the transcript level, which can be the net amount of transcriptional and post-transcriptional (e.g. RNA turnover) regulations. Virtual time-lapse profiling may reveal unknown mechanisms that modulate gene expression at the transcriptional or post-transcriptional level. We would like to stress that accumulation of genetic evidence in former studies since the 1970s are not '*old*' but '*dormant*' for a long time. Development of the new technologies is therefore not the goal, it is a '*wake-up alarm*' for us to realise former precious results in preceding studies.

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Declarations

Conflict of interest The authors declare no conflict of interest in this work.

Ethical approval This study does not involve any human or animal subjects and followed all ethical standards of research.

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