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REVIEW

Cellular quiescence in budding yeast

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Abstract

Cellular quiescence, the temporary and reversible exit from proliferative growth, is the predominant state of all cells. However, our understanding of the biological processes and molecular mechanisms that underlie cell quiescence remains incomplete. As with the mitotic cell cycle, budding and fission yeast are preeminent model systems for studying cellular quiescence owing to their rich experimental toolboxes and the evolutionary conservation across eukaryotes of pathways and processes that control quiescence. Here, we review current knowledge of cell quiescence in budding yeast and how it pertains to cellular quiescence in other organisms, including multicellular animals. Quiescence entails large-scale remodeling of virtually every cellular process, organelle, gene expression, and metabolic state that is executed dynamically as cells undergo the initiation, maintenance, and exit from quiescence. We review these major transitions, our current understanding of their molecular bases, and highlight unresolved questions. We summarize the primary methods employed for quiescence studies in yeast and discuss their relative merits. Understanding cell quiescence has important consequences for human disease as quiescent single-celled microbes are notoriously difficult to kill and quiescent human cells play important roles in diseases such as cancer. We argue that research on cellular quiescence will be accelerated through the adoption of common criteria, and methods, for defining cell quiescence. An integrated approach to studying cell quiescence, and a focus on the behavior of individual cells, will yield new insights into the pathways and processes that underlie cell quiescence leading to a more complete understanding of the life cycle of cells.

Take Aways

- Quiescent cells are viable cells that have reversibly exited the cell cycle
- Quiescence is induced in response to a variety of nutrient starvation signals
- Quiescence is executed dynamically through three phases: initiation, maintenance, and exit
- Quiescence entails large-scale remodeling of gene expression, organelles, and metabolism
- Single-cell approaches are required to address heterogeneity among quiescent cells

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1 | INTRODUCTION

Living cells exist in one of two distinct states: (1) a proliferative state in which cell size and number increase through growth and the mitotic cell division cycle and (2) a nonproliferative state in which cell growth and division has ceased. Nonproliferative cells can be in either an irreversible state or a reversible state defined by the capacity of the cell to re-initiate the mitotic cell cycle and resume growth and division (Valcourt et al., 2012). Cells that are incapable of re-initiating the cell division cycle include post-mitotic cells that are terminally differentiated in multicellular organisms. Senescent cells are also unable to resume cell growth and division. Cells that have ceased to grow and have temporarily exited the cell cycle, but nonetheless maintain the potential to reinitiate the mitotic cell cycle, are quiescent cells (Figure 1) (Cheung & Rando, 2013; Laporte, Gouleme, Jimenez, Khemiri, & Sagot, 2018). Quiescence is the predominant cellular state for all living cells as cells in both single-celled and multi-celled organisms only rarely undergo periods of rapid proliferation and division over the course of their lifespan (O'Farrell, 2011; Valcourt et al., 2012).

Cell quiescence plays an essential role in organismal development and impacts human disease in a variety of ways (C. F. B. Kim et al., 2005; Lin, Fu, & Sakamoto, 2007; Suda, Arai, & Hirao, 2005). In multicellular organisms, development, tissue renewal, and long-term survival are dependent upon the persistence of quiescent stem cells that maintain the ability to re-enter the cell cycle to self-renew or to produce progeny that can differentiate and re-populate tissues (Cheung & Rando, 2013). The aberrant exit from quiescence and initiation of dysregulated proliferative growth is common in cancer (Hanahan & Weinberg, 2011). Conversely, many cancer-related deaths are the result of quiescent tumor cells that are resistant to therapeutics and frequently underlie tumor recurrence (Borst, 2012; Yano et al., 2017). In infectious disease, quiescent prokaryotic and eukaryotic single-celled pathogens are recalcitrant to many drug treatments for diseases including tuberculosis (Parrish, Dick, & Bishai, 1998), cryptosporosis (Alexander & Perfect, 1997), anthracis (Murray, 1999), candidiasis (Hall, 2015; Traven et al., 2012), and aspergillosis (Latgé & Chamilos, 2019). Thus, understanding the regulation and consequences of cellular quiescence and how cells transition between proliferative and quiescent states is of critical significance to our understanding of development, tissue homeostasis, and disease.

Budding (*Saccharomyces cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeasts have been key model organisms for advancing our understanding of quiescence in eukaryotic cells. Given the large evolutionary distance between these two species, the identification of shared mechanisms, properties, and consequences of quiescence point to conserved evolutionary processes. Moreover, many features of quiescence in yeast are found in quiescent human, and other animal, cells such as a reduced cell size, cell cycle arrest, condensed chromosomes, reduced rRNA synthesis and protein translation, increased autophagic activity, and increased stress resistance (Figure 2) (Dhawan & Laxman, 2015; Ho et al., 2017; Roche, Arcangioli, & Martienssen, 2017; S. S. Su, Tanaka, Samejima, Tanaka, & Yanagida, 1996; Valcourt et al., 2012; van Velthoven & Rando, 2019; Yanagida, 2009). Quiescence in multicellular organisms is difficult to study because of the complex interactions between

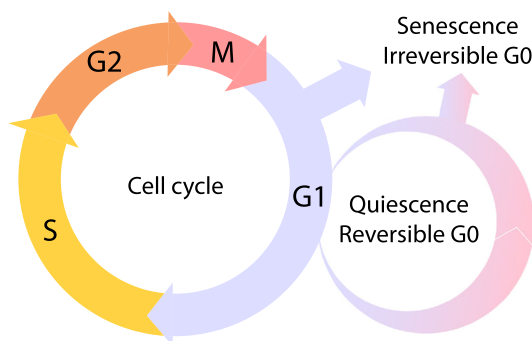

FIGURE 1 The mitotic cell cycle and quiescence. Quiescent cells have exited the cell division cycle but maintain the capacity to resume growth and re-enter the mitotic cell cycle in response to appropriate signals. This reversible state is in contrast to terminally differentiated or senescent cells, which cannot recommence the cell division cycle. In budding yeast, most quiescent cells exit the cell cycle in G1 and thus typically present as unbudded cells. However, in some cases, yeast cells can initiate quiescence from other cell cycle stages

	Quiescent cell		Proliferative cell
Morphological	↓	Cell size	↑
	↑	Cell wall	↓
	↑	Cell density	↓
Molecular	↑	Cell cycle arrest	↓
	↑	Autophagy	↓
	↓	Metabolism	↑
	↓	RNA content	↑
	↓	Ribosome biosynthesis	↑
	↑	Translational repression	↓
Cellular	↑	Vacuole/lysosome	↓
	↓	Cytoplasm mobility	↑

FIGURE 2 Contrasting properties of quiescent and proliferative cells. Quiescent yeast cells are characterized by a combination of factors including altered cell morphology and remodeling of multiple cellular processes. Key features that distinguish proliferative and quiescent cells, with an indication of whether they are upregulated (up arrow) or downregulated (down arrow), are summarized

metabolic and hormonal signals that mediate quiescence. By contrast, in budding and fission yeast, quiescence entry and exit are solely determined by nutrient availability (De Virgilio, 2012; Gray, Petsko, & Johnston, 2004; Yanagida, 2009). Nonetheless, the genes and pathways that control quiescence are conserved from yeast to humans, justifying the use of yeast model systems to make rapid progress in studying the mechanisms that underlie quiescence in human cells.

The study of quiescence in yeast may also be informative about chronological aging - defined as the period of elapsed time in a nonproliferative state (in contrast to replicative aging, which is the total number of divisions a cell has undergone). Historically, quiescence in budding yeast has referred to cells in liquid culture grown to stationary phase (Gray et al., 2004; Herman, 2002). The ability of stationary phase yeast cells to maintain viability, and reinitiate proliferative growth upon addition of nutrients, has also been presented as a model of cellular chronological lifespan (CLS) (Paola Fabrizio & Longo, 2003; Kaeberlein, 2010; N. Zhang & Cao, 2017). Because quiescent cells are defined as those nonproliferative cells that can re-enter the cell cycle, CLS is equivalent to the proportion of quiescent cells in stationary phase cultures (Allen et al., 2006; N. Zhang & Cao, 2017). Cells with a shortened CLS have reduced reproductive capacity upon replenishment of nutrients (Garay et al., 2014) and thus are functionally the same as cells that are defective in the regulation of quiescence. Therefore, successful programming into quiescence can extend the CLS of cells (Cao et al., 2016). For example, null mutations in quiescence-regulating genes including TOR1 and SCH9 (a major target of TOR1) are reported to extend the CLS of yeast cells (Fabrizio, Pozza, Pletcher, Gendron, & Longo, 2001; Paola Fabrizio & Longo, 2003; Powers, Kaeberlein, Caldwell, Kennedy, & Fields, 2006; M. Wei et al., 2008). Mechanisms linking chronological aging to cellular quiescence in budding yeast in response to carbon starvation have been discussed in a recent review (Mohammad, Baratang Junio, Tafakori, Orfanos, & Titorenko, 2020).

The study of quiescence in the lab is also likely to be informative about the life of yeast cells in nonlaboratory and natural environments. As with bacteria, nonpathogenic and pathogenic yeast can form biofilms (Chandra et al., 2001; Lynch & Robertson, 2008; Ramage, Rajendran, Sherry, & Williams, 2012). The complex structure of biofilms can result in an inadequate supply of nutrients to some individuals, which can lead to initiation of a quiescent state. Antibiotic and antifungal resistance in biofilms can be recapitulated by starvation in nonbiofilm conditions (Anderl, Zahller, Roe, & Stewart, 2003; Bojsen, Regenberg, & Folkesson, 2014; Nguyen et al., 2011) indicating that quiescence is an important determinant of drug resistance in biofilms. In this review, we focus on studies of quiescence in the lab.

Despite the central importance of studying quiescence in yeast, several factors have impeded research progress. These include a lack of consensus among researchers on the appropriate strategies for inducing quiescence, the absence of specific molecular or physical markers of quiescence, and insufficient consideration of the role of environmental signals and genotypes on quiescence. In this review, we summarize our current understanding of the pathways and processes that underlie quiescence initiation, maintenance, and exit; and the molecular and physical properties of quiescent cells. We highlight key features of quiescence that are evolutionarily conserved, identify key questions in quiescence that await solutions, and propose approaches to their resolution. We conclude that cellular quiescence is dynamically regulated and exhibits significant within population heterogeneity requiring a renewed focus on the behavior of individual cells, rather than population aggregates, to attain a comprehensive understanding of this important cellular state.

2 | DEFINING QUIESCENCE—NOT ALL NONPROLIFERATIVE CELLS ARE IN A QUIESCENT STATE

Cellular quiescence has been an area of active research for at least 50 years. For many years, there was significant debate about whether quiescent cells are simply in a prolonged G1 phase or whether cellular quiescence is a distinct cell cycle phase (Patt & Quastler, 1963). However, quiescence ultimately came to be understood as a distinct state outside of the replicative cell cycle referred to as G0 (Epifanova & Terskikh, 1969). Evidence for this distinct state came from early studies in human cells that showed that quiescent G0 cells take longer to reinitiate the cell division cycle compared to G1 cells (Zetterberg & Larsson, 1985). Subsequent studies provided further evidence of a clear distinction between G1 and G0 cells; for example, an artificially prolonged G1 arrest (e.g., through inhibition of cyclin-dependent kinases) does not recapitulate the establishment of quiescence in either yeast or mammals (Coller, Sang, & Roberts, 2006; Laporte et al., 2011).

It is important to note that two additional types of nonproliferative cells, terminally differentiated and senescent cells, are also considered to be in a nonproliferative G0 state, but these are not quiescent cells according to the formal definition (Figure 1). Senescent and differentiated cells cannot re-enter the cell cycle and therefore are not quiescent cells. Cellular quiescence can be viewed as a continuum that dynamically changes with time. Quiescent cells may ultimately lose the ability to re-enter the cell cycle and thereby become senescent cells (Figure 1), but this fate is not inevitable and most quiescent cells can maintain viability for extremely long periods of time. Thus, it is useful to distinguish a variety of quiescent states that are defined by the time since cell cycle arrest or by the time it takes to re-enter the cell cycle (Kwon et al., 2017; Laporte, Jimenez, Gouleme, & Sagot, 2018). It is worth noting that certain differentiated cells, such as mature hepatocytes, are capable of entering the cell cycle in response to injury or stressful conditions. These cells should also be considered quiescent cells according to the definition (Baserga, 1968) that quiescence is a reversible growth arrested state. This definition allows us to distinguish quiescent cells from senescent and most terminally differentiated cells and emphasizes the commonalities (cell cycle arrest) and differences (reversibility) among the broader classification of cells in a G0 state.

TABLE 1 Methods used for studying quiescence in budding yeast

Isolation/identification methods				Quiescence induction		Strain genotype		Viability verification			
Density	Cell wall	Outgrowth	Imaging	Undefined[a]	Defined[b]	Auxotrophic	Prototrophic	Staining	CFU	Ploidy	Selected publications
✓				✓		✓		✓	✓	haploid	Allen et al. (2006); Davidson et al. (2011)
✓				✓			✓	✓	✓	haploid	Swygert et al. (2019)
✓	✓			✓			✓		✓	haploid	L. Li et al. (2015); L. Li et al. (2013)
✓				✓		✓	✓	✓	✓	haploid	Laporte, Gouleme, et al. (2018)
		✓			✓		✓	✓		haploid	Gresham et al. (2011); Sun et al. (2020)
		✓		✓		✓	✓		✓	haploid	Bontron et al. (2013)
					✓		✓		✓	haploid	Klosinska et al. (2011)
			✓		✓	✓				diploid	Argüello-Miranda, Liu, Wood, Kositangool, and Doncic (2018)

Note: A variety of different methods have been used to isolate or identify quiescent cells that rely on their altered properties. The conditions used for inducing quiescence, the genotypes used, and the method for determining the viability of quiescent cells vary widely among studies.

[a]Undefined indicates cellular quiescence induced using starvation in nutrient rich (glucose-containing) medium;

[b]Defined indicates cellular quiescence induced using chemically defined medium and definition of nutrient starvation signal.

3 | STUDYING QUIESCENCE IN THE LAB

3.1 | Inducing quiescence using nutrient starvation

Quiescence in yeast is initiated in response to nutrient starvation. Haploid yeast cells grow at a constant rate proportional to the population size (i.e., exponentially) when nutrients are abundant and enter stationary phase when nutrients become scarce. Stationary phase is defined at the population level and is characterized by the absence of an increase in detectable population growth. Studies of quiescence in haploid budding yeast have typically been performed using cells in stationary-phase cultures following growth in rich, glucose-containing medium (Table 1). In this case, most yeast cells have undergone the diauxic shift from fermentative to respirative carbon metabolism and exhausted the carbon supply in the media. In this case, carbon starvation is the signal for cell cycle arrest and initiation of quiescence. It has been argued that starvation for glucose is the relevant condition for studying quiescence (Sagot & Laporte, 2019), and indeed, the majority of quiescence studies use carbon starvation as the quiescence induction signal (Laporte et al., 2011; Laporte, Gouleme, et al., 2018; Young et al., 2017).

However, early studies of the yeast cell cycle showed that starvation for several different nutrients results in cell cycle arrest (Johnston, Singer, & McFarlane, 1977; Unger & Hartwell, 1976). Subsequent studies have shown that yeast cells respond to a variety of nutrient starvations by exiting the cell cycle and initiating quiescence (Gresham et al., 2011; Klosinska, Crutchfield, Bradley, Rabinowitz, & Broach, 2011; Lillie & Pringle, 1980; Schulze, Liden, Nielsen, & Villadsen, 1996; Sun et al., 2020; Yanagida, 2009). Starvation for essential nutrients including nitrogen, phosphorus, and sulfur results in many of the same characteristics as carbon starved cells including cell cycle arrest as unbudded cells, thickened cell walls, increased stress resistance, and an accumulation of storage carbohydrates (Klosinska et al., 2011; Lillie & Pringle, 1980; Schulze et al., 1996). Defining the nutrient starvation signal that initiates quiescence is a simple process: if all other nutrients are in excess, a single growth-limiting nutrient will determine the final population size of a stationary phase culture. A linear relationship between nutrient concentration and population density in stationary phase is indicative of starvation for that nutrient.

An exclusive focus on cellular quiescence in response to carbon starvation is not justified and may impede our understanding of quiescence. Indeed, one of the central questions in studying quiescence is how different starvation signals converge on the same consequence of cell cycle arrest and induction of quiescence. Moreover, many important biological processes are likely to be missed—autophagy being a pre-eminent example—if carbon starvation is the only condition studied (Kawamata, Horie, Matsunami, Sasaki, & Ohsumi, 2017; Lang et al., 2014). Organisms in the natural world experience a range of nutrient limitations and nitrogen and phosphorus appear to be the predominant growth-limiting nutrients in most ecologies (Elser et al., 2007). Although fission yeast can also initiate quiescence in response to a variety of starvation signals (Dedo et al., 2015; Ohtsuka et al., 2017; Petrini et al., 2015; Pluskal, Hayashi, Saitoh, Fujisawa, & Yanagida, 2011; S. S. Su et al., 1996), the diauxic shift characteristic of budding yeast is not observed in fission yeast starved for glucose highlighting key differences in the metabolic states of the two yeast species immediately prior to quiescence. Thus, a comprehensive understanding of cellular quiescence requires explicit consideration of the different nutrient starvation signals that initiate quiescence and determination of the similarities and differences in the inputs and outputs of these signals.

The ability of a cell to initiate quiescence in response to nutrient starvation has evolved over hundreds of millions of years. As a result, cells have mechanisms to sense nutrient starvation signals and mount the appropriate response by initiating quiescence. This is in contrast to laboratory engineered starvations that occur when genetically modified auxotrophic strains deplete nutritional supplements that complement the auxotrophic mutation. For example, commonly used mutations that function as auxotrophic markers, such as mutations in *URA3* or *ADE2* in budding yeast, are chemically complemented by the addition of uracil or adenine to the media. If an auxotrophic yeast cell starves for the nutritional supplement, population growth is arrested and the population enters stationary phase; however, these cells do not effectively become quiescent as there is no evolved response to this unnatural starvation signal. As a result, population viability rapidly declines (Boer, Crutchfield, Bradley, Botstein, & Rabinowitz, 2010; Gresham et al., 2011). Even when the chemical supplement is present at high concentrations and cells initially starve for carbon, continued metabolic activity in quiescent cells may result in subsequent starvation for the auxotrophic requirement, resulting in a rapid decline in viability (Mülleder et al., 2012; Santos et al., 2020). The use of auxotrophs and undefined starvation conditions creates considerable ambiguity in the interpretation, and generality, of results and therefore should be avoided.

3.2 | Quiescence in diploid yeast cells

Cell ploidy has important consequences for quiescence. *S. cerevisiae* in the wild is usually diploid (Landry, Townsend, Hartl, & Cavalieri, 2006; Neiman, 2011); however, quiescence in yeast has most frequently been studied using haploid cells (Table 1). Diploid yeast cells can either enter quiescence or, typically in response to nitrogen starvation and the presence of a nonfermentable carbon source, undergo meiosis and sporulation to form haploid spores (Honigberg, 2016; Tomova, Kujumdzieva, & Petrova, 2019). Both quiescence and sporulation facilitate survival during extended periods of nutritional stress (Esposito & Klapholz, 1981; Freese, Chu, & Freese, 1982). Phenotypically, the products of meiosis in yeast—four haploid spores—are in a growth arrested state from which they can exit and resume proliferative growth. Quiescent budding yeast cells share key features with spores, including increased thermostability, low metabolic activity, reduced transcription and translational activity, and resistance to various environmental stresses. A whole proteome study of quiescent cells and spores in the SK1 strain background showed similar protein expression states in both cell types (Kumar & Srivastava, 2016).

Given the similar properties, haploid spores could be considered a specialized type of quiescent cell. However, quiescent cells and spores

do exhibit some differences. Some diploid strains can only enter cellular quiescence, whereas others can only sporulate. For example, W303 haploids can enter quiescence, but their diploid counterparts cannot. Interestingly, disabling sporulation or introducing quiescence-related genes by genetic manipulation results in diploids entering quiescence in response to sporulation signals (Miles, Li, Melville, & Breeden, 2019). These results suggest that different genes are involved in regulating quiescence and sporulation, and the regulatory mechanisms leading to these two states may be very different (Honigberg, 2016; Miles et al., 2019). Both quiescence (haploid or diploid) and sporulation are strategies that cells utilize to survive long-term under stress (Figure 3). However, further studies are required to understand the relationship between these two quiescent states. In this review, we focus on quiescence in haploid yeast cells.

3.3 | Identification and isolation of quiescent cells

Although quiescent cells have many distinct features compared to actively proliferating cells (Figure 2), no individual feature is unique to quiescent cells. Upon nutrient starvation, a clonal population of haploid budding yeast differentiates into quiescent and nonquiescent cells (Allen et al., 2006) and populations can exhibit different degrees of heterogeneity depending on genotype and starvation conditions (Laporte et al., 2011; Laporte, Gouleme, et al., 2018; Laporte et al., 2018; Miles & Breeden, 2017; Palková, Wilkinson, & Váchová, 2014). A consequence of this heterogeneity is that considering an entire stationary phase culture to be composed of quiescent cells is often inappropriate. Nonetheless, several studies define quiescence as the entire population of cells in a stationary phase culture and do not use methods for isolating or identifying quiescent cells (Table 1).

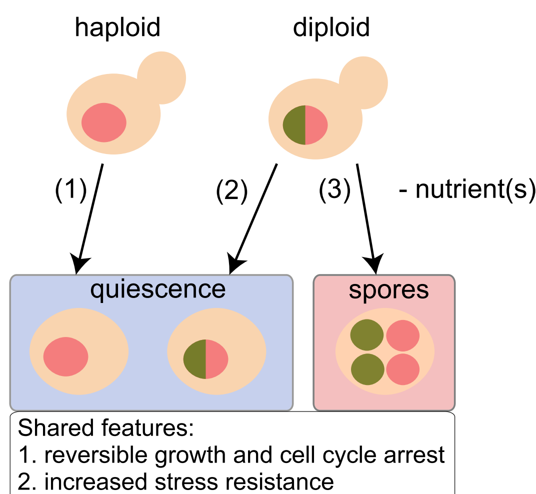

FIGURE 3 Alternative fates of haploid and diploid yeast cells in response to nutrient starvations. *S. cerevisiae* can differentiate to form quiescent cells in haploid (1) or diploid (2) cells or sporulate to form four haploid spores (3). Green and red represent the ploidy of cells

Different methods have been used for identifying, enriching, and isolating quiescent cells (Table 1). From a heterogeneous stationary phase culture, a population-level measure of the fraction of quiescent cells in the population can be determined by plating cells and counting the number of colony forming units (CFUs). The fraction of viable cells in the population, defined as the number of CFUs divided by the number of plated cells, is an estimate of the fraction of quiescent cells. A related approach quantifies the time a starved population takes to reinitiate detectable population growth. In this "outgrowth" approach, a population of cells that contains a small fraction of quiescent cells will take longer to exhibit detectable growth compared with a population comprising a large fraction of quiescent cells. Outgrowth is also a useful approach to enrich for quiescent cells when using complex mixtures of genotypes and analysis using multiplexed methods such as Barcode sequencing (Bar-seq) (Gresham et al., 2011; Sun et al., 2020). Alternative methods for quantifying the fraction of quiescent cells in a population using viability cell dyes are also widely used, often taking advantage of flow cytometry. Viable cells can be detected using propodium iodine (PI) and Syto9, which make use of the increased membrane permeability of inviable cells to specifically stain nonquiescent cells (Sun et al., 2020). Alternatively, a staining method using SytoxGreen (a DNA intercalating dye) makes use of the fact that quiescent cells have fortified cell walls (Figure 2) and therefore are resistant to penetration by the dye. In this case, quiescent cells appear as a discrete peak of reduced fluorescence in a heterogenous population (L. Li et al., 2015). These methods are useful to quantify the fraction of quiescent cells in a population, which provides an efficient means of comparing the impact of different genotypes and conditions. In principle, these methods also enable fractionation of the yeast population using Fluorescence-Activated Cell Sorting (FACS), although there are few examples of this in the literature.

Fractionation methods have been developed to isolate quiescent cells from heterogeneous cultures based on their physical properties (Figure 2). For example, quiescent cells can be isolated using density centrifugation (Allen et al., 2006). Quiescent cells are denser than nonquiescent cells due to increased storage carbohydrates (e.g., trehalose and glycogen), thickened cell walls, and reduced cell volume. The use of a percoll gradient enables isolation of the denser, more rapidly migrating quiescent cells using centrifugation. One limitation of this method is that it requires a large number of cells (2×10^9). Recently, a new density-based separation approach was developed using iodixanol, for which the input cell number can be as low as 3×10^6 (Quasem, Luby, Mace, & Fuchs, 2017). An important caveat to these methods is that they enrich the quiescent population that is largely derived from new daughter cells as these cells are the smallest and most dense. Additionally, the fraction defined as nonquiescent cells actually comprises both quiescent (i.e., viable) and nonquiescent (dead) cells. Thus, a potential limitation of this method is that it isolates a specific subclass of quiescent cells.

A fundamental limitation to fractionation methods is the lack of specific gene expression markers that would make FACS-based fractionation straightforward. The identification of such a marker in yeast would have significant advantages for identifying and isolating

quiescent cells in heterogeneous cultures. This would also enable studies of quiescent cells in multicellular eukaryotic model organisms. For example, *Drosophilia melanogaster* (*D.melanogaster*), *Caenorhabditis elegans* (*C.elegans*) have been developed as useful models for studying stem cell quiescence (Baugh & Ryan Baugh, 2013; Cheung & Rando, 2013; Guo, Flegel, Kumar, McKay, & Buttitta, 2016; Sun & Buttitta, 2015; van Velthoven & Rando, 2019; C. Wang & Spradling, 2020). *C.elegans* and *in vitro* mammalian cell culture (fibroblast cells) are also useful for studying stress-induced quiescence (Baugh & Ryan Baugh, 2013; Coller et al., 2006; Mitra, Ho, & Coller, 2018; Salmenperä, Karhemo, Räsänen, Laakkonen, & Vaheri, 2016; Tenen & Greenwald, 2019; Yao, 2014). Cells in structured environments in muti-cellular organisms, such as tissues, also face various micro-environments, including differences in oxygen supply or physical constraints (Cheung & Rando, 2013; Rumman, Dhawan, & Kassem, 2015) resulting in significant heterogeneity. Identification of conserved quiescent-specific gene expression markers for identifying and isolating quiescent cells would be a significant advance. However, finding a universal gene expression marker is challenging as quiescence can be induced by numerous different input signals. More likely, combinatorial markers comprising multiple genes possibly in combination with cellular features, such as mitochondrial morphology (Laporte et al., 2018), may be an efficient means of identifying and isolating quiescent cells.

4 | THE THREE PHASES OF QUIESCENCE

Cellular quiescence can be viewed as comprising three distinct phases: initiation, maintenance, and exit from quiescence. The ability to effectively initiate, maintain, and exit quiescence confers a significant selective advantage across diverse environments resulting in a powerful evolutionary drive for effective cellular quiescence (O'Farrell, 2011). A key challenge of studying cellular quiescence in populations is that individual cells are frequently unsynchronized with respect to these phases resulting in significant temporal heterogeneity. Nonetheless, the processes active during these phases differ making their distinction of practical utility in designing and interpreting quiescent studies.

4.1 | Quiescence initiation

Starving prototrophic yeast strains for various essential nutrients, such as carbon, nitrogen, phosphorus, or sulfur, can result in quiescence entry. In this case, cells undergo cell cycle arrest in G1 and exit the cell cycle to adopt a G0 state. This is apparent under the microscope as these cells arrest as uniformly unbudded cells. There is some variation in the fraction of unbudded cells depending on the nutrient starvation signal (Saldanha, Brauer, & Botstein, 2004) suggesting that the efficiency of quiescence initiation varies as a function of the starvation signal. A characteristic of unnatural starvations that occur when an auxotroph is starved for its auxotrophic requirement is that only a small fraction of the population arrests as unbudded cells consistent with a failure to effectively initiate quiescence in this scenario (Saldanha et al., 2004).

Quiescence initiated in response to different nutritional starvations results in significant changes in mRNA expression, histone modifications, the proteome, and metabolome. Whereas some of the changes are independent of the starvation signal, there are also specific responses that depend on the exhausted nutrient (Boer et al., 2010; Klosinska et al., 2011; Sun et al., 2020). Quiescence entry is also accompanied by subcellular reprogramming, including chromatin reorganization, protein relocalization, and cytoskeletal rearrangement (McKnight, Boerma, Breeden, & Tsukiyama, 2015; Sagot & Laporte, 2019; Swygert & Tsukiyama, 2019). Cells anticipate the exhaustion of essential nutrients well before population growth ceases. This is evident in the accumulation of storage carbohydrates well before cell cycle arrest (Lillie & Pringle, 1980) suggesting that cell quiescence is initiated in response to sensing a decline in nutrient availability.

Although regulated establishment of quiescence typically entails cell cycle exit through adoption of a G0 state (Figure 1), cells can enter quiescence at different stages of the mitotic cycle. Several yeast species (Costello, Rodgers, & Beach, 1986; Takeo, Tanaka, Miyaji, & Nishimura, 1995), stem cells (Otsuki & Brand, 2018; Sutcu & Ricchetti, 2018), and cancer cells (Drewinko, Yang, Barlogie, & Trujillo, 1984; Pearl Mizrahi, Gefen, Simon, & Balaban, 2016) can enter quiescence in G2. Yeast cells also appear to be able to enter quiescence when arrested in cell cycle phases other than G1 or G2 (Daignan-Fornier & Sagot, 2011; W. Wei, Nurse, & Broek, 1993). The relationship between G0 quiescent cells and quiescent cells that have initiated from other stages of the cell cycle remains largely unknown.

4.2 | Quiescence maintenance

Once a cell is in a quiescent state, it must maintain basal activities to provide protection against long-term cellular stress and environmental insults. Therefore, quiescence is not simply a passive state in which all cellular activities have ceased but is an actively maintained state (Cheung & Rando, 2013; Coller et al., 2006; Sang, Coller, & Roberts, 2008). The amount of time that a cell spends in a quiescent state contributes to the underlying changes in cellular and molecular properties. For example, remodeling of cellular machinery in "early" quiescent cells may not be maintained over time and can change if quiescent cells become senescent (Figure 1) (Laporte, Gouleme, et al., 2018; Sagot & Laporte, 2019).

Genes that have been identified as being important for CLS may be informative for understanding the mechanisms underlying quiescence maintenance. Multiple genome-wide screens performed in both budding and fission yeast have identified genetic factors that determine CLS using either nitrogen or carbon starvation. In budding yeast, a competitive screening approach revealed that 6.8% of single-gene knockouts had a long-lived phenotype (e.g., genotypes mutant for chromatin-modification and DNA repair genes), while 7.2% had significantly reduced CLS (e.g., genotypes mutant for autophagy, mitochondrial, protein trafficking, and protein degradation genes) under carbon starvation

in rich medium (Garay et al., 2014). Similar screens have also been performed in fission yeast under nitrogen starvation (Sideri et al., 2014), which found 48 long-lived mutants although none of their orthologs have been identified in budding yeast screens (Paola Fabrizio et al., 2010; Matecic et al., 2010; Powers et al., 2006). This discrepency might indicate differences between the two CLS model systems.

In mammalian systems, quiescent cells can move progressively "deeper" into quiescence and display an elongated pre-DNA replication phase upon stimulated exit from quiescence (Augenlicht & Baserga, 1974; Kwon et al., 2017; Owen, Soprano, & Soprano, 1989; Yanez & O'Farrell, 1989). Thus, over time the quiescent state deepens and as a result it takes longer for cells to re-enter the mitotic cycle. Deep quiescent cells also exhibit greater gene expression changes than early quiescent cells (Coller et al., 2006) suggesting that gene expression changes occur during the maintenance of quiescence.

However, little is known about the mechanism that controls the maintenance and depth of quiescence. A recent study in fibroblast cells identified a Retinoblastoma (Rb)-E2F network switch, whose activation appears to control the depth of cellular quiescence (Kwon et al., 2017). Whether this network switch represents an evolutionary conserved mechanism is unknown, but testing the function of SBF (the yeast homolog of E2F) in regulating the depth of quiescence in budding yeast would be a means of addressing this question.

4.3 | Quiescence exit

Quiescent yeast cells that are exposed to nutrients exit from quiescence and reinitiate the cell division cycle. Although little is known of the molecular mechanisms and processes that control exit from quiescence, one prerequisite for quiescence exit is that cells maintain mitotic competence (Sajiki et al., 2018). The ability to re-enter the mitotic cycle appears to require the function of multiple processes including the ability to store trehalose and glycogen for use as a future energy source (Shi, Sutter, Ye, & Tu, 2010), the transcriptional repression of specific growth and cell cycle-related genes (Miles, Li, Davison, & Breeden, 2013), post-transcriptional regulation of mRNAs (L. Li et al., 2013), and transcriptional initiation preparation by maintaining poised RNA Polymerase II upstream of genes (Radonjic et al., 2005). Acetyl-CoA, a metabolite of carbon sources, induces entry into growth. Metabolic activation during exit from quiescence results in the rapid accumulation of acetyl-CoA, which stimulates cell growth by driving the acetylation of histones at specific loci that encode for growth regulatory genes (Cai, Sutter, Li, & Tu, 2011; Kuang, Pinglay, Ji, & Boeke, 2017; Shi & Tu, 2013). Whether this metabolic requirement exists for different nutrient starvation signals in yeast and if it is conserved in quiescent mammalian cells remains unknown.

The efficiency and dynamics of quiescence exit is highly heterogeneous. In a clonal culture of quiescent cells, individual cells exhibit significantly different kinetics in restarting the cell cycle upon stimulated exit (Brooks, 1976; Temin, 1971; Zetterberg & Larsson, 1985). For instance, upon adding serum back to serum-starved cells using a short pulse, some cells re-enter the cell cycle while others remain quiescent (Brooks, 1976; Temin, 1971; Tsuruo, 2008). It has been proposed that this heterogeneity in exiting quiescence is beneficial *in vivo* as it avoids exhausting a pool of quiescent cells with a single stimulus. However, there do appear to be some factors that are predictive of quiescence exit. For example, studies in both yeast and mammalian cells have shown that cell volume and size can affect the efficiency of quiescence exit (Laporte, Jimenez, et al., 2018; X. Wang et al., 2017).

Although there is utility in separating quiescence into three phases, they exist along a continuum and are not mutually independent. A recent study in mammalian cells showed that heterogeneity of quiescence exit reflects a memory of the cell growth and division history immediately prior to quiescence initiation (X. Wang et al., 2017). Understanding the mechanisms that regulate the initiation, maintenance, and exit from quiescence, and the interrelatedness of those mechanisms, is of central importance. Given the heterogeneity among cells at each of these stages of quiescence, studies of single-cell behavior using quantitative time-lapse microscopy will likely be critical to our understanding of the different phases of quiescence.

5 | THE CELL BIOLOGY OF QUIESCENCE

Cellular quiescence is associated with dramatic reorganization of multiple cellular complexes and organelles. The significance of these large-scale changes has recently gained increased recognition, and advances in this area have been summarized in a recent review (Sagot & Laporte, 2019). In brief, multiple organelles and macromolecular structures are remodeled in quiescent cells including accumulation of actin cytoskeleton into actin bodies (Sagot, Pinson, Salin, & Daignan-Fornier, 2006), microtubule stabilization (Danowski, 1998; Laporte et al., 2015; Laporte, Courtout, Salin, Ceschin, & Sagot, 2013; Pitaval et al., 2017), reorganization of mitochondria (Aulestia et al., 2018; Laporte, Gouleme, et al., 2018), formation of ribonucleoprotein granules (Buchan, 2014; Ramachandran, Shah, & Herman, 2011; Sfakianos, Whitmarsh, & Ashe, 2016; Shah et al., 2014), proteasome storage granule (PSG) accumulation out of the nucleus (Laporte, Salin, Daignan-Fornier, & Sagot, 2008; Marshall & Vierstra, 2018), relocalization of enzymes and stress response proteins (Chughtai, Rassadi, Matusiewicz, & Stochaj, 2001; Narayanaswamy et al., 2009; O'Connell, Zhao, Ellington, & Marcotte, 2012; Tapia & Morano, 2010), and cytosolic protein aggregation (O'Connell et al., 2014). Many of these phenomena increase the stress resistance of quiescent cells. These large-scale cellular changes may be a means of minimizing the damaging effects of prolonged quiescence and allow the cell to efficiently respond to quiescence exit signals. Moreover, organelle-organelle or organelle-cytosol communication and their spatiotemporal dynamics play essential roles in chronological aging of yeast cells (Dakik & Titorenko, 2016). Therefore, proper spatiotemporal dynamics of intercommunication among compartments is likely to be essential for cells to program into quiescent states under different starvation conditions.

The vacuole, the lysosome-like organelle in yeast, appears to be a key organelle for coping with external stimuli (Aufschnaiter &

Büttner, 2019; Baba, Takeshige, Baba, & Ohsumi, 1994; S. C. Li & Kane, 2009). A recent study in quiescent fibroblast cells shed light on the role of lysosomes on regulating quiescence depth (Fujimaki et al., 2019). In yeast, many vacuolar or functionally associated genes are essential for quiescence establishment (L. Li et al., 2015; Sajiki et al., 2009). For example, in *S. pombe* under nitrogen starvation YPT5 (orthologue of VPT52/VPT53/PS21 in *S. cerevisiae*), VAM6 and VPS11 (orthologue of PEP5 in *S.cerevisiae*) which are involved in vacuole fusion were found to be essential for quiescence entry and maintenance (Sajiki et al., 2009). Genes that function in vacuolar targeting (GMH1), transportation (VPS20), and biogenesis (KCS1) are required for quiescence in *S. cerevisiae* (L. Li et al., 2015). Furthermore, a recent genetic study in *S. cerevisiae* found evidence of the functional requirement of vacuole-associated genes in regulating quiescence under multiple environmental conditions (Sun et al., 2020). Vacuoles are important regulators of cellular homeostasis, metabolism, and lifespan (Aufschnaiter & Büttner, 2019). Vacuoles are also highly dynamic organelles, which can undergo distinct morphological changes in response to different environmental conditions and throughout ageing. For example, nutrient limitation induces vacuolar fusion, resulting in one enlarged vacuole (Baba et al., 1994). A recent publication illustrated the importance of vacuoles in coordinating arginine import and export across vacuole membrane under nitrogen starvation, suggesting that vacuoles can store and transport amino acids during cellular quiescence (Cools et al., 2020). The significance of morphological dynamics and vacuole reorganization in quiescent cells across different environmental conditions warrants further investigation.

The large-scale reorganization of cellular structures in quiescence is coupled with changes in the biophysical properties of the cytoplasm. Molecular crowding in the cytoplasm is highly dynamic and changes in response to stress conditions such as heat shock, osmotic stress, energy depletion, and nutrient starvation (Delarue et al., 2018; Marini, Nüske, Leng, Alberti, & Pigino, 2020; Mourão, Hakim, & Schnell, 2014; Munder et al., 2016; Riback et al., 2017). Dysregulated homeostasis of cytoplasmic crowding can contribute to cell death (Neurohr et al., 2019). The transition from proliferation to quiescence is coupled with various physicochemical changes, such as lowered cytosolic pH, reduced cell volume, and decreased macromolecule mobility in the cytoplasm (Ashe, De Long, & Sachs, 2000; Joyner et al., 2016; Munder et al., 2016), as well as physiological changes, such as reduction in protein synthesis, enzymatic activities, and signal transduction (De Virgilio, 2012; Fuge, Braun, & Werner-Washburne, 1994, Gray et al., 2004, Miermont et al., 2013). In yeast, the cytoplasm appears to undergo a transition from a fluid-like material to a glass-like material under glucose starvation, which may be important for long-term survival under stress conditions (Munder et al., 2016). As the majority of metabolic reactions and protein translation take place in the cytoplasm, induced changes in its physicochemical properties of the cytoplasm may be required for the cell to transition into a quiescent state. However, the extent to which the biophysical properties of the cytoplasm change in response to different quiescence inducing signals and the dynamics, functional consequences, and regulators of these changes are largely unknown.

6 | GENE EXPRESSION PROGRAMS IN QUIESCENT CELLS

6.1 | Regulation of RNA expression

Quiescent cells maintain basal transcriptional activity consistent with the maintenance of an operational transcriptional machinery. In both yeast and mammalian cells, quiescent cells have distinct transcriptomes compared to actively dividing cells (Coller et al., 2006; Klosinska et al., 2011; Marguerat et al., 2012; Shimanuki et al., 2007). Specifically, the transcription factors responsible for inducing autophagy and stress responses are upregulated in quiescent cells, whereas growth-related genes, many of which contribute to protein synthesis, are strongly repressed upon quiescence initiation (Broach, 2012; De Virgilio, 2012; N. Zhang & Cao, 2017).

Cells undergo global downregulation of transcription when entering quiescence. In yeast, the overall mRNA and rRNA levels are reduced in quiescent cells; however, the diversity of transcripts remains high (Marguerat et al., 2012; Shimanuki et al., 2007). An absolute quantification of transcripts in quiescent cells revealed a 30-fold repression of global mRNA levels compared with cells in log phase (McKnight et al., 2015). The global transcriptional repression is correlated with chromatin remodeling during quiescence initiation. RPD3, a lysine deacetylase, is a key mediator of chromatin remodeling leading to global repression of gene expression and the activation of quiescence-specific transcription factors, such as XBP1 and STB3 (McKnight et al., 2015). The global transcriptional repression in quiescent cells under carbon starvation also correlates with chromatin condensation (Swygert et al., 2019). This condensin-dependent chromatin compaction is conserved in quiescent human fibroblasts (Swygert et al., 2019) suggesting a conserved regulatory mechanism for repressing transcription in quiescent cells.

Quiescent cells exhibit a transcriptional profile that has a unique component, reflecting the signal that induced quiescence, and a common component that reflects the quiescent state. The transcriptional state continues to evolve during quiescence. In mammalian cells, the transcriptional profiles of human fibroblasts differ in "early" quiescent cells depending whether the signal is contact inhibition, loss of adhesion, or serum starvation but gradually converge on a common quiescent transcriptome profile as cells enter into "deep" quiescence (Coller et al., 2006). Similarly, budding yeast cells subjected to different starvations exhibit acute signal-specific transcriptional responses that initially become increasingly similar as the period of quiescence increases (Klosinska et al., 2011). One caveat to these findings is that transcriptome studies are not typically performed on fractionated quiescent cells, and thus, the contribution of senescent cells to the gene expression state is unknown. As characterization of the transcriptome in "early" quiescent cells has allowed identification of several genes essential for quiescence establishment (Shimanuki et al., 2007), an important future direction is to characterize the transcriptome as a function of time spent in quiescence using fractionated samples as this may lead to identification of factors involved in long-term maintenance and better define the trajectory of quiescence development.

Recently developed single-cell RNA sequencing (scRNAseq) approaches in yeast (Jackson, Castro, Saldi, Bonneau, & Gresham, 2020) may aid this goal.

6.2 | Regulation of protein expression

Quiescence results in reduced overall protein production and remodeling of the proteome. On one hand, survival in quiescence requires upregulation of proteins that function as stress protectants such as chaperones or heat shock proteins (Iwama & Ohsumi, 2019; Onodera & Ohsumi, 2005; Tapia & Morano, 2010; Tsukada & Ohsumi, 1993; Verghese, Abrams, Wang, & Morano, 2012). Conversely, protein abundance measurements using GFP-tagged proteins in quiescent cells starved for carbon (Davidson et al., 2011) indicate that several proteins are decreased in abundance, many of which are involved in biosynthetic processes, especially in protein translation. Thus, both degradative and synthetic processes contribute to remodeling the proteome of quiescent cells. However, the relative role of synthetic and degradative processes in regulating proteome homeostasis in quiescent cells and the signaling pathways that regulate these different aspects of proteostasis are largely unknown (Figure 4).

Although both transcription and translation are reduced during quiescence entry, there appears to be a significant discrepancy between the transcriptome and proteome of quiescent cells. An absolute quantitative study in quiescent *S. pombe* cells starved for nitrogen

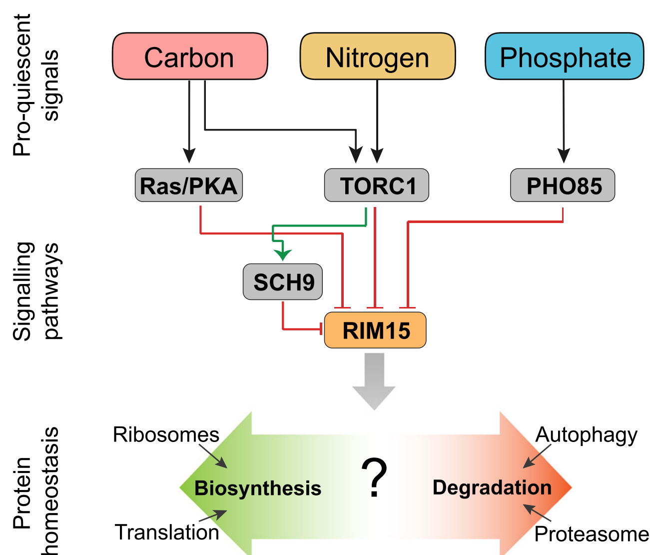

FIGURE 4 Conserved signaling pathways regulate cellular quiescence in budding yeast. Different nutritional starvation signals (carbon, nitrogen, and phosphorus) are sensed and transmitted via distinct signaling pathways. These pathways converge on regulation of the protein kinase, RIM15, which is considered the master regulator of quiescence. Regulation of protein homeostasis is a major downstream target of these pathways. Red arrow indicates global upregulation, and green arrow indicates an overall downregulation of major activities that contribute to protein degradation (autophagy and proteasome) and biosynthesis (ribosomes and translation)

found that the cell size normalized total transcriptome decreases globally, but the cell size normalized proteome does not (Marguerat et al., 2012). This highlights the importance of studying gene expression at both the protein and RNA level to define the roles of post-transcriptional, translational, and posttranslational regulation of cellular quiescence. Moreover, the causal connections between proteome homeostasis, cytoplasmic crowding, and changes in organelle morphology during cellular quiescence warrant further investigation.

7 | METABOLISM IN QUIESCENT CELLS

The metabolic activity of quiescent cells is also globally suppressed. However, quiescent cells require basal catabolic activity to ensure energy homeostasis and to facilitate effective quiescence exit. Quiescent cells exhibit distinct metabolic profiles compared with proliferative cells. A defining characteristic of quiescent yeast cells is an increase in the storage carbohydrates glycogen and trehalose (Lillie & Pringle, 1980), which are subsequently degraded upon exit from quiescence (Shi et al., 2010; J. Zhang, Martinez-Gomez, Heinzle, & Wahl, 2019). These compounds appear to be critical for quiescence as mutants defective in trehalose synthesis are defective in quiescence and adding external trehalose can rescue some diploid mutants that are otherwise not able to establish quiescence (Miles et al., 2019).

Quiescent yeast cells starved for different nutrients exhibit different metabolic profiles that reflect the starvation signal. For example, nitrogen starved cells that have uniquely reduced levels of amino acids, whereas nucleotide triphosphates are uniquely depleted in phosphorous starved cells (Klosinska et al., 2011). Tri-carboxylic acid (TCA) cycle intermediates accumulate in quiescent cells starved for nitrogen and phosphorus but not in cells starved for glucose (Klosinska et al., 2011). Interestingly, the metabolic state of quiescent cells differs from slowly growing cell populations, which are composed of large fractions of cells in G1, consistent with quiescence entailing a distinct metabolic state (Klosinska et al., 2011).

During quiescence initiation, genes required for respiration, fatty acid metabolism, glyoxylate cycle reactions, and antioxidant defenses are turned on to allow scavenging and destruction of reactive oxygen species (ROS) (Ashrafi, Farazi, & Gordon, 1998; Costa & Moradas-Ferreira, 2001; Cyrne, Martins, Fernandes, & Marinho, 2003; Gasch et al., 2000; Jamieson, 1998). Quiescent cells isolated by density fractionation (Allen et al., 2006; Werner-Washburne, Roy, & Davidson, 2012) maintain low ROS, and maintaining low ROS (e.g., superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen) is essential for long-term survival as ROS can damage DNA and cellular macromolecules (Gangloff & Arcangioli, 2017). Antioxidants and NADPH, which is used for the recycling of many antioxidants, can protect cells from mitochondrial-generated ROS to delay cell ageing (Bradshaw, 2019). In addition, transcriptional regulators (e.g., MSN2/MSN4) accumulate in starved cells under the control of the signaling kinase RIM15 (Lee et al., 2013) and upregulate targets including superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2), which aid in managing stress induced by nutrient

deprivation (Auesukaree et al., 2009; Cameroni, Hulo, Roosen, Winderickx, & Virgilio, 2004; P. Fabrizio, Pletcher, Minois, Vaupel, & Longo, 2004). *SOD1* and *SOD2* are upregulated at least twofold in quiescent cells (Davidson et al., 2011), and SOD1 or SOD2 deletion results in increased loss of viability when stationary cells are aerated (Longo, Gralla, & Valentine, 1996).

Metabolism is closely related to protein production (Litsios, Ortega, Wit, & Heinemann, 2018) as ribosome biogenesis requires most of the cellular biosynthetic capacity of a cell, and protein translation is by far the most expensive biosynthetic process in the cell. Central metabolism is essential for amino acid production and therefore determines the rate of protein synthesis and degradation (Ljungdahl & Daignan-Fornier, 2012). Understanding the regulatory connections between metabolism and protein production is central to understanding the role of metabolism in quiescence. In addition, dissecting metabolic regulation during different stages of quiescence is required for understanding how metabolism drives maintenance of quiescence and exit from quiescence (Kaplon, van Dam, & Peeper, 2015). Identifying the causal relationships between regulatory mechanisms, gene expression, and metabolism in quiescence represents a major challenge in understanding quiescence.

8 | SIGNALING PATHWAYS IN QUIESCENCE

The transition from a proliferative to quiescent state requires signaling pathways that sense environmental signals and transmit that information within the cell. Multiple evolutionary conserved pathways are known to regulate cell quiescence including the Ras/protein kinase A (PKA), SNF1 (AMPK in humans), TORC1 (the target of rapamycin complex I), and PHO80-PHO85 (cyclin-dependent kinase 5 in humans) pathways. These pathways are conserved from yeast to humans (De Virgilio, 2012). As central regulators of quiescence and cell growth, these pathways are frequently dysregulated in human diseases including cancer and diabetes (Beristain et al., 2015; Broach, 2012; Faubert, Vincent, Poffenberger, & Jones, 2015; L. C. Kim, Cook, & Chen, 2017; Laplante & Sabatini, 2012).

In yeast, these pathways respond to specific nutrients. Initiation of quiescence relies on information transmitted by three nutrient signaling pathways: TORC1 pathway, regulated by nitrogen sources, the glucose-responsive Ras/PKA pathway, and the phosphorus-responsive PHO pathway (Figure 4) (Bontron et al., 2013; De Virgilio, 2012). When nutrient availability declines, decreased activity of these pathways results in reduction of growth-related processes and derepression of growth-repressed processes. Similarly, PKA, PHO85, or TORC1 inhibition causes growth arrest and promotion of a G0-like state (De Virgilio & Loewith, 2006; Menoyo et al., 2013; Tatchell, 1986; Thevelein & de Winde, 1999; Wullschleger, Loewith, & Hall, 2006). These pathways are essential for quiescence as cells mutant for these pathways rapidly lose viability when starved for specific nutrients (Gresham et al., 2011; Sun et al., 2020). Conversely, cells with uncontrolled, elevated PKA activity typically fail to acquire many physiological characteristics of quiescence in stationary phase.

Quiescence regulating pathways converge on common targets. In yeast, there is evidence of interactions between signaling pathways (Jorgensen et al., 2004; Laplante & Sabatini, 2012; Laplante & Sabatini, 2009; Marion et al., 2004; Oliveira et al., 2015) and convergence on a common regulatory kinase, RIM15 (Pedruzzi et al., 2003; Sun et al., 2020; Swinnen et al., 2006). Systematic approaches are needed to study the coordination between signaling pathways in quiescent cells. One efficient approach to define functional relationships between genes and pathways is through quantitative genetic interaction mapping (Billmann et al., 2016; Billmann, Chaudhary, ElMaghraby, Fischer, & Boutros, 2018; Costanzo et al., 2016; Ryan et al., 2012). Quantitative maps of genetic interactions between signaling pathways in multiple environmental conditions allow identification of context-specific functional relationships between signaling pathways. We recently developed an approach to quantifying genetic interactions in quiescent cells (Sun et al., 2020) using Bar-seq and found that TOR1, RIM15, and PHO85 exhibit signal-dependent genetic interaction profiles in quiescence, but that RIM15 genetically interacts with protein degradation and synthesis genes in different quiescent conditions consistent with its function as a master regulator of quiescence via modulation of protein homeostasis (Figure 4). How RIM15 regulates protein homeostasis and whether RIM15 phosphorylates the same set of targets under different starvation conditions is unknown.

9 | CLINICAL RELEVANCE OF QUIESCENCE

Understanding the regulation of quiescence is of significant clinical relevance as many pathogenic microbes exist in a quiescent state that makes them difficult to kill. Although mutation and selection frequently underlie drug resistance, there is growing appreciation of the role of nongenetic variation in drug resistance (Balaban, Merrin, Chait, Kowalik, & Leibler, 2004). Many microbes form complex community structures, such as biofilms, which are an increasing problem in hospitals and invasive therapeutic devices such as catheters and stents (Lynch & Robertson, 2008; Ramage et al., 2012). The complex structure of biofilms can result in the inadequate supply of nutrients to some cells leading to initiation of a quiescent state.

Understanding quiescence in pathogenic fungi such as *Candida albicans* (*C. albicans*) and *Candida aureus* is likely to be useful for developing new antifungal drugs and strategies that target nonproliferative cells. *C. albicans* typically undergoes morphological changes in response to different nutritional starvation signals (Kadosh & Mundodi, 2020; Sudbery, 2011). Recently, studies from several groups have shown that signaling pathways essential in regulating cellular quiescence in yeast, such as the TORC1 and Ras/PKA pathways, play similar roles in regulating morphological changes in *C. albicans*, conferring drug resistance (Chen, Zeng, & Wang, 2018; Flanagan et al., 2017; C. Su, Lu, & Liu, 2013). Therefore, extending studies to other eukaryotic microorganisms will be beneficial for devising

therapeutic strategies and informing our understanding of the conservation of quiescence in different species.

Quiescence also has a significant role in human cancers. Recent studies have shown that quiescent cancer stem cells (CSCs) are able to evade immune surveillance and promote tumor development (Agudo et al., 2018; Bruschini, Ciliberto, & Mancini, 2020; Laughney et al., 2020). Studies have also shown that quiescent stem cells, such as those in hair follicles and muscle, are resistant to T-cell killing (Agudo et al., 2018). Furthermore, in ex-vivo experiments, quiescent stem cells appear to be protected from natural killer (NK) cells (Laughney et al., 2020). These results suggest that the immune privileged status is not an intrinsic property of CSCs but is linked to the ability to enter a quiescent state. Understanding the interaction between quiescent CSC and the microenvironment will potentially contribute to more effective cancer treatments. Studies in yeast have the potential to rapidly develop and test hypotheses regarding therapeutic strategies that leverage an enhanced understanding of cellular quiescence.

10 | CONCLUSION AND PERSPECTIVE

Although significant progress has been made in understanding quiescence in yeast and other organisms, there are several key questions and issues that await resolution. Given the increasing appreciation of the importance of cellular quiescence, addressing these questions as a coordinated community-wide effort is clearly the most expedient approach to making progress in our understanding cellular quiescence. Central to this aim is consensus among researchers on the definitions, characteristics, and analytical methods for studying quiescence in yeast, which we hope will be stimulated by this review.

First, the identification of an evolutionarily conserved marker of quiescent cells would represent a significant technical advance. Although myriad cellular alterations occur in quiescent cells (Figure 2), none of them is individually well suited as a marker of quiescence because (1) they are not specific to quiescence, (2) their relevance in different quiescence inducing conditions has not been demonstrated (Sagot et al., 2006), and (3) they are not amenable to high-throughput studies (e.g., FACS-based isolation). We believe that expanding and refining the molecular characterization of quiescent cells will be useful for identifying specific quiescent cell markers.

The turnover of molecules (e.g., RNAs and proteins) during quiescence establishment, maintenance and development is unknown. For example, it would be informative to distinguish newly synthesized RNA or proteins from extant molecules. Because of the heterogeneity of quiescent population, single-cell-based high-throughput sequencing approaches may be well suited to defining the dynamics of gene expression during quiescence. Approaches to metabolic labeling of mRNAs (Neymotin, Athanasiadou, & Gresham, 2014) and proteins (de Godoy et al., 2008) would allow distinction of mRNA synthesis and degradation rates during the different stages of quiescence.

The significance of remodeling of cellular organelles in quiescence is a particularly exciting area of research. Cell biological studies of organelle function have typically been performed in proliferating cells and many organelles—including mitochondria and vacuoles—exhibit striking differences in quiescent cells. Studies have shown that the cytoplasm of quiescent cells undergoes dramatic changes in its physical properties. Ribosome concentration appears to have important impacts on this property in a TORC1-dependent manner (Delarue et al., 2018). Understanding the regulatory and functional connections between quiescence regulating pathways and organelle and cytoplasmic properties during quiescence presents a major challenge for future studies.

Expansion of current approaches to assess the role of natural variation warrants increased attention. The vast majority of quiescence studies in yeast have been performed in laboratory adapted strains. As quiescence is of primary importance in many natural environments, natural variation is likely to be informative. Both QTL mapping and methods such as insertional mutagenesis (Michel et al., 2017; Segal et al., 2018) would be informative approaches. Efforts should also be made to expand the study of quiescence in fungal species beyond model organisms. Human pathogens, such as *C. albicans*, are readily amenable to many of the approaches that have been used in *S. cerevisiae* and *S. pombe*, and studies of such organisms may yield findings of clinical utility. Expansion of studies to other fungal species will also facilitate identification of evolutionarily conserved mechanisms.

Quiescence also provides a unique opportunity to understand how signaling pathways sense different signals and converge on related processes. As yeast cells respond to diverse nutritional signals to initiate quiescence, it seems likely that these pathways converge on related cellular processes required for quiescence. This begs a systems-level approach, using the power of genome-wide methods and the resolution of single-cell approaches, to understand how this signal integration occurs. Ultimately, studies of quiescence in yeast and other organisms will yield a deeper understanding of the life cycle of cells with potential therapeutic insights.

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