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Review

Life in lockdown: Orchestrating endoplasmic reticulum and lysosome homeostasis for quiescent cells

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SUMMARY

Cellular quiescence—reversible exit from the cell cycle—is an important feature of many cell types important for organismal health. Quiescent cells activate protective mechanisms that allow their persistence in the absence of growth and division for long periods of time. Aging and cellular dysfunction compromise the survival and re-activation of quiescent cells over time. Counteracting this decline are two interconnected organelles that lie at opposite ends of the secretory pathway: the endoplasmic reticulum and lysosomes. In this review, we highlight recent studies exploring the roles of these two organelles in quiescent cells from diverse contexts and speculate on potential other roles they may play, such as through organelle contact sites. Finally, we discuss emerging models of cellular quiescence, utilizing new cell culture systems and model organisms, that are suited to the mechanistic investigation of the functions of these organelles in quiescent cells.

INTRODUCTION

Evolution has endowed cells with numerous mechanisms to dynamically respond to changes in their environment throughout their life. Conditions that are not suitable for growth induce cells to reversibly exit from the cell cycle, entering a G_0 state known as cellular quiescence. The reversibility of cell cycle arrest in quiescent cells distinguishes it from permanent cell cycle arrest in senescent cells and also specialized, terminally differentiated post-mitotic cells (Di Micco et al., 2021). Reversible cell cycle exit is actively maintained by cell cycle inhibitors such as p21, p27, and p57. Disruption of these cell cycle inhibitors in quiescent cells leads to their premature proliferation, over time depleting the pool of quiescent cells that are later needed to repopulate injured or diseased tissues. Although "quiescence" indicates that quiescent cells are dormant or inactive, active homeostatic mechanisms influence the viability and re-entry of quiescent cells into active proliferation (Marescal and Cheeseman, 2020; Swartz et al., 2019; van Velthoven and Rando, 2019). In simple eukaryotes, environmental conditions, such as a lack of nutrients, are the main cues inducing quiescence. Cells in higher eukaryotes build on these ancient mechanisms to restrain the growth of adult stem cells, such as hematopoietic stem cells (HSCs), muscle stem cells (MuSCs), and neural stem cells (NSCs), as well as differentiated cells, such as hepatocytes, fibroblasts, lymphocytes, and oocytes, via endocrine and paracrine signals until their proliferation is needed (Marescal and Cheeseman, 2020). However, aging and disease can compromise the survival, retention, or re-activation of quiescent cells. Thus, a more comprehensive understanding of cellular quiescence may lead to new treatments for aging-related diseases (Chakkalakal et al., 2012; Choi and Tanzi, 2019; Hidalgo San Jose et al., 2020; Kalamakis et al., 2019; Leeman et al., 2018; Liu et al., 2018; Moreno-Jiménez et al., 2019; Navarro Negredo et al., 2020; Xie et al., 2020).

The term "quiescence" suggests that these cells are dormant, but this cell state is actively maintained by numerous epigenetic factors, metabolic adaptations, regulated protein turnover, and through interactions with their cellular environment (Marescal and Cheeseman, 2020; van Velthoven and Rando, 2019). Quiescent cells can also have a range of metabolic activities, from the very inert population of long-term HSCs to very metabolically active hepatocytes. The mechanisms quiescent cells utilize to maintain cellular homeostasis are likely different than those extensively studied in proliferating cells *in vitro* or in specialized post-mitotic cells. For instance, although asymmetric inheritance of cellular damage, such as dysfunctional mitochondria and protein aggregates, in proliferating cells can help maintain stemness and youthful cell function, quiescent cells must manage cellular damage on their own (Figure 1; Aguilaniu et al., 2003; Higuchi et al., 2013; Katajisto et al., 2015; Liu et al., 2010). The mechanisms quiescent cells use to maintain organelle functions and minimize the accumulation of cellular damage is crucial for the future regenerative ability of quiescent cells: cellular damage, such as dysfunctional mitochondria, reactive oxygen species (ROS), and protein aggregates, is correlated with defects in re-activation of quiescent cells and may underly defects in the re-activation of quiescent cells during aging and in disease (Hidalgo San Jose et al., 2020; Leeman et al., 2018; Roux et al., 2016). Often, stressors that progressively lock

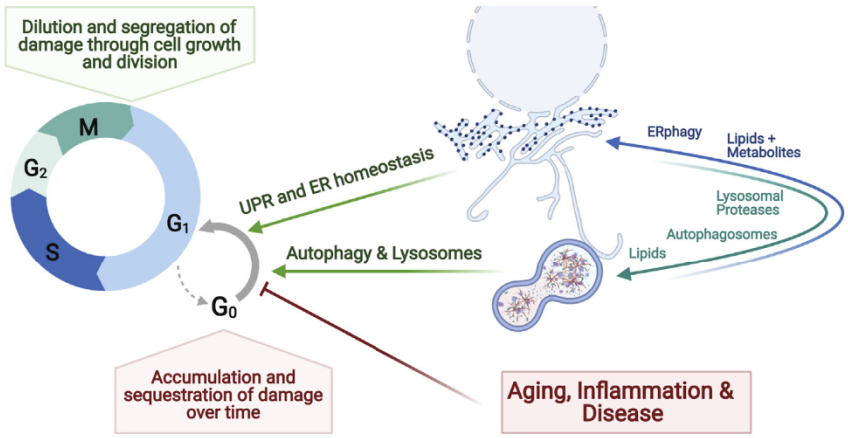

Figure 1. Cellular quiescence
Cell growth and division as part of the cell cycle dilutes damaged and toxic cellular components, such as ROS, protein aggregates, and dysfunctional mitochondria. In the case of cells that divide asymmetrically, such as stem cells, it also allows for the retention higher functioning components in long-lived stem cells and portioning of damaged components to shorter lived daughter cells. Cells that enter G_0 and exit the cell cycle, such as terminally differentiated cells (not shown), senescent cells (not shown), and quiescent cells must repair and contain the accumulation of damaged and toxic cellular components. Key to the function of quiescent cells is their ability to re-enter the cell cycle and repopulate injured tissues, which is compromised by aging and disease states in many divergent types of quiescent cells. The ER and lysosome function together in the maintenance of cellular quiescence and re-entry into the cell cycle. Figure created with BioRender.com.

quiescent cells into permanent growth arrest can be uncoupled from cell death, which suggests that interventions identified in proliferating or post-mitotic cells that simply protect them from cell death may miss some of the ways to preserve the functional *regenerative* capacity of quiescent cells (Kalamakis et al., 2019; Leeman et al., 2018; Liu et al., 2015; Roux et al., 2016). Finally, quiescent cancer stem cells, because they are not proliferating, are resistant to many traditional chemotherapies and could underly disease relapse (Batlle and Clevers, 2017; Goddard et al., 2018; Sachdeva et al., 2019; Schewe and Aguirre-Ghiso, 2008). Thus, further studies of quiescent cells utilizing emerging techniques will illuminate unique ways these cells can survive and thrive in the absence of growth, paving the way toward new therapies for many human diseases.

Although quiescence is a feature of many cell types that have important roles in human health and disease, its study has remained relatively refractory to mechanistic cell biological studies as the ability to generate and maintain cells in a physiologically relevant quiescent state *in vitro* has remained limited. *In vitro* cultivation of cells offers obvious advantages, including increased control of experimental conditions, such as culture media, and is far more tractable for certain techniques such as super resolution fluorescence microscopy and live-cell imaging. However, *in vitro* systems do not always recapitulate the important intercellular interactions that maintain cells in a quiescent state and often do not account for high oxygen and nutrient levels in culture that are not physiologically relevant to the environment of quiescent cells *in vivo*. Technological advances in cell culture methods that allow researchers to more closely mimic *in vivo* conditions of quiescent cells *in vitro* will aid in new ways of exploring the biology of these cells, from functional genomics to biochemistry to live-cell microscopy, and aid in the preclinical efforts to identify interventions that preserve their capacity for proliferation in aging and disease.

Studies examining the survival and re-activation of quiescent cells in a variety of paradigms *in vitro* and *in vivo* point to important roles for two physically and functionally interconnected cellular organelles: the endoplasmic reticulum (ER) and lysosomes. Aging and disease compromise the functions of these two organelles, possibly contributing to the age-associated decline in the re-activation of quiescent cells and subsequent tissue repair (Carmona-Gutierrez et al., 2016; Hughes and Gottschling, 2012; Martínez et al., 2017; Orenstein and Cuervo, 2010; Sun et al., 2020; Taylor and Dillin, 2013). In this review, we will explore the roles of these two ends of the endomembrane/secretory pathway in regulating the survival and re-activation of quiescent cells.

ER functions and the UPR in quiescent cells

The ER is perhaps the most anabolic organelle. About one-third of the human proteome is synthesized by ribosomes on the surface of the ER before being trafficked throughout the cell and beyond. The ER is also responsible for synthesizing the majority of the cell's lipids, which are exported to the rest of the cell through both vesicular trafficking and via membrane contact sites. Membrane contact sites between the ER and other organelles also regulate the timing and location of organelle growth and division, most notably for endosomes and mitochondria (Friedman et al., 2011; Rowland et al., 2014).

Cells sense and respond to changes in the integrity of the ER by an ancient and highly conserved signal transduction system: the unfolded protein response (UPR) (Frakes and Dillin, 2017). The most ancient and conserved branch of the UPR is a two-component system comprising Inositol-Requiring Enzyme 1 (Ire1), a kinase/endoribonuclease that resides in the ER membrane, and X-box binding protein 1 (Xbp1) (Figure 2). Ire1 dimerization/oligomerization and trans-autophosphorylation in response to an accumulation of misfolded proteins in the ER lumen or lipid bilayer stress in the ER membrane activates it toward non-canonical splicing of Xbp1 mRNA, which encodes a functional transcription factor, Xbp1s, that upregulates ER-resident chaperones, ER-associated degradation (ERAD), and lipid biosynthesis. Ire1 also degrades ER-localized mRNAs that encode proteins to be synthesized in the ER in a process termed regulated-Ire1 dependent decay (RIDD) (Hollien and Weissman, 2006; Hollien et al., 2009). Xbp1s-dependent transcriptional changes increase the protein folding capacity, and RIDD lessens the protein folding burden placed on the ER. Ire1 also regulates c-Jun kinase (JNK) and interacts with other signaling pathways, such as those involved in antioxidant responses (Urano et al.,

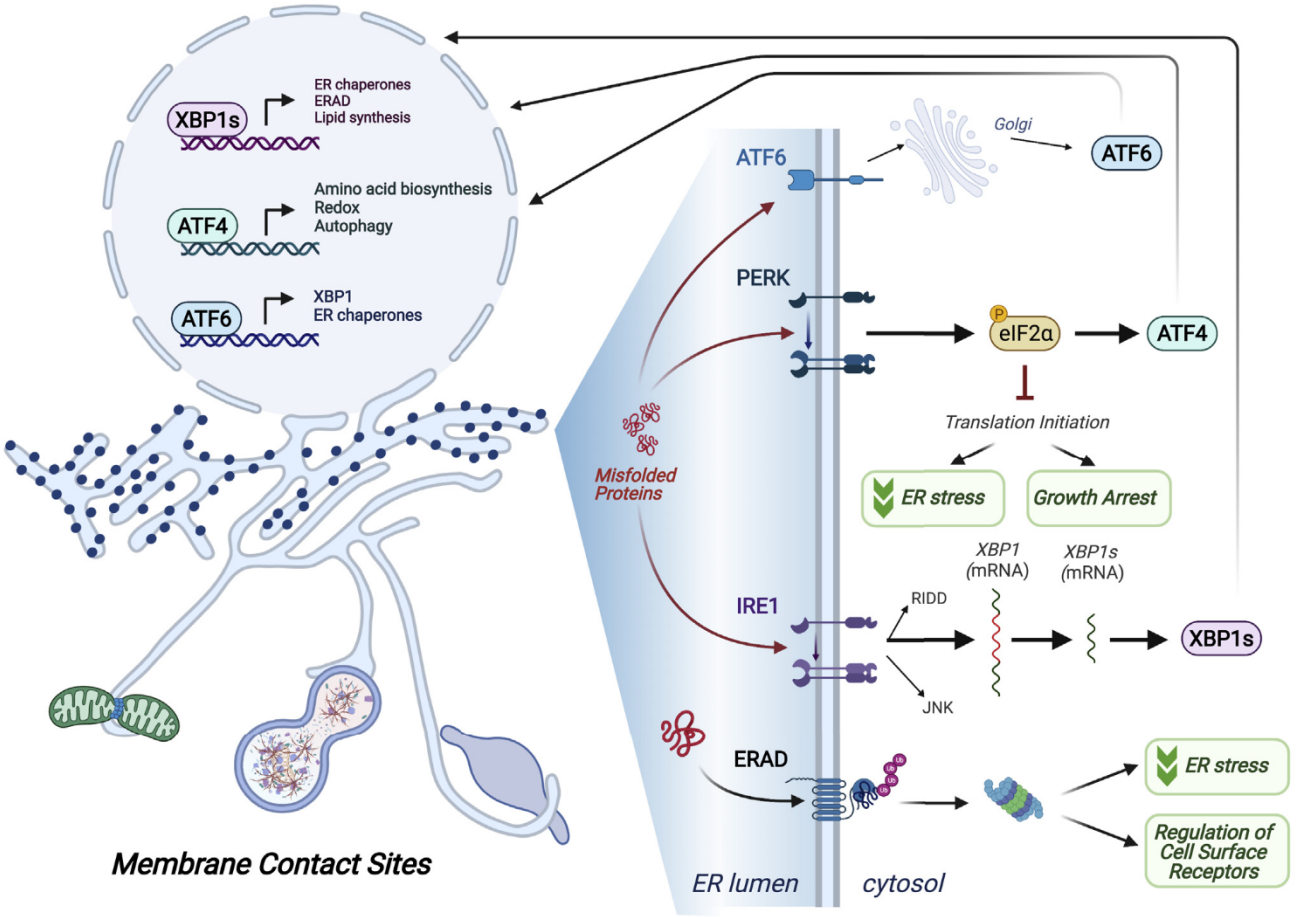

Figure 2. ER functions in cellular quiescence and re-activation

The ER forms a continuous network that makes contact sites (from left to right) with mitochondria, lysosomes, and recycling endosomes, among other organelles. ER homeostasis is sensed and maintained by the UPR, which detects an accumulation of misfolded proteins in the ER lumen and lipid bilayer stress (not shown) and mounts an adaptive transcriptional response. As part of the integrated stress response, PERK contributes to the maintenance and viability of some quiescent cells, such as MuSCs, by phosphorylating eIF2alpha, thereby inhibiting translation. ERAD facilitates the removal and subsequent proteosomal degradation of terminally misfolded proteins in the ER, which mitigates ER stress and likely contributes to the maintenance of quiescent cells. Figure created with BioRender.com.

2000). ER integrity is also sensed by the integral ER membrane protein Activating transcription factor 6 (Atf6), which in response to ER stress traffics to the Golgi where it is cleaved to release a transcription factor that promotes expression of Xbp1 and activates processes partially complementary to Xbp1s (Haze et al., 1999; Lee et al., 2002; Yamamoto et al., 2007; Figure 2). Finally, ER stress is also sensed by (PKR)-like endoplasmic reticulum kinase (PERK), which phosphorylates eIF2alpha as part of the integrated stress response that represses global translation while increasing the translation of privileged mRNAs, such as that encoding the transcription factor Atf4 (Figure 2). Atf4 promotes expression of genes influencing cellular metabolism and oxidative stress as well as the transcription factor C/EBP homologous protein (CHOP) which modulates cellular commitment to apoptosis in response to ER stress (Harding et al., 2003).

ER homeostasis and the UPR play an important role in regulating the survival and re-activation of quiescent cells, often to a greater extent than in mitotic cells or terminally differentiated cells (Table 1; Figure 2). In many cases, cells from yeast to *C. elegans* to mammals without a functional UPR have compromised survival during or while exiting from quiescence. For instance, in budding yeast, Ire1 is dispensable for growth and replicative lifespan (how many times a cell can divide) but is crucial for the survival of cells during quiescence-like chronological aging (Chadwick et al., 2020). Likewise, although somatic tissues in adult *C. elegans* are *de facto* post-mitotic and do not have quiescent cells, L1 larvae that are born in the absence of nutrients undergo a developmental arrest that requires certain cells to enter a cellular quiescence that is remarkably similar to quiescence in higher eukaryotes (Baugh, 2013). Over time, these cells accumulate damage, impairing the ability of the animal to recover from this prolonged developmental arrest. IRE-1/XBP-1 are very important for maintaining the ability of L1 larvae to recover from prolonged L1 arrest and re-enter development (Roux et al., 2016). The authors also propose that IRE-1 influences recovery of arrested L1 larvae through p38

Table 1. Proposed roles of UPR^{ER} branches and effectors in regulating quiescent cells

UPR^{ER} branch	Role in quiescence
PERK	Beneficial *-PERK activation maintains quiescent MuSCs and prevents premature differentiation* (Zismanov et al., 2016). *-promotes tissue regeneration in skeletal muscle post injury by restraining activation of p38 MAPK signaling* (Xiong et al., 2017) Detrimental *-PERK signaling predisposes HSCs to apoptosis following ER stress. May prevent propagation of damage to stem cell progeny* (Van Galen et al., 2014)
Ire1	Beneficial *-Ire1 signaling via STAT3 is required for hepatocyte proliferation following liver injury* (Liu et al., 2015). *-IRE-1 signaling via KGB-1 and XBP-1 is required for exit from quiescent L1 arrest in C. elegans* (Roux et al., 2016). *-Ire1 is required for survival during quiescence in budding yeast but does not affect replicative lifespan* (Chadwick et al., 2020)
Atf6	Beneficial *-Atf6a is required for the survival of dormant tumor cells in vivo via Rheb-mTORC1 signaling* (Schewe and Aguirre-Ghiso, 2008).
ERAD	Beneficial *-ERAD suppress mTORC1 activation in HSCs, preventing cell activation* (Liu et al., 2020). *-maintenance of key cell-surface receptors and stem cell niche communication* (Xu et al., 2020).

mitogen-activated protein kinase (p38 MAPK)/JNK signaling via KGB-1. It should be noted that in yeast and nematodes, some of the effects of UPR-deficiency on survival or recovery from quiescence could be explained by nutrient levels or composition of the media, since starvation is required to induce quiescence (Chadwick et al., 2020; Roux et al., 2016). However, mammalian hepatocytes are quiescent in that they have the remarkable ability to proliferate in response to liver injury and regenerate a functional liver yet are very metabolically active. Studies in mice have found that liver injury activates the UPR and that liver-specific knockout of Ire1a, although not dramatically compromising homeostatic liver function or causing cell death, almost completely blocks the proliferation of hepatocytes in response to CCl_4 liver injury or partial hepatectomy (Liu et al., 2015). The authors also found that adenovirus-mediated expression of Xbp1s in liver-specific Ire1 knockout mice did not suppress defects in regeneration, suggesting that Ire1a may function through additional pathways, such as STAT3 activation as the authors suggest, or through JNK signaling similarly to the role of IRE-1 during *C. elegans* L1 arrest (Roux et al., 2016). Although Ire1-JNK signaling has primarily been studied with focus on cell death, the involvement of JNK in several signaling pathways could indirectly link Ire1 signaling to stem cell maintenance (Brown et al., 2016; Semba et al., 2020). For instance, JNK may modulate cellular metabolism through its role in regulating in insulin signaling: UPR activation and Ire1-JNK signaling inhibits insulin receptor signaling via serine phosphorylation of insulin receptor substrate-1 (IRS-1) (Hirosumi et al., 2002; Ozcan et al., 2004).

Other branches of the UPR also play a role in regulating the survival and re-activation of quiescent cells. Freshly isolated quiescent MuSCs (also called satellite cells) bear hallmarks of an activated UPR, with increased phosphorylation of PERK and expression of the ER-resident protein chaperone BiP (Zismanov et al., 2016). The authors found that phosphorylation of PERK is necessary for maintaining MuSCs in a quiescent state, and in its absence, MuSCs lose their stemness and activate the myogenic program (Figure 2; Zismanov et al., 2016). These effects can primarily be attributed to PERK's downstream effect on eIF2alpha phosphorylation, as inhibiting de-phosphorylation of eIF2alpha reverses the effects of PERK knockout on satellite cells. Further evidence in skeletal muscle shows tissue regeneration and myogenesis is directly promoted by the action of PERK. MuSCs increase expressions of both PERK and Ire1a after muscle injury. In contrast to the *ex vivo* experiments of Zismanov et al., *in vivo* experiments with satellite cell-specific PERK knockout found diminished skeletal muscle renewal after injury (Xiong et al., 2017). Despite an observed increase in Ire1a expression, Xbp1 deficiency had no effect on satellite cell proliferation or survival. Evidence from this paper introduces a possible mechanism by which PERK affects the myogenic program involving p38 MAPK signaling. Post injury, PERK KO satellite cells have increased p38 MAPK phosphorylation, preventing muscle recovery. These studies together reveal the importance of PERK in maintaining MuSC function. A similar conclusion can be drawn from studies in HSCs (Van Galen et al., 2014). Gene expression analysis of HSCs showed increased expression of PERK and decreased expression of Ire1a in comparison with progenitors when treated with ER stressors. Interestingly, activation of the UPR was greater in HSCs when compared with their progeny. This differential UPR expression was linked to reduced HSC viability and reconstitution in recipient bone marrow, indicating that ER stress and UPR activation in HSCs negatively affected maintenance of quiescence and cell fate outcomes. Moreover, the increased expression of PERK appeared to result in preferentially induced apoptosis of HSCs compared with their progeny. The authors proposed that the UPR maintains the health of stem cell populations by ensuring that individual cells encountering disproportionate levels of stress readily enter the cell death program, thereby preventing propagation of damaged cells. This proposed model of HSC pool resilience is challenged in a following report that questions how HSCs could remain viable following repetitive stress events while maintaining high levels of pro-apoptotic PERK signaling (Liu et al., 2019). HSCs in pharmacologically stressed mice upregulate both Ire1a and PERK, but UPR activity did not correlate with apoptotic signaling. Moreover, HSCs with increased Ire1a signaling are better able to maintain reconstitution potential. The contrasting

results observed in these two studies may be attributed to the limitations of studying ER stress responses *in vitro*. Indeed, prolonged culture of HSCs resulted in decreased Ire1a activity (Liu et al., 2019). Although these studies point to different mechanisms for how the UPR contributes to re-activation of quiescent cells, a parsimonious interpretation of their results suggests an important role for ER homeostasis in regulating the survival, retention, and re-activation of quiescent cells.

The UPR tunes the protein folding capacity of the ER to meet the needs of the cell, but as many types of quiescent cells, such as adult stem cells, tend to have much lower protein synthesis rates that presumably do not place stresses on the protein folding capacity of the ER, what is the role of UPR in quiescent cells during normal homeostatic conditions? Consistent with low rates of *de novo* synthesis of secreted proteins in many types of quiescent cells, ER-resident protein chaperones are not differentially expressed between quiescent and activated NSCs (Leeman et al., 2018). In contrast, Xbp1 and components of ERAD are significantly upregulated in quiescent NSCs in comparison with their proliferating counterparts. Consistently, ERAD is also required for normal chronological lifespan (quiescence) in budding yeast (Chadwick et al., 2020). ERAD, which functions to remove terminally misfolded proteins from the ER, may therefore have a crucial role in removing misfolded or damaged proteins from the ER in quiescent cells, which may serve several important functions. First, it would mitigate ER stress, stifling the activation of pro-apoptotic branches of the UPR, thereby maintaining a functional pool of quiescent cells. Second, by shuttling misfolded and aggregation-prone proteins to the proteasome for degradation, it may also prevent the accumulation of protein aggregates, which are correlated with decreased capacity of quiescent cells to re-enter the cell cycle (Hidalgo San Jose et al., 2020; Leeman et al., 2018; Roux et al., 2016). Finally, disruption of ERAD may also lead to cell-wide changes in protein homeostasis, disturbing the balance of synthesis and turnover of quiescence regulators, contributing to aberrant exit from quiescence (Hidalgo San Jose et al., 2020). Consistently, ERAD functions in HSCs to suppress mechanistic target of rapamycin complex 1 (mTORC1) activation, premature proliferation, and subsequent HSC pool exhaustion (Liu et al., 2020). ERAD also directly connects ER function to extrinsic signals from the stem cell niche that maintain appropriate stem cell decisions. Depletion of key ERAD complex protein Sel1L in HSCs can lead to aggregation of cell-surface receptors at the ER, preventing necessary communication with the niche, leading to cell migration and a reduction in quiescent stem cell population (Xu et al., 2020).

The transition from quiescence to proliferation also places enormous strain on the anabolic functions of the ER in protein and lipid synthesis. Indeed, studies of quiescent cell re-activation in diverse contexts have found activation of the UPR in hepatocytes following liver injury, MuSCs following muscle injury, HSCs following hematological stress, and *C. elegans* recovering from L1 arrest (Van Galen et al., 2014; Liu et al., 2015; Roux et al., 2016; Zismanov et al., 2016). Thus, ER homeostasis in quiescent cells may be regulated primarily by removing damaged or misfolded proteins via ERAD, and conversely, anabolic requirements placed on the ER as quiescent cells resume proliferation may rely on upregulation of ER chaperones and lipid synthesis genes for *de novo* protein and lipid synthesis.

Potential functions of ER-organelle contact sites in quiescent cells

A surge of research over the past decade has illuminated another key role of the ER in cells: regulation of other organelles' function through contact sites. First observed in electron micrographs and in the biochemical co-fractionation of "mitochondrial-associated membranes" (MAMs), membrane contact sites are regions where the membranes of two organelles are closely and actively apposed at a distance of 10–30 nm (Wu et al., 2018). The molecular and physiological characterization of membrane contact sites has greatly expanded in the last 10 years, and although they can exist between any two organellar membranes, the ER seems to play a central role in cells by making membrane contact sites with every other organelle, including the cell membrane (Wu et al., 2018). By bringing two membranes close together, these sites serve as major sites of ion and lipid exchange between organelles and, indeed, are essential for the delivery of membrane phospholipids from their site of synthesis in the ER to mitochondria, which are not connected to the secretory pathway via traditional modes of vesicular traffic. Membrane contact sites also dictate the timing and location of organelle division, fusion, and trafficking events, most notably for mitochondria and endosomes (Friedman et al., 2011, 2013; Rowland et al., 2014). Although few studies have yet examined the role of ER contact sites in quiescent cells, they may play key roles in regulating lipid homeostasis or, for example, by regulating the replication of mitochondrial DNA when cells exit quiescence (Lewis et al., 2016; Murley et al., 2013). ER contact sites may also regulate metabolic adaptations of cells to quiescence and influence the structural changes in organelles that accompany them (Lim et al., 2019; Murley et al., 2015, 2017; Zhang et al., 2020). Thus, another way a dysfunctional ER may lead to defects in quiescent cells is through its broad-ranging effects on the functions of other organelles via contact sites. Examining ER contact sites with other organelles in quiescent cells may illuminate new roles for these structures across the broader range of lifestyles that cells adopt.

The ER likely also contributes to the function of quiescent cells through membrane contact sites with, and by regulating the production of proteins that reside in, lysosomes, which reside at the opposite end of the secretory pathway, and whose function in quiescent cells is especially important.

Lysosomes, autophagy, and TFEB in quiescent cells

Mitochondria and lysosomes are the most catabolic organelles in cells and are central metabolic signaling hubs. Although certain mitochondrial functions such as oxidative phosphorylation are attenuated in quiescent adult stem cells, the function of lysosomes is increased (de Almeida et al., 2017; Fujimaki et al., 2019; Kobayashi et al., 2019; Leeman et al., 2018; Liang et al., 2020; Simsek et al., 2010). Lysosomes are acidic organelles that are the sites where much of the superfluous or damaged components of cells are degraded via endocytosis, phagocytosis, and autophagy. Lysosomes also store nutrients

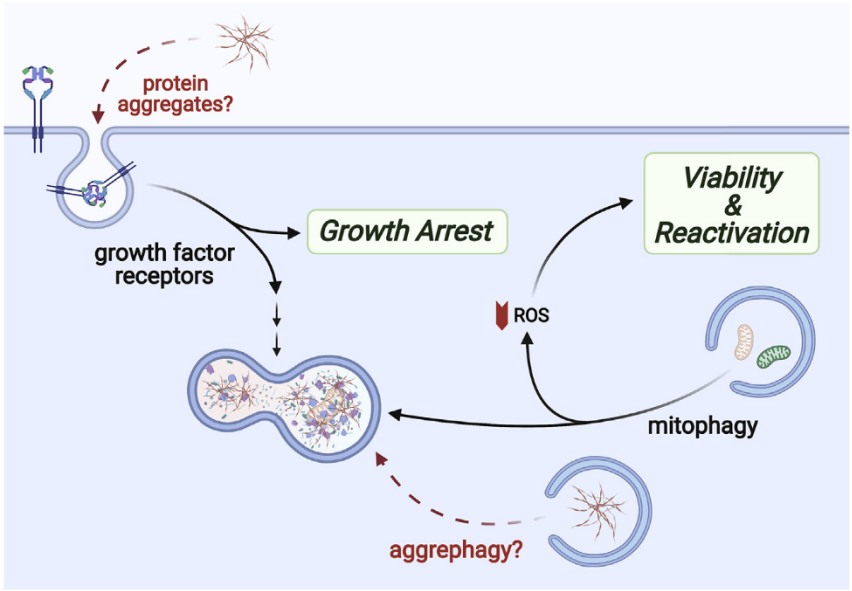

Figure 3. Lysosomal and macroautophagic contributions to cellular quiescence and re-activation

Lysosomes contribute to both the maintenance of cellular quiescence (growth arrest) and re-entry of quiescent cells into the cell cycle. For instance, autophagic degradation of mitochondria suppresses the production of reactive oxygen species (ROS), which ensure cell viability and eventual re-entry into the cell cycle. Endocytic degradation of growth factor receptors also contributes to robust maintenance of quiescence. Protein aggregates also accumulate in lysosomes of some quiescent cells, such as NSCs, which may originate intracellularly and/or extracellularly and reach lysosomes via aggrephagy or endocytosis, respectively. Increased protein aggregates in quiescent NSCs are correlated with impaired ability to re-enter the cell cycle. Figure created with BioRender.com.

and serve as major coordinators of cell metabolism and proliferation through the regulation of mTORC1 (Lawrence and Zoncu, 2019).

Lysosomes are essential to all cells, but they are especially relied on by quiescent cells (Figure 3). A feature of quiescent adult stem cells, such as NSCs, MuSCs, and HSCs is upregulation of lysosomal biogenesis and a concomitant downregulation of proteasomes (García-Prat et al., 2021; Leeman et al., 2018; Liang et al., 2020). These data suggest that quiescent stem cells rely more on lysosomes to degrade and/or sequester damaged proteins than on proteasomes. Indeed, protein aggregates are sequestered primarily in the lysosomes of quiescent NSCs (Leeman et al., 2018). Increased lysosome biogenesis and function play key roles in the maintenance of quiescent cells, although they seem to be context dependent. Inhibition of the vacuolar ATPase (V-ATPase) of lysosomes, which causes lysosome de-acidification, thereby blocking autophagy and lysosomal degradation, prevents the re-activation of quiescent NSCs (Leeman et al., 2018). Conversely, stimulating lysosome biogenesis by overexpression of the transcription factor TFEB or treating cells or mice with rapamycin, an inhibitor of mTORC1, enhances the re-activation of aged quiescent NSCs (Leeman et al., 2018). Likewise, serum starvation of rat embryonic fibroblasts increases expression of lysosome-related genes over time, although hampered lysosomal function over time increases the quiescence depth of these cells and attenuates their re-activation (Fujimaki et al., 2019). In contrast to these findings are those concerning quiescent HSCs, where inhibiting protein degradation in lysosomes via leupeptin or de-acidification of lysosomes actually increases the potency with which they are re-activated in competitive transplantation experiments (Liang et al., 2020). Lysosomal inhibition causes a small number of quiescent NSCs to re-enter the cell cycle, possibly by modulating cell-surface expression of epidermal growth factor receptor (Kobayashi et al., 2019). Finally, TFEB expression enforces quiescence in long-term HSCs *in vivo* by regulating expression of cell-surface receptors, with TFEB overexpression deepening quiescence and its knockdown leading to cell cycle re-entry (García-Prat et al., 2021).

A major mechanism for delivery of cellular components to the lysosomal lumen is macroautophagy, which is crucial for the maintenance of quiescent cells and becomes more dysfunctional with age (Chang, 2020). Selective and non-selective macroautophagy is accomplished through the *de novo* formation of a double membrane structure termed an autophagosome that engulfs cytoplasmic components and other organelles, reviewed extensively elsewhere (Galluzzi et al., 2017). In most cases, genetic and pharmacological perturbations that decrease macroautophagic flux compromise the survival, retention in a quiescent state, and/or re-activation of quiescent adult stem cells, whereas interventions that increase autophagic flux promote their survival, retention in a quiescent state, and subsequent re-entry into the cell cycle (Chang, 2020; García-Prat et al., 2016; Leeman et al., 2018; Wang et al., 2022). A decrease in proteasome activity in quiescent cells suggests that they rely more on autophagic mechanisms to sequester or degrade protein aggregates (Leeman et al., 2018). Indeed, as mentioned above, protein aggregates accumulate in lysosomes of quiescent NSCs; however, whether these aggregates are delivered to lysosomes via autophagic processes is not clear. Treatment with compounds such as bafilomycin A or chloroquine that de-acidify lysosomes (and inhibit macroautophagy) actually increase the number of lysosomal aggregates in cells, although this does not preclude the possibility that aggregates are delivered to lysosomes by autophagy and turned over slowly in a manner dependent on lysosomal acidification (Leeman et al., 2018). Moreover, rapamycin treatment and lysosomal de-acidification have other effects on lysosomal and cellular function that could be independent of autophagy, and genetic perturbations that specifically inhibit macroautophagy should be considered. For example, like macroautophagy, chaperone-mediated autophagy (CMA) requires lysosome function and has been found to play an important role in HSC quiescence and re-activation: during quiescence, CMA reduces oxidative stress by degrading oxidized protein, whereas during re-activation, it facilitates metabolic

reprogramming necessary for expansion (Dong et al., 2021). Indeed, knockout of autophagy genes Atg5 and Atg16L1 using *GFAP-Cre* mice had no effect on NSC maintenance, although knockout of other autophagy genes such as *FIP200* did, although the authors performed their studies on young mice, and autophagy inhibition may take time to exert its effects on quiescent NSCs (Wang et al., 2016). Furthermore, recent studies of knock-in mice that attenuate the canonical autophagy function of FIP200 found that it is the non-canonical role of FIP200 in regulating phosphorylation of p62 that is required for maintenance of NSCs and neurogenesis, not its essential role in autophagy (Liu et al., 2021).

Although increases in macroautophagy and lysosomal function are generally correlated to increased resiliency of quiescent adult stem cell populations, their roles may be nuanced and context dependent. Inhibition of macroautophagy pathways causes depletion of HSC pools, likely both from increased cell death and HSCs inappropriately exiting quiescence (Chang, 2020). The inappropriate exit from quiescence for HSCs with compromised macroautophagy may be linked to increased oxidative phosphorylation caused by mitochondria that are not correctly degraded (Ho et al., 2017). However, mitochondria are abundant in HSCs, and autophagy of mitochondria is low (de Almeida et al., 2017). Furthermore, oxidative phosphorylation and a functional electron transport chain are essential for maintaining the quiescence of adult HSCs (Ansó et al., 2017). Macroautophagy is also beneficial to the long-term maintenance of MuSCs by preventing the loss of proteostasis and accumulation of dysfunctional mitochondria and ROS (García-Prat et al., 2016). However, although mitophagy (the autophagic degradation of mitochondria) might eliminate dysfunctional mitochondria that generate increased ROS, it might, unabated, have broad-ranging effects on cellular metabolism that can affect re-activation of quiescent cells (Liang et al., 2020). The sometimes contradictory (or context dependent) benefits or detriments of macroautophagy in quiescent cells could in part be due to the diversity of substrates for autophagy. For instance, aggrephagy (the autophagic digestion of protein aggregates) could result in the accumulation of difficult-to-degrade protein aggregates that might cause membrane rupture or other kinds of damage to lysosomes, negatively affecting quiescent cell homeostasis over time, especially in contexts where lysosomal degradation pathways are perturbed (Johmura et al., 2021). In certain cell types and contexts, the benefits of stimulated autophagy might outweigh the downsides, whereas the opposite may be true in other cell types and contexts. The molecular basis for many types of selective autophagy are understood; therefore, in future studies, it will be possible for the field to systematically address which kinds of autophagy are beneficial or detrimental to quiescent cells in various cell types, disease contexts, and throughout life (Galluzzi et al., 2017). It is also important to note that certain interventions that inhibit or boost autophagy can have other effects on cell function; hence, results should be interpreted carefully. For instance, Bafilomycin A1 treatment de-acidifies lysosomes, blocking autophagy, but also interferes with other lysosomal functions and endocytic degradation. Likewise, rapamycin boosts autophagy through regulation of TFEB, discussed below, and by de-inhibiting autophagosome formation but also inhibits protein translation initiation.

At the transcriptional level, lysosomal biogenesis and macroautophagy are primarily regulated by the transcription factor TFEB that in nutrient-replete conditions is phosphorylated by mTORC1, which prevents its nuclear translocation and stimulates its degradation (Martina et al., 2012; Napolitano and Ballabio, 2016; Roczniak-Ferguson et al., 2012; Sardiello et al., 2009; Settembre et al., 2011, 2012). Recent studies of mTORC1-TFEB regulation in other cellular contexts has raised questions as to how TFEB is activated in quiescent cells. In contrast to other mTORC1 substrates, such as S6K and 4EBP1, TFEB is only activated when nutrients such as amino acids are limiting via the lysosomal Rag-Ragulator complex but is not affected by the Akt-Tsc1/2-RHEB signaling axis that transmits signals from growth factors (Napolitano et al., 2020). Activated TFEB is a hallmark of quiescent cells, but these cells are cultured in media that is, except for changes in growth factors, very similar to the media used to for growing mitotic cells that have low TFEB activity (Fujimaki et al., 2019; Leeman et al., 2018; Liang et al., 2020). Furthermore, evidence suggests that TFEB promotes transcription of RagD, part of the Rag-Ragulator complex that activates mTORC1 in response to nutrients, which should feedback to inhibit TFEB over time (Di Malta et al., 2017). Thus, it is not clear how TFEB is activated in quiescent cells for prolonged periods, since some of the main signals that regulate its activation are the same in quiescent cells and their activated counterparts and because of a feedback loop that should restrain its activity. One possible explanation is that CDK4/6, which promote G1-S transition, was recently shown to phosphorylate TFEB and promote its nuclear export, and worked together with the Rag-Ragulator complex to regulate TFEB nuclear accumulation, but whether reduced CDK4/6 activity in quiescent cells regulates TFEB is not known (Yin et al., 2020). Another explanation is that reduced signaling through Akt1 in quiescent cells could promote TFEB activation independently of mTORC1 (Palmieri et al., 2017). It is also possible that protein aggregates that reside in lysosomes or unique metabolites (or metabolic signatures) that accumulate in quiescent cells regulate the Rag-Ragulator complex or that TFEB activation is regulated by changes in ER-lysosome contact sites (Lim et al., 2019). Finally, TFE3/TFEB are regulated by PERK as part of the integrated stress response, which is sometimes active in quiescent cells (Martina et al., 2016). Although it is unlikely that one pathway alone controls TFEB activity in quiescent cells, future studies examining this conundrum may provide new insights into the regulation of mTORC1 and TFEB in cells.

Coordination between ER and autophagy/lysosomes

As major anabolic and catabolic hubs in cells, the ER and lysosomes, which rely on one another for proper function, should, respectively, coordinate their activities, and indeed, they do. Functionally, upregulation of ER function through overexpression of XBP-1s improves lysosomal function, coordinating with TFEB, possibly by increasing the ability of cells to produce lysosomal enzymes that are first synthesized in the ER (Imanikia et al., 2019). Presumably, reduced ER homeostatic capacity with age may compromise lysosomal function as well; however,

as mentioned above, ER stress also regulates TFEB/TFE3 through PERK, which promotes lysosomal function and the upregulation of ERphagy receptor FAM134B (Cinque et al., 2020; Martina et al., 2016). Lysosomes also contribute to ER homeostasis: increasing lipophagy improves ER homeostasis and lysosomal regulation of amino acid metabolism influences ER stress resistance (Daniele et al., 2020; Higuchi-Sanabria et al., 2020). Autophagy, including reticulophagy (also called ERphagy), is upregulated in response to ER stress, providing, among other things, a back-up system for failures in the degradation of terminally misfolded proteins via ERAD (Houck et al., 2014; Rashid et al., 2015). The ER also impacts lysosome function directly through contact sites with the endolysosomal system, controlling the timing and location of endosomal sorting events and non-vesicular trafficking of lipids to and from lysosomes (Allison et al., 2017; Friedman et al., 2013; Hoyer et al., 2018; Lim et al., 2019; Murley et al., 2015; Rowland et al., 2014). Although the interplay between lysosomes and ER is not routinely explored in quiescent cells, their shared dysfunction with age and role in quiescent cell functions therein may link back to shared mechanisms through their extensive physical and functional interconnectedness. Assessing the temporal relationship between deficiencies in ER and lysosomal homeostasis with age may illuminate strategies to mitigate the age-associated failures in quiescent cell function before they become irreversible.

Changes in lysosomal structure in quiescent cells

tChanges in lysosomal structure and function in quiescent cells have a deep evolutionary origin. For instance, in quiescent budding yeast cells, vacuoles (yeast analogs of metazoan lysosomes) are dramatically re-organized. In proliferating yeast, vacuolar membrane proteins are distributed homogenously throughout the membrane. However, in quiescent yeast cells, large, stable, lipid raft-like domains form that segregate all tested vacuolar membrane proteins to one of two domains: a sterol-enriched, liquid-ordered domain and a sterol-depleted liquid-disordered domain (Toulmay and Prinz, 2013). Sterol lipid transport proteins, which may facilitate exchange of sterol lipids between the ER and vacuoles, as well as vesicular trafficking and lipophagy (autophagy of lipid droplets) all play a role in the formation of vacuole membrane domains (Murley et al., 2015; Toulmay and Prinz, 2013; Wang et al., 2014). Vacuole membrane domains have been shown to be major sites of lipophagy, which is required to preserve vacuole membrane domains over time (Wang et al., 2014). Upstream regulators of TORC1 are among the few proteins that are enriched in the raft-like domains, and forced formation of vacuole membrane domains is sufficient to significantly inhibit TORC1 and render cells hyper sensitive to rapamycin (Murley et al., 2017). Lysosomes in mammalian cells are significantly smaller than vacuoles in yeast, making it difficult to determine if similar raft-like structures exist in mammalian cells. One exception are the enormous lysosomes in quiescent NSCs, which can reach up to 5 μm in diameter (Leeman et al., 2018). Although the formation of lysosomal membrane domains has not been directly examined in these cells, the large size of their lysosomes gives one a good chance of ascertaining whether lysosomal membrane domains exist in mammalian cells.

CONCLUSIONS AND FUTURE DIRECTIONS

Quiescent cells face a unique challenge to persist in a non-proliferating state in a dynamic environment, although remaining primed to divide in the event of tissue injury or other conditions that require their growth and proliferation. Cell extrinsic networks (e.g., stem cell niche signaling) and intrinsic networks like the endomembrane system discussed in this review are required to preserve functional cellular quiescence (Kalamakis et al., 2019; Leeman et al., 2018; Liu et al., 2018; Morrow et al., 2020). These processes interact with each other: lysosomal degradation of growth factor receptors is required for maintenance of quiescence in NSCs, and ERAD can degrade Rheb to enforce quiescence of HSCs downstream of Tsc1/2 signaling (Kobayashi et al., 2019; Liu et al., 2020).

As an organism ages, the regenerative potential of its tissues diminishes. Studies have attributed quiescent cell exhaustion to accumulated DNA damage, telomere shortening, and inflammatory signaling. Links between the ER and lysosomes and their role in homeostatic regulation of quiescent cells, likely also contribute to age-related deterioration of quiescent cell function, considering the parallels between age-associated dysfunction in the endomembrane system and failures in quiescence (Carmona-Gutierrez et al., 2016; Hughes and Gottschling, 2012; Martínez et al., 2017; Orenstein and Cuervo, 2010; Sabath et al., 2020; Sun et al., 2020; Taylor and Dillin, 2013). The numerous pharmacological tools that have been developed to modulate the UPR and lysosomal function provide the means of addressing the functional role of these organelles in quiescent cells and address whether the age-associated decline in the function of ER and lysosomes can be reversed to the benefit of re-activating quiescent cells (Bonam et al., 2019; Doultsinos et al., 2017; Ferri et al., 2020).

Although ER and lysosomal homeostasis counteract the transition of quiescent cells to an irreversible non-proliferative state in a variety of contexts, the mechanistic basis for this is not completely understood. Quiescent cells without robust ER and lysosomes/autophagy may begin to resemble senescent cells over time, expressing well-defined markers of senescence such as $p16^{Ink4a}$ that prevent their re-entry into the cell cycle (García-Prat et al., 2016). However, perturbed ER homeostasis mitigates the re-activation of quiescent cells in *C. elegans* that do not possess the machinery of cellular senescence that has been elucidated in mammals (Roux et al., 2016). Thus, other pathways leading from perturbed ER and lysosomal homeostasis to permanent cell cycle arrest may exist, and their elucidation may provide new hallmarks for loss of quiescence and possibly new targets for intervention in the age- and disease-associated dysfunction of quiescent cells.

Finally, technological advances on many fronts, from functional genomics and single-cell profiling, to improved *in vitro* systems enabling high-resolution and live-cell imaging promise to allow researchers to study quiescent cells *in vivo* and *in vitro* in every greater detail (van Velthoven and Rando, 2019). *In vitro* approaches to study quiescent cells, especially stem cells, would enable researchers to address aspects of their biology with greater mechanistic detail and control; however, current models would benefit from improvements. First, *in vitro* studies of

quiescent cells utilize cellular monoculture and do not recapitulate their native environment, which includes interactions with other cells, mechanical forces, and oxygen levels (Fujimaki et al., 2019; Leeman et al., 2018; Martynoga et al., 2013; Morrow et al., 2020). Artificial "niches" can aid in the maintenance of quiescent cell states of MuSCs *ex vivo*, at least for a few days, suggesting that further efforts to more closely recapitulate the cell-cell and mechanical interactions of quiescent cells with their niche, including the use of organoids, can improve *in vitro* modeling of cellular quiescence (Quarta et al., 2016). Given the importance of metabolic regulation to cellular quiescence, it will also be important to improve cell culture media formulations to reflect the *in vivo* environment of quiescent cells more closely. Indeed, physiological cell culture media can dramatically impact the outcome of experiments when used instead of traditional cell culture media (Cantor et al., 2017; Rossiter et al., 2021). These techniques could prove useful in understanding how ER and lysosomal function regulate quiescence under physiological conditions. The studies discussed in this review highlights the endomembrane system as a foundational network in regulating functional quiescence. Further study will offer detailed mechanistic insight of cellular quiescence potentially leading to the development of new therapeutic strategies for age-associated and degenerative disease.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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