States of G_0 and the proliferation-quiescence decision in cells, tissues and during development

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ABSTRACT While cellular proliferation is fundamental to the development of all multicellular organisms, the slowing or stopping of proliferation at the right places and times is equally important for proper tissue and organ development. The non-cycling state of cellular quiescence or "G_0" is relatively understudied compared to proliferation, given its prevalence in nature. It may seem that actively proliferating cells undergo a series of dynamic events, while quiescent cells are in a passive, static state. However, studies over the last 10-15 years suggest that quiescence may be more dynamic than previously thought and must also be actively regulated and maintained. This review focuses on recent advances in understanding quiescence or G_0 and in particular, on observations about the proliferation-quiescence decision in cell lines, in tissues and during development. We also discuss novel, advanced molecular tools that are likely to enable the field to address outstanding, unresolved questions about cellular quiescence and its regulation.

KEY WORDS: *cell cycle arrest, terminal differentiation, restriction point*

Quiescence and G_0

G_0 is a term broadly used to refer to a prolonged cell cycle arrest, or a sustained non-dividing state. G_0 can encompass distinct states, distinguished by their range of reversibility: from easily reversible to non-reversible (Fig.1). The term reversible quiescence has sometimes been used to describe cells that are not actively cycling, but may re-enter the cell cycle upon external stimuli. Examples include stem cells that respond to signals upon wounding to maintain tissue homeostasis or dormant cancer cells that can suddenly re-enter the cell cycle to seed recurrent tumors. This term also is commonly used to describe cells that are not dividing due to nutrient or growth factor starvation, for example in cell culture. In contrast, cells that acquire their final fate and undergo terminal differentiation during development, often enter a prolonged or sometimes permanent cell cycle arrest, which is also referred to as G_0. This is a feature characteristic of neurons, mature epithelia and differentiated muscle to name a few examples. In some organisms however, certain mature, differentiated cell types maintain a reversible G_0 and can re-enter the cell cycle upon damage. This includes examples such as mature muscle from amputated axolotl limbs (Sugiura *et al.*, 2016), the extraocular muscles of zebrafish (Saera-Vila *et al.*, 2015), Müller glia of the zebrafish retina (Wan and Goldman, 2016), and the sensory epithelium of the avian inner ear (Tsue *et al.*, 1994). A third state of cell cycle arrest, senescence, is also often referred to as G_0. In senescence, cells exit from the cell cycle in response to telomere loss, stress, accumulation of DNA damage or aberrant oncogenic activity, and undergo permanent arrest accompanied by metabolic, nuclear and morphological changes associated with DNA damage (Salama *et al.*, 2014). Senescence often represents a general response to aging and stress (Chandler and Peters, 2013), but recent work has shown that senescence also occurs during normal development (Munoz-Espin *et al.*, 2013, Storer *et al.*, 2013).

While most G_0 cells are thought to exit the cell cycle in G_1 phase or with a G_1 DNA content (2C), not all cells do so. For example, in human epidermis, differentiating keratinocytes undergo additional replication without cell division and finally exit the cell cycle with polyploid DNA (Zanet *et al.*, 2010). Cells in the liver, heart and placenta can be quiescent in G_0 with 4C, 8C or greater DNA content (Fox and Duronio, 2013), and in plants, several rounds of endocycling (cell cycles lacking mitosis and consisting of Gap and S-phases only) occur before terminal differentiation in trichome cells, resulting in cell cycle exit with 32C DNA (Orr-Weaver, 2015).

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Furthermore, cells that normally exit the cell cycle with a G_1 DNA content, in many cases can be induced to exit the cell cycle in G_2. For example, mammalian cells lacking all three of the Retinoblastoma family pocket proteins respond to serum deprivation with massive apoptosis and a prolonged cell cycle arrest in G_2, and tissues in *Drosophila* that normally exit the cell cycle with a G_1 DNA content can be induced to exit the cell cycle permanently with partially replicated DNA or in G_2 by genetic manipulations of certain cell cycle regulators (Buttitta *et al.*, 2007, Foijer *et al.*, 2005). Altogether, these observations raise a big question regarding how we diagram and discuss the G_0 state and cell cycle exit (Fig. 1). If cells do not necessarily leave the cell cycle from G_1, is this still considered G_0? Or are non-dividing cells with hyperploid DNA in a distinct state from G_0? This question may also apply to the G_0 associated with senescence, as recent work suggests mitotic slippage occurs prior to senescence and many senescent cells in fact do not exhibit a 2C DNA content (Dikovskaya *et al.*, 2015, Restall *et al.*, 2015).

Potential molecular markers of quiescence

Molecular markers that can distinguish G_0 cells from cycling G_1 cells are of great interest in the cell cycle field. Most cell cycle assays such as immunostaining for markers of proliferating cells or flow cytometry cannot distinguish G_0 from early G_1 cells since both G_0 and G_1 cells generally contain a 2C DNA content, and there may not be any obvious change in cell morphology or size (Pozarowski and Darzynkiewicz, 2004, Zambon, 2010). In some cell types such as skeletal muscle, neurons or keratinocytes, specific molecular markers can be correlated with G_0, but these often don't translate to other cell types. Without a universal marker for G_0, it has been very challenging to reliably identify G_0 cells both *in vitro* and *in vivo*. Below is a discussion of existing molecular approaches used to distinguish G_0 from G_1 cells.

Cyclin-dependent kinase inhibitors as markers of G_0

Cell cycle progression is controlled by oscillations of the activity of different cyclin/Cyclin-dependent kinase complexes. The active kinases and the activation process are conversely suppressed by the binding of cyclin-dependent kinase inhibitors (CKIs). The CKI members of the Cip/Kip family (p21^{Cip1}, p27^{Kip1}, p57^{Kip2}), share a homologous inhibitory domain, which is responsible for the inhibition of the G_1 cyclin complexes containing Cdk4 and Cdk2, by direct binding. The Cip/Kip family acts on Cdk2 preferentially *in vivo*, though they can target all G_1 cyclin complexes *in vitro* (Ortega *et al.*, 2002, Parry *et al.*, 1999, Soos *et al.*, 1996). Disruption of p27^{Kip1} in mice leads to multiorgan hyperplasia and an increase in the fraction of S phase cells in the thymus, demonstrating that p27^{Kip1} functions as a negative regulator of cell proliferation in specific contexts during development (Fero *et al.*, 1996, Kiyokawa *et al.*, 1996). Similarly, loss of p57^{Kip2} leads to a partial loss of quiescence in the neural stem cells of the hippocampus, and delayed cell cycle exit of terminally differentiating lens cells of the eye and chrondrocytes in

limbs (Furutachi *et al.*, 2013, Yan *et al.*, 1997, Zhang *et al.*, 1997). Although mice lacking p21^{Cip1} exhibit normal tissue development, they display defects in quiescence of hematopoietic stem cells and mouse embryonic fibroblasts (MEFs) lacking p21 exhibit defects in cell cycle arrest in response to DNA damage (Cheng *et al.*, 2000, Deng *et al.*, 1995). Consistent with the tissue specific differences observed in the mutant mice, the Cip/Kip family members exhibit tissue specific expression and can act redundantly to limit proliferation in many cases (reviewed in Pateras *et al.*, 2009).

The CKIs p21^{Cip1} and p27^{Kip1} are highly expressed during G_0 (Coller *et al.*, 2006, Oki *et al.*, 2014), but p21^{Cip1} also transiently accumulates in the nucleus at the G2/M boundary to inhibit CycA/Cdk2 complexes in response to DNA damage (Dulic *et al.*, 1998). The levels of p27^{Kip1} may therefore be a useful and specific marker for G_0. The highest levels of p27^{Kip1} expression are maintained in G_0 by the sequential activity of distinct ubiquitin ligases during the cell cycle. As cells enter the cell cycle, cytoplasmic p27^{Kip1} is ubiquitinated by KPC (Kip1 ubiquitination-promoting complex) during G_1. KPC-mediated p27^{Kip1} proteolysis depends on p27^{Kip1} nuclear export, which occurs at G_0 exit/ G_1 phase entry. A bit later in the cell cycle, proteolysis of the remaining nuclear p27^{Kip1} is triggered by phosphorylation on T187 and ubiquitination via interaction with the F-box protein Skp2 during early S and G_2 phase (Kamura *et al.*, 2004, Tsvetkov *et al.*, 1999). In addition a third RING-H2-type ubiquitin ligase, Pirh2 ensures p27^{Kip1} ubiquitination both in the nucleus and the cytoplasm at late G_1 phase (Hattori *et al.*, 2007). Based on the high levels of p27^{Kip1} at G_0 and its destruction at G_1 entry and G_1 progression, a novel fluorescent cell cycle reporter was generated to separate G_0 and G_1 cells by fusing a fluorescent protein to an inactive form of p27^{Kip1} protein that cannot bind to Cdk2, which we discuss in detail later.

Using the pRb family to identify cells in G_0

Cyclin/Cdk complexes and the activator forms of the transcription factor complex E2F/DP promote the progression of the cell cycle. By contrast, the Cyclin-dependent Kinase Inhibitors (CKIs) and the retinoblastoma protein-family members (pRB, p107 and p130) are negative regulators of cell cycle progression (Cobrinik, 2005, Vidal and Koff, 2000). Investigations into the function of the retinoblastoma proteins (pRB), the first identified tumor suppressor (Huang *et al.*, 1988, Trimarchi and Lees, 2002), showed that pRB is important for inhibiting cell cycle entry by binding and suppressing the transcription factor complex E2F/DP, which transcriptionally
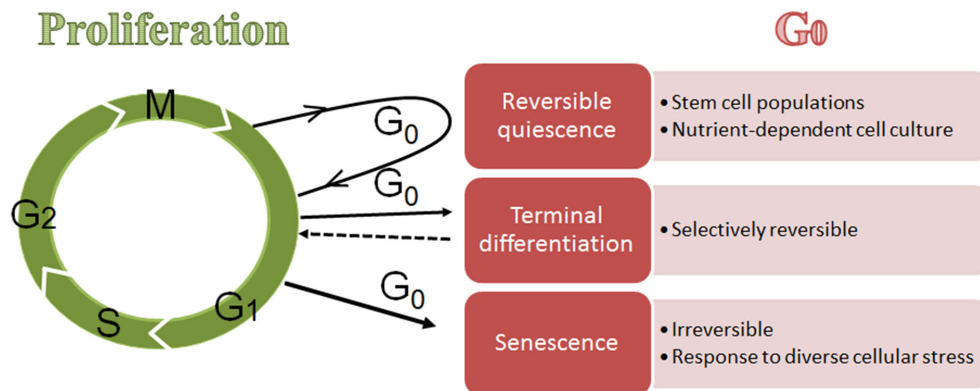

Fig. 1. Distinct states of G_0. *There are at least 3 distinct states of G_0 that vary in their reversibility.*

regulates hundreds of cell-cycle regulators (Burkhart and Sage, 2008, Tamrakar *et al.*, 2000, van den Heuvel and Dyson, 2008). This repression is counteracted by the G_1 Cyclin/Cdk complexes Cyclin E/Cdk2 and Cyclin D/Cdk4 which catalyze the phosphorylation of RB family members, resulting in the release of E2F/DP binding, allowing E2F/DP transcriptional activity (Du and Pogoriler, 2006). The active E2F/DP complexes promote Cyclin/Cdk expression transcriptionally, along with hundreds of other cell cycle genes, thus creating a positive feedback loop that promotes G_1 progression and robust commitment to cell cycle entry (Blais and Dynlacht, 2004, Trimarchi and Lees, 2002).

In the prevalent model of G_0-G_1 progression, G_1 CyclinD-Cdk4/6 and CyclinE/Cdk2 complexes progressively phosphorylate RB resulting in the transition from an active, unphosphorylated RB, through a less active, hypo-phosphorylated form to drive G_1 entry, to a fully inactive hyper-phosphorylated RB at the G_1-S transition. However, recent biochemical studies in mouse embryonic fibroblasts (MEFs) showed that RB is exclusively mono-phosphorylated by CyclinD-Cdk4/6 on any one of 14 Cdk phosphorylation sites that are spread throughout the protein at early G_1 phase. Surprisingly, the mono-phosphorylated RB still functions as a suppressor of E2F activity, in direct contrast to the prevalent model. At late G_1 phase, the mono-phosphorylated RB is further hyper-phosphorylated by the active CyclinE/Cdk2 complex to prevent its suppression of E2F activity, thereby allowing G_1/S progression (Narasimha *et al.*, 2014). Importantly, in quiescent or differentiating cells, RB remains in a repressive, un-phosphorylated form, which suggests that appearance of mono-phosphorylated RB could be an early mark of the G_0-G_1 transition. Any one of the potential 14 phospho-sites may be phosphorylated to generate the "active" form of RB, and therefore it is impossible to predict which one of the sites out of 14 will be mono-phosphorylated. Moreover, the mono-phosphorylated RB and un-phosphorylated RB co-migrate on a traditional 1D SDS-acrylamide gel and require resolution by a two-dimensional isoelectric focusing (2D IEF) gel (Narasimha *et al.*, 2014). Thus, it will be challenging to monitor mono-phosphorylated RB as a molecular marker for the G_0-G_1 transition in individual cells or in fixed tissue samples.

A different RB family member, p130 (RBL2), is the primary E2F complex repressor in certain quiescent cells (Sadasivam and Decaprio, 2013, Takahashi *et al.*, 2000). The highly conserved p130/E2F complex termed DP, RB-like, E2F, and MuvB (DREAM) is thought to be responsible for cell-cycle dependent gene repression in certain G_0 cells, including human glioblastoma cells and human primary fibroblasts (Litovchick *et al.*, 2011, Litovchick *et al.*, 2007). Chromatin immunoprecipitation (ChIP) and Multidimensional Protein Identification Technology (MudPIT) have revealed that DREAM complex directly binds to 800 human promoters, and that most E2F cell cycle targets are repressed by p130/DREAM. The binding of p130 to DREAM is significantly stronger in G_0-arrested cells than in proliferating cells (Litovchick *et al.*, 2007). At the G_0-G_1 transition, G_1 Cdks phosphorylate p130, which triggers ubiquitination of p130 via Skp2, thus leading to the release of E2F (Smith *et al.*, 1996, Tedesco *et al.*, 2002). Because p130 is high during quiescence and destroyed upon cell cycle entry, un-phosphorylated p130 could be an excellent positive marker for G_0 in certain reversibly quiescent cell types. For example, quiescent hematopoietic stem cells (HSCs) predominantly express p130 over other RB family members, and as HSCs exit from quiescence p130 decreases, possibly due to degradation (Passegue *et al.*, 2005).

Using a T-loop phosphorylation cascade to monitor the G_0-G_1 transition

During G_1 phase, CycD-Cdk4/6 kinase activity is responsible for mono-phosphorylation of RB to progressively promote the progression into G_1 phase. Therefore, the steps leading to activation of the Cdk4/6 complex are critical determinants of the G_0-G_1 transition. How is the Cdk4 or Cdk6 activated other than binding to CycD?

The typical Cdk catalytic subunit contains a 300 amino acid catalytic core which is inactive when monomeric (Morgan, 1995). Binding to a cyclin and phosphorylation of a conserved Thr residue in the activation loop (T-loop) are required to activate Cdk kinase activity. Phosphorylation of the Cdk T-loop is catalyzed by the Cdk-activating kinase (CAK) complex, composed of Cdk7, cyclin H, and MAT-1. The catalytic component Cdk7 is expressed uniformly during the cell cycle, as Cdk7 also plays an essential role as a component of the general transcription factor TFIIH, which phosphorylates the C-terminal domain (CTD) of the largest subunit of Pol II (Fisher, 2005, Larochelle *et al.*, 1998, Merrick *et al.*, 2008). *Drosophila* Cdk7 was first identified based on its requirement for proliferation, as it acts as a CAK to activate the Cdk1 complex *in vivo* (Larochelle *et al.*, 1998). Indeed, the effects of Cdk7 loss on cell proliferation can be largely rescued by Cdk2^{T160E} or Cdk1^{T161E} phosphomimetic mutants in mouse embryonic fibroblasts (Ganuza *et al.*, 2012). Thus, Cdk7 is essential for the activation of the cell cycle by phosphorylation of T-loop residues in Cdk2 or Cdk1.

It was recently uncovered that at early G_1, Cdk4 or Cdk6 activation is also largely dependent upon Cdk7 kinase activity *in vivo* (Schachter *et al.*, 2013). Upon mitogenic signals, the Cdk7 activity is induced via the phosphorylation of its T-loop site by an unknown endogenous kinase, enabling Cdk7 to act in a T-loop cascade towards Cdk4/6. Once Cdk4 or Cdk6 are phosphorylated on their T-loop sites and bound to CycD, they become active, but require continued Cdk7 activity to combat an unknown endogenous phosphatase that targets the unprotected T-loop site. Cdk7 plays an essential role in the activation and maintenance of Cdk4/6 activity to promote the G_0-G_1 transition. T-loop phosphorylation of Cdk7 and Cdk4 both increase at G_0 exit/ G_1 entry *in vivo*, which suggests a CDK activation cascade via sequential T-loop phosphorylation could underlie the G_0-G_1 transition. Consistent with this, stem cells lacking Cdk7 also lack the ability to repopulate tissues with rapid turnover suggesting this cascade is critical for cell cycle re-entry of stem cells (Ganuza *et al.*, 2012).

This also suggests important roles for yet to be characterized kinases and phosphatases in the proliferation-quiescence decision. For example, what initiates Cdk7 activation at the G_0-G_1 transition? Is there another CAK? Or as *in vitro* studies have shown (Garrett *et al.*, 2001), is Cdk7 a target of active Cdk2 *in vivo*? If so, Cdk2 activity levels very early after mitosis will be critical for the proliferation-quiescence decision, a time much earlier in the cell cycle than its best known major role in the G_1-S transition.

Developmental regulation of the proliferation-quiescence decision

As tissues mature in development, most cells differentiate into specialized cell types and slow or stop proliferation and contribute to organ function. Most adult animal cells have stopped dividing and enter G_0, where many will remain for lifetime of the organism. However, it remains unclear why or how cells choose to enter G_0

during development, and how certain developmental signals, tissue damage or nutrient conditions can trigger specific quiescent cells to re-enter the cell cycle.

The double–repression of E2F and Cyclin/Cdk activity

Despite the central role for the RB family proteins in most models of cell cycle exit and quiescence, cell cycle exit can still occur without functional pocket proteins. In mouse embryonic development, removal of all three RB family members (in a triple knockout or TKO background) does not prevent cell cycle exit and differentiation in epithelial or neuronal progenitor cells (Wirt *et al.*, 2010). In conditional triple knockout (cTKO) hepatocytes, deletion of the RB family causes a temporary cell-cycle re-entry, but eventually cTKO hepatocytes stably exit the cell cycle (Ehmer *et al.*, 2014). This is consistent with the finding that hyperactivation of the E2F transcription factor complex in *Drosophila* can only temporarily delay cell cycle exit, with cells arresting in G_0 after only 1-2 extra rounds of division (Buttitta *et al.*, 2007). Thus additional mechanisms must exist to actively promote quiescence in differentiating cells *in vivo*.

CKI expression is commonly associated with G_0. Not surprisingly, TKO mouse embryonic fibroblasts still exit cell cycle under serum starvation, but with high p27^{Kip1}, p21^{Cip1} levels. This implies that CKIs may act as central mediators of cell cycle arrest in the absence of functional pocket proteins (Foijer *et al.*, 2005). In mice deletion of p21^{Cip1} induces proliferation in hippocampal neurons, indicating that p21^{Cip1} is required for G_0 in specific differentiating neurons during development (Pechnick *et al.*, 2008). Loss of p21^{Cip1} or p27^{Kip1} also disrupts the proper cycle exit of differentiating cardiomyocytes of newborn mice (Tane *et al.*, 2014), resulting in altered DNA content. However these disruptions do not eliminate cell cycle exit altogether. For example, loss of all three CKI proteins (p21^{Cip1}, p27^{Kip1} and p57^{Kip2}) in the spinal cord delays, but does not prevent cell cycle exit during neurogenesis (Gui *et al.*, 2007). This is consistent with the finding in *Drosophila* that, the sole Cip/Kip-type CKI, Dacapo (Dap), is largely dispensable for most cell cycle exit in *Drosophila* tissues after embryogenesis, including eye and wing development (Buttitta *et al.*, 2007, Firth and Baker, 2005). Instead the Rb family and CKIs play overlapping roles in control of cell cycle exit *in vivo*. In animals ranging from mice to *Drosophila* to *C. elegans*, the loss of Rb family members and CKIs simultaneously leads to further proliferation in tissues that should enter G_0 during development (Boxem and van den Heuvel, 2001, Firth and Baker, 2005, Yeh *et al.*, 2007, Zindy *et al.*, 1999).

Looking beyond the pRb family and CKIs in quiescence

The proteolysis of cyclins and their regulators ensures the proper temporal order of cell cycle events as well as the entry into quiescence. The two major protein degradation complexes involved in cell cycle regulation are Skp-Cullin-F-box (SCF) complexes for G_1-S phase progression and the APC/C (the Anaphase-Promoting Complex/Cyclosome) for the completion of mitosis and entry into quiescence. The SCF complex consists of four subunits: Skp1 (scaffold protein), Cul1 (scaffold protein), RING-finger component (Rbx1), and a variable adaptor protein or F-box protein. The F-box proteins are E3 ubiquitin ligases that target a discrete number of specific substrates through protein–protein interactions. The F-box proteins Skp2 and Fbw7 target multiple cell cycle regulators. Skp2 mediates the degradation of the CDK inhibitors p21^{Cip1}, p27^{Kip1} and p57^{Kip2}, as well as the Rb-family member p130, to promote
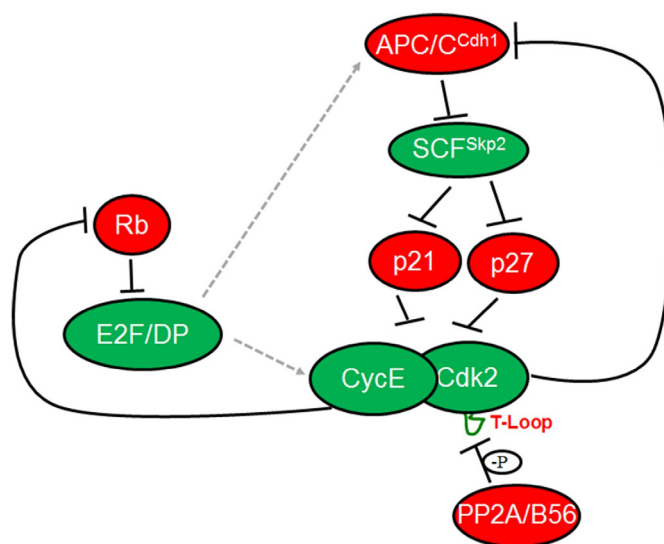

Fig. 2. A regulatory network influencing the proliferation-quiescence decision. *Complexes impacting the proliferation-quiescence transition during development are shown with negative (red) and positive (green) cell cycle regulators. Several studies suggest redundancy of negative regulators ensures robust cell cycle exit upon terminal differentiation. Regulation may act transcriptionally (dashed lines) as well as post-transcriptionally (solid lines) and includes both positive and negative feedback loops.*

G_1 to S progression (Cardozo and Pagano, 2004, Tedesco *et al.*, 2002, Willems *et al.*, 2004). While Fbw7 targets several proto-oncogenes, including CycE, MYC, JUN and Notch (Koepp *et al.*, 2001, Welcker and Clurman, 2008). In the regulation of the cell cycle, Fbw7 specifically targets phosphorylated CycE for degradation, which is high during the G_1-S transition due to Cdk2 kinase complex activity (Clurman *et al.*, 1996, Koepp *et al.*, 2001). Mutations in Fbw7 have been found in several human cancers (Moberg *et al.*, 2001), and *Fbw7* mutations in mice and *Drosophila*, lead to additional proliferation and can delay entry into G_0 in certain cell types (Moberg *et al.*, 2001, Onoyama *et al.*, 2007).

The APC/C is an E3 ubiquitin ligase complex whose activation requires the phosphorylation of specific subunits and the binding of a co-activator, either Cdc20 (or Fizzy; Fzy) or Cdh1 (or Fizzy-related; Fzr) for full activity. The APC/C complex is important to coordinate mitotic exit and quiescence (Clijsters *et al.*, 2013, Sigrist and Lehner, 1997). During mitosis, APC/C^{Cdc20} complex promotes mitotic exit by degrading key substrates such as the mitotic cyclins and the replication licensing inhibitor Geminin, which usually accumulate during the S, G2, and early mitotic phases (McLean *et al.*, 2011, Penas *et al.*, 2011). In contrast, APC/C^{Cdh1} plays a major role after mitotic exit in maintaining quiescence and preventing precocious DNA replication (Eguren *et al.*, 2011). Work from different groups also suggests the proliferation-quiescence transition and cell cycle exit requires the activity of APC/C^{Cdh1} complex (Buttitta *et al.*, 2010, Cappell *et al.*, 2016, Garcia-Higuera *et al.*, 2008, Ruggiero *et al.*, 2012, Tanaka-Matakatsu *et al.*, 2007, The *et al.*, 2015). What roles could the APC/C play to promote G_0? One important target of the APC/C is Skp2, which leads to the ubiquitination and degradation of the negative cell cycle regulators, p27^{Kip1} and p21^{Cip1}. Thus high activity of APC/C not only leads to degradation of mitotic cyclins (Sigrist and Lehner, 1997), but also stabilizes CKIs to prevent cell

cycle progression (Binne *et al.*, 2007). Therefore, the cell cycle protein degradation machinery adds yet another layer of G_0 regulation.

Inhibition of APC/C activity also cooperates with the loss of other negative cell cycle regulators such as loss of RB family members to compromise cell cycle exit. For example, in *C. elegans* double mutants of RB and the APC/C activator Cdh1 exhibit aberrant expression of S phase genes in differentiated muscle cells (The *et al.*, 2015). In *Drosophila*, repression of APC/C activity together with aberrant E2F activity is able to bypass permanent cell cycle exit in the wing (Buttitta *et al.*, 2010). This indicates that APC/C activity is required to limit the accumulation of cyclins and other essential E2F targets that promote active proliferation. CycE/Cdk2 activity has been shown to inhibit APC/C activity (Reber *et al.*, 2006, Sigrist and Lehner, 1997) thus, the high APC/C activity in quiescent, differentiating cells may also increase the threshold level of CycE required to suppress APC/C and initiate re-entry into G_1. Furthermore, the CycD/Cdk4 kinase complex is able to phosphorylate both RB and APC/C^{Cdh1} *in vitro*, resulting in their inactivation (The *et al.*, 2015). Thus, one attractive model for cell cycle re-entry from G_0 is that D-type cyclins phosphorylate and abolish Cdh1 activity towards Skp2, and thereby allowing SCF^{Skp2} complex to degrade CKIs, resulting in high CycE/Cdk2 complex activity, which further inhibits the APC/C activity to push cell cycle re-entry (Bashir *et al.*, 2004, Wei *et al.*, 2004). Thus, Rb-mediated transcriptional repression and stable CKI expression, protected by the APC/C complex, all impinge upon the restriction of G_1 cyclin/Cdk activity to promote cell cycle exit and a stable state of G_0 or quiescence (Fig.2).

Threshold models of the Proliferation-Quiescence decision suggest different states of G_0

A recent single-cell study using a live-cell sensor for Cdk2 activity revealed an unexpected bifurcation in Cdk2 activity after mitosis in individual cells. In this study, individual cells within a clonal population were shown to exhibit different levels of Cdk2 activity after mitosis, and the level of Cdk2 activity was strongly correlated with the subsequent decision of whether to enter the next cell cycle or enter into temporary quiescence. The conclusion is that cells with higher Cdk2 activity after mitosis commit to enter the next cycle while cells with low Cdk2 activity enter a transient G_0-like state, even in a clonal population, in the presence of abundant nutrients (Spencer *et al.*, 2013). The relative percentage of cells that enter G_0-like state after mitosis varies in different cell types and culture conditions, suggesting many signaling inputs likely influence the proliferation-quiescence decision. This is consistent with findings in several cancer cell lines, suggesting some cancer cells enter quiescence, even under full nutrient conditions while others do not (Dey-Guha *et al.*, 2011). This creates a heterogeneous population even within clonal cell lines, where some cells are actively cycling while others enter and leave temporary quiescent states. This leads to a situation termed 'proliferative heterogeneity', where different cells within a genetically identical population proliferate at different rates - which is readily visible in live cell imaging studies but becomes masked in non single-cell studies. A study in search of the regulatory mechanism for proliferative heterogeneity in clonal cell lines suggested that the relative levels of p21^{Cip1} and Cdk2 in individual cells and cell lines are responsible for the level of proliferative heterogeneity and the decision to proliferate or to enter quiescence in a human breast epithelial cell line (Overton *et al.*, 2014). This Cdk2- p21^{Cip1} model suggests that cells with high p21^{Cip1}/low Cdk2 activity are more likely to remain in a steady quiescent state, while cells with low p21^{Cip1}/high Cdk2 activity tend to rapidly commit to the next cycle after mitosis. Thus the threshold of Cdk2 activity set by its inhibitor p21^{Cip1} determines the proliferation-quiescence decision. This raises intriguing questions about how p21^{Cip1} is regulated and whether its levels oscillate between cycles or are stochastically regulated to create the proliferative heterogeneity observed in many cell lines, including cancer cells. While p21^{Cip1} may be critically important for the proliferation-quiescence decision in some cell contexts, many cells deficient in p21^{Cip1}, such as MEFs can still enter quiescence even in low serum conditions. This suggests that mechanisms independent of p21^{Cip1} must also contribute to the decision to enter quiescence or proliferate in the next cell cycle.

Indeed, another regulatory threshold model for the proliferation-quiescence decision, has been proposed (Yao *et al.*, 2008). In this Rb-E2F bistable model, the two steady states are based upon E2F complex transcription factor activity (E2F-Off and E2F- On), which define cellular quiescence and proliferation, respectively. The 'barrier' that separates the two states is the E2F activation threshold, and cells need to accumulate enough E2F activity to transit from quiescence to proliferation. Cells under different environmental conditions or with different cell types are likely to have variable E2F activation thresholds. The model predicts that cells with a higher E2F activation threshold are in a deeper
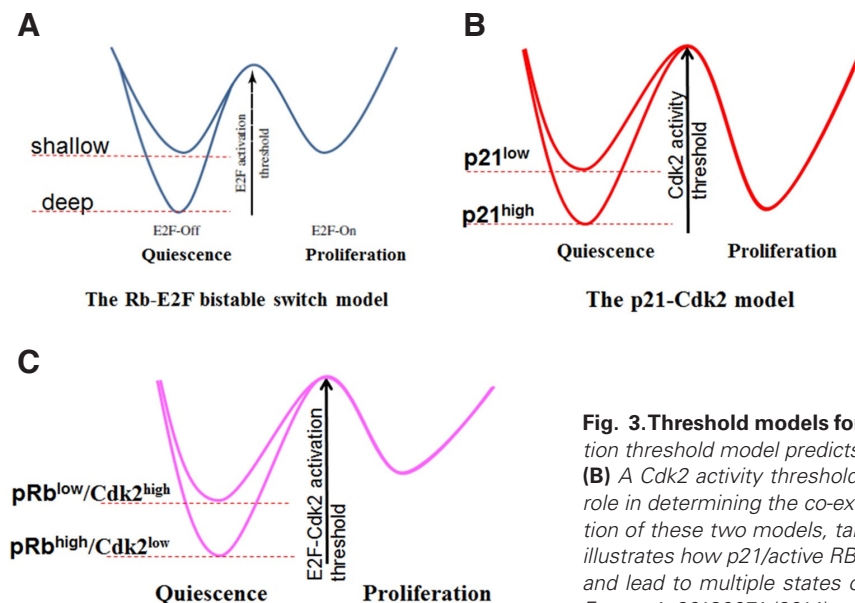

Fig. 3. Threshold models for the proliferation-quiescence decision. (A) *An E2F activation threshold model predicts multiple states of quiescence (shallow or deep) may exist.* **(B)** *A Cdk2 activity threshold model predicts the ratio of endogenous p21:Cdk2 plays a role in determining the co-existence of proliferative and quiescent states.* **(C)** *An integration of these two models, taking into account the impact of Cdk2 activity on RB activity, illustrates how p21/active RB levels could influence the proliferation-quiescence decision and lead to multiple states of quiescence. The figure is adapted from G. Yao, Interface Focus. 4: 20130074 (2014).*

quiescence. This means that cells spend longer time to gain enough E2F activity in order to switch from E2F-Off/quiescence to E2F-On/proliferation, whereas cells in shallow quiescence are more likely to re-enter cell cycle at a faster rate. Thus varying levels of quiescence may exist in an asynchronous cell population, which may explain the observation of a bifurcation of the proliferation-quiescence decision observed by Spencer *et al.,* under full nutrient conditions. These observations raise the question of whether multiple states of quiescence may be a source of proliferative heterogeneity. These two models both suggest that the proliferation-quiescence decision depends upon a bistable model with thresholds set by both positive and negative cell cycle regulators (Fig.3). Does this suggest that asynchronously growing cell populations constantly make the proliferation-quiescence decision, even under full nutrient conditions (Spencer *et al.*, 2013)? If so, we may want to redraw the cell cycle to include varying lengths of G_0 as a normal part of the cell cycle (Fig. 4).

A distinct proliferation-quiescence decision point (Q-point) in the cell cycle

In cell culture normal proliferation is driven by environmental mitogenic stimuli. This includes mitogens such as EGF and other signaling molecules provided in serum. While *in vivo,* cell cycle progression is driven (or inhibited) by local signaling molecules used during development, in wound healing, or to maintain normal tissue homeostasis. As cells terminally differentiate *in vivo*, the signaling environment changes to downregulate mitogens and promote signaling that favors cell cycle exit. Thus, understanding the cell cycle response to the deprivation of mitogenic stimuli in cell culture may be useful to understand some of the mechanisms contributing to cell cycle exit *in vivo* during development. Serum starvation is commonly used to synchronize cell proliferation to
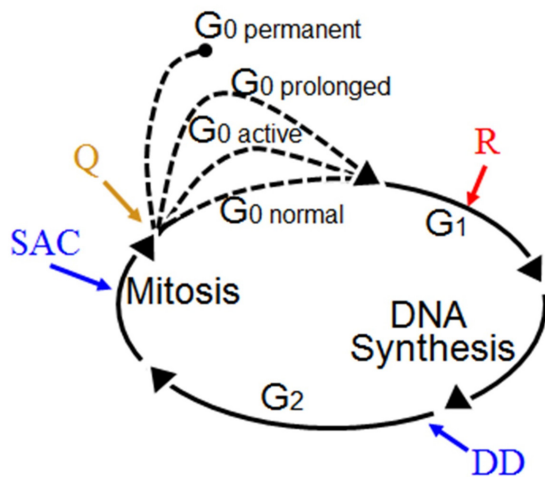
arrest cells in G_0 phase and the response to serum starvation was used to establish the restriction point (R) in the cell cycle, commonly assumed to be the proliferation-quiescence decision point. To define R, work by Dr. Pardee in the 1970s, suggested that cells can shift between proliferation and quiescence up to a certain point in the G_1 phase, the barrier or checkpoint R. If cells progress beyond R in G_1, even with serum deprivation, cells will commit to the next cycle (Pardee, 1974). A bit later in the 1980s, time-lapse imaging of mouse Swiss 3T3 cells revealed that a brief serum deprivation right after mitosis will arrest cells in quiescence /G_0, accompanied by the reduction in protein synthesis. Importantly, only cells less than 3-4 hours beyond the completion of the last mitosis entered into quiescence in response to serum withdrawal, whereas cells beyond 4hr from the last mitosis were able to complete the rest of cell cycle in the absence of serum (Larsson *et al.*, 1985). The G_1 phase was therefore subdivided into two physical phases: G_1 "postmitosis" and G_1 "pre-DNA synthesis", which are separated by the restriction point, serving as the presumed decision point for cells responding to mitogenic signals. But recent studies suggest that the proliferation-quiescence decision point actually occurs prior to the passage of cells through G_1, either during G_2 phase, where postmitotic Cdk2 activity levels may be set up or immediately after mitosis when the bifurcation in Cdk2 activity can be measured (Naetar *et al.*, 2014, Spencer *et al.*, 2013). This indicates the existence of another proliferation-quiescence decision point, one we label as Q, that is distinct from the later R in G_1 (Fig. 4). Q is associated with a bifurcation in Cdk2 activity, while R is associated with a bifurcation in Rb-E2F activity (Yao *et al.*, 2008). Both decision points are regulated by a shared pathway, the Cdk2-Rb-E2F pathway and thus can reflect different behaviors of the same regulatory pathway under different conditions (Zhang, 2013). Q operates during cycling under full nutrient conditions while R becomes evident in response to serum starvation. However results from Spencer *et al.,* suggest that Q and R remain distinct, in that cells with high Cdk2 at the Q point do not enter quiescence if they are subsequently deprived of serum before reaching R. Instead such conditions lead the Cdk2-high cells to enter and complete an additional cell cycle before entering quiescence at R in response to serum starvation. This is in contrast to prior models of R and a model where Q and R act as distinct, subsequent "quiescence checkpoints" during the cell cycle (Fig.4), and may instead be the result of an "On" or "Off" state of the Cdk2-RB-E2F axis from M-G_1 (Zhang, 2013). It may also be that the G_0-like state of Cdk2-low cells and G_0 entry at R in response to serum starvation are in fact distinct G_0 states. This will require further live, single-cell studies with G_0-quiescence sensors described in the following section to resolve what constitutes a prolonged M-G_1 phase from entry into G_0 or multiple states of G_0.

We recently revealed a function for the PP2A/B56 complex in modulating Cdk2 activity and quiescence entry during development, just after the final mitosis preceding a developmentally regulated G_0 entry, in *Drosophila* wings and eyes (Sun and Buttitta, 2015). Our work suggests that PP2A/B56 acts during the final cell cycle *in vivo*, independent of RB/E2F levels, to promote timely entry into quiescence. Our results are consistent with a model where PP2A/B56 regulates Cdk2 activity by removing an activating phosphorylation in the Cdk2 T-loop activation site during or right after mitosis. This would cause a rapid drop in Cdk2 activity during the bifurcation described by Spencer *et al.*, which we believe essential for timely

Fig. 4. Checkpoints throughout the cell cycle. *The red arrow denotes the Restriction Point (R), while the yellow arrow denotes the proliferation-quiescence decision point (Q). Other cell cycle checkpoints, such as the DNA damage checkpoint in G_2 (DD, which may also occur in G_1), and spindle checkpoint (SAC) are in blue. The variation in G_0 length in vivo or under full nutrient conditions is depicted as dashed paths between M and G_1 phases. This model suggests all cells may go through a transient G_0 state labeled "G_0 normal".*

quiescence entry in developing tissues. Of course it is also possible that in different biological contexts PP2A acts upon different targets to modulate the proliferation-quiescence transition. For example, an alternate model for PP2A/B56 acting via Ras signaling and Myc during G$_2$ phase to modulate quiescence in human cells has also been proposed (Naetar *et al.*, 2014). However in all these recent models, the regulatory machinery seems to ultimately impinge upon Cdk2 activity levels, either directly or indirectly at the proliferation-quiescence decision point (Q) just after mitosis, and prior to the Restriction point in G$_1$ phase (Fig.4).

Biosensor techniques to monitor the Proliferation-Quiescence decision

In order to further investigate the features of the proliferation-quiescence decision, and to test whether multiple states of quiescence may be a source of proliferative heterogeneity, we need to be able to identify G$_0$ cells and monitor G$_0$ entry at the proliferation-quiescence transition during a designated period of time. Recently, a novel cell cycle indicator, mVenus-p27K$^-$ (p27K$^-$), was generated to specifically label quiescent cells (Oki *et al.*, 2014). This probe is a fusion protein consisting of a fluorescent protein mVenus and a Cdk binding defective mutant of p27. p27 accumulates during quiescence, and its degradation is triggered upon cell cycle entry by two ubiquitin ligases, KPC and SCF^{Skp2}. The Kip1 ubiquitination-promoting complex (KPC) is an E3 ligase complex that triggers p27 degradation at G$_0$-G$_1$ transition, while the SCF^{Skp2} complex triggers degradation of nuclear p27 during the S/G2/M phases (Kamura *et al.*, 2004). When p27K$^-$ is combined with another cell cycle reporter for G$_1$ phase, mCherry-hCdt1(30/120), G$_0$ vs. G$_1$ can be distinguished (Oki *et al.*, 2014). The mCherry-hCdt1(30/120) reporter is a fusion of mCherry to the degron from the human replication licensing factor Cdt1 (hCdt1) which is targeted for degradation by the SCF^{Skp2} complex at the S/G2/M phases. Thus, mVenus-p27K$^-$ together with mCherry-hCdt1(30/120) label cells from just after the completion of mitosis until early S phase in distinct colors, which now provides the possibility to examine the proliferation-quiescence decision under different conditions via live imaging or FACS analysis without chemical or nutrient-deprivation synchronization. In addition this reporter works in transgenic mice to reveal resting stem cells, such as muscle satellite cells, opening the door for *in vivo* examinations of stem cell quiescence (Oki *et al.*, 2014).

Unfortunately the mVenus-p27K$^-$ probe may be not feasible for all cell types. It is reported the osteosarcoma cell line, U2O2 cell line transduced with this reporter displays defects in proliferation, while NIH3T3 cells as well as several other cell lines transduced with mVenus-p27K$^-$ proliferate normally without detectable defects in cell cycle regulation (Oki *et al.*, 2014). This could be due to the ectopic expression of the mVenus-p27K$^-$ fusion protein, which could be expected to maintain or carry out Cdk-independent functions of p27 raging from cytoskeletal roles to unexpected sequestration of non-Cdk binding partners (Serres *et al.*, 2012, Sharma *et al.*, 2012). Thus, in certain contexts unexpected activities of ectopically expressed reporters may generate confounding effects on the cell cycle.

To get around the problem of expressing an ectopic fusion reporter protein one would rather tag the endogenous protein. An advanced toolkit called "eFlut" has been recently established to do just that (Stewart-Ornstein and Lahav, 2016). This toolkit uses a novel plasmid set and Cas9 reagents to efficiently deliver fluorescent tags into endogenous gene sites in cell lines. This provides several advantages over transducing cells with viruses containing ectopic cell cycle reporters. First, there is less variability in expression levels since the endogenous protein is tagged versus the random integration of a virally encoded reporter into the cell's genome. This will likely favor more precise analysis of protein dynamics during the cell cycle. In addition this avoids the issues described above, where expression of the reporter itself has unexpected biological effects possibly leading to alterations in the cell cycle. However one obvious drawback of fluorescently tagging endogenous proteins via eFlut may be that important cell cycle proteins with lower expression levels will be difficult to monitor.

To show the potential of the eFlut toolkit, Stewart-Ornstein and Lahav used eFlut to investigate the regulation of the CKI p21^{Cip1} by quantitative p21^{Cip1}-YFP measurements at the single cell level. Interestingly, the p21^{Cip1} protein accumulation dynamics and timing varies substantially between individual cells, and even between sister cells. This further indicates the existence of proliferative heterogeneity within clonal cell populations, and importantly is consistent with the threshold model of p21-Cdk2 suggested to be responsible for the proliferative heterogeneity (Overton *et al.*, 2014). The eFlut toolkit should provide further opportunities to systematically characterize the dynamics of cell cycle networks that impinge upon the proliferation-quiescence transition, which will help us to finally understand how cell cycle machinery responds to environmental cues or developmental signals upon the proliferation-quiescence decision.

Future outlook

With the new molecular markers, live cell imaging and single cell assays discussed here, several outstanding questions regarding G$_0$ will hopefully be addressed. Perhaps most pressing is the need to finally understand the differences between reversible quiescence and permanent G$_0$. Are there distinct cell cycle regulatory mechanisms required for permanent or prolonged cell cycle exit of postmitotic cells versus the readily reversible quiescence of stem cells or nutrient deprived cells? One possibility is that the prolonged or permanent G$_0$ of terminally differentiated cells may simply be due to multiple redundant mechanisms, cooperating simultaneously to promote a more robust cell cycle exit in postmitotic cells. Indeed, some of the studies described here, using double or triple mutants support this idea. But there is also evidence that additional factors involved in terminal differentiation, such as differentiation-associated chromatin changes, may come into play to impact cell cycle regulation and promote prolonged or permanent G$_0$ (Pajalunga *et al.*, 2010, Peric-Hupkes *et al.*, 2010, Ruijtenberg and van den Heuvel, 2015). A second outstanding question regards proliferative heterogeneity in clonal cell culture lines and whether the choice to enter states of temporary G$_0$ are stochastic, or whether there is some sort of inheritance (possibly epigenetically) of a slowly vs. rapidly proliferating state. Cell lineage tracing tools may be combined with eFlut or the new quiescence marker mVenus-p27K$^-$ to address this issue. This may be a particularly important question for cancer cells, where slow cycling sub-populations may contribute to issues of chemotherapy-resistance or tumor dormancy and recurrence. Finally, we hope future studies will begin to address the question

of how a stable G_0 state is maintained long-term during the lifetime of an organism, such as that of terminally differentiated neurons, and whether/how the G_0 state may change with age to possibly become more or less flexible.

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