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Regulation of Cell Cycle Entry and Exit: A Single Cell Perspective

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Abstract

The transition between proliferating and quiescent states must be carefully regulated to ensure that cells divide to create the cells an organism needs only at the appropriate time and place. Cyclin-dependent kinases (CDKs) are critical for both transitioning cells from one cell cycle state to the next, and for regulating whether cells are proliferating or quiescent. CDKs are regulated by association with cognate cyclins, activating and inhibitory phosphorylation events, and proteins that bind to them and inhibit their activity. The substrates of these kinases, including the retinoblastoma protein, enforce the changes in cell cycle status. Single cell analysis has clarified that competition among factors that activate and inhibit CDK activity leads to the cell's decision to enter the cell cycle, a decision the cell makes before S phase. Signaling pathways that control the activity of CDKs regulate the transition between quiescence and proliferation in stem cells, including stem cells that generate muscle and neurons.

Introduction

The ability of cells to adopt two distinct stable states, a proliferating state and an arrested state, is critical for the development of multicellular organisms. Proliferation is essential while an organism is growing and developing, when additional specialized cells are required for specific physiological functions, and for replacement of lost cells. When cells proliferate, they proceed through a carefully orchestrated series of events that involve DNA replication, the assignment of chromosomes to daughter cells, and cell division. Cells can also exist in a state of cell cycle arrest that permits the organization of tissues and organs with complex, three-dimensional structures. Forming complex tissues requires cells to enter both proliferating and quiescent states at different times and locations. To form complex structures, a sophisticated and carefully regulated molecular machinery is used to allow cells to transition between proliferating and quiescent states. This molecular machinery ensures that there is a barrier between proliferation and quiescence, and thus, that cells do not transition inappropriately to quiescence or proliferation. Indeed, the molecular mechanisms

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controlling the transition between proliferation and quiescence result in a system in which a clear and persistent signal is required for cells to transition between proliferation and quiescence, and a stronger signal is required for switching between states than simply maintaining an existing state.

In complex, multicellular organisms, multiple different nondividing cellular states are represented. One nondividing state is terminal differentiation, in which cells have exited the proliferative cell cycle in conjunction with the adoption of a final and irreversible cell fate. An example of terminally differentiated cells is red blood cells that eject their nucleus upon differentiation. Cell division is also arrested when cells senesce, that is, reach the end of their lifespan because they have exceeded their maximal number of cell divisions or from stress from oncogenes. Here we focus on a temporary, reversible cell cycle exit or quiescence. While quiescence may be perceived as a state of stasis or arrest, in fact, quiescent cells, while not dividing, can actively engage pathways that enforce their nondividing and reversible state. Quiescent cells have been shown to activate genes that protect them from differentiation and senescence (83, 426). Quiescent cells induce pathways that reduce their levels oxidized proteins and maintain their viability (278, 280). Quiescent cells also engage microRNAs (464) and epigenetic changes (142) that reinforce their nondividing state.

In 1974, Lee Hartwell and his colleagues described yeast with mutations in genes important for cell cycle control. This collection of mutants allowed him to define a “start” as a point within the cell cycle at which yeast accumulate when they deplete their nutrients (198). In the same year, Arthur Pardee reported on the existence of a restriction point, or a point of no return, in the mammalian cell cycle (380). Pardee discovered that if he subjected populations of cells to cell-cycle-arresting signals before the restriction point, the cells did not proceed into the cell cycle (380, 516). Once the cells had passed this point in the cell cycle, they were committed to completing the cell cycle, even if cell-cycle-arresting signals were applied. Pardee recognized that a decision point in the G1 phase of the cell cycle, prior to S phase, would prevent cells from starting a round of DNA replication that they could not properly complete, thus reducing the risk of chromosome breaks (514). In a pioneering study involving time lapse microscopy, in 1985, Zetterberg and Larsson corroborated these findings in mammalian cells. They confirmed that removing serum in the first 3 to 4 h of the cell cycle resulted in cell cycle arrest. In contrast, when serum was removed from the same cells for the same duration, but at a different time in the cell cycle, the cells continued cycling (515).

This article reviews the mechanisms that control cell cycle entry and exit, with an emphasis on single cell analysis in mammalian model systems. In the first section, the molecules that enforce cell cycle progression and cell cycle exit are introduced and their mechanistic roles are described. Principles of cell cycle control are explained including hysteresis and positive feedback. After providing this background, the article focuses on more recent studies that investigate the control of cell cycle entry and exit using single cell analysis. Detailed analysis of cell cycle control in individual cells has become more prevalent in recent years as multiple technologies have been developed and applied. Some studies have used real-time protein-based fluorescent reporters for the restriction point coupled with flow cytometry

analysis to follow the behavior of individual cells and correlate them with other parameters. Other studies have coupled cell-cycle-based fluorescent reporters with live cell imaging to monitor the levels of key proteins before and after mitosis in cells that continue to cycle and in cells that exit the proliferative cell cycle. These studies have resulted in a more complete and rich understanding of the molecular basis of cell cycle regulation than was possible with analyses of cell populations. In the final section of the article, the regulation of proliferation-quiescence decision-making in muscle and neural stem cells (NSCs) is reviewed. Stem cell compartments in both tissues are regulated by multiple different inputs with significant overlap. An “alert” state of quiescence characterized by increased metabolism but lack of cell division was discovered in muscle stem cells. Single cell analysis of NSCs revealed that there is a continuum of states between quiescence and proliferation.

The Cell Cycle Molecular Machinery

The cell cycle is driven forward by oscillating activities of kinases activated by their interaction with their associated cyclins (343, 349, 363, 454). While cell-cycle-driving kinases are mostly constitutively present, levels of their cognate cyclins fluctuate with the cell cycle. The activities of the cyclin-dependent kinases (CDKs) vary over the cell cycle as each is activated based on its physical association with its cognate cyclin (289, 309, 341). The cyclins serve as an internal signal for whether a cell is cycling or arrested and, if cycling, its position within the cell cycle (141, 368). Cyclins also allow cells to perform cell cycle state-specific activities by activating CDKs. The central role of CDKs in coordinating cell cycle progression is highly evolutionarily conserved. CDKs were first identified in yeast (25, 198). Subsequent studies revealed that homologues of these kinases control cell cycle position and the commitment to cell division in yeast, worms, flies, and mammalian cells (25, 128, 133, 151, 197–199, 275, 295, 361).

In a mammalian cell, the initiation of a new cell cycle in resting cells is triggered by exposure to mitogens and other stimulatory signals (93, 343). These stimuli result in activation of cell cycle stage-specific CDKs. For mammals, the transition from G_0, a state outside the cell cycle, to the G1 cell cycle phase is controlled by cyclin-dependent kinases 4 and 6 (Cdk4, Cdk6) (299). These CDKs bind the G1 cyclin, cyclin D (224, 408). Cdk4 and Cdk6 phosphorylate target proteins needed for DNA synthesis. Activities of Cdk4 and Cdk6 also result in elevated levels of the next cyclin in the cell cycle, cyclin E. Cyclin E, in turn, interacts with CDK2, a critical kinase for progression to S phase (258, 368, 369). Cyclin E-Cdk2 is particularly important for promoting entry into the cell cycle by initiating DNA replication and centrosome duplication (69, 167, 211, 317, 368, 369, 450). Progression through S phase is mediated by S-phase cyclin, cyclin A, in conjunction with Cdk2. Cdk2-cyclin A complexes facilitate DNA replication, chromosome duplication, and progression through S phase (174, 343). Near the end of S phase, the M-phase cyclin, cyclin B, is expressed and its protein levels accumulate through G2 phase. Cyclin B in conjunction with its cognate cyclin, cyclin-dependent kinase 1, promotes the transition through G2/M (94, 308, 309).

CDKs, like other kinases, have an amino-terminal lobe and a carboxy-terminal lobe with a cleft between the two lobes (102, 343, 382). The last phosphate of a molecule of ATP

positioned inside the cleft is donated to substrate proteins. This activity is catalyzed by amino acids near the cleft (102, 254, 343, 382). When cyclin is not bound to a CDK, the kinase catalytic cleft is sealed off by a portion of the kinase referred to as the T loop, making the enzyme's catalytic site inaccessible (102, 308, 382). When the appropriate cognate cyclin is present and sufficiently abundant, the cyclin binds to the CDK via hydrophobic interactions (308, 382). The binding to cyclin results in increased accessibility of the active ATP binding site, a reorientation of residues in the active site (102, 343, 382), and exposure of a threonine in the T loop.

Phosphorylation of the threonine exposed by cyclin binding is performed by Cdk-activating kinase (CAK). CAK activity results in a significant increase in CDK activity (88, 107, 140, 149, 180, 194, 223, 232, 283, 318, 343, 382, 454). The catalytic portion of the CAK kinase complex in vertebrates is Cdk7, and the complex also contains cyclin H and Mat1 (149, 194). CAK activity is maintained at a high level throughout the cell cycle and is not considered a gating or regulatory step for CDK activation (341). Once the cyclin binds to a CDK, CAK rapidly phosphorylates the exposed threonine on the Cdk T loop (341). This phosphorylation event by CAK is an important step in the activation of CDKs to their fully active form. Phosphorylation of the threonine leads to a conformational change that facilitates interaction with substrates. Cyclin binding combined with CAK phosphorylation of CDKs can increase their activity by as much as 80-fold (88). The phosphorylation events that follow are often the addition of a phosphate to a serine or threonine that is directly before a proline in the amino acid sequence. The canonical recognition site for CDKs is [S/T]PX[K/R] (308, 482).

The amino acid sequences of CDK proteins include not only threonines that are phosphorylated to activate the kinase, but also sites of inhibitory phosphorylation. These inhibitory phosphorylation events keep the activity of the kinases in check until the appropriate time (150, 300, 420). CDKs can be phosphorylated at tyrosine residues (Tyr15 in humans) and, in animals, at an adjacent threonine (Thr14) (283, 300, 341, 343, 362, 420). The added phosphates are present on the "roof" of the kinase and may prevent proper orientation of ATP in the kinase's active site (102, 223, 343). The Wee1 kinase, present in all eukaryotes, and Myt1 in vertebrates, are responsible for phosphorylating these inhibitory sites. The phosphatases of the Cdc25 family remove these phosphates. Through their phosphatase activity, Cdc25 family members activate CDKs (150, 300, 343, 354, 420).

Cyclin degradation

While the levels of CDKs are relatively constant over the cell cycle, the levels of cyclins themselves vary with cell cycle entry and exit and over the course of the cell cycle (141). Because cyclins are key regulators of cell cycle progression, their levels are carefully controlled at the level of both synthesis and degradation. Transcript levels of multiple cyclins fluctuate over the cell cycle, and some cyclins are periodically transcribed by the E2F transcription factors as described below (105, 129, 166). Also critical for regulating cyclin levels is their degradation via the proteasome degradation pathway (176, 350). Proteins are targeted for degradation by the proteasome after a series of enzymatic reactions that add ubiquitins to the proteins (108). In these reactions, ubiquitin is first activated by an E1

ubiquitin-activating enzyme and then transferred to an E2 ubiquitin-conjugating enzyme. Substrate specificity is then conferred by an E3 ubiquitin ligase, which binds the substrate and either allows for the direct transfer of ubiquitin from the E2 to the substrate, or ubiquitin is transferred first to the E3 and then to the substrate. Substrates with multiple ubiquitins can become targets for degradation by the proteasome. The timely synthesis and degradation of cyclins is essential for the oscillatory nature of the cell cycle (98, 176, 343).

At the appropriate time in the cell cycle, cyclins become ubiquitinated (176, 207). The presence of multiple ubiquitins on cyclins marks them for degradation at the proteasome. There are several times during the cell cycle at which cyclin destruction is critical for cell cycle state (343). Destruction of mitotic cyclins and proteins that control sister-chromatid cohesion is essential for the transition from metaphase to anaphase (193, 216, 217, 249, 386, 463, 480, 512). Elimination of cyclins at the end of G1 allows cells to progress to S phase (73).

There are two complexes that facilitate cell-cycle-dependent protein ubiquitination and subsequent degradation by the proteasome (206): the SCF complex (named for three of its components—Skp1, cullin and the F-box protein that binds to the target) (292, 301, 510) and the anaphase-promoting complex (APC) (193, 216, 217, 249, 343, 386, 463, 480, 512). The SCF complex is present in similar amounts throughout the cell cycle. To achieve cell-cycle-dependent regulation of its activity, the SCF usually targets proteins only when they are phosphorylated at one or multiple sites (343, 371).

The SCF is important for cell cycle control because of its role in the degradation of cyclin E. Cyclin E levels peak at the G1/S boundary and then decline as DNA is replicated (125, 258). Cyclin E is both required and rate-limiting for G1 progression (368, 369, 408). Cyclin E levels decline in S phase because cyclin E is actively targeted for degradation by the proteasome (73, 191, 257, 498, 501, 508). The F box protein FBW7 in the SCF complex recognizes phosphorylated cyclin E and targets it for degradation (73, 257, 335, 462, 498, 501). Cyclin E becomes a target for degradation after being phosphorylated at conserved motifs, the Cdc4 phosphodegrons (73, 257, 335, 462, 498, 501). FBW7 has high affinity specifically for cyclin E molecules that are phosphorylated at both T380 and S384 (498). Phosphorylation, ubiquitination, and subsequent destruction of cyclin E are necessary for cyclin E's periodic expression and for cell cycle progression. If the sites on cyclin E that are phosphorylated by FBW7 are mutated, or FBW7 is inactivated, there is constitutive cyclin E-CDK2 activity throughout the cell cycle. Uncontrolled cyclin E-CDK2 activity results in abnormal S phase, chromosomal instability, and excess proliferation (135, 294, 330, 451). Consistent with Fbw7's role in preventing excessive cell division, the Fbw7 subunit of SCF^{Fbw7} is mutated in some tumor cell lines and tumors (5) and is a haploinsufficient tumor suppressor in mice (310).

The other complex that controls cell cycle protein degradation is the APC, a large multiprotein complex that promotes the ubiquitination and degradation of proteins that mediate the metaphase to anaphase transition (193, 249, 343, 463, 512). APC-mediated destruction of securin leads to activation of separase, an enzyme that catalyzes the separation of sister chromatids from each other in anaphase (217, 386). The APC is also responsible for

degrading S-phase and M-phase cyclins at the end of mitosis, through cytokinesis and during G1 (217, 343, 512). By eliminating cyclins during the portions of the cell cycle when they are not required, the APC ensures cell cycle progression.

While the SCF is activated by phosphorylation of its targets, APC activity is largely regulated via the binding of activator subunits. Cell-cycle-specific phosphorylation of core APC subunits by CDKs affects the association of specific activator proteins, resulting in different forms of the APC, with different target affinities, at different stages of the cell cycle (46, 343, 385, 386, 489, 512). In order to generate cell-cycle-specific APC activity, the activator proteins themselves are substrates of CDKs. As M-phase CDKs are activated in mitosis, they phosphorylate APC subunit CDC20 (74, 342, 343, 433, 489). An autoinhibitory loop in CDC20 prevents its binding until it is phosphorylated during mitosis (398). APC^{Cdc20} is thus formed, and this form of the APC is active during mitosis when it destroys securin, thus releasing separase. Separase then cleaves cohesin, thereby allowing sister chromatids to segregate from each other, and the cell to complete mitosis (343, 489). APC^{Cdc20} also targets M-phase cyclins for degradation. Thus, activation of the APC by M-phase CDK activity promotes M-phase cyclin destruction, leading to lower M-phase CDK activity, and less phosphorylation of the APC (343, 463). This negative feedback loop ensures that cells receive a strong, sharp peak of M-CDK activity that rapidly declines.

As the cell progresses to late mitosis and early G1, the accessory subunit associated with the APC changes from CDC20 to Cdh1, and the dominant form of the APC becomes APC^{Cdh1}. Cdh1, in contrast to Cdc20, binds better when APC subunits are not phosphorylated (343, 489). APC^{Cdh1} ubiquitinates and degrades Cdc20, ensuring an orderly progression of the APC from its APC^{Cdc20} form in metaphase and anaphase to its APC^{Cdh1} form in G0/G1 (210, 397, 412, 447, 456). APC^{Cdh1} also ensures that S and M cyclins are destroyed during quiescence and G1 until the cell commits to the next cell cycle (343, 386, 489). In early mitosis, phosphorylation of Cdh1 prevents its interaction with the APC (188, 513). At this time in the cell cycle, Cdh1 is phosphorylated, and APC^{Cdc20} accumulates again, continuing the cycle (386). Later in mitosis, activation of phosphatases results in dephosphorylation of Cdh1 and restoration of APC^{Cdh1} activity (222, 488).

Cdh1 is associated with quiescence and differentiation in multiple species. In *Drosophila* and *Xenopus*, Cdh1 is not expressed in rapidly dividing embryos (296, 386, 448). Cdh1 expression is induced with differentiation and establishment of a G1 phase of the cell cycle (296, 386, 448). Fission yeast cells that do not have Cdh1 can proliferate but, when challenged with nutrient starvation, they fail to arrest properly in G1 (252, 260, 386). APC^{Cdh1} is expressed in postmitotic cells, including the brain (170). In conjunction with other methods for reducing CDK activity, APC^{Cdh1} plays an important role in quiescence and the restriction point as described further below (386).

Checkpoints

Cells also have the ability to quickly, effectively, and reversibly inactivate the cell cycle. These mechanisms, called checkpoints, can be invoked to induce cells to exit the proliferative cell cycle, to maintain cells in G_0, or to arrest cell cycle progression. Cell cycle progression can be halted by gating events that occur at multiple different points within the

cell cycle, a process referred to as checkpoints (137, 200, 343, 349, 363). These checkpoints help to ensure that a cell does not progress through the cell cycle if it has not completed the previous phase successfully, and that cells do not enter cell cycle phases for which they are not prepared (285, 429, 497). One important checkpoint regulates whether a cell will enter the proliferative cell cycle (G1) or remain quiescence (G_0) (200, 380). There are additional checkpoints that monitor the transition between cell cycle phases in cycling cells. In yeast, there is evidence for a cell size checkpoint that ensures that only cells that have reached a certain size progress through G1 and G2 (20, 243, 244). DNA damage can also induce a checkpoint arrest during interphase or G2 (20, 172, 265, 497). In some eukaryotes, the tumor suppressor transcription factor p53 can enforce a prolonged G2 arrest if DNA damage is persistent (20, 55, 460). During S phase, if DNA replication stalls, checkpoints are in place to ensure mitosis does not progress until DNA replication is complete (8, 20, 138, 291, 410). A checkpoint also occurs during the metaphase to anaphase transition in mitosis when the APC triggers the separation of sister chromatid chromosomes (285, 343). Activation of this spindle checkpoint prevents accumulation of the APC^{Cdc20} if the chromosomes are not attached to spindle microtubules or if the microtubules are not under tension (351). The spindle checkpoint protein Mad2 inhibits Cdc20 activity until the spindle is properly assembled (63, 212, 219, 248, 285). In addition to the ability to induce a pause in the cell cycle, in some situations, activation of a cell cycle checkpoint can result in cell death, thus protecting the rest of the organism from a cell that could contain pro-tumorigenic chromosomal aberrations (20, 56).

Cyclin-dependent kinase inhibitors

One mechanism to halt the cell cycle is the activation of kinases that introduce inhibitory phosphorylations to CDKs as described above (410, 479). In addition, there are also protein inhibitors that block CDK activity (28, 195, 393, 444, 445). These inhibitors can bind to and inactivate CDKs, which results in reduced CDK activity, and can arrest the cell cycle (382, 444, 445). Eukaryotic organisms as diverse as yeast, *Drosophila*, and mammals encode at least one CDK inhibitor (103, 343). In the budding yeast *Saccharomyces cerevisiae*, there are two well-studied CDK inhibitors: Far1 and Sic1 (343). Far1 was recognized as a pheromone-induced inhibitor of G1 cyclin-CDK complexes (62). Far1 protein levels are elevated in early G1 and decline after yeast cells pass through START (323). In response to pheromones, Far1 is induced, and this induction arrests the cell cycle (384). Sic1 inhibits cyclin-CDK complexes that are required for progression through DNA replication and mitosis (434). Phosphorylation of Sic1 by CDKs leads to its ubiquitylation and proteolysis, thereby activating CDK activity and promoting S-phase entry (487). This feedback mechanism, like periodic cyclin destruction, contributes to bursts of CDK activity that progress cells through the cell cycle.

In mammals, there are two families of CDK inhibitors defined based on their evolutionary origins, structures, and mechanisms of inhibiting CDK activity: the CIP family and the INK4 family (28, 103, 445). Members of the CIP family of CDK inhibitors block the activity of cyclin-CDK complexes by binding to both the cyclin and the CDK thereby significantly impairing the ability of the complex to phosphorylate target proteins (28, 393). CIP family CDK inhibitors can bind to cyclin-CDK complexes when the associated cyclin is

cyclin D, E, A, or B (28, 445). For this reason, CIP family CDK inhibitors are effective throughout the cell cycle. There are three members of the CIP family: CDKN1A ($p21^{Cip/Waf1}$) (136, 185, 192, 503), CDKN1B ($p27^{Kip1}$) (392, 393, 475), and CDKN1C ($p57^{Kip2}$) (276, 319). The crystal structure of the p27 CDK inhibitor has been determined (421). When p27 binds to a cyclin-CDK complex, p27 interacts with the amino terminal lobe of the kinase, thereby distorting the active site, blocking ATP binding, and rendering the enzyme incapable of performing its function (382, 421).

The members of the CIP family are important for restraining cell proliferation during development and differentiation and reducing the cycling of cells under stress (445). Each inhibitor in the CIP family has different contexts in which it is active. p27 levels are elevated in cells that are induced into quiescence, and it is rapidly degraded as cells enter the cell cycle (29, 75, 280). Mice with loss of p27 are larger than wild-type controls and exhibit hyperproliferation in multiple organs (145). $p27^{-/-}$ mice spontaneously develop pituitary adenomas and are more likely to develop cancer when treated with chemical carcinogens (144, 145, 253, 353). One of the mechanisms that control p27 degradation is its phosphorylation at threonine 187. The T187 phosphorylated form of p27 is recognized by the Skp2 protein of the SCF complex, which ubiquitinates p27 and targets it for degradation (355, 376, 440, 478, 490). The CIP family CDK inhibitor p21 also plays an important role in arresting the cell cycle, but in different contexts. p21 can block G1/S- and S-CDKs and is often induced by tumor suppressor p53 in response to DNA damage (136, 164). p21 induction provides cells with time to repair DNA damage before the cell cycle proceeds. Cells that lack p21 fail to undergo cell cycle arrest in response to DNA damage (106). Finally, the third CDK inhibitor of the CIP family, p57, has an important role in reducing cell proliferation during development. p57 is induced in cells that are committing to a differentiated lineage (168, 179, 483). Mice with knockout of p57 have delayed differentiation and hyperplasia (518).

The INK4 family of CDK inhibitors includes INK4A (p16), INK4B (p15), INK4C (p18), and INK4D (p19) (3, 28). These proteins are more restricted than the CIP family of CDK inhibitors in the cyclin-CDK complexes they recognize. INK4 family members bind to CDK4 and CDK6 and prevent their association with D-type cyclins (28, 43, 343, 382, 422) through an interaction that is distinct from the interaction that occurs between cyclins, CDKs, and CIP family inhibitors (43, 382, 422). When INK family proteins inhibit CDKs, they bind both the amino and carboxy terminal lobes of the CDKs and twist the lobes with respect to each other in a way that disrupts the interface between the CDKs and their associated cyclins (43, 382, 422). They may also sterically block access of cyclins to CDKs (43, 343, 382, 422). INK family members can result in cell cycle arrest in several situations including in response to signaling from the cytokine TGF-β (409) and during senescence (7). p16INK4A, a member of the INK family of CDK inhibitors, is a tumor suppressor that is homozygously deleted or silenced via methylation at high frequency in melanoma and multiple different tumors (30, 233, 234, 259, 286, 359, 413, 521). Thus, inactivation of CDK inhibitors is associated with inappropriate proliferation in the context of development and tumor formation.

Retinoblastoma (RB) and the E2F transcription factors

The G1/S cyclins, the D-type cyclins in humans, are unusual because their levels do not respond to a cell cycle position, but rather increase as cells grow and in response to extracellular mitogenic signals. The level of D-type cyclins is an important regulator of the transition from quiescence to proliferation (273, 459, 506). When cyclin D associates with CDK4 or CDK6, these kinases can be activated through subsequent positive and negative phosphorylation events to phosphorylate their targets. Targets of cyclin D-CDK4/CDK6 include the tumor suppressor retinoblastoma protein (RB) (45, 67, 87, 131, 143, 235, 343, 419, 443). Proteins in the retinoblastoma protein family (Retinoblastoma, p107 and p130) are important for restraining the activity of the E2F activator transcription factors (16, 65, 72, 104, 343, 461). When these Retinoblastoma family proteins are phosphorylated on multiple amino acids by cyclin D-CDK4/CDK6, E2F transcription factors are released (44). RB inhibits E2F-dependent gene expression by binding the transcriptional activation domain at the carboxy terminus of E2F activator transcription factors (419). In early G1, RB is in a hypophosphorylated state that binds and inhibits E2F (3, 329). Entry into S phase is accompanied by RB's conversion to a hyperphosphorylated state (3). The initial stages of RB phosphorylation are mediated by cyclin D-CDK4/CDK6 complexes (208, 299). Mitogens can contribute to this process by increasing transcription of cyclin D, thereby promoting the activity of cyclin-CDK complexes that phosphorylate RB and enable RB's dissociation from E2F (114, 132, 333, 419). RB family members also bind to E2F-bound chromatin and recruit repressive chromatin remodeling complexes (231, 343, 405, 419).

The activity of E2F transcription factors is regulated by binding to retinoblastoma family proteins (3), as well as partner proteins of the DP family (270, 271). Phosphorylation of E2F can also increase its binding to DNA and stability. Release of activator E2F transcription factors from retinoblastoma family proteins leads to expression of genes involved in cell cycle progression and DNA replication (16, 114, 132, 155, 208, 437). Transcriptional targets of E2F family activators include cyclin E. Thus, E2F activation increases the levels of cyclin E-CDK2 activity, the key regulator of the next phase of cell cycle progression. Cyclin E-CDK2 can then further phosphorylate RB, resulting in even more molecules of E2F being released from RB. As a result of this positive feedback loop, E2F activity rises sharply at the G1/S boundary (3, 201, 290). Activation of E2F-regulated transcripts, including transcripts encoding the S-phase cyclin, cyclin E, promotes cell cycle entry and entrance into S phase (16, 34, 105, 114, 129, 130, 166, 255, 343).

Through evolution, the activities of E2F gene family members have evolved to activate or repress different gene sets, including those needed later in the cell cycle (407). Activator E2Fs include E2Fs 1 to 3, while E2F3b, E2F4, and E2F5 are repressors that bind to promoters in quiescent cells and inhibit gene expression (114, 156, 343, 468, 476). As cells enter the cell cycle and RB family members are phosphorylated, repressor E2Fs are removed from G1/S gene promoters and are replaced with activator E2Fs (114, 132). Cells with defects in RB family proteins have the ability to proceed through the cell cycle, but can not stop proliferating in response to extracellular signals or DNA damage (10, 196). Consistent with its critical role in cell cycle arrest, RB is a tumor suppressor (123, 154, 162) and was named for its association with the eye tumor retinoblastoma (256). The majority of human

cancers have mutations in the retinoblastoma gene or other genes that result in functional retinoblastoma inactivation (72, 343, 443, 486).

Characteristics of the network controlling cell cycle commitment

An important principle of cell cycle progression is that cells should not be in multiple cell cycle states simultaneously (146, 343, 394). In order to achieve this, CDK activity rises sharply to its maximal activity at the appropriate stage of the cell cycle (146, 343, 394). Achieving this almost switch-like behavior results from the presence of multiple feedback loops in the cell cycle that ensure that increasing levels of mitogenic signaling result in an abrupt increase in CDK activity rather than a gradual, linear increase in CDK activity (146, 316, 343, 481). One example described above is the positive feedback loop that results as partial phosphorylation of RB releases E2F proteins. These E2F proteins then transcriptionally induce cyclin E (315), which leads to increased cyclin E-CDK2 activity. Cyclin E-CDK2 then further phosphorylates RB, leading to a more complete release of E2F, thus further activating its cell-cycle-promoting targets (299). Through this positive feedback loop, the commitment to S phase is reinforced (3). The multiple phosphorylation events needed to activate G1/S transcription result in a system that has properties of ultrasensitivity —small increases in the amount of signal result in no response, while increasing the signal above a threshold level results in maximal response (91, 146, 181). The example also demonstrates another important attribute of the cell cycle network. Events within a specific cell cycle phase activate signals for the following phase, ensuring that the cell proceeds through cell cycle phases in the correct order (343).

A second example of positive feedback in the cell cycle involves the addition and removal of inhibitory phosphates covalently attached to CDKs. The Wee1 kinase was identified as a gene regulating mitosis in fission yeast (364) and was discovered to add inhibitory phosphates to CDKs (367, 394, 420). This ensures that CDKs can be synthesized and held in check until they are needed. M-phase CDKs can phosphorylate and inactivate Wee1 (322, 343). This will make it less likely that new phosphates will be added to M-phase CDKs once a cell is committing to entering mitosis. M-phase CDKs can also phosphorylate Cdc25, which removes the inhibitory phosphates (150, 453). Phosphorylation of Cdc25 activates it, resulting in even faster and more robust removal of inhibitory phosphates from M-phase CDKs (209, 220, 267, 343). Thus, any M-phase CDKs that are synthesized and held in check by inhibitory phosphates during G2/M will be rapidly dephosphorylated and converted into their active form once the cell enters M phase, resulting in a burst of M-phase CDK activity (343, 453). Cell cycle signaling is also characterized by redundancy. For most of the important steps in cell cycle control, there are multiple layers of regulation, so that even if the gene encoding one molecule is faulty, for most molecules, cells will nevertheless cycle due to the activity of redundant pathways (343). A final important attribute of the biological network underlying the cell cycle is its adaptability to different conditions via modifications to the levels and activities of different proteins at different times and in different contexts (343).

Mitogenic signaling regulates cell cycle entry and exit

One important control step for cell cycle decision-making involves the regulation of cyclin D levels. Cyclin D levels can be controlled through receptor tyrosine kinase signaling, which responds to the levels of extracellular mitogens and serves an important role in determining cell cycle entry (2, 226, 277, 396). Receptor tyrosine kinase ligands such as fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) can serve as potent stimulators of cell cycle progression (228, 441, 504). The signaling pathway from receptor tyrosine kinase activation to activation of cyclin D can be mediated through the Ras-effector pathway (3, 152, 312). Upon activation, Ras binds to and activates the mitogen-activated protein kinase (MAPK) Raf (169, 339, 491). Raf, in turn, begins a kinase cascade by phosphorylatig mitogen-activated protein kinase (MEK), which phosphorylates extracellular signal-regulated kinase (ERK) (3, 152, 312, 339). ERK can then translocate into the nucleus and activate transcription of a set of genes termed “immediate early” genes (3). Immediate early genes include Ets and AP1 transcription factors, such as Fos and Jun, that increase cyclin D1 expression (3, 182, 202, 205, 312, 366). The transcription factor Myc is also induced as an immediate early gene (18). Myc forms heterodimers with its partner protein Max (32, 33, 187, 492) and activates transcription of cell-cycle-promoting genes cyclin D2 (40, 82), CDK4 (82, 204), Cdc25A (163), E2F2 (436), and cyclin E (383).

Integrins and cell cycle progression

Integrins are heterodimers of alpha and beta chains that provide information about the extracellular matrix to the actin cytoskeleton (213). Integrins can signal to the cell cycle machinery through the ERK signaling pathway (417). In order for ERK signaling to result in cell cycle progression, short-term treatments are not sufficient; cyclin D1 induction will only result from ERK signaling that is sustained for hours (324, 496, 520). Further, growth factors do not induce cyclin D if they are administered to cells that lack an extracellular matrix (37). Based on these results, Assoian and colleagues found that in fibroblasts, integrin and receptor tyrosine kinase (RTK) signals synergize to determine the fraction of ERK molecules that are activated. In particular, attachment of fibroblasts to fibronectin, or stimulation of $\alpha_5\beta_1$ integrin, in conjunction with mitogenic signals, resulted in sustained ERK activity that successfully resulted in activation of cyclin D1 and cell cycle progression (417).

Relationship between cell cycle proteins and DNA replication

Activation of cyclins is important for the initiation of DNA synthesis. In early G1 phase, the prereplication complex of proteins assembles at locations on DNA that are destined to be origins of replication (113, 171, 241, 343, 469). These prereplication complexes contain a multiprotein complex, the origin recognition complex (279, 343, 469). In early S phase, Cdc6 and Cdt1 are recruited to existing prereplication complexes to form active preinitiation complexes (279, 343, 403). Subsequently, the minichromosome maintenance (MCM) complex, a helicase that unravels the DNA helix (71, 279, 343, 403), is recruited. The MCM complex will initiate replication at origins of replication and progress in both directions. The DNA polymerase replicates the DNA as the MCM complex unwinds chromatin (279, 343).

Each origin will be used once, and then the origin will be inactive until the next S phase (79, 343, 358).

Cell cycle molecules, cyclins and CDKs, are important regulators of DNA synthesis (279, 358, 469). M-phase cyclin-CDK complexes help to prevent origins from firing at the wrong time through multiple mechanisms (95, 357). When CDKs phosphorylate Cdc6, Cdc6 can then be recognized by the SCF, leading to Cdc6 ubiquitination and proteolysis at the proteasome (121, 122, 343, 357). As a result, Cdc6 only accumulates in G1 phase when M-phase CDKs are inactive (343, 357). M-phase cyclin-CDK complexes can also phosphorylate MCM proteins, which results in exclusion of MCM proteins from the nucleus (272, 287, 343, 356, 357, 470). In addition to mechanisms that inhibit the firing of origins during the portions of the cell cycle in which they should be inactive, cyclins and CDKs also promote prereplication complex formation during S phase (109, 343, 469). Activation of S-phase cyclin-CDK complexes, such as cyclin E-Cdk2, phosphorylates Cdc6 in a manner that stabilizes it and promotes prereplication complex assembly during late G1 (89, 92, 307, 343, 358, 388, 493). Thus, the waves of different cyclin-CDK complexes that are active at different times during the cell cycle facilitate the commitment to DNA replication at the appropriate time and prevent DNA over-replication.

Single Cell Analysis of Cell Cycle Entry and Exit

Most studies performed on the cell cycle have been performed on populations of cells (316). Results from these studies will be an average compiled from many different cells. Studies that involve analysis of cell population averages will not provide information on the variability from cell to cell. Cell-to-cell heterogeneity can play an important role in biological decision-making under conditions where the different cells within a population diverge in their fate. Heterogeneity in cell cycle progression can result from differences in the gene or protein level of individual genes important for cell cycle commitment (70, 321, 452). In addition, each time a cell divides, the two daughters will receive different amounts of critical cell cycle regulators (124). Recent studies have sought to gain more insight into cellular decision-making by employing single cell approaches to tackle the question of cell-to-cell heterogeneity in the commitment to proliferation and the restriction point (97, 316). These studies have shed light on the molecular basis for the heterogeneity in cell division among cells within a clonal population.

RB-E2F axis as a critical determinant of the restriction point

Some influential, early single cell studies of the cell cycle focused on the role of the RB-E2F axis in controlling the restriction point. Previous studies had shown that cells in which all RB family members are knocked out proceed through the restriction point inappropriately (96, 424), while cells with inactivation of all activator E2Fs are not able to pass the restriction point (502). Further, introducing E2F activity was found to be sufficient to induce quiescent cells to enter S phase (225). Motivated by these studies, Guang Yao and his colleagues in the laboratory of Joseph Nevins developed a reporter for E2F activity that could be used to monitor E2F activity in individual cells with flow cytometry (507). Yao and colleagues engineered cells to express green fluorescent protein (GFP) transcribed under the

control of the E2F promoter. They ensured that the GFP that was synthesized would be rapidly degraded. Destabilized GFP driven by the cyclin D promoter rather than the E2F promoter served as a control. For GFP driven by the E2F promoter, but not GFP driven by the cyclin D promoter, they observed a bistable system in which each cell either had very low GFP expression or a significant amount of GFP expression. Adding different amounts of serum resulted in E2F activity that was either “off,” when serum levels were lower than a threshold, or “on,” when serum levels were high. In contrast, levels of GFP driven by the cyclin D promoter were proportional to the amount of serum present. This type of analysis would not have been possible if only cell populations were analyzed because the all-or-nothing behavior of the E2F promoter would be obscured by averages that would show an apparent linear response in E2F activity with serum as more cells switched from low to high activity.

Yao and colleagues also investigated whether the system displayed hysteresis. Hysteresis is a property of a system in which it has a memory, that is, the same amount of stimulus will result in a different effect depending on the system’s history. Previous studies by the Ferrell laboratory had shown that CDKs exhibit hysteresis in their activation (394). Yao and Nevins confirmed and extended these findings. They found that a pulse of 0.3% serum was not sufficient to activate E2F in cells in which E2F was off. However, once E2F had been turned on by 10% serum, even if serum levels were reduced to 0.3% serum, E2F activity remained on. Indeed, in the range of 0.2% to 1% serum, most cells had low levels of E2F if they had previously experienced low serum, but high E2F activity if they had previously been maintained in higher serum conditions. Further, once E2F activity was turned on by sufficiently high levels of serum, E2F activity was maintained at a high level even if serum levels were lowered, thus demonstrating hysteresis in the system.

By combining the analysis of E2F-driven GFP expression with DNA content analysis, Yao and colleagues could test whether the specific cells with high E2F activity were more likely to progress through the cell cycle. Indeed, they found that the individual cells with high E2F expression were the same cells as the cells that contained greater than 2N DNA content after serum stimulation. Thus, high levels of E2F activity correlated with the ability of the cells to transit through the restriction point and proliferate, a conclusion that was much stronger when supported by single cell data. Yao, Nevins, and colleagues also investigated the temporal effects of serum treatment. They found that when quiescent cells were treated with 20% serum, a treatment of 2 to 4 h was required to activate E2F expression. The results were consistent with a model in which the cells pass through the restriction point after 2 to 4 h of continuous activation. The authors concluded that E2F activity shares essential properties with the restriction point (380, 515) because both E2F activity and the restriction point are gating for cell cycle entry.

In a more recent work, Yao and his colleagues investigated the molecular basis for the heterogeneity in the transition from quiescence to proliferation in individual cells (494). Yao and colleagues noted that in a clonal culture, individual cells will reenter the cell cycle in response to serum at different rates (41, 472, 515). Further, when challenged with non-saturating serum stimulation, some cells will reenter the cell cycle, while others will remain quiescent (41, 472, 507). Based on previous studies showing that cell size is an important

determinant of whether a cell will progress through the cell cycle (19, 112, 120, 298), they tested whether cell size correlates with exit from quiescence. The authors labeled quiescent cells with a membrane-permeable dye that binds to intracellular amines and provides information on cell size. They found that after a serum pulse, large quiescent cells with high dye intensity were more likely to switch from an E2F “off” to an E2F “on” state after a serum pulse. Further experimentation with multiple pulses revealed that the size of the cells when they first entered quiescence correlated best with whether the cell would transition to an E2F-positive cell (80, 81).

Yao and his colleagues also built upon their single cell analysis platform to investigate the relationship between RB-E2F pathway activity and quiescence depth (269). Previous studies had shown that cells that have been quiescent for longer periods of time require a longer time to reenter the cell cycle (17, 42, 80, 374, 455, 505). There is evidence that this distinction between shallow and deep quiescence is also important in organisms; cells in some tissues exhibit slower cell cycle entry and cells in other tissues reenter the cell cycle more readily (269). Yao and his colleagues showed that for some cells, the minimum serum concentration required to activate RB-E2F transcription factors defines the depth of quiescence (269). Cell populations that had been maintained in quiescence by serum starvation or contact inhibition for longer periods of time had fewer cells actively dividing in response to the same serum stimulus. Further, when E2F activity was monitored with a reporter, cells that had been maintained in quiescence for longer required additional time to activate E2F transcriptional activity, and a smaller percentage of cells were “on” for E2F activity in response to the same serum concentration and the same time. They concluded that cells that are more deeply quiescent have a higher threshold for E2F activation. Yao and colleagues then tested the effects of specific molecules that control the RB-E2F axis on quiescence depth. They found that introducing p21, RB, or RB homologue p130 all resulted in deeper quiescence indicated by reduced incorporation of the modified nucleotide EdU upon stimulation with 3% serum. In particular, introduction of p21 greatly reduced the extent of EdU incorporation. Conversely, ectopic expression of either cyclin D or Myc resulted in more shallow quiescence, in which cells incorporated more nucleotides in response to the same amount of serum stimulation. Taken together, the findings indicate that on a cell-by-cell basis, the RB-E2F axis, and its regulators—cyclins, Myc, and CDK inhibitors—can control the depth of cellular quiescence.

Monitoring cell cycle in cells in real time with a CDK2 sensor

An alternative and complementary approach to understanding the relationship between quiescence and proliferation in individual cells was taken by the Meyer laboratory. In 2013, Spencer, Meyer, and colleagues reported on a live cell sensor for CDK2 activity (459). They engineered a fluorescent sensor that contains four consensus CDK phosphorylation sites, a nuclear localization signal, a nuclear export signal, and a yellow fluorescent protein (186, 459). The sensor localizes to the nucleus at the beginning of the cell cycle and moves out of the nucleus as the cell cycle progresses. The sensor localizes to the cytoplasm at the end of G2. CDK2 activity can be determined as the ratio of fluorescence signal in each cell in the cytoplasm to the nucleus.

Cells expressing this CDK2 sensor were monitored over time with real-time imaging and individual cells were tracked with computer algorithms (459). Previous studies reported that there is an increase in CDK2 activity in late G1 and again in S phase (125, 258, 369), and the authors observed these two peaks of activity. As the cells emerged from mitosis, the authors discovered that there was a bifurcation point, with each cell adopting one of two distinct fates (459). Most of the cells exited mitosis with an intermediate amount of CDK2 activity and continued to accumulate CDK2 activity as they transitioned into the next cell cycle. In addition, there was a small number of cells that failed to accumulate CDK2 activity at the end of the previous cell cycle and did not immediately enter a new S phase. Using an siRNA against cyclin A, they found that the initial rise in CDK2 activity after mitogen stimulation did not require cyclin A, but the later rise in CDK2 activity was dependent on cyclin A (459). Spencer, Meyer, and colleagues further demonstrated that the population of cells that do not immediately enter S phase after stimulation with mitogens had low levels of CDK2 activity from the previous mitosis (459). While cells with average CDK2 activity levels exhibited intermitotic times of 16 to 20 h, cells with low levels of CDK2 activity had intermitotic times of 20 h to greater than 50 h (459). Whether a cell had high or low CDK2 activity was clear by 4 h after anaphase, thus CDK2 activity predicted later cell cycle progression. If an inhibitor of the MAP Kinase signaling pathway was added 1 to 3 h after anaphase, it could inhibit CDK2 reactivation. In contrast, treatment with a MAP Kinase inhibitor at four or more hours after the previous anaphase did not affect the upcoming increase in CDK2 activity (459). In determining the source of the variability in CDK2 activity between cells, they found that cells had the same CDK2 activity level as their sister cells (either increasing or low) 98% of the time, suggesting daughters derived from the same mother had shared characteristics that determined whether to progress through the cell cycle.

Spencer and colleagues developed a method to perform immunofluorescence on the cells at the end of the time lapse movies and used this approach to monitor the levels of different cell cycle proteins. They discovered that phosphorylated RB and p21 were statistically significantly different in cells with increasing CDK2 activity compared with cells with low CDK2 activity. RB phosphorylation correlated with CDK2 activity, which was expected because it is a substrate of CDK2. p21 levels were found to anticorrelate with CDK2 activity: high p21 was associated with low CDK2 and cell cycle exit. When $p21^{-/-}$ cells were monitored, very few individual cells exited the cell cycle. By adding back different levels of p21, the commitment to cell cycle could be titrated; cells with higher levels of p21 had lower likelihoods of dividing. The findings therefore demonstrate the importance of p21 for the commitment to proliferation in the model system studied.

In a follow-up paper, Yang, Meyer, and colleagues investigated how cultured cells with the same genetic background make different choices about continuing to cycle or leaving the cell cycle (506). They found that cells do not erase their signaling histories when they reach cell cycle checkpoints, but rather, “remember” their history. More specifically, they found that mother cells transmit p53 induced by DNA damage to their daughter cells and cyclin D1 mRNA to newly born daughters. Information about the levels of these two factors, cyclin D1 and p21 produced by p53, can predict whether daughter cells will cycle. The daughters therefore control the decision whether to cycle by converting mitogen and stress signals, mediated by a competition between cyclin D and p21, into a binary decision.

The anaphase-promoting complex (APC) as a component of a commitment point

In another line of experimentation, Cappell, Meyers, and colleagues investigated in more detail the specific molecular event that defines the point of no return within the cell cycle (52). Cappell and colleagues investigated inactivation of the E3 ubiquitin ligase anaphase-promoting complex APC^{Cdh1} that occurs between pRB-E2F activation and DNA synthesis. As described above, APC^{Cdh1} degrades many of the proteins necessary for S phase. Cycling cells activate APC^{Cdh1} at the end of mitosis and quiescent cells maintain high levels of APC^{Cdh1}, which helps them remain outside the cell cycle (134). Cells with inactivation of APC^{Cdh1} exhibit a shortened G1 phase, accumulate DNA damage and apoptose (387). Cappell, Meyer, and colleagues found that APC^{Cdh1} levels are reduced to less than 1% of their previous G1 activity levels within an hour before the cells start to replicate their DNA (52). Exposing cells to stress between RB phosphorylation and APC^{Cdh1} inactivation resulted in a halt of the cell cycle (322). However, if the cells were exposed to stress after APC^{Cdh1} inactivation, the cells progressed through the cell cycle and ignored the stress (322). Their findings support APC^{Cdh1} inactivation as a novel commitment point between the restriction point and DNA replication.

Cappell, Meyer, and colleagues showed that CDK2 can inhibit APC^{Cdh1}, and thus promote the accumulation of proteins needed for DNA synthesis. This would predict that APC^{Cdh1} activity would decline linearly with increasing CDK2 activity (53). In contrast, they found that APC activity demonstrates hysteresis: doses of CDK inhibitors that were sufficient to activate the APC when it was already “on” were not sufficient to activate the APC when it had been “off” (53). Investigating further, they discovered that during G1, cells have low early mitotic inhibitor (EMI1) levels and high APC^{Cdh1} activity, while during S and G2 phases, cells have high EMI1 levels and low APC^{Cdh1} activity. Cell cycle commitment was found to correlate with EMI1 transitioning from serving as a substrate of APC^{Cdh1} to becoming an inhibitor of APC^{Cdh1} (53). At low concentrations, EMI1 binds to a low affinity site on APC^{Cdh1} and EMI1 becomes mono- and polyubiquitinated by the activity of the APC. As EMI1 levels increase, EMI1 binds to an inhibitory site of APC^{Cdh1}, thereby suppressing polyubiquitination of unbound EMI1 and other substrates. Under these conditions, the proteins required for cell cycle progression can accumulate. In this way, the commitment to cell cycle progression becomes irreversible as high EMI1 irreversibly inhibits APC^{Cdh1}. The results offer the molecular basis for a new commitment point in the cell cycle.

Monitoring quiescence entry and exit in individual cancer cells

The laboratory of Sridhar Ramaswamy has investigated similar questions about the control of cell cycle progression focusing on cancer cells. Using cell sorting and microscopy, Dey-Guha, Ramaswamy, and colleagues found that cancer cells could divide asymmetrically to produce a daughter cell that transiently exits the proliferative cell cycle (111). Slowly proliferating progeny were more abundant after treatment with chemotherapy agents and resulted from asymmetric suppression of AKT kinase signaling in one daughter cell during telophase (111). AKT kinase is recruited to the plasma membrane in response to tyrosine kinase receptor signaling, and its activity can initiate signaling pathways leading to mTORC1 kinase, a crucial regulator of cell growth. While one daughter cell with normal

AKT activity would rapidly enter the next cell cycle, the other daughter cell with low AKT activity required a longer period of time before it proceeded through the cell cycle (111). The AKT^{low} cancer cells were more resistant to treatment with chemotherapeutic agents, thus making them a potential reservoir of treatment resistance (111). Cells with mutations in AKT1 did not produce asymmetric divisions with one dividing cell and one G_0-like cell, while treating cancer cells with compounds that displace AKT1 from the cell membrane increased the frequency of asymmetrical divisions (110). Further studies addressed the prevalence of $AKT1^{low}$ quiescent cancer cells in tumors (230). Identifying AKT^{low} cells using immunofluorescence microscopy revealed that AKT^{low} cancer cells are distributed in clusters in primary tumors (230). Whereas many cells within the tumor died after treatment with neoadjuvant chemotherapy, AKT^{low} cells persisted in primary tumors and metastases, though many cells had died. The findings are consistent with quiescent cells being resistant to chemotherapy and low AKT levels being a marker for quiescence.

Proliferation-quiescence Decisions in Stem Cells

Many types of mammalian cells can enter a reversible, nondividing state including hepatocytes, T cells, fibroblasts, and stem cells. Here we focus on adult stem cells as an example of a cell type that is reversibly quiescent (439). Using the molecular framework described above, we consider existing knowledge on the role of cell cycle proteins in controlling the regulation of adult stem cells. Adult stem cells have the ability to both self-renew and generate specialized progeny that can replace lost cells from cell death, aging, or damage (66, 83, 336, 370, 432, 511). Adult stem cells are usually found in a reversible quiescent state. After being activated to proliferate, they retain the ability to return to a quiescent state. This ability of stem cells to enter and exit quiescence allows for the maintenance of the form and architecture of complex tissue, despite numerous rounds of injury, throughout an organism's life. Adult stem cells often reside within a specific structure, called a niche, that creates a suitable microenvironment. The stem cell niche can be important for providing signals that maintain stem cells in a quiescent state and that regulate stem cell division (263, 284). We consider here the contributors to the proliferation-quiescence transition in muscle and nerve stem cells.

Muscle stem cells are in the satellite position

Muscle fibers are composed of sarcomeres rich in actin and myosin that serve as the contractile units. These sarcomeres are surrounded by a sarcolemma, which functions as a plasma membrane and plays additional roles involving action potential propagation and excitation–contraction coupling (50). Surrounding the sarcolemma is a basement membrane composed of a basal lamina. Between the sarcolemma and the basal lamina reside muscle satellite cells that were first identified using electron microscopy and named based on their location in the periphery of rat and frog muscle fibers (236, 320). The basolateral side of the satellite cells is adjacent to the myofiber plasma membrane, while the apical side lies next to the basal lamina.

These muscle satellite cells are adult quiescent muscle stem cells. Skeletal muscle undergoes a phase of intense growth after birth (177, 313). During this time, the muscle satellite cells

are abundant (30% of nuclei) and highly proliferative (9, 431). The progeny of satellite cells differentiate into myoblasts (320, 428). The myoblasts can continue to proliferate and differentiate into postmitotic myocytes that fuse with existing fibers (313, 415).

Adult muscle stem cells transition from quiescence to proliferation in response to injury

Skeletal muscle can repair itself after damage and maintain its contractile abilities (26, 313). After injury, newly formed satellite cells are the primary cells in muscle tissue that contribute to the regeneration and repair of skeletal muscle in adults (64, 439). Upon stimulation, quiescent muscle satellite cells will enter the cell cycle, proliferate to form myoblasts, and regenerate satellite cells (495).

Lineage trace and transplantation studies have shown that subpopulations of satellite cells can self-renew and differentiate to reform muscle fibers during repair of muscle injury (59, 84, 264, 338, 423, 439). Adult skeletal muscle stem cells can be identified based on their expression of paired-box protein Pax7 (439). Pax7 is a critical regulator of satellite cell survival and is required for muscle tissue homeostasis (262, 373, 406, 435). $Pax7^+$ cells in uninjured muscle exhibited no detectable bromodeoxyuridine (BrdU) labeling, demonstrating that Pax7-positive muscle satellite cells are quiescent (439). This finding is consistent with other studies showing that muscle stem cells in the satellite position are proliferatively quiescent (467, 500). These cells have been further characterized as having low cytoplasmic to nuclear ratio, low metabolic activity, and few mitoses (68). The reduced metabolic activity of stem cells has been proposed to protect them from stress induced by oxidative respiration (68). In contrast, sections from regenerated muscle that were injured and then labeled with BrdU revealed that quiescent $Pax7^+$ cells had incorporated and retained BrdU, showing that these cells had progressed through a cell cycle during injury repair (439).

Single cell analysis of muscle regeneration reveals a possible helper cell population

A recent single cell analysis of mouse hindlimb muscle tissue was performed with RNA-sequencing and mass cytometry (173). This analysis revealed an additional, distinct population of cells the authors named smooth muscle and mesenchymal cell markers (SMMCs). These SMMCs were identified as positive for integrin α7 and negative for vascular cell adhesion molecule (VCAM). By single cell analysis, these cells were clearly distinct from the muscle satellite cells. SMMCs had myogenic potential and could contribute to myofibers when injected in muscle that was then injured. When muscle satellite cells were transplanted alone or in the presence of SMMCs into muscle that was then injured, muscles that received both types of cells had a higher number of myofibers, suggesting the SMMCs may act as helpers for the satellite muscle cells (173). The findings reveal how single cell analysis can help to delineate cell populations with regenerative potential.

Regulatory signals for muscle stem cells: Receptor tyrosine kinases

Multiple mechanisms have been identified that allow satellite muscle stem cells to maintain quiescence. One important molecular basis for quiescence in muscle stem cells is by limiting the activity of mitogenic signaling pathways. Receptor tyrosine kinase ligands FGF and HGF are potent activators of cell division in muscle satellite cells (227, 441, 504). Signaling

downstream from receptor tyrosine kinases to the p38α/β MAPK functions as a molecular switch that can activate satellite cells in the muscle (229). ERK1/2 kinase in the MAPK pathway is required for myoblast proliferation (228). Thus, growth factor signaling can play an important role in controlling the proliferation-quiescence decision in satellite cells.

One important mechanism that muscle satellite cells use to limit proliferation is to inhibit the activity of growth factors. Muscle satellite cells limit FGF-mediated ERK signaling by increasing expression of the receptor tyrosine kinase inhibitor Sprouty1 (439). Sprouty1, a negative regulator of RTK signaling (190, 246, 314), is expressed in quiescent, $Pax7^+$ satellite stem cells in uninjured muscle. Sprouty1 expression decreases in proliferating myogenic cells after injury, and its expression increases again when the $Pax7^+$ cells return to quiescence (439). Sprouty1 was found to be required for $Pax7^+$ cells to return to quiescence. When muscles were injured in mice with Pax7 cell-specific inactivation of Sprouty1, there was a significant 50% reduction in the number of $Pax7^+$ satellite cells 50 days after injury, demonstrating an important role for Sprouty1 in satellite cell homeostasis after injury (439). Without Sprouty1 reducing receptor tyrosine kinase signaling, there were fewer quiescent $Pax7^+$ satellite cells and an increase in the number of apoptotic myogenic cells (439). Thus, downregulation of receptor tyrosine kinase signaling is an important mechanism ensuring that muscle satellite cells properly return to quiescence rather than apoptosing.

FGF and Sprouty1 signaling are also important for aging muscle (61). FGF2 levels increase in skeletal muscle with age. Sprouty1 levels, in contrast, decrease as mice age. Because aged muscle has lost the inhibitory function of Sptrouty1 in satellite cells, the satellite cells begin to divide (61). Further, activating the FGF receptor (FGFR1) increases the proliferation of older satellite cells (27). Conversely, if FGF signaling is inhibited in older satellite cells, the cells become quiescent and less regenerative (61).

Regulatory signals for muscle stem cells: Integrins—Another important mechanism regulating the proliferation of quiescent muscle stem cells is via integrin-mediated signaling. Based on their localization between the plasma membrane of the muscle fiber on one side and the basement membrane on the other side (313), satellite cells engage in multiple contacts. Interaction between muscle stem cells and the basement membrane is mediated through integrins including $\alpha_7\beta_1$ integrin, which interacts with the transmembrane protein dystroglycan (35, 77, 127, 418). Loss of β_1 integrin in $Pax7^+$ muscle stem cells in adult mice resulted in a reduction in the number of satellite cells 21 days after β_1 integrin was deleted and continued depletion of satellite cells over time (418). Labeling with BrdU revealed that satellite cells with inactivation of β_1 integrin incorporated more BrdU and divided inappropriately compared to satellite cells from control mice (418). β_1 integrin inactivation also resulted in an increase in the fraction of cells expressing the muscle differentiation marker MyoD (418). Thus, β_1 integrin is important for maintaining satellite cells in a quiescent state; without it, satellite stem cells lose their protected quiescent status, enter the cell cycle, and differentiate (418). The myoblasts formed are later incorporated into existing muscle fibers (418). When muscles with β_1 integrin inactivation in satellite cells were injured, the ability of the muscle to regenerate was compromised (418). Isolated satellite cells had lower levels of cyclins D1 and D2, an increased fraction of cells in the G1 phase and fewer S-phase cells compared with controls (418). The results demonstrate the

importance of β_1 integrin and the connection to the basement membrane for muscle stem cell quiescence.

Based on earlier studies showing that activation of integrins by the ECM cooperates with the FGF receptor to activate MAP kinases and drive proliferation (15), Rozo and colleagues investigated the role of FGF signaling in mice with satellite-cell-specific β_1 integrin inactivation. In control cells, the presence of either fibronectin or FGF-2 alone only weakly activated ERK and AKT, while the presence of both fibronectin and FGF-2 resulted in stronger ERK phosphorylation. Integrin β_1-deficient satellite cells did not respond to low levels of FGF-2 even in the presence of fibronectin, though high levels of FGF-2 partially restored cooperativity. The findings support the importance of cooperation between mitogenic signaling and integrin signaling for the commitment of satellite cells to proliferate, similar to the coordinated response to mitogen and integrin signaling observed in cultured fibroblasts (417).

Rozo and colleagues noted that integrin β1 loss resulted in a phenotype similar to aging. During aging, satellite cells are gradually lost from the niche (61) and fail to self-renew (27). The authors found that while stem cells in muscles from young animals contained mostly active membrane-bound β_1 integrin, satellite cells from older animals exhibited disorganized or undetectable β_1 integrin (418). Further, injecting injured muscles with an antibody that activates β_1-integrin had no effect on regeneration in young mice, but improved regeneration in older mice to a level comparable to young mice (418). Treatment with a β_1 integrin activator increased the fraction of satellite cells in injured, aged muscle with phosphorylation of the FGF receptor, supporting the importance of the extracellular matrix for the response to mitogens (418). The findings demonstrate roles for loss of integrin signaling and loss of mitogen signaling as contributors to changes in the proliferation-quiescence transition of satellite cells during the aging process.

Regulatory signals for muscle stem cells: Syndecans—Other transmembrane proteins that mediate the communication between stem cells and their niche have also been implicated in muscle satellite cell quiescence (390). Syndecan-3 is a transmembrane protein containing heparan sulfate sugars on its extracellular domain that regulates cell-cell adhesion and matrix-cell adhesion through integrins (404). The extracellular portion of syndecan-3 interacts with extracellular matrix proteins and growth factors, while the intracellular portion of syndecan-3 interacts with signaling molecules. Syndecan-3 was initially determined to be generally important for myogenesis (57, 90, 157, 391) and subsequently discovered to be specifically important for maintenance of quiescence in muscle stem cells (391). Mice with inactivation of syndecan-3 fail to replenish satellite stem cells after muscle injury (391). Loss of syndecan-3 results in spontaneous muscle stem cell activation in adult muscles (90). Mice with syndecan-3 inactivation contain higher levels of satellite stem cells migrating away from their niche that were more likely to proliferate (90). Regenerating muscle with inactivation of syndecan-3 had higher levels of activated satellite cells, more proliferating myoblasts, and more myogenic cells (390). As a result, syndecan-3 inactivation actually resulted in improved regeneration in repeatedly injured muscle in normal mice and in mice with muscular dystrophy (390).

Syndecan-3 null satellite cells may be more likely to proliferate because they have been shown to be more sensitive to receptor tyrosine kinase ligands FGF2 and HGF (90, 157), mitogens that promote satellite cell activation and proliferation (227, 441, 504). In this case, the mechanism through which syndecan-3 affects muscle stem cells would be through MAP kinase signaling. Consistent with this hypothesis, stimulation with FGF-2 or HGF leads to higher levels of ERK phosphorylation in syndecan-3 knockout cells (90). The results suggest that the transition between proliferation and quiescence in muscle satellite cells is regulated by mitogens and molecules that affect the potency of these mitogens, including both integrins that cooperate with mitogen signaling and extracellular matrix proteins that mediate the diffusion of mitogens through the extracellular environment and the accumulation of mitogens at their target.

Regulatory signals for muscle stem cells: Notch—Notch receptors are heterodimers that interact with ligands presented on the surface of nearby cells (446). Upon activation, the notch receptor is cleaved, leading to the release of the notch intracellular domain, which can translocate to the nucleus (218, 221). The intracellular domain can activate transcription factors to induce gene expression. Notch signaling regulates stem cells in skeletal muscle (31, 86, 161, 499), brain, skin, intestine, and blood vessels (36, 54, 86, 153, 160, 297, 517).

Notch ligands found at the surface of myofibers can bind to receptors at the surface of muscle stem cells and promote quiescence maintenance. Using a transgenic notch reporter mouse, notch signaling was observed in quiescent muscle stem cells (31). Notch targets Hairy enhancer of split 1 (Hes1), Hairy enhancer of split 5 (Hes5), Hes-related with YRPF motif 2 (Hey2), and Hes-related with YRPF motif L (HeyL) were found to be highly expressed in the quiescent state and downregulated during activation (31). Notch 3, in particular, is expressed by quiescent stem cells (160), and its disruption leads to misregulation of muscle stem cell proliferation (251). Notch promotes Pax7 expression and a return to quiescence (31, 86, 161, 499). Inactivating the notch effector RBP-Jκ specifically in muscle stem cells results in spontaneous activation followed by terminal differentiation (31, 347). These muscle stem cells lacking Recombination signal binding protein for immunoglobuin kappa J region (RBP-Jκ) fail to self-renew (31). Conversely, constitutive expression of the notch intracellular domain in myoblasts inhibits S-phase entry and Ki-67 expression (499). Further, myofibers expressing an E3 ubiquitin ligase family member that activates notch signaling, mind bomb 1, also facilitates muscle satellite cell quiescence (247). Thus, notch signaling is required to maintain the muscle stem cell population in a quiescent state and prevent inappropriate stem cell activation (31). Taken together, these multiple studies show that notch signaling regulates the maintenance of the quiescent state and the ability of satellite cells to reenter quiescence after activation (31).

Notch signaling may also be important for the effects of syndecan-3 on muscle stem cells. Syndecan-3-null satellite cells, in addition to increased sensitivity to mitogens, also exhibit reduced notch signaling (391), which may contribute to their failure to return to quiescence upon activation (391). Muscles in syndecan3-null mice have lower levels of Pax7 and higher levels of Myf5, a myogenic transcription factor, after injury (390). Three months post injury, syndecan3$^{-/-}$ mice have more Myf5^{+}, Pax7^{-} cells, which are thought to continue to proliferate (390). This is consistent with their reduced notch signaling preventing a return to

quiescence. Reduced notch signaling in syndecan-3-null mice may reflect a requirement for syndecan-3 in notch processing and signaling (391). Instead of returning to their niche, in the absence of syndecan-3, the Myf5+ myoblasts proliferate and increase in abundance over time (390). This leads to a larger muscle progenitor population, which contributes to large myofibers after injury and therefore improved muscle function (390). The loss of stem cells is slow, and the syndecan-3-deficient mice do not exhaust their satellite cells (390). Syndecan-3-null mice exhibit improved muscle regeneration that may reflect both mitogenic and notch signaling. These results suggest reduced syndecan-3 as a potential new approach reducing muscle aging.

Multiple quiescent states in muscle stem cells

Rodgers and colleagues from the Rando laboratory discovered that there are multiple possible quiescent states for satellite cells. They discovered that muscle satellite cells can be in traditional G_0 or a more “alert” state of cell cycle pause that they termed Galert (416). Rodgers and colleagues found that if they injured an animal on one side of its body, quiescent muscle stem cells on the contralateral side had a higher likelihood of dividing compared to stem cells in an uninjured animal (416). When the stem cells from the contralateral side of an injured mouse were cultured, they entered their first cell cycle faster than stem cells isolated from an uninjured mouse. The stem cells from the contralateral side were larger than quiescent stem cells from an uninjured mouse. Contralateral stem cells also had higher levels of total RNA, incorporated more labeled ribonucleotides, had higher levels of mitochondrial activity, and contained more ATP (416). Investigating further, Rodgers and colleagues found that the mammalian Target of Rapamycin complex 1 (mTORC1) signaling pathway was induced in the contralateral skin (416). mTORC1 is regulated by a wide variety of signals including inflammation, DNA damage, hypoxia, and amino acids (274). When active, the mTORC1 kinase regulates the activity of multiple processes including protein synthesis, lipid synthesis, autophagy, and energy metabolism (274). When mTORC1 inhibitor tuberous sclerosis was inactivated specifically in muscle satellite cells with a Pax7 driver, the muscle stem cells had attributes of contralateral stem cells without injury, cells in the Galert state (416). When Raptor, an essential component of the mTORC complex, was eliminated, the contralateral stem cells did not respond to injury by entering an activated quiescent state and instead, remained in deep quiescence (416). Investigating further to identify the signaling pathways that result in mTORC activation, the authors found that HGF, an mTORC1-regulator, was also important for the commitment to Galert, as knockout of the HGF receptor of mesenchymal epithelial transition factor (cMET) in contralateral stem cells resulted in cells that did not respond to injury elsewhere by converting into a G_{alert} state. The results support a role for the mTORC1 pathway in controlling the depth of quiescence.

Neural stem cells can regenerate neurons in adult mammals

For many years, it was believed that neurons do not divide in adult brains. Histological and anatomical studies by early neuroscientists led to the conclusion that a constant and unchangeable structure of brain neurons is required for long-term memory storage (400, 401). They concluded that no new neurons could be generated after birth because brain architecture is fixed during development (47, 85, 184). In the past three decades, the ability

to monitor cell division in intact animals with BrdU allowed scientists to recognize that, in fact, neurons are generated anew in adult rodent brains. In the adult rodent brain, NSCs continuously generate functional neurons in specific brain regions throughout life (237). These NSCs are controlled by signals provided by surrounding cells, as well as intrinsic factors such as cell cycle proteins (139, 288, 458). In response to physiological, pathological, and pharmacological stimuli (302), NSCs show plasticity; they can remain quiescent, divide, return to quiescence, or differentiate into different lineages (302). We now know that NSCs are a critical reservoir of regenerative cells that help the brain recover from injury and limit neurodegeneration with aging. Inducing NSCs to divide is important for the brain's ability to regenerate damaged tissue (76, 100, 118, 293, 328). Adult NSCs can integrate into existing neuronal circuits and contribute to neural functions (49).

Two neural stem cell locations in the brain

Adult brains contain NSCs in two distinct regions: the ventricular-subventricular zone (V-SVZ) of the lateral ventricle wall (379) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (302). Neurogenesis in the SVZ is important for learning new odorants (306, 414). Data from fluorescence-activated cell sorting (FACS) and lineage trace support the presence of NSCs in the subependymal zone of the lateral ventricles (4, 22, 117, 442). Quiescent adult NSCs reside in niches underneath the ependymal layer, where they are in contact with the ventricle through their apical surfaces (403, 404, 416, 417). The ependymal cells are multiciliated cells that form pinwheel structures with the V-SVZ NSCs in the centers of the pinwheels (417, 418, 465). Foot-like protrusions emerging from the apical end of the NSCs provide access to extracellular signals in the cerebrospinal fluid (449, 474). The NSCs are also in contact with neurons: their apical protrusions contact the axons of serotonergic neurons (449, 474), while choline acetyltransferase-positive neurons terminate near the NSCs of the subventricular zone (288). In addition, NSCs have basal processes that terminate at blood vessels (288, 375).

Within the V-SVZ, there is heterogeneity among the NSCs (288, 326). The NSCs that reside in geographically distinct regions of the ventricle walls are produced by different types of glia (11). Pools of adult V-SVZ in spatially different regions can be regulated by different stimuli and distant neural circuits (381). When they differentiate, NSCs in different locations produce different types of olfactory bulb neurons with different functions (11). Thus, depending on where in the brain they reside, different V-SVZ NSCs may be more likely to generate different types of olfactory bulb interneurons or glia (60, 148).

There are also NSCs in the SGZ of the adult hippocampus (13). Neurogenesis from NSCs in the hippocampal SGZ is important for spatial learning and memory (215). While there is a clear niche for the NSCs of the V-SVZ that provides information on the different signaling inputs to the NSCs, in the adult SGZ, there is a less obvious niche for the NSCs that reside there (438). NSCs in the hippocampus dentate gyrus generate granule neurons (12).

Neural stem cells are derived from radial glia

Adult NSCs are derived from radial glia (327), the NSCs of the developing brain, and are a form of glial cell rather than neurons (117, 203, 261). V-SVZ NSCs share morphology and

epithelial apico-basal organization with radial glial cells (332). They also share markers with radial glial cells including glial fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST), Nestin, Sox2, and brain lipid binding protein (BLBP) (76, 288, 328). The presence of these radial glial markers in the adult V-SVZ NSCs suggests that adult NSCs may derive directly from radial glia of the developing brain (11, 261, 473). The NSCs of the hippocampal SGZ also express markers associated with radial glial cells including GFAP, Nestin, and Sox2, and, like V-SVZ NSCs, are negative for S100β (302).

Neural stem cells transition from quiescence to activation

Retroviral labeling and BrdU label retention experiments support a model in which NSCs of the V-SVZ are predominantly quiescent and outside the proliferative cell cycle (288). Primary adult SVZ NSCs divide slowly and retain label for long periods of time. For instance, Morizur and colleagues performed BrdU labeling of NSCs and found low levels of BrdU labeling indicating little division (344). Cells that were labeled with BrdU retained the label for 4 weeks (344), showing they are largely quiescent. To further explore the generation and division of adult NSCs, Fuentealba and colleagues injected BrdU into mice at different days of embryonic development. They found that adult V-SVZ NSCs were descendants of radial glia that divided at E14.5 to E16.5 and not after (159). Thus, these cells were set aside during embryogenesis and remained quiescent until they were reactivated in adulthood (159). The results are consistent with a model in which some cells are designated early in embryonic development to become NSCs in the adult. NSCs in the SGZ of the adult hippocampus also rarely divide (13).

While most adult NSCs are quiescent, they can be activated to divide (76, 288, 328). At the appropriate time and in response to specific external and internal signals, quiescent NSCs can be stimulated to divide and generate activated NSCs that express epidermal growth factor receptor (76). The transition between quiescence and proliferation for adult NSCs is carefully regulated, as defects that lead to inappropriate proliferation can result in depletion of NSCs (250, 331, 337, 372). Activated NSCs can then produce neural progenitor cells or transit amplifying progenitors that express markers of neuronal differentiation (119). The transit amplifying cells can, in turn, produce migrating neuroblasts (288, 395). These migrating neuroblasts move anteriorly along blood vessels in a path that brings them to the anterior ventricle where they follow the rostral migratory stream leading to the olfactory bulb (116, 288). In the olfactory bulb, the neuroblasts differentiate into multiple types of interneurons (302). The new neurons of the olfactory bulb generated by adult V-SVZ NSCs contribute to an animal's ability to learn new smells during adulthood (282, 288, 425).

NSCs of the adult hippocampus are also mostly quiescent. Like V-SVZ NSCs, hippocampal NSCs can, on occasion, divide to form proliferating intermediate progenitors (268, 297, 465). Like NSCs of the V-SVZ zone, quiescent adult hippocampal NSCs can be activated by physiological activities and pathological changes (519). Also similar to V-SVZ NSCs, NSCs of the adult hippocampus can form committed neuroblasts that transition to postmitotic neurons.

Quiescence in NSCs has been associated with high levels of the CDK inhibitor $p27^{Kip1}$. Indeed, the CDK inhibitor $p27^{Kip1}$ has been shown to be an important regulator of the

transition between quiescence and proliferation of NSCs. The expression pattern of p27 is consistent with a role for p27 in maintaining stem cell quiescence (13). $p27^{Kip1}$ was found to be present in the dentate gyrus of adult mice in the granule cell layer and the SGZ. The vast majority of the radial NSCs ($SOX2^{+}GFAP^{+}$) with a long process spanning the granule cell layer were positive for $p27^{Kip1}$ and had high levels of the protein in the nucleus. Only 2% of the radial NSCs were negative for p27. Further, p27 functions as a suppressor of cell proliferation in immature neurons. Loss of p27 leads to more cell proliferation in the SGZ under basal and ischemic conditions, seizures, and chronic stress (399).

Mouthon and colleagues developed a method to prospectively identify quiescent NSCs from the V-SVZ based on the intensity of staining for NSC marker LeX and the EGF receptor (EGFR) (100, 101). They isolated quiescent $LeX^{bright}EGFR^{-}$ and activated $LeX^{bright}EGFR^{+}$ cell populations (344). They sorted V-SVZ NSCs by FACS analysis and analyzed the populations by microarray. Activated NSCs expressed higher levels of genes associated with cell cycle, DNA repair, DNA/RNA metabolism, transcription, and translation compared with quiescent NSCs. Cell-cycle-associated genes that were expressed at higher levels in activated than quiescent NSCs include E2Fs, E2F1 and E2F2, cyclin E, and immediate early gene Fos. Genes expressed at higher levels in quiescent compared with activated NSCs were enriched for lipid metabolic processes, transport, response to stimulus, cell localization, cell communication, and cell adhesion. For example, quiescent NSCs had high levels of adhesion molecules, neural cell adhesion molecules and cadherins/protocadherins. Many of the genes enriched in the quiescent cells encode adhesion proteins and other membrane-associated proteins, including sonic hedgehog receptor patched, consistent with an important role for the niche in regulating adult NSCs (344). Transcripts for Achaete-scute homologue 1 (Ascl1), an evolutionarily conserved transcription factor necessary and sufficient to generate new neurons (245), were 200 times higher in activated than quiescence NSCs.

Many regulatory signals for NSCs

Adult neurogenesis in the SVZ and the SGZ responds to environmental cues, physiological stimuli, and neuronal activity (239, 304, 484, 519). The different elements of the V-SVZ NSC niche are likely contributors to the signals that direct the NSCs toward activation or quiescence. One source of signals for the V-SVZ NSCs (442, 471) and SGZ NSCs (378) are molecules in blood (288), as the stem cells in both regions are in contact with blood vessels. Kazanis and colleagues found that the periventricular area where new neurons are formed has higher levels of blood vessels than other regions of the brain, supporting an important role for factors circulating in the blood supporting NSCs and their activation (238). Indeed, NSCs of the V-SVZ are regulated by multiple signaling molecules that are likely provided through the bloodstream, including EGF (119), FGF (509), betacellulin (an EGF-like growth factor secreted by endothelial cells) (178), sonic hedgehog (115), wnt (48), notch (14), bone morphogenetic proteins (BMPs) (78), pigment epithelium derived factor (PEDF) (402), ephrins (158, 214), leukemia inhibitor factor (24), transforming growth factor alpha (477), cytokines (23, 348), and brain-derived neurotrophic factor (430). In addition to soluble factors, the ECM surrounding NSCs is also considered an important determinant of NSC fate, as it provides a platform for the presentation of molecular signals (51, 281). These

signaling pathways can control the transitions between quiescence, activation, proliferation, and differentiation.

While Kazanis and colleagues found that the entire region of the brain that supports neurogenesis is rich in vasculature (238), whether a cell divided or not is not closely associated with its distance to the nearest blood vessel. The likelihood of a cell dividing did correlate with the distance from the ventricle, implicating the ependymal cells as a source of proliferation and self-renewal signals (402). Ependymal cells may provide these cues through direct contact (402) or through paracrine signaling pathways to the NSCs, including pigment epithelium-derived factor (402). Molecules of the BMP family have also been implicated as factors released by ependymal cells that can affect NSC proliferation (78). Conditional deletion of BMP mediator Smad4 in adult NSCs, or introduction of BMP antagonist Noggin, led to increased progeny that migrated and differentiated into oligodendrocytes (78). Thus, BMP-mediated signaling via Smad4 is required for adult NSC neurogenesis (78). Finally, the beating of the motile ependymal cilia has also been found to promote neuroblast migration (427), demonstrating another mechanism through which the ependymal cells can affect NSC activation. On the other hand, in the hippocampus, neurogenesis occurs even though there are no ependymal cells present (411). In the hippocampus, neuronal signals are considered important (240, 303, 304).

Regulatory signals for NSCs: Growth factors—Our understanding of the stem cell potential of NSCs has been advanced by studies of NSCs in culture. Both SVZ- and SGZ-derived NSCs can be grown in culture as “neurospheres” that exhibit self-renewal and multi-lineage neural potentiality (38, 346). The ability to culture and maintain neural cells *in vitro* is based on the success of scientists in cloning and purifying mitogenic factors such as FGF and epidermal growth factor (147, 165). These studies have demonstrated the importance of receptor tyrosine kinase signaling for NSC proliferation (119, 345, 360). FGF-2 helps to maintain the self-renewal of adult rat hippocampal NSCs (305) and neural progenitors (311) in culture. Adult V-SVZ-derived NSCs, in the presence of FGF-2, can proliferate and differentiate into neurons and glia *in vitro* and can then be transplanted back into the central nervous system (1, 377).

Overexpression of FGF-2 promotes the maintenance of cultured neural progenitor cells in a pluripotent, undifferentiated state (99). FGF-2-overexpressing neural progenitors migrated better when introduced into the cortex of 3-day-old pups (99). Further, FGF-2 overexpression resulted in an increased pool of new interneurons in the olfactory bulb (99). The progenitor cells that did differentiate into neurons had downregulated the FGF-2 transgene, supporting a role for FGF-2 in maintaining undifferentiated progenitors (99). *In vivo*, FGF-2 can promote proliferation of V-SVZ progenitors and increase olfactory bulb neurogenesis (266). After brain ischemia, FGF2 and EGF infused into lateral ventricles can increase neuronal regeneration (352). Further, activation of the FGF receptor can promote self-renewal, increase proliferation, and inhibit spontaneous differentiation (305).

In vitro studies have investigated the importance of specific signaling pathways downstream of tyrosine kinase receptors for the activities of NSCs. Activation of ERK1/2 of the canonical MAPK pathway by FGF-2 is necessary for NSC expansion and the anti-

differentiation effects of FGF-2 (305). In addition, phospholipase C activation by FGF signaling also contributes to the ability of cultured NSCs to differentiate into neurons and oligodendrocytes (305). In this way, NSCs share similarities with muscle stem cells where mitogenic signaling through receptor tyrosine kinase activity is important for the activation of both types of stem cells.

Regulatory signals for NSCs: Notch—The notch signaling pathway is important for maintaining the correct number of NSCs (14, 21, 485). Notch ligands jagged1 and Delta-like canonical notch ligand 1 (Dll1) are presented to NSCs by other surrounding cells, including ependyma, astrocytes, activated NSCs, and neuroblasts (175, 293, 365). Notch ligands expressed by these cells promote NSC self-renewal and maintain NSC numbers through notch signaling (175, 293). Notch3, in particular, is expressed at higher levels in quiescent than activated NSCs (237). Knocking down Notch3 in the lateral wall of the adult V-SVZ led to a significant reduction in quiescent NSCs and increased NSC division (237). In contrast, Notch1 is expressed at higher levels in activated NSCs and transit-amplifying progenitors in the adult mouse subependymal zone (237). V-SVZ NSCs were found to be Notch1 dependent in their active state, but not quiescent state (21). With regard to downstream signaling, Notch represses the expression of differentiation-inducing transcription factors (58), including Ascl1. Thus, in NSCs, like muscle stem cells, notch plays an important role in regulating the proliferation-quiescence transition.

Regulatory signals for NSCs: Integrins—The V-SVZ region has large amounts of ECM molecules including fibronectin and laminins (325), chondroitin sulfate proteoglycans (6), and tenascin-C (238). Because in the V-SVZ, the distance from the ventricle is an important determinant of whether cells are NSCs or precursors, while the distance from the blood is less important, Kazanis and colleagues (238) investigated the role of the extracellular matrix and integrins in the NSC niche. Previous studies had shown that laminins are important for survival and proliferation of neuronal progenitors (189), regulating growth factor concentrations (242), and hippocampal regeneration (183). Kazanis and colleagues found that NSCs and their transit-amplifying cell progeny are in a laminin-rich extracellular matrix. Surprisingly, when they considered laminin-binding integrins, mostly $\alpha_5\beta_1$ integrin, they found that β_1 integrins, which are associated with quiescent stem cells in other tissues, were expressed at low levels in NSCs. Surprisingly, while integrin β_1 is important for quiescence of muscle satellite cells (see above), in the V-SVZ, long-term labeling NSCs expressed low levels of integrin β_1. When V-SVZ NSCs were induced to divide, they turned on integrin β_1 expression. Adding an integrin β_1 blocking antibody induced proliferation of transit-amplifying cells with little effect on the NSCs, consistent with their low expression of β_1 integrin. Further, blocking integrin β_1 also resulted in increased neuroblast migration. The authors presented a model in which laminins are present throughout the niche, but the quiescent NSCs express low levels of the proteins that bind laminin, including integrins, and thus do not react to the surrounding laminin. In contrast, the transit amplifying cells and neuroblasts do express laminin-binding integrins and therefore interact with the laminins, an interaction that promotes their migration and proliferation.

Regulatory signals for NSCs: Syndecan—As described above, in a recent study, Morizur and colleagues sorted quiescent NSCs from the V-SVZ of adult mice based on high expression of LeX (344). Syndecan-1 was found to be more highly expressed in activated than quiescent NSCs at the RNA and protein level (344). Similarly, Kazanis and colleagues also found that syndecan-1 protein was mostly found on transit-amplifying cells and neuroblasts (238). The authors further demonstrated that syndecan-1 plays a functional role in the proliferation of activated NSCs (344). Activated NSCs expressing syndecan-1 could generate larger neurospheres than controls (344). Thus, in both muscle and neuronal stem cells, extracellular matrix proteins of the syndecan family play an important role in regulating division, possibly by modulating diffusion and accessibility of proliferative signals.

Single cell sequencing of quiescent and activated NSCs

In an early single cell analysis of NSCs, NSCs were identified based on their expression of both CD133 and Prominin (293). Isolating $CD133^{+}Prom1^{+}$ cells and performing single cell RNA sequencing revealed two types of quiescent NSCs and two types of activated NSCs. Quiescent NSCs expressed transcription factors Sox9, Id2, and Id3. Dormant V-SVZ NSCs were found to enter a primed quiescent state before they were activated. This entry into a primed state was associated with upregulation of genes involved in protein synthesis, downregulation of notch and BMP signaling, and upregulation of lineage-specific transcription factors. Indeed, activated NSCs had a 7.9-fold increase in the rate of protein synthesis compared with quiescent NSCs. In the activated state, glial-associated genes were reduced compared with quiescent cells. The gene expression signature associated with activated NSCs included upregulation of genes associated with cell cycle including EGFR and Cdk2, immediate early genes Egr1 and Fos, protein synthesis, and mitosis (293). Neuroblast-associated transcription factors including Dlx1 and Dlx2 were detected in activated NSCs. Among the cells that had transitioned to neuroblasts, there was an increase in genes associated with neuronal differentiation. The notch2 receptor was enriched in quiescent NSCs and Dll-expressing activated NSCs were hypothesized to directly control the dormancy of quiescent NSCs through direct contact. The authors further showed that in culture, treatment of NSCs with notch inhibitor DAPT resulted in a shift from quiescence to activation. In this study, the authors also performed carotid artery occlusion and monitored the effects on NSCs. This treatment resulted in an increase in the proportion of primed quiescent and activated NSCs. Activation resulted in increased protein synthesis and cell cycle gene expression. The results are consistent with studies from the Rando laboratory characterizing an “alert” state of quiescence in muscle stem cells in response to injury to a distant location in the same animal (416).

A subsequent single cell sequencing analysis provided additional information on the continuum from quiescent to activated NSC states (126). In this single cell analysis, 329 freshly isolated cells were sequenced with good quality data. These cells included niche astrocytes, quiescent NSCs, activated NSCs, and neural progenitor cells (126). Quiescent NSCs were confirmed to express high levels of previously identified targets including Id3 (126). When quiescent NSCs were activated, they upregulated expression of EGFR (126). As the cells transitioned from quiescence to activation, they first upregulated ribosomal

genes that would increase their translational potential (126). In the next stage of activation, they upregulated cell cycle genes (126). Further studies confirmed the existence of activated NSCs with low levels of cell cycle markers (126) as the authors could identify cells that were EGFR-positive, but Ki-67 negative (126). Application of machine learning models produced an ordered continuum of states: quiescent ($EGFR^-$), early ($EGFR^+$ but $CDK1^-$), mid and late activated NSCs. Among the NSCs that do express cell cycle markers, some also expressed a factor known to be required for neuronal differentiation (119, 389, 466), Dlx2. Dlx2 expression distinguished mid from late activated NSCs. The transition from mid to late activated NSCs was also associated with reduced expression of genes associated with the astrocyte lineage. The final states were quiescent NSC ($EGFR^-$), early activated NSC ($EGFR^+$, $CDK1^-$), mid activated NSC ($EGFR^+$, $CDK1^+$, $DLX2^{low}$), late activated NSC ($EGFR^+$, $CDK1^+$, $DLX2^{high}$), and neural progenitor-like ($DLX2^+$, DCX^+), where doublecortin (DCX) is a neuronal marker (126).

In another single cell analysis study of regional differences in adult V-SVZ cell lineages, Mizrak and colleagues investigated NSCs along the lateral ventricles and compared those closer to the striatum with those closer to the septal wall. Sequencing greater than 41,000 V-SVZ cells dissected from the lateral and septal walls of male and female adult mice, they discovered that cells on the lateral walls were more likely to differentiate into astrocytes while those on the septal wall were more likely to differentiate into oligodendrocytes (334).

Basak and colleagues employed single cell RNA-Seq analysis to show that the Wnt target Tnfrsf19/Troy can be used to identify NSCs (22). They sought to use this information to distinguish among several possible models for how stem cells contribute to the activated NSC population. In the “classic model,” each stem cell divides to give rise to one stem cell and one transit-amplifying cell (39). A second possible model is that, on average, each stem cell divides to give rise to one stem cell and one transit-amplifying cell, but any specific stem cell division could give rise to more or fewer stem cells. And a final possible model is that once an NSC divides, both daughters give rise to transit-amplifying progeny; the stem cell pool is never replenished and becomes exhausted. Visualizing Troy-positive cells in the brain and using genetic labeling to trace the progeny of individual Troy+ cells over time revealed clones with Troy-positive cells, showing that NSCs are capable of symmetric division upon activation (414). Because the composition of Troy+ clones did not change over time, overall NSC proliferation must have been balanced by differentiation and loss through migration (414). A model of the cells’ behavior predicted that NSCs go through an average of 2.7 rounds of division before they return to quiescence or differentiate, and that these rounds of division result in an average of 3.4 transit-amplifying cells (22). To test more directly whether actively cycling NSCs can return to quiescence, they labeled cells with Ki-67 expression and found that some of them did, in fact, exit the cell cycle and return to a quiescent state (414). They concluded that after an initial expansion of NSCs within a niche, some can reenter quiescence depending upon the presence of other stem cells in the niche (22). They imagined a model in which there is competition at the molecular level to ensure that there is a single quiescent stem cell per niche, for instance, perhaps only a single cell can fit in the pinwheel center of ependymal cells so only one quiescent stem cell is present in each niche (22). The competition that they envision for the single niche cell could involve

limited access to the ventricle or ependymal cells, limited supplies of niche factors, or an inhibitory signal secreted by other stem cells (22).

Neural stem cells in humans

Whether or not new neurons can be generated in human adults has been a source of debate. A paper in *Nature* in 2018 raised doubts about whether neurogenesis occurs in adults as young neurons were not found in the dentate gyrus of adults (457). More recently, a paper in *Nature Medicine* suggested that neurogenesis is possible in human adults (340). In this article, the authors carefully analyzed human brain samples and used state-of-the-art processing methods. With this approach, the authors observed thousands of immature neurons in the dentate gyrus of adult humans up to 90 years of age. They also observed a decrease in the number and maturation of these neurons in Alzheimer's disease patients. The reduction in new neurons became more extreme as Alzheimer's disease progressed.

Conclusion

The molecular machinery of CDKs, the cyclins that bind them, their inhibitors, and their substrates provide a molecular basis for cell cycle control. CDK activity accumulates during quiescence and mediates the transition into the cell cycle (Figure 1). This process exhibits an abrupt transition, after which, cells are committed to cell cycle entry. Analysis of single cells has allowed investigators to determine the importance of cyclins, CDK inhibitors, molecules that degrade cyclins, and the AKT kinase in these cell fate commitments. Single cell analysis has also contributed to our understanding of the hysteresis that establishes a barrier for switching between proliferation and quiescence. Muscle stem cells and neuronal stem cells are typically quiescent and are stimulated by mitogens to divide through pathways that impinge on the activity of CDKs. Single cell analysis has supported a continuum of quiescent to activated states for NSCs once they are activated to divide. In the future, the application of single cell analysis may be able to provide yet more detailed information on the molecular basis for heterogeneity among cells in the transition between proliferation and quiescence *in vitro*, during development, in regenerative tissues and in normal and diseased tissue.

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Didactic Synopsis

Major Teaching Points

- The restriction point is a point of no return in the cell cycle: after the restriction point, cells will continue to cycle even if pro-proliferative signals are withdrawn.
- Cyclin-dependent kinases are activated by cognate cyclins to phosphorylate target proteins.
- Cyclins are tagged with ubiquitins that mark them for degradation at the proteasome.
- The anaphase-promoting complex and the SCF complex add ubiquitins to cell cycle proteins and regulate their degradation.
- Cyclin-dependent kinases are regulated by activating and inhibitory phosphorylation events.
- The CIP and INK4 families of cyclin-dependent kinase inhibitors arrest the cell cycle.
- The retinoblastoma protein is a substrate of cyclin-dependent kinases; when it is hyperphosphorylated, it releases E2F transcription factors that activate transcription of cell-cycle-promoting target genes.
- Single cell analysis with E2F reporters reveals that E2F signaling is bimodal: some cells have low E2F activity and some cells have high activity, with few cells having intermediate levels.
- Single cell analysis reveals that E2F signaling demonstrates hysteresis: E2F signaling can be “on” or “off ” when cells are stimulated with the same amount of serum depending on whether the cells were quiescent or proliferating prior to treatment.
- Real-time monitoring of a population of cycling cells revealed that cells with low CDK activity fail to divide, and the levels of cyclin-dependent kinase inhibitor p21 in each cell predict whether that cell will continue to divide or arrest.
- The quiescent versus activated state of muscle stem cells is regulated by multiple signaling pathways including fibroblast growth factor, hepatocyte growth factor, ERK, notch and integrin signaling.
- Muscle stem cells can be in a G_0 quiescent state or a G_{alert} state depending on mTORC1 activity.
- Neural stem cells can be activated from a quiescent to an active state by signaling pathways that include FGF, EGF, and notch.
- Single cell sequencing analysis of neural stem cells revealed a continuum of activation states between quiescent and fully activated.

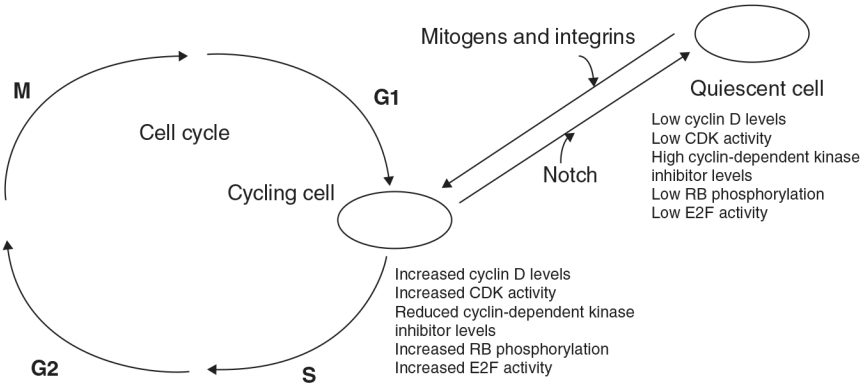

Figure 1.

Molecular changes in proliferating versus quiescent cells. A schematic of quiescent cells and a summary of the molecular changes are provided. Compared with proliferating cells, quiescent cells have lower levels of receptor tyrosine kinase and MAP kinase signaling, lower cyclin D levels, lower CDK activity, higher levels of cyclin-dependent kinase inhibitor levels, lower levels of retinoblastoma phosphorylation, and reduced E2F activity. The activity of mitogens and integrins can shift quiescent cells to proliferation. Proliferating cells have higher levels of receptor tyrosine kinase and MAP kinase activity, increased cyclin D levels, increased CDK activity, lower levels of cyclin-dependent kinase inhibitors, increased retinoblastoma phosphorylation, and increased E2F activity. The notch signaling pathway can promote the quiescent state.