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The Temporal Regulation of S Phase Proteins During G₁

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Abstract

Successful DNA replication requires intimate coordination with cell cycle progression. Prior to DNA replication initiation in S phase, a series of essential preparatory events in G₁ phase ensures timely, complete, and precise genome duplication. Among the essential molecular processes are regulated transcriptional upregulation of genes that encode replication proteins, appropriate post-transcriptional control of replication factor abundance and activity, and the assembly of DNA-loaded protein complexes to license replication origins. In this chapter we describe these critical G₁ events necessary for DNA replication and their regulation in the context of both cell cycle entry and cell cycle progression.

Keywords

Cell cycle; origin licensing; RB; E2F; cyclin; CDK; APC/C; quiescence; DREAM complex

1. INTRODUCTION

Proper progression from the G₁ cell cycle phase into S phase and the accurate duplication of chromosomal DNA are key for successful completion of the cell division cycle. In this chapter we describe the molecular events of G₁ that are critical for successful initiation and completion of DNA replication and, thus, S phase. We primarily focus on events that have been studied in human cell lines, but when appropriate, we expand our focus to other model systems. While the ultimate goal of G₁ is to prepare for S phase, there are many sequential G₁ processes required for the proper transition into S phase including appropriate control of gene expression, protein accumulation, and protein-DNA complex assembly. We have divided our discussion of G₁ into three temporal segments: G₀, early G₁, and late G₁.

Because error free DNA replication is so critical, many different cell cycle checkpoints and levels of regulation ensure that this process is precise. Many of these checkpoints happen in G₁ before DNA replication begins. Given that DNA replication is essentially irreversible, it

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makes sense that multiple inputs are integrated into the decision to begin replication. If cells receive appropriate inputs in G_1 , and the external signals and environment are compatible with another round of the cell cycle, then an intracellular signaling cascade ultimately results in irrevocable commitment to S phase entry; this commitment is known as passage through the “restriction point.” The restriction point, as canonically known, is the point at which cells transition from requiring mitogens (extracellular growth factors) for S phase entry to no longer requiring sustained mitogenic signaling for progressing into S phase and through the rest of the cell cycle. If mitogenic stimuli are removed before cells pass the restriction point, they may enter G_0 , or quiescence, a temporary withdrawal from the cell cycle. Quiescence is a normal part of organismal development and homeostasis and is important for regenerative processes and the long term maintenance of stem cell populations.

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When reduced to its simplest form, progression through G_1 is relatively straightforward: there is a period of low cyclin-dependent kinase (CDK) activity after mitosis or during G_0 . Then mitogenic signals trigger intracellular signal transduction networks that ultimately activate the heterodimeric protein kinase cyclin D/CDK4 or cyclin D/CDK6; CDK4 is the catalytic subunit responsible for phosphotransfer, but is inactive in the absence of cyclin binding. (Current evidence indicates that cyclin dependent kinases 4 and 6 are functionally redundant in G_1 ; thus, to simplify, we will only designate CDK4.) Cyclin D/CDK4, in turn, upregulates cyclin E/CDK2, which both drives expression of genes required for DNA replication and triggers the initiation of DNA synthesis (Figure 1). These two major cyclin/CDK pairs drive cells through early then late G_1 and into S phase. There are three different human genes for cyclin D (1, 2, and 3), and two different genes that encode cyclin E (1 and 2); we refer to the class of genes (*e.g.* cyclin D) instead of specifying each gene product.

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Regulatory events or genetic perturbations can prevent, delay, speed up, or enhance any of the essential G_1 processes. Both positive and negative regulation during G_1 influence the rate of cell cycle progression. For example, cyclins fluctuate with peak abundance at different cell cycle phases that are driven by accumulation (largely from gene induction) and induced protein degradation. The catalytic subunits of CDKs are present at relatively stable levels throughout the cell cycle, but are not active unless bound to a cyclin. Once bound by cyclin, CDK undergoes a series of modifications (protein domain remodeling, phosphorylations, dephosphorylations, etc.) that either activate or inhibit kinase activity. The importance of controlling these events in the cell cycle is illustrated by the fact that most of the proteins involved are misregulated in some way during tumorigenesis.

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Complete and precise genome duplication in organisms with large genomes divided among many discrete chromosomes requires a complex network of pathways and molecules. Decades of research in both the cell cycle and the DNA replication fields has identified a great many proteins that act at key points in G_1 entry and progression. To aid readers, we provide a list of the principle G_1 proteins and their general functions relevant to our discussion in Table 1.

2. QUIESCENCE (G_0) AND DNA REPLICATION

Before passing through the restriction point, cells can enter a non-proliferative phase, called G_0 or quiescence, which is considered to be outside of the cell cycle. This non-cycling state is fundamentally different from other cell cycle exits such as senescence or terminal differentiation, which are irreversible; cells can re-enter the cell cycle from G_0 by transitioning into and through G_1 . G_0 is critically important to organismal health and maintenance because, when needed, populations of stem cells in G_0 can re-enter the cell cycle to replenish the overall cell population. The availability of G_0 cells is especially important in wound healing, the immune response, in the routine regeneration of the intestinal epithelium, and in bone marrow (where hematopoietic stem cells produce blood cells). Similarly, populations of stem cells in G_0 provide protection against acute toxic events such as radiation or chemotherapy. Many types of chemotherapy are designed to induce toxic damage in cycling cells, often by forming DNA adducts that prevent cells from successfully replicating their DNA. It has been proposed recently however, that some populations of cancer cells are able to escape genotoxic drugs and thus the effects of chemotherapy by entering a G_0 state. After treatment has ceased, these cells can re-enter the cell cycle and form a new tumor, causing a cancer relapse (O'Connor et al. 2014). Thus quiescence provides a reservoir of proliferative capacity that maintains adult tissues, but also presents a challenge for cancer treatment.

2.1 Global transcriptional control in G_0

One characteristic of quiescent cells is the active repression of transcriptional activity, especially the repression of genes involved in cell cycle progression. The molecular mechanisms that occur during the transition from the cell cycle into G_0 are largely unknown, but investigators have identified some key molecular characteristics of cells that are already in G_0 (Litovchick et al. 2011; Litovchick et al. 2007). Whole genome transcriptomics studies revealed unique quiescence-associated transcriptional programs (Coller et al. 2006). This microarray-based approach catalogued groups of up- or down-regulated genes in primary human fibroblasts driven into G_0 by three different methods (serum starvation, contact inhibition, or “loss of adhesion”). The differences among these datasets suggests that there are different transcriptional programs active in quiescence depending on the mechanism of cell cycle exit. Nonetheless, a set of transcripts behaved the same in the three different populations of quiescent cells, indicating that there is at least one common transcriptional program in all quiescent cells (Coller et al. 2006). Many of the genes that were downregulated in at least one condition are central to DNA replication and cell cycle control such as the mitotic cyclin B1, enzymes required to create DNA synthetic precursors, and subunits of the major E3 ubiquitin ligases that act in S phase or mitosis. Among the commonly-upregulated genes were transcriptional repressors such as *E2F4* and *E2F5*, which cooperate with the so-called “pocket proteins” to suppress the expression of DNA replication and cell cycle genes during G_0 .

2.2 E2F and pocket proteins

The majority of genes that encode proteins needed for cell cycle progression, DNA synthesis, and mitosis are regulated by a class of transcription factors known as the E2F

family. The activating subclass of E2F transcription factors (E2F1, E2F2, and E2F3) is one of the main drivers for this transcriptional program. A second subclass of E2F transcription factors, known as the repressor E2Fs (E2F4, E2F5, E2F6, E2F7, and E2F8), generally inhibit expression of target genes (Frolov and Dyson 2004). We will refer to the class of activating transcription factors and their binding partners, DP1 or DP2, as E2F, instead of the individual gene products. Each E2F protein has a DNA binding domain, an RB binding domain, and a DP1 binding domain that consists of a leucine repeat sequence and the marked box domain (Figure 2a). E2F is primarily functional during late G₁ and early S phase as cells accumulate proteins in preparation for DNA replication. E2F transcriptionally activates the expression of genes encoding proteins involved in positive feedback loops, such as E2F itself, and cyclin E that drive the cell into S phase. During mid-S phase through mitosis and early G₁, E2F is bound to and repressed by the pocket protein RB which is encoded by the human retinoblastoma gene, the first tumor suppressor identified (Knudson 1971; Benedict et al. 1983; Murphree and Benedict 1984). E2F-regulated genes are actively repressed during G₀ by the action of E2F-associated co-repressors, and that repression is mediated by E2F interaction with the p130 and RB pocket proteins.

Each member of the activating E2F subclass can largely compensate for the loss of the other two members; however, loss of all three E2F proteins results in severely impaired cell proliferation (Wu et al. 2001). *E2f1* null mice are viable and reproduce normally, indicating that the other two activating E2F transcription factors are able to compensate for the loss of E2F1 (Field et al. 1996). Likewise, *E2f1*^{-/-} *E2f2*^{-/-} mice also survive to adulthood. Interestingly, *E2f3*^{-/-} mice die early in development, indicating E2F3 plays a critical role in mouse development, but *E2f3*^{-/-} cells proliferate nonetheless (Wu et al. 2001). Using conditional knockout mice, Wu *et al.* generated mouse fibroblast lines with combinations of two or three null alleles in the three activating *E2f* genes. The cells deficient for two of the three E2F transcription factors proliferated slowly, whereas cells lacking all three had no measurable proliferation (Wu et al. 2001).

There are three E2F-binding “pocket proteins” encoded by mammalian genomes: the retinoblastoma protein (RB), p107 (RBL1), and p130 (or RBL2). They are named for a structural domain containing a protein binding cleft. Though they do not bind DNA directly, these proteins mediate transcriptional repression of many cell cycle genes and are key regulators of cell cycle progression. Each pocket protein consists of five major domains: the N- and C- terminal domains, the A and B pocket domains, and a linker region between the two pocket domains (Figure 2b).

The three pocket proteins have both overlapping and distinct roles, patterns of expression, regulation, and binding partners during the cell cycle (Classon and Harlow 2002). RB is primarily active in G₁ by binding to and inactivating the activating family of E2F transcription factors (E2F1, E2F2, and E2F3). p130 is highly expressed in G₀, whereas p107 expression increases after mitogenic stimulation to peak in S phase (Figure 3) (Litovchick et al. 2007; Devoto et al. 1992; Cobrinik et al. 1993; Burkhart et al. 2010). If one pocket protein is lost through mutation, the others can functionally compensate in some settings. For example, unlike in humans where RB loss alone causes retinoblastoma, *Rb* deficient mice do not spontaneously develop retinoblastomas (MacPherson et al. 2004; Chen et al.

2004; Zhang et al. 2004). However, double knockout *Rb/p107* or *Rb/p130* mice develop retinoblastoma, indicating that p107 is able to compensate for *Rb* loss (Robanus-Maandag et al. 1998).

2.3 Pocket proteins in G₀

During G₀, p130 is abundant and bound to DNA through E2F4 as a member of a multisubunit complex, known as the DREAM complex (Litovchick et al. 2007; Guiley et al. 2015). In contrast, RB is much less abundant and p107 is almost undetectable in quiescent cells (Hurford et al. 1997; Moberg et al. 1996). The p130-containing DREAM complex only assembles during G₀ and is named for its components: DP, RB-like (e.g. p130), E2F, and the MuvB subcomplex, which itself consists of subunits LIN9, LIN37, LIN52, LIN54, and RBAP48. The DREAM complex represses both E2F target genes and genes that are expressed in late S phase and G₂. This repression prevents aberrant expression that could lead to improper cell cycle re-entry (Litovchick et al. 2007; Muller et al. 2012; Muller et al. 2016).

DREAM complex assembly requires phosphorylation of the LIN52 subunit of MuvB at serine 28. In G₀, Lin52 Ser28 is phosphorylated by DYRK1A kinase, a protein kinase with multiple roles in cell proliferation and neuronal development (Chen et al. 2013; Soppa et al. 2014). Phosphorylation of p130 also contributes to DREAM assembly in G₀ by increasing p130 stability. Glycogen synthase kinase 3 β (GSK3 β), a protein kinase that is highly active during G₀, phosphorylates three sites in the loop region in the B-pocket domain of p130. These three phosphorylations stabilize p130 to promote DREAM assembly but are removed during cell cycle re-entry to facilitate DREAM disassembly (Litovchick et al. 2004). DREAM assembly is also facilitated in G₀ because cyclin D/CDK4 (low in G₀) can phosphorylate p130 to disrupt its incorporation into the DREAM complex (Sandoval et al. 2009). These p130 phosphorylations occur in the p130 interdomain linker region and disrupt the association between p130 and LIN52 to derepress DREAM-regulated genes (Guiley et al. 2015).

The MuvB subcomplex associates with DP, RB-like, and E2F proteins to form the DREAM complex in G₀, but it also binds to BMYB during S phase to form the MMB complex. The MMB complex is important for transactivation of genes involved in G₂ and M phase progression in tandem with the transcription factors BMYB and FOXM1 (Sadasivam et al. 2012). Somewhat paradoxically, the MuvB subcomplex is critical for the maintenance of quiescence as part of the transcriptionally repressive DREAM complex, but is also critical for the proper transcriptional expression of G₂/M genes.

Consistent with the idea that cells are sometimes considered to be “resting” during quiescence, overall mRNA abundance is reduced, as are global levels of translation (Williams and Penman 1975; Levine et al. 1965; Degen et al. 1983). These reductions in both mRNA and protein may be due to the ability of RB and p130 to downregulate rRNA gene expression by inhibiting RNA polymerase I (Hannan et al. 2000), or the unique ability of RB to reduce tRNA levels by inhibition of RNA polymerase complex TFIIIB (Scott et al. 2001; White et al. 1996). The resulting reduction in ribosomal RNAs and tRNAs slow protein translation globally.

3. EARLY G₁

Despite its temporal distance from S phase, many events that occur in early G₁ influence S phase progression. These early G₁ events may ultimately impact when a segment of DNA is replicated in S phase, help determine the length of G₁, or ensure that proteins required for S phase do not accumulate prematurely. As described briefly in the introduction, G₁ progression requires the function of two heterodimeric kinases, cyclin D/CDK4 and cyclin E/CDK2. Cyclin E/CDK2 is also essential to initiate DNA synthesis in S phase. All forms of cell cycle driving CDKs are inactivated at mitosis or kept inactive during G₀ through multiple mechanisms targeting both the cyclin and CDK subunits individually and the complexes themselves. These inactivating mechanisms are reversed in early G₁.

3.1 Cyclin D/CDK4 in early G₁

During G₀ and also between anaphase in mitosis and mid G₁ in actively proliferating cells, CDK activity is minimal due to low transcriptional activation and targeted destruction of cyclins. Immediately after cell division, cyclin D protein levels are very low due to a combination of inactive *cyclin d* gene expression and active cyclin D protein degradation. Unlike cyclins E, A, or B, the protein levels of cyclin D do not rise and fall in a cell cycle-dependent fashion, but instead are regulated by the presence of growth factors in the environment (Reviewed in (Choi and Anders 2014)). There are three primary mechanisms of cyclin D accumulation. The first is transcriptional activation by transcription factors controlled by mitogenic signaling pathways (*e.g.* c-Jun, c-Fos, or MYC) activated by cytokines (*e.g.* NF- κ B) or other signaling factors (*e.g.* Notch or CREB). The second method of cyclin D accumulation is through the PI3K-AKT-mTOR- S6K1 signaling pathway, which downregulates glycogen synthase kinase β and upregulates cyclin D translation (Diehl et al. 1998) (Koziczak and Hynes 2004; Fornoni et al. 2008; Katoh and Katoh 2006). The third method is stabilization and nuclear localization of cyclin D, which is regulated by phosphorylation of cyclin D itself. Cyclin D phosphorylation by GSK3 β creates a phospho-dependent binding site for an E3 ubiquitin ligase complex, SCF (Alt et al. 2000; Barbash et al. 2009; Diehl et al. 1998). SCF family enzymes are multisubunit ubiquitylases that use a variety of substrate targeting subunits and mechanisms. Substrate binding stimulates polyubiquitylation which typically targets proteins to a degradative protein complex called the 26S proteasome. Since GSK3 β is inhibited in G₁, cyclin D1 is not phosphorylated and therefore not targeted for ubiquitylation and destruction; hence cyclin D1 accumulates in G₁.

Many different cofactors associate with cyclin D/CDK4 complexes to regulate kinase activity either positively or negatively depending on context. These cofactors include the Cip/Kip family of proteins (p21, p27, and p57). Although Cip/Kip proteins are strictly inhibitors of cyclin E/CDK2 and mitotic CDKs, they can act as positive regulators of cyclin D/CDK4 (Bao et al. 2006; Cheng et al. 1999; LaBaer et al. 1997; Chen et al. 1995; Fotadar et al. 1996; Luo et al. 1995). Specifically, phosphorylated forms of these proteins play integral roles in cyclin D/CDK4 assembly, activation, and nuclear localization. The Cip/Kip family of proteins is required for cyclin D/CDK4 complex assembly, which is a precursor for activation. After the cyclin D-CDK4- Cip/Kip tertiary complex is formed, a substrate-blocking loop in CDK4 is exposed for an essential activating phosphorylation by the CDK7/

cyclin H/MAT1 complex, known as CAK (Schachter et al. 2013). This phosphorylation event physically relocates the T-loop from the active site of the CDK subunit, allowing substrates access to the active kinase site. Finally, cyclin D/CDK4 complexes do not have nuclear localization sequences themselves, whereas Cip/Kip proteins have bipartite nuclear localization sequences. Thus, the association between cyclin D/CDK 4 complexes and Cip/Kip proteins promotes the assembly and nuclear localization of the cyclin D/CDK4 complex, and therefore access to nuclear substrates, most notably RB.

3.2 Pocket proteins in early G₁

Perhaps the most consequential event leading to the transition from G₁ into S phase is passing through the restriction point, a cell cycle checkpoint between early G₁ and S (Pardee 1974; Blagosklonny and Pardee 2002). Cells that have not reached the restriction point can either progress through the cell cycle or can transition into quiescence, G₀. Cells that have progressed past the restriction point are destined to initiate DNA replication and complete the cell cycle. Progression past the restriction point is generally correlated with the phosphorylation state of RB. RB phosphorylation is dependent on cyclin dependent kinases, in particular cyclin D/CDK4 in early G₁ and cyclin E/CDK2 in late G₁. Prior to the restriction point, RB is not fully phosphorylated and binds activating E2F transcription factors (E2F1-3) to prevent activated E2F target gene expression. Once RB is fully phosphorylated, it releases E2F, allowing activated transcription of most of the genes that encode DNA replication proteins.

RB activity effectively represses the transcription of E2F target genes, particularly those necessary for DNA replication, during early G₁ and prevents cells from prematurely passing the restriction point and irreversibly committing to S phase entry. The mechanism of repression is primarily through local chromatin changes at E2F-dependent promoters. RB recruits HDAC1 or HDAC2, two histone deacetylases that repress gene transcription by maintaining a closed chromatin state at the promoters of E2F target genes (Brehm et al. 1998; Takaki et al. 2004). RB is also linked to the mSin-HDAC complex (containing RBAP46, RBAP48, SAP18, and SAP45) via the RBP1 and SAP30 linker proteins (Figure 4) (Suryadinata et al. 2011). The ultimate effect of these interactions is not only the inability of E2F to stimulate transcription, but also active gene repression.

RB is phosphorylated by the G₁ cyclin/CDK complexes, and these phosphorylation events induce structural changes in RB that, in turn, weaken (but do not fully disrupt) the interaction of RB with E2F. The human retinoblastoma protein has 16 potential CDK phosphorylation sites (S/T-P sites) (Figure 2b). 12 of the 16 RB phosphorylation sites are optimal CDK consensus sites (S/T-P-X-K/R motifs) and can be readily phosphorylated by cyclin/CDK complexes (Ubersax and Ferrell 2007). Each of the 16 phosphorylation sites is also detectably phosphorylated *in vivo* (Brown et al. 1999; Lents et al. 2006; Zarkowska and Mittnacht 1997; Lees et al. 1991; Harbour et al. 1999; Huttlin et al. 2010; Dephoure et al. 2008; Connell-Crowley et al. 1997). Using a combination of *in vitro* kinase assays and two-dimensional tryptic phosphopeptide maps, Zarkowska and Mittnacht found that RB is preferentially phosphorylated by different cyclin/CDK pairs (Zarkowska and Mittnacht 1997). Cyclin A/CDK2 best phosphorylates RB positions T5, S612, S795, and T821. Cyclin

D/CDK4 preferentially phosphorylates T5, S249, T252, T356, T373, S788, S795, S807, S811, and T826. Finally, cyclin E/CDK2 phosphorylates T5, T373, S612, S795, and T821. While some positions are only phosphorylated by a single cyclin/CDK pair (for instance, S811 by Cyclin D/CDK4) many can be phosphorylated by two different pairs or even, like T5, all three pairs (Zarkowska and Mittnacht 1997).

These cyclin/CDK RB phosphorylation site preferences, combined with the temporal regulation of cyclin abundance (Figure 1), suggest a temporal order for when individual sites are likely phosphorylated *in vivo*. In early G₁, RB is phosphorylated by cyclin D/CDK4 at eight sites (S249, T252, T356, S608, S788, S807, S811, and S826) (Zarkowska and Mittnacht 1997). After these initial eight sites are phosphorylated, a next round of phosphorylation occurs via either cyclin D/CDK4 complexes or cyclin E/CDK2 complexes at threonine 373. Finally, cyclin E/CDK2 phosphorylates serine 612 (Zarkowska and Mittnacht 1997). However, to date there has been no biochemical proof that these phosphorylations occur in the temporal order suggested, nor are there biochemical data precisely defining what functionally constitutes hypophosphorylated vs. hyperphosphorylated RB (Narasimha et al. 2014). Using two-dimensional isoelectric focusing (2D IEF), Narasimha *et al.* showed that during early G₁, RB is monophosphorylated by cyclin D/CDK4 at any one of 14 different sites, but there was no evidence for individual RB molecules with more than one phosphate until very late in G₁. Later in G₁, upon upregulation of cyclin E expression and activation of cyclin E/CDK2, the vast majority of RB molecules were hyperphosphorylated on at least 14 sites and no longer bound to E2F (Figure 5). Reducing RB hyperphosphorylation with different concentrations of the CDK2 inhibitor roscovitine, a compound that has little effect on Cyclin D/CDK4, suggested that cyclin E/CDK2 is the principle processive RB kinase that rapidly converts monophosphorylated RB molecules to hyperphosphorylated RB molecules in a single binding step.

While phosphorylation of either S788 or S795 somewhat reduces RB affinity for E2F, simultaneous phosphorylation of both residues is additive. Characterizing the effects of multiple phosphorylation events on RB, Burke *et al.* showed that phosphorylation of S788/S795 destabilizes the interaction of RB and the E2F1-DP1 complex as well as inducing a conformational shift in RB, allowing a disordered linker, RB amino acids 787-816, to bind to the pocket domain. This phosphorylated linker region competes with E2F to bind the pocket domain of RB (Burke et al. 2014). These phosphorylation events weaken the overall interactions between RB and the E2F-DP1 complex as cells progress to late G₁, thus stimulating the production of gene products necessary for DNA replication.

Why is RB phosphorylation so complex (Figure 2b)? For example, monophosphorylation at four sites (S230, S249, T356, or S612) may in fact increase the affinity of RB for E2F1, while phosphorylation at three other sites (T373, S608, or S795) decrease RB-E2F1 affinity (Burke et al. 2010; Burke et al. 2012; Burke et al. 2014; Narasimha et al. 2014). Currently, the biological significance of the various individual phosphorylation sites on RB-interactions affinity is unclear. These different phosphorylation sites may modulate binding affinities for the different members of the activating E2F transcription factor family. Another possibility is that the differences in RB affinity for E2F1 allow for activation of some subset

of E2F1 target genes, but not other subsets. Furthermore, CDK-dependent phosphorylation affects not only RB-E2F binding but also induces conformational changes in RB that disrupt interactions with histone deacetylases and associated proteins (Takaki et al. 2004). The abundance of phospho-sites on RB may also facilitate integration of different signaling pathways and prevent aberrant E2F release and activation.

During early G₁ the other two pocket proteins, p107 and p130, are complexed with E2F4 at promoters contributing to gene repression, though they are less abundant than RB. In situations where RB has been lost, such as in an *Rb* null mouse, transcriptional repression and G₁ restraint is partially covered by the action of p130 and p107 (Cobrinik et al. 1996). Because RB phosphorylation and the subsequent E2F release stimulates progression from G₁ into S phase, this process is susceptible to oncogenic perturbation. Interestingly, there are only a few cancer types, such as retinoblastoma, osteosarcoma, and small cell lung cancer, which are associated with inactivating *RB* mutations, possibly reflecting the partial redundancy with p107 and p130 (Wikenheiser-Brokamp 2006; Kaye and Harbour 2004). In contrast, many tumors have mutations in upstream regulators of pocket proteins that render them constitutively inactive in cell cycle control (Wikenheiser-Brokamp 2006; Paternot et al. 2010).

3.3 APC^{CDH1}-mediated protein degradation in early G₁

In addition to the complex network of transcriptional control described above, post-transcriptional regulation ensures that early G₁ is free from both mitotic and S phase activities, to provide essential protected time to properly prepare for S phase. Prior to the cascade of RB phosphorylation events, both during cell cycle re-entry from G₀ and in early G₁ after mitosis, somatic cells experience a period of low cyclin dependent kinase activity and high phosphatase activity (Figure 1). This period removes mitosis-associated phosphorylations and introduces a cell cycle pause to be receptive to inputs from the environment, such as growth factors. Activities that ensure this low CDK period, which ultimately determines the length of G₁, are the APC/C and protein phosphatases.

The anaphase-promoting complex/cyclosome, APC/C, is a multisubunit E3 ubiquitin ligase named for its essential role in mitotic progression, but APC/C also has critical functions throughout G₁. The substrate targeting subunit of APC/C varies such that substrates in anaphase are recognized by the CDC20 targeting subunit whereas substrates in G₁ are recognized by the CDH1 subunit (Manchado et al. 2010). As cells exit mitosis, APC/C^{CDC20} targets the mitotic cyclins for degradation via the 26S proteasome, reducing the cellular kinase activity and allowing the activation of mitotic exit phosphatases, such as PP1 or PP2A (Wu et al. 2009; van Leuken et al. 2008). The reduced mitotic kinase activity promotes the assembly of APC/C^{CDH1}. Like APC/C^{CDC20}, the newly formed APC/C^{CDH1} targets mitotic cyclins and kinases for degradation. APC/C^{CDH1} also targets CDC20 for destruction, allowing for the complete switch from APC/C^{CDC20} to APC/C^{CDH1}. Once cells have progressed through telophase and have completed the division into two daughter cells, APC/C^{CDH1} continues to target mitotic proteins for destruction throughout G₁. One of the mitotic cyclins is cyclin A, which is active from mid-S phase until early mitosis (Figure 1),

and since cyclin A/CDK2 can trigger DNA synthesis, its removal before and during G₁ helps prevent premature DNA replication (Coverley et al. 2002; Erlandsson et al. 2000).

Alongside the elimination of cyclin/CDK activity, phosphatases reverse mitotic (or G₀) phosphorylations. During mitosis, protein phosphatase 1 (PP1) is inactivated by CDK-mediated phosphorylation at threonine 320. In anaphase, PP1 removes the inhibitory phosphorylation on threonine 320 by auto-dephosphorylation as CDK activity drops. PP1 also inactivates its own inhibitor, inhibitor-1, a protein uniquely expressed in G₂/M (Wu et al. 2009). Activated PP1 then removes the phosphates from hyperphosphorylated RB until it is in the hypophosphorylated state which re-establishes RB-mediated repression. This dephosphorylation activity lasts from anaphase to mid-G₁ when the kinase activity towards substrates outpaces the phosphatase. Another phosphatase, the PP2A holoenzyme, also dephosphorylates the pocket proteins. The PP2A holoenzyme can also dephosphorylate the three pocket proteins after a variety of signals that, if ignored, can result in genome instability. These signals include oxidative stress, UV radiation, and DNA damage (Avni et al. 2003; Cicchillitti et al. 2003; Magenta et al. 2008; Voorhoeve et al. 1999). The dephosphorylation of RB delays activating E2F activity, allowing time to recover from the genomic insult.

In early G₁, APC/C^{CDH1} also targets the substrate receptor of the SCF E3 ubiquitin ligase, SKP2 (Bassermann et al. 2014; Bashir et al. 2004). The resulting loss of SCF activity leads to accumulation of SCF substrates including the cyclin dependent kinase inhibitors p21^{Cip1} and p27^{Kip1}. High levels of p21 and p27 help to maintain low CDK activity in early G₁. In human cells and mouse models lacking the CDH1 substrate targeting subunit for APC/C, the exit from mitosis occurs normally but cells begin G₁ with aberrantly high CDK activity. High CDK activity shortens G₁, *i.e.*, causes premature entry into S phase. Early entry into S phase, in turn, can result in increased endogenous DNA damage, presumably from inadequate G₁ preparation or an uncoordinated G₁/S transition.

APC/C^{CDH1} substrates include other proteins involved in controlling the length of G₁. ETS2, one such target, induces cyclin D expression. APC/C^{CDH1} directed destruction of ETS2 delays *cyclin D* gene expression and prolongs G₁. Two of the repressor E2F transcription factors, E2F7 and E2F8, are also targets of APC/C^{CDH1} and repress transcription of the CDH1 inhibitor, the EMI1 pseudosubstrate. The APC/C^{CDH1} directed destruction of these two E2F repressors and subsequent de-repression of EMI1 contributes to CDH1 inactivation in late G₁. APC/C^{CDH1} also targets CDC6 and ORC1, two proteins involved in origin licensing for DNA replication which itself contributes to CDK2 activation in late G₁ (described below). Altogether, APC/C targets a cohort of proteins in early G₁ including both cyclins and CDK regulators, to ultimately set the timing of the onset of DNA replication.

3.4 Nuclear and chromatin architecture changes in G₁

Transcriptional and post-transcriptional control are not the sole determinants of DNA replication parameters that are established during G₁. One special aspect of DNA replication in eukaryotes is the phenomenon of replication timing. Genomic DNA replication does not initiate simultaneously at all sites at the beginning of S phase but rather some loci are replicated early in S phase and others much later in S phase. Much of this replication timing

pattern is, in fact, established during early G₁. Local chromatin structure is an important contributor to replication timing and progress towards understanding this phenomenon is described in parts VI and VIII of this book.

As a test of when replication timing is determined, Lu, Li *et al.* (2010) introduced nuclei isolated from cells in specific cell cycle phases (*e.g.* early or late G₁) into a cell-free DNA replication initiation system that triggers initiation immediately. They found that nuclei from cells in mid- to late G₁ displayed a pattern of temporal regulation of DNA in replication domains similar to their pattern in intact cells, but nuclei from cells in early G₁ or G₂ lacked the temporal replication (Lu *et al.* 2010). They proposed that DNA replication timing is determined in a ~1 hour window during early G₁, a model strengthened by more recent live cell imaging analysis by Wilson *et al.*, (2016).

Wilson *et al.* (2016), used live cell imaging to show that as cells differentiate, the replication domains coalesce into larger regions of dense chromatin that are replicated in a coordinated fashion (Wilson *et al.* 2016; Dixon *et al.* 2015). It is well known that the nucleus has a three dimensional architecture and that this architecture is modified as animal cells progress through development (Hiratani *et al.* 2010). The interior of the nucleus generally contains highly- transcribed genes that are replicated early in S phase, while portions of chromosomes that are closer to the nuclear periphery are likely to be transcriptionally silent and replicated later in S phase. For example, in mammalian females, the inactivated X chromosome is compacted and localized to the nuclear periphery and is replicated much later in S phase than the active X chromosome (Lyon 1961; Morishima *et al.* 1962; Barr and Bertram 1949). This architectural compaction, DNA replication delay, and localization to the periphery also applies to pluripotency genes when they are silenced during differentiation (Meshorer *et al.* 2006; Hiratani *et al.* 2010). Thus both chromatin and nuclear architecture that are established in early G₁ influence subsequent S phase events.

4. LATE G₁

The transition from “early G₁” to “late G₁” is not readily defined by any discrete molecular markers; nonetheless, a collection of molecular events are typically confined to a period of G₁ just before the onset of S phase. Many of these late G₁ events depend on successful completion of early G₁ steps, such as initial RB phosphorylation to begin the accumulation of essential DNA replication proteins. In particular, a burst of cyclin E/CDK2 activity coincides with both induced E2F-dependent gene expression and with DNA replication origin licensing in late G₁. A sequence of events in late G₁ reflects passage through the restriction point and leads inexorably to the initiation of DNA synthesis in S phase. Disruptions to this sequence, particularly after cells are already committed to S phase initiation, increase the likelihood of replication failure or genome instability. Recent advances in live cell imaging have begun to elucidate stereotypical orders of molecular events in a window of 1-2 hours (in somatic human cells) at the G₁/S transition; these tools will ultimately support investigations of how that order promotes normal S phase completion (Cappell *et al.* 2016; Coleman *et al.* 2015).

4.1 Transcriptional activation via E2F

As cells pass the restriction point, commit to S phase, and therefore commit to progression through the cell cycle, they must accumulate the proteins required for DNA replication. Full release of E2F does not occur until late G₁ upon full RB hyperphosphorylation and is one indicator that the requirements of the restriction point have been fulfilled. Once fully released by RB hyperphosphorylation, activating E2F family members stimulate transcription of key genes involved in DNA replication and S phase onset (Grant et al. 2013; Muller et al. 2016; Helin 1998; Johnson et al. 1994; Slansky and Farnham 1996). Additionally, the dissociation of RB from the activating family of E2F transcription factors allows for the association of other transcriptional coactivators with E2F, such as p300 or CBP (Ait-Si-Ali et al. 2000; Ferreira et al. 1998).

E2F1 specifically is part of two positive feedforward loops that help to drive the cell into S phase with little possibility of turning back to a G₁-like state. The first of these loops involves *cyclin E* and was described in the previous section: cyclin E/CDK2-mediated RB hyperphosphorylation stimulates E2F1-mediated transcription of the *cyclin E* gene to further increase cyclin E/CDK2. The second feedforward loop is autoregulatory: E2F1 binds to and activates its own promoter, driving expression of more E2F1. Additionally, E2F1 is part of a negative feedback loop by activating transcription of the gene encoding the E3 ubiquitin ligase subunit SKP2. SKP2 is a substrate targeting subunit for the SCF (SKP1-Cullin-F-box) class of E3 ubiquitin ligases and is active from late G₁ through G₂ (Marti et al. 1999). SCF^{SKP2} is responsible not only for the eventual ubiquitin-mediated degradation of E2F1 in S phase, but also for ubiquitylating the cyclin E/CDK2 inhibitor, p27. This p27 destruction leads to higher cyclin E/CDK2 activity, and yet more robust RB hyperphosphorylation creating another feedforward loop (Sheaff et al. 1997; Yung et al. 2007). The result of these reinforcing relationships is maximal cyclin E/CDK2 activity and E2F protein levels at the G₁-to-S phase transition.

The E2F-mediated induction of genes involved in the positive feedback loops occurs before the transcriptional activation of genes involved in the negative feedback loops (Grant et al. 2013; Whitfield et al. 2002; Eser et al. 2011). This temporal arrangement provides time for cells to accumulate sufficient DNA replication proteins while still limiting that time so replication protein levels do not increase to unregulatable levels. The offset in expression timing also prevents G₁-S phase genes from being expressed in late S phase or during G₂ when they are not needed or could be disruptive.

Nearly every protein required for DNA replication preparation in G₁ or DNA synthesis in S phase is the product of an E2F-regulated gene. Using a combination of microarray expression analysis of cell cycle-regulated transcription in synchronous cells and genome-wide chromatin precipitation followed by high-throughput sequencing (ChIP-seq), E2F1 has been detected at a majority of genes involved in DNA replication in cancer cells and at the promoters of over 2500 genes across the genome (Grant et al. 2013). Since E2F transcription factors regulate the expression of many genes and are active during late G₁ and S phase, it is not surprising that they regulate a large number of genes involved in all stages of DNA replication. E2F-dependent genes encoding proteins critical for DNA replication include DNA polymerase Delta 3 (*POLD3*), replication factor C subunit 4 (*RFC4*), proliferating cell

nuclear antigen (*PCNA*), and the histone mRNA stem loop binding protein (*SLBP*). RFC4 and PCNA are accessory proteins for leading strand DNA replication by Polymerase Delta. SLBP binds to stem loops at the 3' end of histone RNAs; this binding stabilizes the RNAs and promotes histone protein production (Townley- Tilson et al. 2006; Wang et al. 1996; Whitfield et al. 2000). Many genes whose products are involved nucleotide metabolism including thymidine kinase 1 (*TK1*), thymidylate synthetase (*TYMS*), dihydrofolate reductase (*DHFR*), and ribonucleotide reductase subunit M2 (*RRM2*) (DeGregori et al. 1995; DeGregori et al. 1997). Genes whose products are essential for origin licensing that are regulated by E2F transcription factors include *CDC6*, *CDT1*, *ORC1*, and *MCM2-7* (Yoshida and Inoue 2004b; Ohtani et al. 1999; Tsuruga et al. 1997; Leone et al. 1998).

4.2 Origin licensing

Creating a single exact copy of the genome is, of course, an S phase event and is a critical process for somatic cell viability and genome stability. To replicate the vast quantity of DNA in each eukaryotic cell in a timely fashion, cells initiate DNA replication at thousands of origins of DNA replication located throughout the genome. Eukaryotic origins are not strictly defined by specific DNA sequences and are strongly influenced by local chromatin structure. For example, histone H4 lysine 20 monomethylation shows a strong positive correlation with origin activity (Tardat et al. 2010; Abbas et al. 2013; Jorgensen et al. 2013). Discussions of the molecular features associated with eukaryotic origins, and ongoing efforts to identify molecular determinants of mammalian origins, can be found in Parts II, VII, and VIII and therefore are not addressed here.

Origin licensing is DNA loading of the core component of the replicative DNA helicase, known as the mini-chromosome maintenance complex, or MCM (Bell and Kaguni 2013; Remus and Diffley 2009). MCM complexes are stable heterohexameric ring-shaped complexes of subunits MCM2-MCM7, and their sequences are conserved not only throughout eukarya but also in archaeal species (Bell 2012). The majority of knowledge to date about the mechanism of MCM loading comes from pioneering studies with purified *Saccharomyces cerevisiae* or *Xenopus laevis* proteins to reconstitute the MCM loading reaction (Remus and Diffley 2009; Gillespie et al. 2001), but the strong evolutionary conservation among licensing proteins gives confidence that insights into the functions of licensing components are readily extrapolatable to mammalian licensing systems. Despite this strong functional conservation, the regulation of origin licensing proteins themselves varies among different eukaryotic species. In addition, some mammalian licensing proteins have non-replication functions including roles in transcriptional control, centrosome duplication, CDK regulation, chromosome segregation, and cell division (Kawasaki et al. 2006; Varma et al. 2012; Prasanth et al. 2010; Traisupa et al. 1990; Bacon and Grantham 1989; Hemerly et al. 2009; Prasanth et al. 2004; Prasanth et al. 2002; Tachibana and Nigg 2006; Hossain and Stillman 2012; Demols and Schrooyen 2003).

During G₁, MCM complexes are loaded such that double-stranded DNA passes through their central channels (Figure 6) (Bell and Botchan 2013; Gambus et al. 2011; Remus and Diffley 2009; Evrin et al. 2009); a detailed discussion of MCM structure can be found in the chapter by B. Tye. Functionally licensed origins have at least two MCM complexes loaded in

anticipation of their lead roles at bidirectional replication forks in S phase (Sun et al. 2014; Li et al. 2015). MCM complexes are loaded to create these double hexamers by the concerted action of three essential loading factors: the heterohexameric origin recognition complex (ORC), the CDC6 protein, and the CDT1 protein (Siddiqui et al. 2013; Nishitani and Lygerou 2004). ORC and CDC6 are each members of the AAA+ family of ATPases (Duncker et al. 2009; Lee and Bell 2000; Speck et al. 2005; Clarey et al. 2006), and bear some sequence similarity to replication factor C (RFC) which is responsible for the DNA loading of another ring-shaped complex, the PCNA sliding clamp (Schepers and Diffley 2001). By analogy to RFC function, ORC and CDC6 are thought to bind and open MCM rings to allow double-stranded DNA to pass into their central channels (Samel et al. 2014; Bochman and Schwacha 2008). Non-productive MCM hexamer loading attempts are removed through the ATPase activity of CDC6 (Cocker et al. 1996; Frigola et al. 2013). The CDT1 protein binds MCM and is required for MCM loading, but CDT1 has no known enzymatic activity (Xouri et al. 2007; Zhang et al. 2010; Jee et al. 2010; Khayrutdinov et al. 2009). At least one role for CDT1 in origin licensing is to recruit MCM complexes to ORC and CDC6, which are resident at origins in G₁ (Xouri et al. 2007).

Based on the reconstituted yeast MCM loading reaction, once a first MCM hexamer is properly loaded onto DNA, CDT1 and CDC6 are released and can presumably recruit additional MCM complexes (Figure 6) (Randell et al. 2006; Chen et al. 2007; Duzdevich et al. 2015; Ticaú et al. 2015). This dynamic behavior of CDT1 relative to ORC implies that CDT1 and CDC6 can readily participate in MCM loading at many origins over the course of G₁ (Xouri et al. 2007). Perhaps for this reason, human CDT1 in particular is the most highly-regulated of the origin licensing proteins and this regulation is critical to restrict licensing to only G₁. CDT1 is degraded during S phase, bound by the geminin inhibitor protein during S phase and G₂, and inhibited by phosphorylation during G₂ and M phase (Wohlschlegel et al. 2000; Xouri et al. 2007; Chandrasekaran et al. 2011; Coulombe et al. 2013; Nishitani et al. 2006). A second molecule of CDC6 assists with loading a second MCM hexamer. During this process, the second molecules of CDC6 and CDT1 are removed from the complex, completing origin licensing (Ticaú et al. 2015; Duzdevich et al. 2015).

An important feature of origin licensing is that once MCM complexes are loaded, none of the loading factors are required for MCM to maintain stable DNA associations or replication competence (Rowles et al. 1999; Yeeles et al. 2015; Tsakraklides and Bell 2010; Ticaú et al. 2015; Bowers et al. 2004). Each replication origin should fire, initiating DNA synthesis at most once per S phase. The complex mechanisms and regulations for converting a licensed origin to an active replication fork are described in several chapters of this book, including Parts III and IX. If an origin fires twice during a single cell cycle, it results in DNA re-replication, a form of endogenous DNA damage that increases genome instability (Green and Li 2005; Vaziri et al. 2003; Davidson et al. 2006; Arentson et al. 2002; Liontos et al. 2007). Origin licensing can only occur during a period beginning in late mitosis (telophase) throughout all of G₁ until the onset of S phase; telophase and G₁ are the only times when all licensing proteins are abundant and active. At all other times from the beginning of S phase until the end of mitosis, origin licensing is prevented by an extensive series of overlapping molecular mechanisms that inactivate or destroy MCM loading proteins. More extensive

descriptions of the mechanisms preventing re-replication after G₁ may be found in several other chapters, such as those contributed Li, by Abbas and Dutta, and by Teixeira and Reed.

4.3 Origin licensing regulation during G₁

Origin licensing is blocked during G₂ and mitosis by several mechanisms; one of these involves tight binding of the licensing inhibitor, geminin, to CDT1 (Wohlschlegel et al. 2000). Geminin accumulates throughout S phase and reaches peak levels in late G₂. Geminin binding to CDT1 interferes with the CDT1-MCM interaction, and since that interaction is essential for origin licensing, high levels of geminin block origin licensing during G₂ and mitosis (Cook et al. 2004; Yanagi et al. 2002). Geminin is subject to ubiquitin-mediated proteolysis during anaphase, so its reduction by the beginning of G₁ releases CDT1 to once again bind MCM (McGarry and Kirschner 1998). CDT1 is also hyperphosphorylated during G₂ and M phase, and at least some of these phosphorylations interfere with CDT1 function, though the mechanism of that interference is not yet known. CDT1 is dephosphorylated in early G₁, which presumably increases CDT1 licensing activity (Chandrasekaran et al. 2011; Coulombe et al. 2013).

Each protein in the licensing system is the product of an E2F-regulated gene. As a consequence, each of the genes for licensing proteins is induced during cell cycle re-entry from quiescence, and at least some of them are also subject to cell cycle-dependent fluctuations during active proliferation (Ohtani et al. 1996; DeGregori et al. 1995; Yoshida and Inoue 2004a; Yan et al. 1998). For example, five of the six subunits of the origin recognition complex (*ORC2-6*) are constitutively expressed throughout the cell cycle, while *ORC1* is cell cycle-regulated with peak expression at G₁/S (Whitfield et al. 2002; Grant et al. 2013).

In addition to the genes encoding proteins that act at origins, E2F-dependent *cyclin E* expression impacts origin licensing during late G₁. Like geminin, the CDC6 protein is targeted for degradation during mitosis. Both geminin and CDC6 are substrates of the APC/C E3 ubiquitin ligase, which is activated in anaphase and remains active until the onset of S phase (McGarry and Kirschner 1998; Petersen et al. 2000). CDC6 escapes APC/C-mediated degradation in late G₁ because cyclin E/CDK2 phosphorylates an amino acid in CDC6 near the CDH1 binding site (required for APC/C binding), and this phosphorylation blocks ubiquitylation (Mailand and Diffley 2005). As a result, cyclin E accumulation and cyclin E/CDK2 activation in late G₁ stabilizes CDC6; the subsequent increase in CDC6 stimulates MCM loading. Cyclin E has also been ascribed non-kinase roles in origin licensing, though the mechanism of these roles remain to be determined (Geng et al. 2007). The relationship between CDK activity and licensing is complex, however, because very high levels of cyclin E/CDK2 activity can block origin licensing through multiple routes that are independent of CDC6 stabilization. These routes include phosphorylation-stimulated CDT1 and ORC1 degradation, and phosphorylation-mediated inhibition of interactions among licensing proteins or between licensing proteins and chromatin (Wheeler et al. 2008; Ekholm-Reed et al. 2004; Mendez and Stillman 2000; Takeda et al. 2005). This licensing inhibitory CDK function helps block origin re-licensing and the consequent genome instability.

4.4 The origin licensing checkpoint

Due to both the enormity of the task and the potential dire consequences of even small errors, there are checks and balances built into replication control, particularly at the transition from G₁ to S phase. Mechanisms that prevent origin re-licensing during S phase set up a separate challenge when considering the need for complete genome duplication. Entering S phase with too few licensed origins to fully duplicate each chromosome results in under-replication which, like re-replication, is also a form of endogenous DNA damage. Reducing the number of licensed origins in G₁ can lead to sections of unreplicated DNA segregating during mitosis which then require DNA repair in the subsequent G₁ (Moreno et al. 2016). In addition, a destabilizing mutation in an MCM subunit causes reduced origin licensing, chromosomal instability and the development of cancer in mice (Shima et al. 2007; Pruitt et al. 2007). Moreover, replication forks can stall if they encounter bulky lesions or interstrand crosslinks, but as long as a fork converges from the other side of the lesion, repair and replication can be completed (Raschle et al. 2008; Moreno et al. 2016). In response to fork stalling, nearby licensed origins are activated to generate such converging forks, but those origins must have been licensed in the previous G₁ (Woodward et al. 2006; Ge et al. 2007; Ge and Blow 2010).

The tight regulation of licensing means that there is no opportunity to license additional origins after G₁, but cells typically license many more origins than are strictly required to accommodate replication fork stalling and to ensure complete replication. Moreover, individual origins can be loaded with more than one MCM double hexamer. At least *in vitro*, loaded MCM complexes can slide away from their initial loading site, freeing origins to receive additional MCM complexes (Remus and Diffley 2009; Gros et al. 2015; Evrin et al. 2009). As long as the MCM complexes remain DNA-loaded as double hexamers, they can be activated in S phase with no need for ORC, CDC6, or CDT1. The additional licensed origins may never be utilized during S phase, but these dormant origins can be activated if needed.

Though it is imperative that enough origins are licensed to support complete replication before S phase begins, it is not clear how cells couple the completion of origin licensing to the timing of S phase onset. In the early years of origin licensing investigations, the consequences of licensing failure were evident, but the existence of an origin licensing checkpoint that can prevent S phase onset before some critical threshold of licensing was reached and sensed was questioned. Budding and fission yeasts with null alleles in essential licensing components do not execute a cell cycle arrest in G₁, but rather proceed through cell division without replication (Piatti et al. 1995; Kelly et al. 1993; Hofmann and Beach 1994). Depleting origin licensing proteins by RNAi in the most commonly used cancer-derived human cell lines results in the predicted DNA synthesis defects, but no G₁ arrest (Nevis et al. 2009; Shreeram et al. 2002). These early results argued against any origin licensing sensing mechanism.

Subsequently, however, several groups found that depleting origin licensing proteins in non-transformed human cells did indeed cause delayed S phase onset. The delay could only be detected in untransformed cell lines, suggesting that growth control pathways disrupted in cancer-derived cell lines are normally required to link origin licensing status in G₁ to S

phase initiation (Nevis et al. 2009; Shreeram et al. 2002; Teer et al. 2006). In each of these studies, reducing origin licensing caused defects in the activation of cyclin dependent kinases, especially cyclin E/CDK2.

Cyclin E/CDK2 is a major driver in the transition from G₁ into S phase; constitutive or ectopic expression of cyclin E leads to a shortened G₁ (Resnitzky and Reed 1995) (reviewed in (Clurman et al. 1996). CDK activity is required for origin firing, and early S phase origin firing is driven by CDK2 complexes with cyclin E or cyclin A (Figure 1); chapters on replication initiation describe the molecular mechanisms of replication initiation. Delayed CDK2 activation from impaired origin licensing consequently delays origin firing, and by definition, S phase entry. No single molecular mechanism to link origin licensing to CDK activity has yet emerged. In different studies, elevated CDK inhibitor proteins, CDK localization or phosphorylation, or expression of G₁ cyclins were each implicated in the S phase delay caused by insufficient origin licensing (Nevis et al. 2009; Shreeram et al. 2002; Liu et al. 2009; Teer et al. 2006; Lunn et al. 2010). It may be that each of these mechanisms can operate in all non-transformed cells, but different cell types are more or less dependent on specific ones.

4.5 APC^{CDH1} inactivation and S phase entry

The CDH1 substrate targeting subunit of APC/C must be degraded for proper entry into S phase to allow full CDK activation and cyclin A accumulation. There are three main mechanisms for inactivating APC/C^{CDH1}. The first mechanism is the E2F-dependent accumulation of the APC/C^{CDH1} pseudosubstrate early mitotic inhibitor-1 (EMI1). EMI1 binds to CDH1 complexed with APC/C but is not degraded, thus acting as a competitive inhibitor. Once EMI1 binds APC/C^{CDH1}, an irreversible cascade towards APC/C inactivation is triggered (Cappell et al. 2016). The EMI1-driven partial reduction in APC/C^{CDH1} activity stabilizes cyclin A. Cyclin A/CDK2 phosphorylates CDH1, preventing it from binding APC/C. Cyclin A/CDK2 phosphorylates CDH1 at four different amino acids (S40, S151, S163, and T121) (Lukas et al. 1999). The phosphorylation of serine 40 and threonine 121 create two binding domains for the protein kinase, PLK1. PLK1 can then bind and phosphorylate CDH1 at serines 138 and 146 (Fukushima et al. 2013). Phosphorylated CDH1 molecules are then targeted for degradation by the SCF complex (Figure 7) (Fukushima et al. 2013).

Cells that lack CDH1 enter S phase early but also experience increased rates of DNA damage. This increased damage could be from insufficient origin licensing if origin firing begins before chromosomes are fully licensed (Ayuda-Duran et al. 2014). In addition CDH1-deficient cells show enhanced mutagenesis. This increased mutation rate could be due to the accumulation of a pair of proteins involved in dTTP formation: thymidine kinase 1 (TK1) and thymidylate kinase (TMPK) (Ke et al. 2007; Ke et al. 2005). The over-accumulation of these two proteins leads to an imbalance in the dNTP pool. This imbalance then leads to an increased rate of dNTP misincorporation, reducing the fidelity of DNA replication and increasing the mutation rate (Ke et al. 2005). Conversely, human osteosarcoma cells that overexpress CDH1 using inducible expression have a delayed S phase entry as well as a

slower rate of DNA replication (Sorensen et al. 2000). Thus, the proper regulation of CDH1 is important for proper cell cycle progression and S phase entry.

5. CONCLUSION

Successful completion of S phase relies on proper progression through G₁. In this chapter we have discussed some of the many different G₁ events and checkpoints that contribute to the proper initiation and completion of DNA replication including quiescence, the restriction point, origin licensing, and cyclin dependent kinase regulation. Many of the topics covered in this chapter are regulated to occur normally in a stereotypical order and were initially studied using assays of bulk cell populations such as immunoblots, microarray analysis, or ChIP-seq. As technology progresses, researchers will be able to analyze cell cycle kinetics of individual cells using single cell assays such as single cell RNA-seq or high content live cell imaging with cell cycle biosensors (Kaida et al. 2011; Pauklin and Vallier 2013; Sugiyama et al. 2009; Wilson et al. 2016; Cappell et al. 2016; Spencer et al. 2013; Coleman et al. 2015; Purvis et al. 2012). These next generation technologies to study cell cycle progression with timescales of mere minutes have begun to reveal new timelines of G₁ progression (Cappell et al. 2016; Spencer et al. 2013; Coleman et al. 2015).

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Figure 1. Activities of cell cycle dependent kinases and phosphatases during G₁ and G₀
Darker shading indicates more activity. Dashed lines indicate entry and exit from G₀.

Figure 2. E2F and RB proteins

A. A diagram of the motifs of the transcription factor E2F1 as an example activator E2F. DBD, DNA binding domain; DP1, DP binding domain; RB, RB binding domain. **B.** RB phosphorylation sites. Blue indicates increased possible binding affinity to E2F transcription factors; red indicates decreased affinity and black indicates no change in affinity to E2F transcription factors. The dashed lines indicate where RB binds E2F1.

Figure 3. Activities of the pocket proteins RB, p107, and p130, and activating E2F transcription factors during G₁ and G₀
Darker shading indicates more activity. Dashed lines indicate entry and exit from G₀.

Figure 4. Inactivation of E2F target genes by the RB associated SAP30-mSin3-HDAC complex in G₁

Monophosphorylated RB binds activating E2F transcription factors at promoters of E2F target genes. The SAP30-mSin3-HDAC complex binds to RB and the associated HDACs deacetylate the E2F target gene, repressing transcription. Hyperphosphorylated RB releases E2F and dissociates from the SAP30-mSin3-HDAC complex, relieving the transcriptional repression of E2F target genes.

Figure 5. Inactivation of E2F by monophosphorylation of RB

In early G_1 , E2F is bound and inactivated by mono-phosphorylated RB. Later in G_1 , cyclin E/CDK2 becomes active and hyperphosphorylates RB, releasing E2F which then activates transcription of target genes.

Figure 6. Origin licensing

DNA replication origins are licensed by loading MCM complexes. The first CDT1 associated MCM complex is loaded onto DNA through the action of the DNA bound origin recognition complex (ORC) and CDC6. Once the first MCM complex is successfully loaded by ORC, CDC6 and CDT1, CDC6 and CDT1 dissociate from the DNA bound MCM and ORC complexes. A second CDC6 molecule then associates with the DNA bound complexes. Finally, a second CDT1 associated MCM complex is loaded onto the DNA in a head to head fashion and CDC6 and CDT1 are released.

Figure 7. Modifications and activation of APC/C^{CDH1} from mitosis to the onset of S phase

In late mitosis, CDH1 replaces CDC20 as the APC/C E3 ligase and is active during early G₁. Two APC/C^{CDH1} substrates in early G₁ are CDC20 and the origin licensing protein, CDC6. In late G₁ Cyclin E/CDK2 phosphorylates and protects CDC6. Also in late G₁, the pseudosubstrate, EMI1, binds to CDH1, inactivating the complex. In late G₁ and early S phase, CDH1 is phosphorylated by cyclin E/CDK2 and cyclin A/CDK2, respectively; this phosphorylation dissociates CDH1 from APC/C, thus inactivating the complex. From S phase through mitosis, the E3 ligase CDH1 is polyubiquitinated by the SCF complex and degraded by the 26S proteasome.

Table 1Important G₁ proteins and their functions

FACTOR	FUNCTION
Kinases	
Cyclin D/CDK4	Rb phosphorylation
Cyclin E/CDK2	Rb phosphorylation, initiating DNA synthesis at origins
DYRK1A	DREAM complex assembly, Lin52 phosphorylation
GSK3 β	p130 stabilization and cyclin D destabilization in G ₀
Protein kinase inhibitors	
p21	CDK2 inhibition (cyclin D/CDK4 assembly)
p27	CDK2 inhibition
Phosphatases	
PP1	Rb dephosphorylation
PP2A	Rb dephosphorylation
Transcription factors	
E2F1,2,3	Cell cycle gene activation
E2F4,5,6,7,8	Cell cycle gene repression
DP1	Obligate binding partner of activating E2F1,2,3
Transcriptional co-repressors	
Rb	Activator E2F repression in G ₁
p107	Activator E2F repression in S phase
p130	Cell cycle gene repression in G ₀ (DREAM complex)
HDAC	Histone deacetylase
DREAM complex	Repression in G ₀ (E2F4/DP1/p130 and Lin52-containing MuvB complex)
Ubiquitylation	
APC/C	E3 ubiquitin ligase complex in mitosis, G ₁ , and G ₀ ;
Cdc20	Substrate recruiting subunit of APC/C in mitosis
Cdh1	Substrate recruiting subunit of APC/C in mitosis, G ₁ and G ₀
Emi1	Pseudosubstrate inhibitor of APC/C ^{Cdh1} in G ₁
SCF	E3 ubiquitin ligase complex throughout the cell cycle
Skp2	Substrate recruiting subunit of SCF
26S proteasome	Degradation of ubiquitylated proteins
Origin licensing	
MCM	DNA helicase (Mcm2-Mcm7)
ORC	DNA binding MCM loading enzyme (Orc1-Orc6)
Cdc6	MCM loading enzyme
Cdt1	MCM recruitment and loading
Geminin	Cdt1 inhibitor
Set8 (PR-Set7)	Histone H4 K20 monomethylase