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REVIEW

Living with or without cyclins and cyclin-dependent kinases

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Entry into, progression through, and exit from the G1 phase of the mammalian cell cycle in response to extracellular mitogenic cues are presumed to be governed by cyclin-dependent kinases (Cdks) regulated by the D-type and E-type cyclins. Studies performed over more than a decade have supported the view that these holoenzymes are important, if not required, for these processes. However, recent experiments in which the genes encoding all three D-type cyclins, the two E-type cyclins, cyclin D-dependent Cdk4 and Cdk6, or cyclin E-dependent Cdk2 have been disrupted in the mouse germ line have revealed that much of fetal development occurs normally in their absence. Thus, none of these genes is strictly essential for cell cycle progression. To what extent is the prevailing dogma incorrect, and how can the recent findings be reconciled with past work?

Mammalian G1 cyclins and their associated kinases are regulators of the cell division cycle that integrate information flow from outside the cell to drive G1-phase progression and initiate DNA replication in response to mitogenic signals. The relevant proteins include three D-type cyclins (D1, D2, and D3) that, in different combinations, bind to, and allosterically regulate, one of two Cdk subunits, Cdk4 and Cdk6, as well as the E-type cyclins (E1 and E2), which, in analogous fashion, govern the activity of a single catalytic subunit, Cdk2 (Morgan 1997). Various combinations of D-type cyclins are expressed in different cell types, whereas cyclin E–Cdk2 complexes are expressed ubiquitously.

Both the D- and E-type cyclins, and their associated kinases, had been thought to be necessary and "rate-limiting" for entry into and progression through the G1 phase of the cell cycle. In cultured cells, the enforced overexpression of D-type cyclins can shorten the G1 interval (Jiang et al. 1993; Quelle et al. 1993; Resnitzky et al. 1994); conversely, attenuation of Cdk4 activity by polypeptide inhibitors of the INK4 gene family (Serrano et al. 1993) or by Cdk4-selective drugs (Tetsu and McCormick 2003) results in G1-phase arrest and cell cycle exit. Microinjected antibodies or antisense constructs directed to D-type cyclins can interfere with G1-phase progression, but are without effect during other cell cycle intervals (Baldin et al. 1993; Quelle et al. 1993; Lukas et al. 1994). Cyclin D1 can also override G1-phase checkpoint arrest in response to DNA damage (Pagano et al. 1994; Agami and Bernards 2000) and the unfolded protein response (Brewer et al. 1999). Similarly, cyclin E overexpression can greatly accelerate G1 progression of cultured mammalian cells (Ohtsubo and Roberts 1993; Resnitzky et al. 1994), as well as those in *Drosophila* embryos (Knoblich et al. 1994; Duronio and O'Farrell 1995). In turn, microinjection of antibodies against cyclin E into cultured mammalian cells prevents the transition from quiescence (G0) into S phase (Ohtsubo et al. 1995), and inactivation of the cyclin E gene in *Drosophila* blocks all mitotic cell cycles and endocycles (Knoblich et al. 1994). Likewise, inhibition of Cdk2 activity by dominant-negative, catalytically inactive versions of the kinase (van den Heuvel and Harlow 1993), by microinjected antibodies to Cdk2 (Pagano et al. 1993; Tsai et al. 1993), and by induction of polypeptide inhibitors of the Cip/Kip family in many physiologic settings (Sherr and Roberts 1995) result in G1-phase arrest. Cdk2 is also essential for S phase in *Drosophila* (Knoblich et al. 1994; de Nooij et al. 1996; Lane et al. 1996) and in *Xenopus* (Fang and Newport 1991; Strausfeld et al. 1994). Although these findings strongly supported the argument that the activities of these regulators are universally required in metazoans for cell cycle entry and G1 phase progression, recent work has now challenged that view. Below, we summarize previous thinking in the field, review the latest results of gene inactivation studies in the mouse, and discuss the implications and limitations of the most recent work.

G1 cyclin-dependent kinases: the basic paradigm

Lessons from yeasts, flies, and frogs

Eukaryotic cells in G1 phase express a relatively low level of net Cdk activity that rises progressively as cells advance through their division cycle, peaking and then rapidly decaying during mitosis (Morgan 1997). The greatly reduced level of Cdk activity during G1 phase is required for the formation of preinitiation complexes at chromosomal origins of DNA replication, after which

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progression into and through S phase and entry into mitosis can only proceed in the presence of more robust enzyme activity.

In yeast, which express only one relevant Cdk subunit (now generally designated Cdk1, equivalent to p34^{Cdc28} in *Saccharomyces cerevisiae* and p34^{Cdc2} in *Schizosaccharomyces pombe*), increases in Cdk activity during cell cycle progression are determined by the periodic synthesis of different cyclin partners that are differentially expressed during various phases. As early as 1989, it was recognized that two of three G1 cyclins in budding yeast could be deleted without affecting normal cell cycle progression, indicating that the cell cycle machinery either possessed considerable redundancy or was capable of robust compensation for absent components (Richardson et al. 1989). In now classic experiments, the elimination of all the then-known G1 cyclins in fission yeast still permitted their ordered progression through the cell division cycle, implying that a single enzymatic oscillator, composed of a B-type cyclin and Cdk1, may be all that is required (Fisher and Nurse 1996). This led to a quantitative model of cell cycle regulation in which the net level of CDK activity dictated the initiation of specific cell cycle events; low CDK activity would promote S-phase and higher levels mitosis. Despite its appealing simplicity, this model does not appear to be generally applicable to other organisms, such as budding yeast, frogs, and flies, in which the cell cycle cannot operate with just one cyclin. Not only do budding yeasts need G1 (Clns) and B-type (Clbs) cyclins, but also the Clns and Clbs differ among themselves. Cln3 acts upstream of Clns 1 and 2 (Tyers et al. 1993), and elimination of all three Clns is required to induce G1 arrest (Richardson et al. 1989). Clb2 (a mitotic cyclin) cannot substitute for Clb5 (an S-phase cyclin) unless it is overexpressed, and Clb5 cannot trigger mitosis at all (Donaldson 2000; Jacobson et al. 2000). The current thinking is that the major differences between cyclins reflect their timing of expression, their subcellular localization, and other biochemical differences that help to mediate Cdk substrate access and selection, thereby allowing a more refined tuning of the cell cycle oscillator (Roberts 1999; Murray 2004).

As a general rule, the G1 cyclin-dependent kinases deliver mitogenic signals to the core cell cycle machinery by disabling cell cycle inhibitory proteins (Fig. 1; Sherr and Roberts 1995, 1999). For example, budding yeast express a stoichiometric inhibitor of S- and M-phase Cdks, named Sic1, which accumulates as cells exit mitosis and thereby helps to institute the low Cdk activity characteristic of the G1 state (Mendenhall 1993; Schwob et al. 1994). One essential function of G1 cyclins in budding yeast is to phosphorylate Sic1, triggering its ubiquitination and proteasomal degradation, and thus enabling the activation of those cyclin-directed kinases that are required for entry into S phase. Hence, while yeast strains with engineered deletions of all three G1 cyclins are inviable because of an Sic1-imposed G1 arrest, this is reversed and proliferation is restored by concomitant deletion of the Sic1 gene (Schneider et al. 1996; Tyers et al. 1996). A second function of the early expressed G1 cyclin–Cdk complexes in budding yeast is to govern the onset of the S-phase program of gene expression by inactivating a different cell cycle inhibitor, Whi5 (Costanzo et al. 2004). G1/S-phase gene expression is positively regulated by the SBF/MBF transcription factors, which, in turn, are negatively controlled through their interaction with Whi5. G1 cyclin–Cdks phosphorylate Whi5 on multiple sites, which frees SBF/MBF from Whi5 constraint, enabling them to stimulate transcription from G1/S-phase-specific promoters, including the genes encoding S-phase cyclins (Fig. 1).

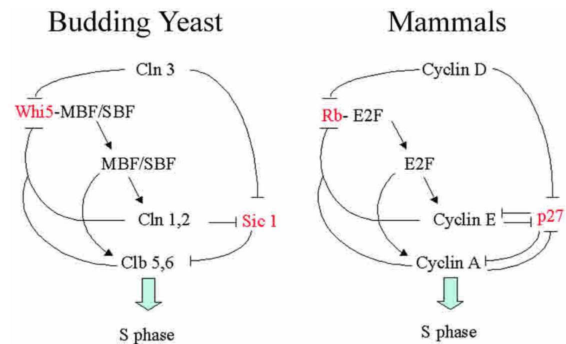

Figure 1. Parallels among the pathways that regulate G1 in budding yeast and mammals. The G1 cyclins (together with their Cdk partners, not shown) promote cell cycle progression by inactivating proteins that are stoichiometric inhibitors of Cdk activity (Sic1 in yeast and p27 in mammals) as well as proteins that inhibit the program of S-phase gene expression (Whi5 in yeast and Rb family members in mammals). Inactivation of these two classes of inhibitory proteins is initiated by the early-expressed G1 cyclins (Cln3 in yeast and D-type cyclins in mammals) and maintained by later-acting G1 cyclins (Clns 1 and 2 in yeast and cyclin Es in mammals). The recruitment of the later-acting cyclins is a feed-forward function that ultimately provides an irreversible switch from a nutrient/mitogen-dependent- to a nutrient/mitogen-independent mode. The pathways converge on the expression and activation of S-phase cyclins (Clbs 5 and 6 in yeast and cyclin A in mammals).

Early frog and fly embryos contain cells exhibiting simple, rapid cycles that consist only of alternating S and M phases. It is not until later in embryogenesis that cell cycles incorporate a G1 phase, which entrains cell cycle progression to the receipt of extracellular cues and allows for coordination of growth and division. "Insertion" of the G1 phase into the cycle during development is assumed to determine when, where, and if cells within various tissues exit the cycle into a quiescent state (G0) during development and, in some circumstances, whether they can re-enter the cycle at some later time in the lifetime of these organisms. In flies, the G1 cyclins include one D-type and one E-type cyclin, which allosterically activate Cdk4 and Cdk2, respectively. Unlike cyclin E and Cdk2, neither cyclin D nor Cdk4 is essential for viability in *Drosophila*, and mutant flies lacking the latter genes develop to adulthood, although they exhibit a loss of fertility and small size (Datar et al. 2000; Meyer et al. 2002). Thus, at least some of the cell cycle proteins

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that contribute to G1-phase regulation are not obligate components of the cell division machinery, but rather couple the activity of the core S- and M-phase oscillator to diverse mitogenic signal transduction pathways.

G1 regulation in mammalian cells

The functions of G1 cyclins and Cdks in mammalian cells closely parallel those in budding yeast, although the mechanisms that link their activation to mitogenic signaling are considerably more elaborate. The D-type cyclins (D1, D2, and D3) and their catalytic partners Cdk4 and Cdk6 act early in G1 phase. Mitogen-induced signal transduction pathways promote the activation of cyclin D–Cdk complexes at many levels, including gene transcription, cyclin D translation and stability, assembly of D cyclins with their Cdk partners, and import of the holoenzymes into the nucleus, where they ultimately phosphorylate their substrates (Sherr and Roberts 1999). Thus, when G0 cells are stimulated by mitogens to reenter the cell division cycle, active cyclin D–Cdk complexes progressively accumulate as cells progress through G1 phase. Usually, this requires de novo synthesis of cyclin D proteins. However, in some quiescent cells, such as memory T cells, cyclin D proteins are present in a latent form that can be activated by mitogenic signals to facilitate cell cycle re-entry (Meyyappan et al. 1998; Veiga-Fernandes and Rocha 2004).

Once DNA replication begins, the activities of cyclin D-dependent kinases are no longer required until cells complete the division cycle and re-enter the following G1 phase (Matsushime et al. 1991). In continuously dividing cells, cyclin D1 exits the cell nucleus during S phase, and its turnover is accelerated throughout this interval (Baldin et al. 1993; Diehl et al. 1998), but Ras-dependent cyclin D1 synthesis and its stabilization in G2 phase allow it to reaccumulate before cells divide (Guo et al. 2002). Hence, when cells are continuously stimulated by mitogens, the second and subsequent cell cycles are shorter than the first. Conversely, withdrawal of extracellular mitogens results in the abrupt discontinuation of cyclin D synthesis, its destabilization, and rapid degradation, leading to a precipitous decay in kinase activity. If this occurs before the G1/S boundary, cells exit the cycle without replicating their DNA, but if it occurs afterward, cells complete mitosis and exit from the cycle in the next G1 interval (Matsushime et al. 1994; Sherr 1994).

Once activated by mitogenic signals, the D cyclins in association with their catalytic subunits Cdk4 and Cdk6 promote cell division by inactivating two classes of cell cycle inhibitors: negative regulators of S-phase gene expression and a class of stoichiometric Cdk inhibitory proteins. These functions of the D cyclins in mammalian cells appear to be directly analogous to those of the G1 cyclins in budding yeast (Fig. 1). The cyclin D-dependent kinases are notoriously fastidious in substrate selection, and until this year, have only been documented to phosphorylate Rb family members (Rb, p107, and p130), thus helping to inactivate their transcriptional corepressor activities. This process controls an E2F-dependent transcriptional program that activates a battery of genes whose products are required for DNA metabolism and replication (Trimarchi and Lees 2002). Interestingly, very recent work has indicated that cyclin D–Cdk4 complexes also phosphorylate Smad3, negatively regulating the functions of transcriptional complexes that mediate cell growth inhibition by proteins of the TGF-β family (Matsuura et al. 2004). Thus, the cyclin D-dependent kinases now appear to cancel the activities of at least two families of proteins that negatively regulate cell cycle-dependent gene expression.

Importantly, several lines of evidence indicate that cyclin D–Cdk complexes play a second noncatalytic role in G1 progression by sequestering proteins of the Cip/Kip family, including $p27^{Kip1}$ and $p21^{Cip1}$, two potent inhibitors of Cdk2 (Polyak et al. 1994; Reynisdottir et al. 1995; Sherr and Roberts 1995). Binding of Cip/Kip proteins to cyclin D1–Cdk4 stabilizes the complex and facilitates its nuclear import, without necessarily inhibiting cyclin D-associated kinase activity (LaBaer et al. 1997; Cheng et al. 1999). Conversely, mitogen withdrawal not only leads to the disassembly of the cyclin D-dependent kinases but also mobilizes the latent, cyclin D–Cdk-bound pool of $p27^{Kip1}$, which blocks the activity of cyclin E–Cdk2 and facilitates cell cycle exit (Sherr and Roberts 1995, 1999). Additional experimental support for this "titration model" stems from observations that multiple phenotypic abnormalities associated with cyclin D1 deficiency in mice are rescued by deletion of $p27^{Kip1}$ (Geng et al. 2001; Tong and Pollard 2001). An even more stringent test would be to determine if knocking in an allele of cyclin D1 that assembles with Cdks but is unable to activate their kinase activities, or of kinase-defective Cdk4 itself, would support normal development. As a harbinger of things to come, it was observed that mouse embryo fibroblasts (MEFs) lacking $p27^{Kip1}$ and $p21^{Cip1}$ did not stably express D-type cyclins and had >90% reduction in net cyclin D-dependent kinase activity, but nevertheless proliferated normally (Cheng et al. 1999). This was reminiscent of the relationship between the G1 cyclins and the Cdk inhibitor Sic1 in budding yeast, and it raised the possibility that D-type cyclins might not be essential for cell cycle progression, at least in a setting where Cip/Kip proteins were absent.

Unlike the cyclin D-dependent kinases, the activity of cyclin E–Cdk2 is periodic and maximal at the G1- to S-phase transition (Dulic et al. 1992; Koff et al. 1992). Nonetheless, cyclins E1 and E2 seem to exhibit many of the classical attributes of G1 cyclins: specifically, their expression and activity is at least in part mitogen-dependent, and their downstream targets include a subset of the G1 inhibitors that are also targeted by the D-type cyclins—Rb and $p27^{Kip1}$. However, the mechanisms by which cyclin E inactivates these inhibitors are different from those of the cyclin D-dependent kinases, suggesting that their actions may be complementary and not strictly redundant (Fig. 1). Cyclin E–Cdk2 does not sequester $p27^{Kip1}$, but rather phosphorylates it on a single threonine residue (Thr 187) (Sheaff et al. 1997; Vlach

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et al. 1997), providing a recognition motif for an E3 ligase (SCF^{Skp2}) that targets phosphorylated $p27^{Kip1}$ for ubiquitination and its subsequent degradation by the proteasome (Elledge and Harper 1998; Bloom and Pagano 2003). The remaining pool of $p27^{Kip1}$ in proliferating cells remains bound to cyclin D–Cdk complexes in an inactive form. Although phosphorylation of Thr 187 contributes to $p27^{Kip1}$ degradation as cells enter S phase, its turnover earlier in G1 phase is also mediated by a second pathway that does not rely on this process (Malek et al. 2001). This suggests that the balance between $p27^{Kip1}$ inhibition of Cdk2 versus Cdk2-catalyzed destruction of the inhibitor is likely influenced by several factors, which include sequestration by cyclin D–Cdk complexes of $p27^{Kip1}$ and its Thr 187-independent destruction. Cyclin E–Cdk2 also preferentially phosphorylates Rb on different sites from the cyclin D-dependent kinases, and these modifications may differentially impact on the interactions of Rb with E2Fs, histone deacetylases, and other chromatin-remodeling proteins (Harbour and Dean 2000). However, the functions of cyclin E–Cdk2 do not appear to be strictly limited to G1 regulation. Cyclin E–Cdk2 phosphorylates a second set of substrates that are more directly involved in cell duplication; these events affect histone gene expression, centrosome duplication, replication origin licensing, and, possibly, origin firing (Yu and Sicinski 2004). Its timing of expression and broader range of substrates suggest that cyclin E–Cdk2 spans the interface between G1 regulation and the core cell cycle machinery, with properties that overlap both types of cell cycle regulators.

Cyclin E and cyclin A2 are E2F-responsive genes, and their synthesis increases after phosphorylation of Rb by the D-cyclin-dependent kinases. Moreover, the increased activities of cyclin E–Cdk2 and cyclin A–Cdk2 maintain $p27^{Kip1}$ at a low level in S-phase cells through phosphorylation-triggered proteolysis (Malek et al. 2001). Once the E2F transcriptional program is initiated and significant cyclin E- and A2-dependent Cdk2 activity is achieved, cells no longer rely on the cyclin D-dependent kinases nor on persistent mitogenic signals to maintain Rb phosphorylation, and they complete the cell cycle. Therefore, activation of Cdk2 may underlie the mechanism by which the inactivation of two cell cycle inhibitors, Rb and $p27^{Kip1}$, irreversibly switch cells from a mitogen-dependent to mitogen-independent state. In this sense, cyclin E acts downstream of cyclin D, consistent with studies in the mouse in which cyclin E coding sequences, when knocked into the cyclin D1 locus, corrected the effects of cyclin D1 deficiency (Geng et al. 1999).

The abrupt decay of cyclin E–Cdk2 activity in early S phase results from cyclin E degradation. Phosphorylation by both GSK-3β and Cdk2 itself is required to target cyclin E for ubiquitination by the SCF^{Fbw7} E3 ligase, leading to its proteasomal destruction (Clurman et al. 1996; Won and Reed 1996; Welcker et al. 2003). The involvement of GSK-3β, an enzyme that is inhibited by the phosphatidylinositol 3-kinase/AKT signaling pathway, in determining the stability of cyclin E implies that its life history, like that of cyclin D1 (Diehl et al. 1998), can be directly influenced by at least one mitogen-dependent kinase signaling cascade. The AKT kinase controls the subcellular localization of Cip/Kip proteins as well (Liang et al. 2002; Shin et al. 2002; Viglietto et al. 2002), providing yet another avenue for cyclin E–Cdk2 regulation in response to extracellular cues.

Cdk2 regulates cell cycle progression through its interactions with both cyclin E and cyclin A2; thus its functions extend beyond G1 regulation to govern events in S and G2/M as well. (Cyclin A1 expression is restricted to germ cells.) Whereas maximal periodic cyclin E–Cdk2 activity is detected at G1/S, low levels of cyclin A–Cdk2 activity are first detected in late G1 phase, steadily increase as cells begin to replicate their DNA, and do not decline until cyclin A is degraded in early mitosis. Notably, while neither cyclin E1 nor E2 is essential for cell cycle progression during mouse development (Geng et al. 2003; Parisi et al. 2003; see below), cyclin A2 disruption results in early embryonic lethality (Murphy et al. 1997). In S phase, cyclin A–Cdk2 is thought to phosphorylate substrates that start DNA replication from preassembled replication initiation complexes (Krude et al. 1997; Hua and Newport 1998; Coverley et al. 2002) and to be required for coordinating the end of S phase with activation of the mitotic Cdks (Mitra and Enders 2004). Because it appears to orchestrate the numerous activities required for S-phase entry, S-phase progression, and entry into mitosis, Cdk2, in particular, has been viewed as a master regulator whose activities were thought to be essential.

Paring away cyclin D-dependent kinases

A series of papers, the first two of which were published almost a decade ago, have challenged the essential nature of mammalian G1 cyclins and Cdks by reporting that mice with engineered deletions of one or combinations of these cell cycle regulators undergo remarkably normal embryogenesis. If any defects are present in the embryo, they emerge only relatively late in fetal development, and seemingly, in only a minority of cell lineages. Before considering these findings in detail, we pause to emphasize that in any genetic analysis, a positive result is often more informative than a negative one. Specifically, although the discovery of a mutant phenotype stemming from gene loss implies that the targeted gene plays a normal physiological role in the implicated processes, the converse is not necessarily true. Redundancy or developmental compensation, whether at the molecular or cellular level, can mask the functions that a deleted gene may ordinarily control. Indeed, redundancy can provide a protective mechanism that safeguards essential processes from genetic attack.

In 1995, two groups of investigators reported the phenotype of mice lacking the gene encoding cyclin D1 (Fantl et al. 1995; Sicinski et al. 1995). Although it was already known that cyclin D1 was expressed in some cell types but not others (Matsushime et al. 1991), mice lacking cyclin D1 were viable and exhibited only focal de-

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velopmental anomalies confined largely to the retina and to developing breast tissue during pregnancy (Table 1). These animals were smaller in size than littermates retaining the gene, and they exhibited a still uncharacterized neuropathy exemplified by altered clasping reflexes. Piotr Sicinski and coworkers followed up on these findings in a fascinating series of experiments in which they disrupted the genes encoding the other D-type cyclins

Table 1. *Phenotypes of mice with disrupted cyclin and CDK genes*

Disrupted gene(s)	Survival	Pathology	References
Cyclin D-dependent kinases			
Cyclin D1	Viable	Small body size, hypoplastic retinopathy, defective beast lobuloalveolar development during pregnancy, and uncharacterized neuropathy with altered clasping reflexes	Sicinski et al 1995; Fantl et al. 1995
Cyclin D2	Viable	Defective ovarian granulosa cell development and female sterility. Males have hypoplastic testes but are fertile. Abnormal postnatal cerebellar development due to a reduced number of granule neurons and loss of stellate interneurons. Impaired proliferation of peripheral B-lymphocytes	Sicinski et al. 1996; Huard et al. 1999; Lam et al. 2000; Liu et al. 2000
Cyclin D3	Viable	Hypoplastic thymus with loss of T-cell maturation from double-negative (CD4–, CD8–) to double-positive (CD4+, CD8+) cells due to cytokine-independent defects in pre-TCR signaling	Sicinska et al. 2003
Cyclin D2 and D3 (D1 only)	Embryonic lethality before E18.5	Death likely is due to severe megaloblastic anemia. Other hematopoietic lineages were not evaluated.	Ciemerych et al. 2002
Cyclin D1 and D3 (D2 only)	Death at P1, but a few survive to 2 mo	Neuropathy leading to meconium aspiration is cause of early death. Survivors fail to thrive and exhibit hypoplastic retinas.	
Cyclin D1 and D2 (D3 only)	Viable but die within first three postnatal weeks	Retarded growth and impaired coordination. Inhibited postnatal cerebellar development, and hypoplastic retinas	
Cyclins D1, D2, and D3 (no D-type cyclin)	Dead by E16.5	Severe hematopoietic deficits affecting number and proliferative capacity of stem cells and multipotential progenitors. Fetal liver lacks progenitors and cannot reconstitute lymphoid or myeloid function after transplantation. Death due to anemia and defects in heart development. MEFs can be propagated in culture but exhibit greatly reduced susceptibility to transformation by oncogenic Ras + Myc, E1A, or DN-p53	Kozar et al. 2004
Cdk4	Viable	Small body size. Most males are sterile due to hypoplastic testes and low sperm counts. Female sterility is due to defects in the hypothalmic–pituitary axis, abnormal estrus, and failure of corpus luteum. Abnormal development of pancreatic β-islet cells leads to insulin-dependent diabetes within the first 2 mo of life. MEFs can be propagated in culture with decreased ability to enter the cell cycle from quiescence; they express aberrantly high levels of p21^{Cip1} and resist transformation by oncogenic Ras + DN-p53.	Rane et al. 1999; Tsutsui et al. 1999; Zou et al. 2002; Moons et al. 2002
CDK6	Viable	Thymic and splenic hypoplasia, and mild defects in hematopoiesis. T-lymphocytes exhibit delayed S-phase entry.	Malumbres et al. 2004
CDK4 and CDK6	Progressive embryonic lethality from E14.5 onward. The few live pups die soon after birth.	Small embryos. Partial failure of hematopoiesis results from reduced multipotential progenitors and multilineage deficits, including severe megaloblastic anemia. MEFs proliferate with increased generation time and reduced S-phase fraction. Some D-type cyclins associate with and activate Cdk2. MEFs resist transformation.	Malumbres et al. 2004
Cyclin E-dependent kinases			
Cyclin E1	Viable	Overtly normal	Geng et al. 2003; Parisi et al. 2003
Cyclin E2	Viable	Hypoplastic testes, reduced sperm count, and male infertility	
Cyclins E1 and E2	Embryos dead by E11.5 due to failure of endoreduplication of placental trophoblasts and loss of giant cells. Tetraploid rescue allows most embryos to develop to term.	Cardiac anomalies of varying severity in rescued embryos. Reduced endoreduplication in megakaryocytes. MEFs proliferate somewhat slowly with an increased G1 phase fraction and undergo senescence; however, quiescent MEFs cannot re-enter the cell cycle due to a failure in loading MCM proteins onto prereplication origins. MEFs resist transformation by oncogenic Ras + Myc, E1A, or DN-p53.	
Cdk2	Viable	Meiotic failure, gonadal hypertrophy, and male and female sterility. MEFs can proliferate and undergo senescence and spontaneous immortalization; quiescent MEFs exhibit delayed entry into S phase and/or decreased ability to re-enter the cell cycle. MEFs can be transformed with oncogenic Ras + E1A, but not as efficiently as wild-type cells.	Ortega et al. 2003; Berthet et al. 2003

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(Sicinski et al. 1996, 2003), and then interbred these animals to obtain strains of mice lacking any two (Ciemerych et al. 2002) or all three genes (Kozar et al. 2004).

Like the cyclin D1-deficient mice, animals lacking cyclin D2 or D3 were viable and exhibited very specific deficits (Table 1). The loss of cyclin D2 compromised female fertility and postnatal cerebellar development, and impaired the mitogen-dependent expansion of peripheral B-lymphocytes (Sicinski et al. 1996; Huard et al. 1999; Lam et al. 2000; Liu et al. 2000). In turn, cyclin D3 inactivation primarily affected the early steps of maturation of lymphocytes in the thymus that depended on the pre-T cell receptor (Sicinski et al. 2003). A common theme supported by detailed analyses of cyclin D gene expression in these mice is that the tissues most affected by gene disruption are those that principally express only one D-type cyclin at the expense of others. For example, cyclin D1 is exclusively expressed at high levels in the retina and breast, but is detected together with other D-type cyclins in many other (but not all) tissues.

If the tissue-specific expression patterns of the different D-type cyclins determine the pathologic manifestations that arise from their individual elimination, mice lacking more than one D-type cyclin should have combinations of defects observed in the single knockout animals. This is true, at least to some extent. However, during early embryonic development, animals retaining only one D-type cyclin lose the tissue-specific expression characteristic of that gene and up-regulate the remaining cyclin in most of their tissues (Ciemerych et al. 2002). Later in gestation, focal abnormalities then arise that compromise the animal's viability, such that "single-cyclin" animals die before or soon after birth (Table 1). Importantly, the tissues that exhibited severe abnormalities were ones in which the residual cyclin was not up-regulated. For example, all mice lacking cyclin D1 exhibited hypoplastic retinas, because neither cyclin D2 nor D3 was induced to compensate for D1 loss in the proliferating zone of this tissue. Animals retaining only cyclin D3 that survived for several weeks post-partum exhibited more severe cerebellar abnormalities than those seen in the D2 knockout strain. (Much of the development of the cerebellum occurs in the first two postnatal weeks of a mouse's life [Wechsler-Reya and Scott 2001]; hence, the same cerebellar defects could not be evaluated in animals expressing only cyclin D1, which died before embryonic day 18.5 [E18.5].)

It was initially proposed that normal organogenesis, at least until midgestation, in single D-cyclin animals might depend on the ubiquitous expression of the remaining cyclin, although the possibility was entertained that normal development, at least in some lineages, might proceed independently of the D cyclins. The generation of a mouse strain lacking all three D-type cyclins has now shown that the latter is likely the explanation, and that D-cyclin-independent cell cycles may be much more widespread than anyone had thought (Kozar et al. 2004). All such mice died by E16.5, but the fact that they developed to midgestation without any overt evidence of pathology demonstrated that complete elimination of cyclin D function did not compromise the cell cycle per se (Table 1). These embryos exhibited only focal abnormalities that appeared after E13.5. Defects in heart development arose that likely compromised the circulatory compartment and resulted in edematous embryos. Like animals lacking both cyclins D2 and D3, mice lacking the three D-type cyclins developed severe megaloblastic anemia, which was the likely cause of their death in utero. Intriguingly, transplantation experiments and in vitro assays of hematopoietic progenitor cells indicated that the loss of all D-type cyclins severely crippled the ability of long-term reconstituting hematopoietic stem cells and multipotential progenitors that represent the committed precursors of the lymphoid or myeloid series, respectively, to cycle. This bellwether finding raises the issue of whether cyclin D may be essential for other self-renewing cell populations to re-enter and progress through the cell cycle during an animal's lifetime, and it remains to be determined whether progenitor cells in other lineages are similarly defective. In fact, the cerebellar defects in mice lacking cyclins D1 and D2 already suggest that other stem cell-containing compartments in the central nervous system are affected (Ciemerych et al. 2002). Are other tissue stem cell pools also compromised? What will be the effects of cyclin D loss on processes such as wound healing, the immune response, or liver regeneration, all of which require entry into the cell cycle from quiescence? Because many such phenomena cannot be studied in utero, the answers will most likely depend on the design of conditional cyclin D knockouts that may allow such studies to be undertaken in living adult animals.

MEFs explanted from E13.5 cyclin D-null embryos could be cultivated in vitro, and these cells exhibited deficits in their ability to exit from quiescence. After a brief period of mitogen starvation that was sufficient to arrest all the cells in G0, 60%–80% of the cyclin D-null MEFs were able to resume proliferation when stimulated with medium containing 10% serum, and those that reentered the cell cycle appeared to complete cell division with normal kinetics. However, when stimulated with lower concentrations of serum, more severe cell cycle re-entry defects were seen. The levels of many other cell cycle regulators, including cyclins E and A, Cdk4, Cdk6, and Cdk2, were unaffected by cyclin D loss. As expected, Cdk4 and Cdk6 did not detectably associate with other cyclins or with $p27^{Kip1}$, and the cells were resistant to effects of the cyclin D-dependent kinase inhibitor $p16^{Ink4a}$.

It is unclear why the response of the cyclin D-null MEFs to mitogenic stimuli was variable, but one possibility is that the requirement for D-type cyclins reflects the time that cells spend in a quiescent mode. Once a cell exits the cell cycle, it may retain only a finite, short-term memory of its former proliferating state. Because the availability of pro-proliferative protein assemblies in their appropriately modified forms decay after exit from the cell cycle, a uniform requirement for D-type cyclins may be evident only after this molecular memory has been erased: that is, when these factors have declined below a critical level or, alternatively, after inhibitors

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have accumulated above a threshold. The virtually complete failure of cyclin D-null hematopoietic stem and progenitor cells to appropriately cycle, in vivo or in vitro, is consistent with this idea. Nevertheless, the nature of the cell cycle defect in the cyclin D-null hematopoietic stem cells has not yet been fully characterized, and it will be important to resolve whether these cells become locked into an irreversibly quiescent mode or, alternatively, are cycling slowly and cannot yield a sufficient number of cells to repopulate the lymphohematopoietic system.

Cdk2 activity was not decreased in proliferating cyclin D-null MEFs, even though these cells cannot sequester p27^{Kip1} into cyclin D–Cdk complexes. Instead, as cells entered cycle from quiescence and progressed through G1 phase, the overall levels of p27^{Kip1} were reduced compared to those in wild-type cells, whereas cyclin E-associated kinase activity was modestly elevated. Perhaps, when not sequestered by cyclin D–Cdk complexes, p27^{Kip1} is more efficiently phosphorylated by cyclin E–Cdk2 and subsequently degraded. Nonetheless, this finding seems paradoxical in light of the fact that the effects of cyclin D1 deficiency in the retina and breast of living mice are rescued by p27^{Kip1} loss (Geng et al. 2001; Tong and Pollard 2001), implying that cell cycle regulatory mechanisms in MEFs may not accurately reflect how these proteins operate in other cell types. Similarly, although the absence of cyclin D-dependent kinases prevented the complete phosphorylation of Rb, E2F target genes were still induced, albeit less efficiently than in wild-type MEFs, correlating with the reduced percentage of cells that exited quiescence. The implication is that, in the absence of cyclin D–Cdk complexes, cyclin E- and A-dependent Cdk2s are necessary and sufficient to counteract Rb and p27^{Kip1} inhibition in proliferating MEFs, although they may be less able to do so once cells become quiescent. In fact, siRNAs directed to Cdk2, while having a negligible effect on wild-type MEFs (see below), strongly inhibited the proliferation of cells lacking all D-type cyclins. These findings alert us to the possibility that Cdk2 may substitute for a lack of cyclin D-dependent kinase activity during mouse development. Conversely, we might infer that stress-induced G1-phase arrest of cell cycle progression in response to p53 and the Cdk2 inhibitor p21^{Cip1} would occur normally in these cells, although this has yet to be tested.

Eliminating Cdk4 and Cdk6

Based on the above findings, we would expect that mice lacking both Cdk4 and Cdk6 would also survive to midgestation and exhibit a phenotype that closely resembles that of cyclin D-null mice. Animals lacking Cdk6 are viable, exhibiting relatively mild defects in hematopoietic compartments (Table 1; Malumbres et al. 2004). Cdk4-null mice are also viable, but are small (Rane et al. 1999; Tsutsui et al. 1999), mimicking Cdk4-null flies in this regard (Datar et al. 2000). Most Cdk4-null males and all females are sterile because of low sperm counts and defects in the hypothalamic–pituitary axis that affect the female estrus cycle. Cdk4 is also essential for the development of β-islet cells in the pancreas, but not other islet cells, so that Cdk4-null animals develop insulin-dependent diabetes within the first few months of life.

Although they express relatively low levels of Cdk6, Cdk4-null MEFs proliferate normally under culture conditions that promote continuous growth. However, like cyclin D-null MEFs, only a fraction of the cells were able to respond to serum stimulation and re-enter the cell cycle from a short-term quiescent state (Tsutsui et al. 1999). As for cyclin D-null MEFs, the net levels of p27^{Kip1} were decreased as compared to those in wild-type MEFs, but induction of the Cdk2 kinase was diminished and delayed, contrasting with the slightly increased Cdk2 activity observed in cyclin D-deficient cells. This was associated with increased binding of p27^{Kip1} to cyclin E–Cdk2 and impaired Rb phosphorylation, a phenotype that was partially rescued in doubly null MEFs lacking both Cdk4 and p27^{Kip1} (Tsutsui et al. 1999). The nature of these rather subtle differences in Cdk4-deficient MEFs versus those lacking D cyclins is not resolved.

Mice lacking both Cdk4 and Cdk6 begin to die in utero from E14.5 onward with a failure of hematopoiesis and megaloblastic anemia resembling that seen in cyclin D-deficient animals (Malumbres et al. 2004). MEFs deficient in both Cdks were only partially able to respond to mitogenic stimulation and to exit quiescence, and the percentage of cells progressing through S phase at any given time was only half that seen in normal MEFs, although the cells that successfully entered the cycle proliferated normally. These cells continued to synthesize D-type cyclins, which, based on earlier studies, can directly bind to and activate Cdk2 in vitro (Matsushime et al. 1992; Ewen et al. 1993). Complexes between cyclin D2 and Cdk2 were more abundant in MEFs lacking Cdk4 and Cdk6 than in wild-type MEFs, and they exhibited Rb, but not histone H1, kinase activity (Malumbres et al. 2004). Like cells lacking D-type cyclins, shRNAs specific for Cdk2 inhibited the proliferation of Cdk4/6-deficient MEFs, but not their wild-type counterparts, again indicating that Cdk2 can compensate for loss of Cdk4 and Cdk6. Perhaps the earlier embryonic lethality of mice lacking three D-type cyclins as compared to those dually deficient in Cdk4 and Cdk6 reflects the ability of the D-type cyclins to activate Cdk2 in the latter setting.

Is Cdk2 less masterful than we thought?

Cdk2 has been assumed to be a crucial regulator that coordinates many different processes required for entry into, progression through, and exit from S phase. Unexpectedly, this enzyme was not found to be required for the proliferation of certain human colon cancer cell lines in culture (Tetsu and McCormick 2003). Still, it was a much bigger surprise when two groups of investigators reported that Cdk2-null mice were viable and, apart from male and female sterility due to meiotic defects, were

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ostensibly free of other pathology and survived for up to 2 yr (Berthet et al. 2003; Ortega et al. 2003). In fact, of all the knockout strains lacking various cyclins or Cdks, the Cdk2-deficient mice were the least compromised. Cdk2-null MEFs in culture also appeared to proliferate relatively normally, albeit with a decreased ability to exit quiescence and/or enter S phase at a normal rate, a phenotype that became more pronounced at later passages. These properties of Cdk2-null MEFs may not result from compensatory processes achieved during early embryonic development, since acute ablation of "floxed" Cdk2 alleles by Cre-mediated recombination did not compromise their continuous proliferation in vitro, although it was not reported whether exit from quiescence was affected (Ortega et al. 2003).

The possibility that other Cdks might compensate for Cdk2 loss requires much further investigation. Unfortunately, deriving mice deficient in Cdk2, Cdk4, and Cdk6 is problematic, because of close linkage of the Cdk4 and Cdk2 genes. Another candidate for study is Cdk1, particularly because it can be activated by both cyclins A and B, which are first synthesized in late G1 and S phase, respectively (Pines and Hunter 1990). However, following re-entry into the cycle from quiescence, cyclin A-associated kinase activity is reduced in Cdk2-null MEFs when compared to wild-type MEFs. Moreover, cyclin A-associated kinase activity, while present in Cdk2-null splenocyte extracts from young mice or in early-passage MEFs, was no longer detected in Cdk2-deficient adult spleen cells and in immortalized MEFs (Berthet et al. 2003). Therefore, although cyclin A2 is essential for embryonic development (Murphy et al. 1997), it may not be required in adult tissues or in immortalized cells. We also think it unlikely that cyclin B–Cdk1 will prove to be the only essential kinase in proliferating mammalian cells. Even if low levels of Cdk activity (irrespective of the particular cyclin partner) are competent to initiate S phase, and if higher amounts are sufficient to initiate mitosis, this would require that the timing of cyclin B expression and its subcellular location be altered in the Cdk2 knockout mouse in order for cyclin B–Cdk1 to be active and located in the nucleus at the G1- to S-phase transition (Moore et al. 2003). There is no evidence that this is the case, but critical experiments have not yet been performed.

Cyclin F, while less well characterized than the D-, E-, A-, or B-type cyclins, is expressed in parallel with cyclin A during the cell cycle, and its amino acid sequence in the region of the "cyclin box" (the segment that contacts Cdks) is most related to that of cyclin A. Although its associated kinase has not yet been identified, cyclin F is essential for mouse development after E10.5, and cyclin F-deficient embryos exhibit a wide array of anomalies, including a failure to complete axial rotation, and defects in neural tube closure and brain development. Both the yolk sac and chorioallantoic placenta also fail to develop normally (Tezlaff et al. 2004). MEFs lacking this protein cycle more slowly than wild-type cells and have difficulty re-entering the cell cycle from G0. It is therefore conceivable that cyclin F-dependent Cdk activity might compensate when other normal cell cycle regulators are absent.

Why, indeed, do higher eukaryotes express multiple Cdks? This diversity provides two advantages during the cell cycle, both of which have received some experimental support. The first is that each cyclin–Cdk combination has become specialized in interacting with particular substrates, potentially enlarging the repertoire of molecules that are governed through Cdk activity. The second is that the various holoenzymes, while retaining a capacity for biochemical interchange, have become subject to different modes of regulation that affect their subcellular localization and interaction with different regulators so as to ultimately dictate when and where they act. The phenotypes of the knockout mice reviewed here emphasize that during mouse development, the G1 cyclin–Cdks are not sufficiently unique so as to disrupt cell cycle progression per se, should one class or another be completely eliminated. But if, in fact, it turns out that none of Cdks 4, 6, or 1 compensates for the loss of Cdk2, are there any other candidates that we might consider? Stretching the conceptual boundaries further, is it possible that Cdks (or cyclins) whose known functions are to regulate transcription (Cdks 7, 8, and 9) can fill in for Cdk2 loss? Or, would other kinases be required? Murray (2004) has suggested that although evolution has allowed Cdks to gain control of the cell cycle machinery, "the defeated remains of (other) ancestrally dominant kinases can be glimpsed in both DNA replication (the Cdc7/Dbf4 kinase/activator couple) and in mitosis (Polo and others)." We might equally reconsider whether these other enzymes play a central role in the absence of Cdk2.

Cyclin E loss: perplexing Cdk2-independent phenotypes

If Cdk2 is nonessential, than cyclins E1 and E2 should be dispensable as well. Both of these cyclins are ubiquitously expressed in somatic cells, but, except for a reduction in male fertility in animals lacking cyclin E2, deletion of either one was without effect. Dual disruption of these genes resulted in embryonic lethality by E11.5 due to defects in the endoreduplication cycles that normally occur in placental trophoblast giant cells (Geng et al. 2003; Parisi et al. 2003). Rescue of this defect in extraembryonic tissues by tetraploid blastocyst complementation yielded animals that survived to term, with cardiac anomalies of varying severity but otherwise normal development (Geng et al. 2003). (Although this technique guarantees that ES cells contribute only to the embryo proper, mice die of respiratory failure after birth, a process that can be bypassed by the use of hybrid ES cell strains [Schwenk et al. 2003]. Therefore, postnatal cyclin E1- and E2-deficient mice derived from an inbred ES cell strain did not survive.) Like placental trophoblasts, megakaryocytes from the doubly mutant mice also failed to undergo a normal number of endocycles. MEFs explanted from cyclin E1- and E2-deficient embryos were capable of continuous proliferation in culture. However, whether the late G1 transition to mito-

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gen-independent cell cycle progression occurred normally in the absence of E-type cyclins has not yet been addressed.

Strikingly, quiescent cyclin E-null MEFs were unable to re-enter S phase from G0, despite normal levels of cyclin A-associated kinase activity (Geng et al. 2003). These cells were evidently able to exit G0 and to initiate the E2F transcriptional program, but they were still blocked from initiating DNA replication. This is probably explained by the requirement for cyclin E to load the MCM helicase onto chromosomal DNA, thereby aiding assembly of the preinitiation complex at origins of DNA replication. This function, previously demonstrated in *Drosophila* endocycles (Su and O'Farrell 1998) and in quiescent mammalian cells entering the cell cycle (Coverley et al. 2002), logically accounts for the cell cycle defects observed in mice lacking E-type cyclins. Notably, cyclin E is not required for MCM loading during mitotic cycles where there is no G0 to G1 transition, thus providing a possible explanation for why very few other abnormalities were observed in the cyclin E-null animals.

However, because E-type cyclins have no known catalytic partners other than Cdk2 and Cdk3, and because Cdk3 is nonfunctional in mice (Ye et al. 2001), the differences between the cyclin E1/E2-null animals and mice lacking Cdk2 are perplexing (Roberts and Sherr 2003). In principle, either the few unveiled essential functions of cyclin E may prove not to be Cdk2-dependent, or a novel Cdk partner for E-type cyclins awaits discovery. Given that no cyclin E-associated kinase activity could be detected in Cdk2-null cells, it might be that an as-yet-unidentified cyclin E-dependent Cdk has escaped detection because it exhibits an unusually narrow substrate specificity, for example, phosphorylating substrates required for MCM loading, but not the already identified array of Cdk2 targets. (There may be some precedent for this—consider the fastidious nature of cyclin D-dependent kinases for Rb proteins.) Nonetheless, the possibility of Cdk2-independent functions deserves equally serious consideration.

Implications for cancer

The cyclin D- and cyclin E-dependent kinases, when analyzed as independent classes, now each appear to be nonessential for somatic cell cycles, although they likely play critical roles in exit from quiescence. This raises the very interesting question of whether activation of these proteins is required for oncogenic transformation, which fundamentally represents the pathological ability of cancer cells to evade the normal signals that stop cell division and thereby escape from the quiescent state. In this regard, a particularly intriguing finding concerns the resistance of MEFs lacking either the D-type or E-type cyclins to transformation by oncogenes. Wild-type MEFs can be transformed by oncogenic Ras in conjunction with other "immortalizing" oncogenes, such as Myc, adenovirus E1A, or dominant-negative (DN) p53. In contrast, MEFs lacking the G1 cyclins resist such transformation, at least when assayed for focus formation in vitro. Thus, these cells have difficulties both in entering the cell cycle from a quiescent state and in responding to elevated and sustained activation of mitogenic signaling pathways.

Cdk4-null MEFs were previously found to senesce rapidly in culture and to be refractory to transformation by oncogenic Ras plus DN-p53 (Zou et al. 2002). Not surprisingly, MEFs lacking both Cdk4 and Cdk6 senesced even more rapidly in vitro, re-enforcing the idea that there is a link between these kinases and a cell's long-term proliferative capacity (Malumbres et al. 2004). Moreover, although wild-type MEFs lacking the Ink4a/Arf locus (encoding two tumor suppressors, $p16^{Ink4a}$ and $p19^{Arf}$) do not senesce in culture, MEFs lacking both Ink4a/Arf and Cdk4 underwent senescence like their wild-type counterparts (Zou et al. 2002). Thus, the loss of Cdk4 surprisingly reconferred "mortality" in this setting. Loss of Cdk4 in Ink4a/Arf-null cells increased the expression and stability of $p21^{Cip1}$. Conversely, suppression of $p21^{Cip1}$ synthesis by siRNA increased the life span of these cells and restored their susceptibility to transformation by oncogenic Ras and DN-p53. Therefore, these cell cycle regulators may contribute to transformation not only by promoting emergence from quiescence, but also by allowing cells to avoid senescence. In summary, a complex functional interplay between Cdk4, the Cip/Kip proteins, and Cdk2 can determine whether MEFs can be transformed or not.

The implication of all these experiments is that cell proliferation and differentiation in response to physiologic levels of growth factors in vivo might be able to proceed with only low levels of net G1 Cdk activity, whereas an increased threshold of sustained mitogenic signals invokes a requirement for these G1-phase regulators in order for transformation to occur. This begs the broader question of whether the tissues of a living animal lacking these G1-phase regulators are also resistant to cancer. Indeed, we already know that animals lacking Cdk4 are completely refractory to Ras-mediated skin carcinogenesis in vivo, whereas Cdk4 deficiency has no effect on the normal proliferation of keratinocytes in vitro (Rodriguez-Puebla et al. 2002). Inactivation of Cdk4 also completely protects transgenic mice expressing c-Myc under the control of the keratin-5 promoter from developing epithelial tumors (Miliani de Marval et al. 2004). Similarly, cyclin D1 inactivation confers resistance to breast cancers induced by oncogenic Neu or Ras transgenes targeted to the mammary gland (Yu et al. 2001) and to intestinal tumors resulting from Apc loss and constitutive β-catenin signaling (Hulit et al. 2004). This has suggested that interference with cyclin D function might be of clinical benefit in cancer treatment. However, it remains unresolved whether resistance to carcinoma development in any of these settings reflects the loss of crucial target cell populations in the tissues of cyclin D-null or Cdk4-null mice or, instead, the refractoriness of still resident target cells to the effects of persistent oncogene stimulation. Even within a single tis-

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sue, the response to cyclin D ablation specifically depends on exactly the nature of the oncogenic signaling pathways involved. Unlike the response to Ras or Neu, cyclin D1 deficiency did not protect mice from carcinogenesis induced by targeted expression of Wnt to the mammary gland (Yu et al. 2001). Strikingly, cyclin D1 loss enhanced rather than ablated tumor formation in mice engineered to stably express a mutant form of β-catenin (a target of Wnt signaling) in their mammary glands (Rowlands et al. 2003). This is consistent with the idea that early β-catenin-driven events in the development of the alveolar lineage do not rely on cyclin D1, whereas only later differentiation steps do (Fantl et al. 1995; Sicinski et al. 1995). Hence, by blocking the late steps in alveolar development, cyclin D1 loss would enhance the number of more primitive cells susceptible to transformation by β-catenin.

A further complication raised by some of the present work concerns the deleterious effects of cyclin D loss on the proliferation of hematopoietic, neuronal, and possibly other tissue stem cells. The cells that give rise to many cancers have both the self-renewal and differentiating capacities of tissue stem cells, raising the idea that mutant "cancer stem cells," rather than their differentiated progeny that may make up the bulk of a tumor, are the only cells strictly responsible for durable tumor maintenance and, in turn, for resistance to therapy (Reya et al. 2001; Taipale and Beachy 2001; Al-Hajj et al. 2003). If this is correct, a critical issue is whether agents that interfere with cyclin D function would have a selective advantage in eliminating mutated tumor cells while sparing normal stem cells.

It also remains to be determined whether the essential transforming function of the D cyclin–Cdk complexes reflects their ability to phosphorylate and inactivate the Rb family of proteins, and/or to bind and sequester the Kip/Cip inhibitors. To what extent will deletion of these inhibitors rescue the various phenotypes of the cyclin D-null mouse, including its susceptibility to transformation in vitro and tumorigenesis in vivo? As discussed previously, we would like to know whether a knock-in of a kinase-defective Cdk4 allele, or of a cyclin D1 mutant that assembles with Cdk4 and Cdk6 without activating them but that can still "titrate" $p27^{Kip1}$, would rescue cyclin D1 deficiency in the breast and retina. Would such mutants, like cyclin D1 loss itself, protect animals against oncogenic Ras and Neu-induced breast tumors? Importantly, only if the essential transforming role of cyclin D-dependent kinases relies on their catalytic function will drugs that specifically target their enzyme activities prove useful as cancer therapeutic agents.

A similar issue arises with respect to cyclin E's function in tumor cells. Cyclin E-null MEFs proliferate normally but cannot re-enter cycle after mitogen starvation, and they are refractory to the same oncogenes that fail to transform cyclin D-null MEFs (Geng et al. 2003). Although this further highlights the importance of the transition between quiescence and proliferation in oncogenic transformation, we need to know whether this represents a Cdk-dependent or independent function of cyclin E, as this may affect the potential efficacy of Cdk2 inhibitory drugs in cancer therapy.

Why do the latest findings seem to contradict the many previous results obtained with cultured cell lines indicating that the roles of G1 cyclin–Cdks are essential? In fact, most of the latter experiments were either performed using quiescent mammalian cells stimulated to enter cycle in which G1 cyclin–Cdks do play a more significant role, or they used enforced overexpression of inhibitors with loose specificity and therefore multiple targets (e.g., Kip/Cip proteins, dominant-negative Cdks, or microinjected antibodies to cyclins). Still, the overexpression of D- and E-type cyclins and Cdk4, and reduction in the expression of $p27^{Kip1}$, commonly occur in many human tumors, suggesting, with the many caveats noted above, that drugs that prevent the assembly or activation of cyclin–Cdk complexes, or that directly inhibit their kinase activities, may have efficacy in the treatment of certain cancers. If, indeed, the requirement for G1 cyclin–Cdk activity is only crucially manifested in cells responding to an increased signaling threshold, then drug-induced inhibition of Cdk activity in tumor cells might still have profound effects on their ability to proliferate, whereas many normal cells might resist drug challenge.

Much work still lies ahead before we rewrite all the textbooks. A more complete understanding of the transition from quiescence to the cell cycle and clear distinction between that and the progression from mitosis to S phase will be necessary to fully appreciate the roles of these G1 regulators in normal cells and their contribution to tumorigenesis. As Yogi Berra (1998) put it, "The future ain't what it used to be."

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