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Minireview

Wagging the Dogma: Tissue-Specific Cell Cycle Control in the Mouse Embryo

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The family of cyclin-dependent kinases (Cdks) lies at the core of the machinery that drives the cell division cycle. Studies in cultured mammalian cells have provided insight into the cellular functions of many Cdks. Recent Cdk and cyclin knockouts in the mouse show that the functions of G1 cell cycle regulatory genes are often essential only in specific cell types, pointing to our limited understanding of tissue-specific expression, redundancy, and compensating mechanisms in the Cdk network.

Eukaryotic cells rely on the presence of cyclin-dependent kinases (Cdks) for their division. In addition to the catalytic subunit (the Cdk itself), each Cdk complex contains one of many activating subunits called cyclins because their levels fluctuate periodically throughout the cell cycle. Distinct cyclin-Cdk complexes power the cell through different phases of the cell cycle. In mammals, these complexes include the D-type cyclins (cyclins D1, D2, and D3), which activate Cdk4 and Cdk6 to execute critical regulatory events in G1; the E-type and A-type cyclins, which activate Cdk2 to effect events in S phase including DNA replication and centrosome duplication; and the A-type cyclins (in a second role) and B-type cyclins, which activate Cdk1 to direct structural and regulatory events in mitosis. Inactivation of Cdk1 in late mitosis contributes to reset the cell in G1. In addition to positive regulation by cyclins, Cdks are regulated by multiple phosphorylation and dephosphorylation events and by several subunits named CKIs (for Cdk inhibitors) that physically associate with cyclin-Cdk complexes to inhibit their activities and promote cell cycle arrest or delay.

Among the cyclins, the seminal observation that different cyclin D family members are transcriptionally induced in response to extracellular signals, including growth factors (Matsushime et al., 1991), led to the hypothesis that D-type cyclins represent crucial intermediaries for transmitting signals from the extracellular environment, through cell signaling pathways, to activate the cell cycle machinery. Cdk4 and Cdk6 catalyze the phosphorylation of the retinoblastoma (Rb) tumor suppressor gene product to disrupt its association with members of the E2F family of transcription factors, thereby promoting transcription of E2F target genes. Many E2F targets (including cyclin A, cyclin E, and various replication factors) are important for the G1/S transition and DNA replication. Cyclin D-Cdk4/6 complexes have also been suggested to drive cell cycle progression by sequestering the CKIs p21 and p27, two potent inhibitors of cyclin E-Cdk2 and cyclin A-Cdk2 as well as cyclin A-Cdk1 and cyclin B-Cdk1. Released from the inhibitory effect of p21 and p27, activated Cdk2/1 complexes have been suggested to further phosphorylate Rb and other downstream substrates and thereby drive progression toward mitosis. Cyclin D may have other roles either requiring or independent of Cdk4, Cdk6, or Rb (see, for example, cyclin D-Cdk4 phosphorylation of Smad3 [Matsuura et al., 2004]). Even the regulation of Rb by cyclin D-Cdk complexes may activate not only transcription, but also other programs including chromatin remodeling and the initiation of DNA replication by directly regulating origin firing.

Cell Cycle Control by D-Type Cyclins in the Developing Embryo

Recent studies from the Barbacid and Sicinski groups report the effect of deleting both Cdk4 and Cdk6 genes (Malumbres et al., 2004) or all three D type cyclins (Kozar et al., 2004) in the mouse. In both cases, lethality is observed at around embryonic day 16, accompanied by a major failure in hematopoesis including erythropoesis, which is the likely cause of death (Table 1). In contrast, most organogenesis and tissue development appears unaffected. Thus, it seems that the majority of embryonic tissues are "cyclin D independent," whereas hematopoesis is "cyclin D dependent." Nonetheless, the embryos show reduced body size, underscoring the importance of these Cdks in controlling overall cell proliferation or tissue growth. The decrease in proliferative potential is particularly visible in vitro, where both T cells and mouse embryonic fibroblasts (MEFs) are reduced in their ability to proliferate in response to mitogens. In addition, in MEFs from the cyclin $D1^{-/-}D2^{-/-}D3^{-/-}$ mouse, a higher dependency on serum is observed and these cells are less susceptible to oncogenic transformation, supporting the overwhelming literature that shows a role for G1 Cdks in serum responses and cellular transformation. These serum response and transformation pathways therefore appear to be different than the embryonic pathways important for organogenesis in many tissues.

The embryonic deficiency in hematopoesis clearly demonstrates the importance of D-type cyclins or their associated Cdks in specific tissues. Analysis of adult mutant mice lacking a single D cyclin, Cdk4 or Cdk6, has demonstrated that most of these proteins support tissue-specific functions (Table 1), although not causing strict lethality. Indeed, some phenotypes observed in the single knockouts are probably masked in the triple knockout because of its earlier lethality. A similar conclusion for tissue-specific functions can be drawn by examining the phenotypes of mice lacking genes encoding other cyclins and Cdks (see comprehensive list in Supplemental Table S1 at http://www.cell.com/cgi/content/full/118/5/535/DC1) as well as Cdk regulators, such as

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Table 1. Phenotypes Associated with Homozygous Loss of D Type Cyclins or Associated Cdks

Genotype[a]	Major In Vivo Phenotype/s	Phenotype Observed in Tissue Culture
$D1^{-/-}$	Postnatal lethality; Reduced body size; Small cerebellum; behavior/neurological abnormalities; Hypoplastic retina; Defects in lactation due to lack of proliferation of the mammary epithelium Reduced susceptibility to breast cancer	MEFs showed slightly reduced growth but normal proliferation
$D2^{-/-}$	Female infertility (inability of granulosa cells to proliferate, but normal oocytes); Males were fertile but displayed small testes and decreased sperm count; Small cerebellum; Pancreatic beta cell hypoplasia	Prolonged G1 in B lymphocytes
$D3^{-/-}$	Decreased expansion of immature T lymphocytes; Reduced susceptibility to certain T cell malignancies	Decreased T cell proliferation
$D1^{-/-}D2^{-/-}$	Reduced body size; Undeveloped cerebellum	Not tested
$D1^{-/-}D3^{-/-}$	Neonatal lethality (likely due to neurological defects); Reduced body size; Hypoplastic retina	Not tested
$D2^{-/-}D3^{-/-}$	Embryonic lethality (~E18.5); Severe megaloblastic anemia; Reduced body size	Not tested
$D1^{-/-}D2^{-/-}D3^{-/-}$	Embryonic lethality (~E16) Multi-lineage hematopoietic failure; Reduced body size; Cardiac abnormality	Reduced proliferation in hematopoietic cells and MEFs Cdk2 silencing reduced dramatically proliferation in MEFs Reduced susceptibility to oncogenic transformation
$Cdk4^{-/-}$	Viable but slightly lower ratio of homozygotes (19.6%); Reduced body size Behavior/neurological abnormalities; Pancreatic beta cell hypoplasia 80% males were sterile with small testes and reduced numbers of sperm cells Females were infertile (small ovaries, defect in corpus luteum formation)	MEFs showed reduced proliferation
$Cdk6^{-/-}$	Decreased expansion of immature T lymphocytes	Decreased T cell proliferation
$Cdk4^{-/-}Cdk6^{-/-}$	Embryonic lethality (between E14.5 and E18.5); Multilineage hematopoietic failure; Reduced body size	Reduced proliferation in T cells and MEFs; Cdk2 silencing reduced dramatically proliferation in MEFs

[a] A more comprehensive list of phenotypes associated with homozygous loss of cyclins or Cdks is presented in the Supplemental Data at http://www.cell.com/cgi/content/full/118/5/535/DC1.

CKI family members (reviewed by Ortega et al. [2002]), Cdc25b (Lincoln et al., 2002), Cdc25c (Chen et al., 2001), Skp2 (Nakayama et al., 2000), βTrcp1 (Guardavaccaro et al., 2003), Dp1 (Kohn et al., 2004), and E2F and Rb family members (reviewed by Yamasaki [2003]). In fact, so far, only cyclin A2, cyclin B1 (see Supplemental Table S1), and Apc2 (Wirth et al., 2004) are essential in early embryogenesis, supporting the fundamental importance of regulation by mitotic cyclins. Differential expression of D cyclins and their inhibitors (the four members of the INK family of CKIs) in human tissues is also consistent with cell type-specific roles.

At a molecular level, the proliferation defects in the cyclin D triple knockout MEFs correspond to a delay in the induction of E2F target genes and a decrease, but not elimination, of Rb phosphorylation on previously described cyclin D-dependent sites (Ser248, Thr252, Thr826, Ser807, and Ser811). The existing model held that these sites would prime phosphorylation by cyclin E- or cyclin A-Cdk2 complexes. However, the levels of phosphorylation on the cyclin E-, cyclin A-specific residue Thr821 were almost identical to wild-type MEFs, arguing against the notion that the phosphorylation on these sites is strictly dependent on cyclin D-Cdk complexes. Indeed, in the cyclin $D1^{-/-}D2^{-/-}D3^{-/-}$ MEFs, the kinetics of timing and rapid activation of cyclin E- and cyclin A-associated kinases was not strongly affected, further suggesting that D-type cyclins are not a necessary prerequisite for activation of the other G1 and S phase cyclins. There was also a reduction in the levels of phosphorylation of the Rb-related p107. The other family member, p130, thought to be important for G1/S control, has not yet been tested.

Another notion challenged by these two studies is the above-mentioned sequestration model. One would naively expect that in the absence of D cyclin complexes, more p21 and p27 would bind to Cdk2, but this was not observed. However, less p21 and p27 were present in the cyclin D- and Cdk4/6-deficient cells, particularly during G1. Functionally, by having lower levels of Cdk inhibitors, there would also be a reduced requirement for cyclin D-dependent sequestration of CKIs. The reduction in p21 and p27 may reflect a failure to sequester and thus accumulate these regulators in complex with cyclin D-Cdk. So, both the sequestration model and the mechanism by which the embryo compensates to reduce p21 and p27 levels will require further testing.

Reordering Models and Expectations?

Despite earlier evidence for cell type-specific expressions and functions, because of their roles in coupling growth factor signaling to cell cycle, loss of D-type cyclins might have been expected to show dramatic proliferation defects in the mouse embryo, similar to those seen in cyclin A2 or cyclin B1 knockouts. There were even higher expectations for E cyclins and Cdk2, which are thought to be expressed in most if not all cells. Nonetheless, mouse knockouts for either cyclin E1 or E2 (see Supplemental Table S1) were also viable. Thus, following the history of the logic, when it became evident that embryos can develop normally and adult mice exist without one, and in some cases two, of the cyclin D or cyclin E genes, it was postulated that loss of all three D-type cyclins (or lacking Cdk4 and Cdk6) or the two E-type cyclins (or lacking Cdk2) would do the trick. Having now found that cyclin E-Cdk2 complexes are not required for viability and that cyclin

D-Cdk4/6 complexes are not required for most tissues to develop, we may need to reexamine the model for how these cyclins contribute to mammalian cell proliferation during development and thereafter.

On the surface, these genetic observations in the mouse seem to require a radical reorientation of current models and expectations. But more likely our surprise may reflect at least three general shortcomings in our current knowledge. First, because many more studies have been performed examining the cell cycle characteristics of hematopoetic cells and fibroblasts than, for example, neural cells or lung cells, we likely do not understand the considerable differences in the cell cycle requirements for specific cell types. Indeed, our knowledge of the expression of many G1 regulators during development and in different tissues is somewhat fragmentary. This differential expression may reflect varying requirements for these regulators. For example, hematopoetic cells require a high proliferative turnover, so their dependency on cyclin D may reflect a requirement for "turbo-charged" proliferation. Second, different tissues may exhibit varying degrees of embryonic plasticity, such that D-type cyclins are only required in tissues where other redundant programs cannot be rewired to compensate. These tissue-specific differences may underlie one of the most critical unsolved problems in cancer biology, i.e., what determines the tissue selectivity of certain cancer genes (as an example, LOH for the *RB* and *INK4* genes are selected for only in a few tissues).

Finally, there may be varying levels of regulatory redundancy in different tissues. Inhibition of Cdk2 by siRNA induced a stronger proliferation defect in $Cdk4^{-/-}Cdk6^{-/-}$ and cyclin $D1^{-/-}D2^{-/-}D3^{-/-}$ MEFs than in wild-type MEFs, suggesting that even if cyclin D-Cdk4/6 or cyclin E-Cdk2 complexes are dispensable, inactivation of both would at least not sustain cell division in embryonic fibroblasts and perhaps in other cell types. In addition, these in vitro results suggest that if mice missing cyclin E-Cdk2 or cyclin D-Cdk4 complexes are viable, perhaps both sets of regulators cannot be removed. This hypothesis might be confirmed or disproved by specific crosses, but the pentuple cyclin knockout will require multiple crosses, and linkage between Cdk2 and Cdk4 makes the probability of a recombinant too low, so a targeted knockout of Cdk4 in a $Cdk2^{-/-}Cdk6^{-/-}$ background is in progress (Malumbres et al., 2004). Lastly, in addition to Cdk2, Cdk4, and Cdk6, mammalian cells have many additional cyclins, Cdks, and Cdk-like proteins that have been only thinly examined, and these may provide compensating mechanisms in G1.

So, if cyclin D-Cdk complexes are not required for embryonic proliferation generally, what do they do? Genetic studies in *Drosophila* and nematodes are revealing. In *Drosophila*, recent studies suggest the idea that cyclin D controls both mass accumulation and cell proliferation (Frei and Edgar, 2004; Meyer et al., 2000). Indeed, a recent analysis suggests that cyclin D participates in a pathway regulating oxygen sensing, possibly linking cyclin D to respiratory homeostasis (Frei and Edgar, 2004). In nematodes, cyclin D does appear to control proliferation, but possibly only in those tissues where cell proliferation is linked to cell growth, such as postembryonic divisions (Park and Krause, 1999). Amazingly, in cells of the moss Physcomitrella, cyclin D appears to function to maintain cell proliferation in the presence of high glucose (Lorenz et al., 2003).

One synthesis of these findings is that cyclin D-Cdk complexes may couple various kinds of time-varying, homeostatic (turbo-charged) signals (growth factors, nutrients, oxygen, etc.) to cell cycle division. This signaling-linked proliferation may be most important in situations where the signaling is associated with cellular growth, such as in regenerating tissues, hemopoetic tissues, or sugar-fed moss cells. In the mouse, where the embryo is fed by placental sources, many of the growth signals may be constitutive and cyclin D would become less important. In regenerating tissues, the ability of cyclin D to respond to signals and trigger new tissue growth would presumably be tightly linked to the growth and signaling state of the cells. A number of critical pathways appear to regulate cell division in regenerating tissues, including the Wnt, Hedgehog (Hh), Notch, and Hox pathways. Both the Wnt and Hh pathways have been suggested to signal in part through cyclin D- and cyclin E-dependent pathways, although it is not clear how much these pathways depend on Cdk activation. It is notable that deficiencies in Wnt and Hh genes do show defects in organogenesis in the mouse embryo. So, as we begin to search for "cyclin D-independent" pathways controlling proliferation during organogenesis, targets of Wnt, Hh, Notch, and Hox regulators would be particularly good candidates.

Models and Reductionism

If G1 cell cycle complexes are not absolutely required, how did we develop that idea to begin with? In many cases, the genes encoding Cdks, cyclins, and many of their regulators were originally characterized in budding or fission yeast. When Cdks were identified in mammals, somatic cell genetic experiments perturbed Cdk activity by expressing Cdk dominant-negative mutants and Cdk inhibitors, or using antisense oligos, microinjection of neutralizing antibodies into living cells and, more recently, by RNA interference. Based on numerous published studies, we learned that Cdk inhibition certainly slows down the cell cycle of cultured mammalian cells. However, these experiments didn't prove that Cdks are "essential" genes and certainly did not show that they are necessary in all cell types and at all times in vivo. Cdk inhibition in asynchronous mammalian cells induces accumulation of cells in G1, but never in 100% of the cells. If Cdk inhibition is induced in synchronized cells, we see a delay in S phase entry (not an arrest!), similar to that observed in $D1^{-/-}D2^{-/-}D3^{-/-}$ MEFs (Figures 5 and 6 in Kozar et al. [2004]) and $Cdk4^{-/-}Cdk6^{-/-}$ MEFs (Figure 5 and 7 in Malumbres et al. [2004]). Historically, it was hard to discriminate whether a partial block reflected an inefficient inactivation technique or that the inhibited regulator was not the only important factor. Although many studies have been done in MEFs and in T cells, the majority of experiments in mammalian cell culture were performed using a limited number of highly transformed cell lines (mostly HeLa cells). In addition, the importance of cyclin-Cdk complexes in experimental systems examining intact organs or tissues has been examined in very few cases.

Experiments in cultured cells may have been overly generalized in some cases. Statements such as "this cyclin is *required* for G1 progression," implying *all* G1 progressions in *all* cell types, are common. The tendency to generalize has many roots, but it may derive in part

from the yeast literature. Here, the simple and sharply controlled growth responses in yeast (no sugar–no growth) and the lesser degree of redundancy cause yeast phenotypes to be stronger and more amenable to a black-and-white description. Subsequent studies in mammals adopted the same language.

There are also limitations of the treatments used to inactivate cyclin D-Cdk or cyclin E-Cdk2 complexes themselves. By employing Cdk inhibitors, antibody neutralization, or dominant-negative variants of various Cdk components, there is a strong possibility that the inhibited complexes may disrupt the cellular machinery, for example by sequestering substrates. In contrast, the complete absence of a complex may allow better compensation, thereby allowing a closely related cyclin-Cdk complex access to substrates. RNA interference should behave more like a genetic null, and many investigators feel that RNA interference is a preferred method to mimic the loss-of-function phenotype. Another issue may be that genetic inactivation in the mouse embryo allows sufficient time for developmental compensation, whereas acute inactivation of gene products (for example by a siRNA oligo or a drug) does not. In this regard, an enlightening study (Sage et al., 2003) clearly illustrates the important point that acute elimination of Rb's function in somatic cultured cells may have consequences different than in the mouse knockout setting where there may be compensation by related proteins during development. Indeed, the genotoxic stress induced by "culture shock" may make the expatriated cells growing in dissociated cell culture more dependent on Cdks or more sensitive to inhibition than their cousins growing together at home in tissues. It remains to be seen whether lack of cell cycle regulatory genes induces a more profound phenotype under stressed conditions in vivo.

A recent review (Gladden and Diehl, 2003) questioned whether the lack of a strict requirement for cyclin E-Cdk2 complexes in the mouse was a cautionary note for efforts to develop Cdk inhibitors as cancer therapeutics. We feel the differences between an inhibited Cdk and an absent Cdk are sufficient to move forward with some confidence, especially when we consider the expanded role for Cdks in cancer cells.

In summary, there are several reasons (not mutually exclusive!) for why homozygous loss of cyclins or Cdks does not produce an effect in all cell lineages as previously thought: (1) lack of expression and function of certain cell cycle regulators (particularly G1 regulators) in some tissues; (2) redundancy and compensation, either within the family of Cdk regulators or through other pathways (even yeast, which has one Cdk but multiple cyclins, can still replicate its DNA without expressing any G1/S cyclins but just a single mitotic cyclin [Fisher and Nurse, 1996]); and (3) embryonic plasticity. Only reproductive organs do not appear to exhibit the high degree of plasticity observed in other tissues. In fact, meiotic defects are observed in mice (particularly in males) missing a number of cell cycle regulators including cyclin A1, cyclin D2, cyclin E2, Cdk2, Cdk4, Ink4d, β-Trcp1, and E2F1. Why are germ cells more often affected than somatic cells? It is possible that the reproductive system needs to be less plastic to avoid transmission of defects to the progeny. Perhaps, because of this, the network of checkpoints in meiosis appears more complex than the already intricate mitotic apparatus.

In conclusion, recent work in the field supports the notion that a deeper understanding of the cell cycle requires multiple model organisms. The mouse, in particular, provides avenues for both genetics and biochemistry. In addition, genetics in this organism provides information about the cell cycle in embryos, adult organisms, and, most importantly, the cell cycle's varied behavior in different tissues. Yet, despite the power of genetics and the common message provided so far by studies in the mouse, fly, worm, and yeast, all confirming that cyclins and Cdks promote cell proliferation, the details among various systems and models are often different. Even within a single organism, there can be considerable physiological differences in various genetic backgrounds. In the mouse, the use of different strains can change—sometimes dramatically—the phenotype derived by the loss of a particular gene. The amazement expressed by some editorials for the results that not all cyclins and Cdks are essential in vivo seems a bit out of proportion, given that the models were mostly derived from cultured cells. While simplifying facts, and delineating apparently "linear" pathways, help to explain our findings, ultimately, we need to avoid turning the models into reductionist dogma.

Selected Reading

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