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Cyclins and cdks in development and cancer: a perspective

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A fundamental aspect of cancer is dysregulated cell cycle control. Unlike normal cells that only proliferate when compelled to do so by developmental or other mitogenic signals in response to tissue growth needs, the proliferation of cancer cells proceeds essentially unchecked. This does not mean that cancer cell cycles are necessarily different from those found in normal cycling cells, but rather implies that cancer cells proliferate because they are no longer subject to proliferation-inhibitory influences arising from the stroma or from gene expression pattern changes consequent to 'terminal' differentiation, nor do they necessarily require extrinsic growth factors to recruit them into or maintain their proliferative state. Finally, cancer cells have also often avoided normal controls linked to cell cycle progression that halt proliferation in the presence of damaged DNA or other physiological insults. The result of these alterations is the inappropriate proliferation commonly associated with cancerous tumor formation. This review will summarize the current understanding of dysregulation of the G0/G1-to-S-phase transition in cancer cells, with particular emphasis on recent *in vivo* studies that suggest a need to rethink existing models of cell cycle control in development and tumorigenesis.
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Introduction

It is commonly accepted that a thorough understanding of the molecular mechanisms that lead to inappropriate proliferation of cancer cells will lead to the identification of 'targets' that can be therapeutically manipulated to arrest or kill tumor cells. To this end, considerable effort over many years has been expended in order to understand the machinery that controls normal cell cycles, thereby aiding the identification of molecules or processes altered in tumor cell cycles. The result of this effort at present is a detailed (yet still incomplete) molecular picture of cell cycle control as a series of kinase activities that promote transit through each phase of the cell cycle. The kinase family that is critical in these transitions is the cyclin-dependent kinase family (cdk), each of which, as the name implies, is dependent on a discrete protein partner, or cyclin, for activity. Activation of each cdk can thus be controlled by availability of a cyclin partner, as well as by a plethora of post-translational modifications, giving the cell exquisite control over decisions to progress from one cell cycle phase to the next. The details of this scheme for cell cycle control have been thoroughly reviewed elsewhere (Morgan, 1997; Murray, 2004) and are not the subject of this review. Here, we will focus on the broader role of cyclins and cdks in the dysregulation of proliferation control that is the signature of cancer cells.

The archetypal cdk, cdk1 (also known as cdc2), controls entry into, and exit from, M phase of the cell cycle, and commonly partners with A- and B-type cyclins in this process. Activation of cdk1 is required for dissolution of the nuclear envelope and subsequent mitotic events; inactivation of cdk1, for example, by cyclin destruction, is a prerequisite for exit from M phase and re-establishment of G1. Many recent studies have implicated aberrations in mitotic regulation in tumorigenesis, but these seem to promote genetic instability on balance, rather than serve as a rate-limiting step for inappropriate proliferation. Although these mitotic defects are clearly critical for tumor biology and are eminently exploitable therapeutically, they will not be considered further here, and the reader is urged to consult recent reviews for more detail (Bharadwaj and Yu, 2004; Draviam *et al*., 2004; Meraldi *et al*., 2004; Rajagopalan and Lengauer, 2004).

Progression to S phase

Alterations in the machinery that controls the decision to progress from a resting state into the cell cycle (the so-called G0→G1 transition) or to progress from G1 into S phase are found in virtually all tumor cells and will be the focus of this review. Thus, we will examine how the functions of cdk4 and cdk6, the partners of the G1 cyclins D1, D2 and D3, are dysregulated in cancer, and also consider the role of cdk2 and its partners cyclins E1 and E2. These regulatory proteins are functionally

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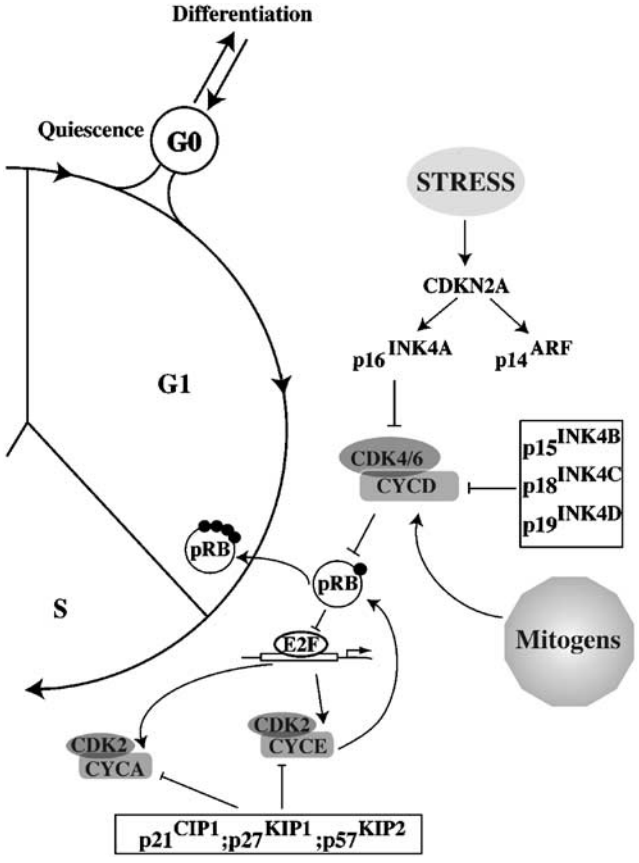

Figure 1 G1-to-S cell cycle control. Production of D-type cyclins and activation of cdk4/6 in response to mitogens results in phosphorylation and inactivation of pRB (and family members; see text) with consequent derepression of E2F-dependent transcription. This results in cyclin E and A synthesis, activation of cdk2 and further pRB phosphorylation. The activity of cdk4/6 is opposed by p16INK4a, produced in response to stress, or by other members of the INK4 family, produced in response to differentiation signals. In a conceptually similar manner, the activity of cdk2 is opposed by members of the CIP/KIP family of inhibitors, also produced in response to stress and differentiation signals. In contrast to INK4 proteins, CIP/KIP inhibitors can act as assembly factors for cycD/cdk4(6) complexes, and can be titrated away from cdk2 by these D cyclin-containing complexes. See text for details

associated with each other through the retinoblastoma protein, pRb, as shown in Figure 1. In this scheme, the core decision-making machinery for progression from G1 to S phase that is commonly altered in cancer is referred to as the pRb pathway. Briefly, D-type cyclins synthesized in response to mitogenic signals form active complexes with cdk4 or cdk6, leading to phosphorylation and inactivation of pRb. This in turn dissolves complexes of pRb with members of the E2F family of transcription factors and associated chromatin-modifying enzymes, allowing transcription of genes required for S phase. One of these target genes encodes cyclin E1, a major G1 partner of cdk2, which can both further phosphorylate pRb and also phosphorylate substrates required for proper replication origin firing, centrosome duplication and histone biosynthesis (Zhao *et al*., 1998; Hinchcliffe *et al*., 1999; Ma *et al*., 2000; Okuda *et al*., 2000; Furstenthal *et al*., 2001; Geng *et al*., 2003).

The activity of D cyclin/cdk4(6) complexes is opposed by members of the INK4 cdk inhibitor family, of which p16INK4a is the founding member. This tumor suppressor gene responds to conditions of cellular stress to shut off cdk4(6) activity, and thus lock pRb in its active, antiproliferative state. The details of this scheme have been extensively described in several excellent, recent reviews (Weinberg, 1995; Bartek *et al*., 1996; Sherr, 1996; Herwig and Strauss, 1997; Grana *et al*., 1998; Malumbres and Barbacid, 2001; Nevins, 2001; Ortega *et al*., 2002; Malumbres *et al*., 2003; Massague, 2004; Ohtani *et al*., 2004). More recently, cdk3 in partnership with cyclin C has been shown to play a role in exit of cells from G0 through early phosphorylation of pRb. Thus, this kinase complex may also play a critical role in aberrant cell cycle decision in cancer cells, but this has not yet been extensively explored (Ren and Rollins, 2004).

Alterations of the G1-to-S regulatory machinery in cancer

It is clear from Figure 1 that improper formation of cyclin D1 complexes with cdk4/6 or other aberrant hyperactivation of these complexes could act equivalently to pRb loss to render a cell insensitive to a need for mitogenic signaling. Further, such activations could promote cell proliferation in the face of physiological insult that would normally result in pRb-mediated inhibition of progression to S phase. Such aberrant cdk activation or loss of pRb has obvious implications for cancer cell generation and, indeed, pRb loss or hyperactivation of cdk4 and/or cdk6 is found in most human tumor cells. Hyperactivation of cdk4 and cdk6 can be achieved through deregulated expression of D-type cyclins, loss of p16INK4a (or other members of the INK4 family more commonly involved in differentiation or TGF-β signaling; Ortega *et al*., 2002) or mutation-based insensitivity to the inhibitory effects of p16INK4a. Hence, every element of the core pRb pathway (p16INK4a, D-type cyclins, cdk4(6) and pRb itself) represents a potential oncogene or a tumor suppressor.

Molecular analyses of human cancers fully support this notion. For instance, amplification or rearrangement of the cyclin D1 gene – located on chromosome 11q13 – as well overexpression of cyclin D1 protein has been described in a wide spectrum of human cancers such as squamous cell carcinomas of head and neck, esophagus, tongue and larynx, carcinomas of uterine cervix, astrocytomas, non-small-cell lung cancers, soft-tissue sarcomas and others (Lammie *et al*., 1991; Reissmann *et al*., 1999; Rodrigo *et al*., 2000; Cheung *et al*., 2001; Fujii *et al*., 2001; Vielba *et al*., 2003). The best documented of these is frequent involvement of cyclin D1 in pathogenesis of human breast cancer. Thus, approximately 15–20% of human mammary carcinomas contain amplification of the cyclin D1 gene (Lammie *et al*., 1991; Buckley *et al*., 1993; Dickson *et al*., 1995), while cyclin D1 protein is overexpressed in over 50% of human breast cancers (Bartkova *et al*., 1994, 1995;

Gillett *et al*., 1994; McIntosh *et al*., 1995; Gillett *et al*., 1996). Cyclin D1 overexpression is seen at the earliest stages of breast cancer progression such as ductal carcinoma *in situ* (DCIS), but not in premalignant lesions (such as atypical ductal hyperplasia). Hence, overexpression of cyclin D1 can serve as a marker of malignant transformation of mammary epithelial cells (Weinstat-Saslow *et al*., 1995). Once cyclin D1 overexpression is acquired by the tumor cells, it is maintained at the same level throughout breast cancer progression from DCIS to invasive carcinoma and is preserved even in metastatic lesions (Bartkova *et al*., 1994; Gillett *et al*., 1996).

Cyclins D2 and D3 genes are also amplified and the encoded proteins overexpressed in many human cancers. Cyclin D2 is involved in B-cell lymphocytic leukemias and lymphoplasmacytic lymphomas (Delmer *et al*., 1995), chronic lymphocytic leukemias (Motokura and Arnold, 1993), as well as in testicular and ovarian germ cell tumors, while cyclin D3 overexpression in seen in glioblastomas, renal cell carcinomas, pancreatic adenocarcinomas and several B-cell malignancies such as diffuse large B-cell lymphomas or multiple myelomas (Buschges *et al*., 1999; Ito *et al*., 2001; Shaughnessy *et al*., 2001; Filipits *et al*., 2002; Hedberg *et al*., 2002).

Likewise, overexpression of CDK4 is found (often as consequence of gene amplification) in breast cancers (An *et al*., 1999), in gliomas, glioblastomas multiforme, sarcomas and meningiomas (Khatib *et al*., 1993; Schmidt *et al*., 1994; He *et al*., 1995; Wei *et al*., 1999; Simon *et al*., 2002). Moreover, in several human malignancies, the kinase activity of CDK4 is hyperactivated due to the loss, mutation or silencing of the gene encoding the CDK4 inhibitor, $p16^{INK4a}$ (Cairns *et al*., 1994; Nobori *et al*., 1994; Spruck *et al*., 1994; Herman *et al*., 1995; Borg *et al*., 2000). Yet, another set of tumors, including retinoblastoma, osteosarcoma, small-cell lung carcinoma and bladder carcinoma, is associated with the loss of the Rb protein (Thomas *et al*., 2003).

pRb pathway in cancer: more than meets the eye

Interestingly, the vast majority of human tumors show alteration of only one member of the pRb pathway. That is, if, for example, cyclin D1 is overexpressed in a tumor, pRb and p16INK4a are normally expressed, presumably because excess cyclin D1 in complex with cdk4/6 is sufficient to evade p16INK4a and neutralize the function of pRb. This observation has been taken as evidence that the pRb pathway is linear, and that cdk4 and cdk6 have pRb as their only critical substrate, at least as far as cancer cell proliferation is concerned. In this view, pRb inactivation and subsequent loss of E2F regulation may be critically and solely responsible for improper cell cycles in tumors. It is interesting to contrast this case with that of cyclins E1 and E2, which can be oncogenic and can contribute, for example, to the pathobiology of breast tumors (Geng *et al*., 2001b; Payton *et al*., 2002). The frequency of cyclin E1/E2 expression defects suggests that they may accompany other mutations in the core pRb pathway, despite the fact that cyclin E1/E2 in complex with cdk2 is thought to participate in pRb inactivation (Lauper *et al*., 1998; Zariwala *et al*., 1998; Gudas *et al*., 1999). This apparent selection for cyclin E aberrancies even in the presence of pRb pathway mutations is likely due to 'downstream' roles of cyclin E1 in replication control, and serves to emphasize the more pRb-centric roles of D-type cyclins and cdk4(6), the functions of which are generally dispensable in cells that lack pRb.

This simple, linear view of the pRb pathway in cancer cell proliferation is complicated by several observations that suggest more complex roles for each member in tumor formation. First, it is likely that pRb is not the sole substrate for cdk4/6, despite the frequent observations that pRb-null cells are insensitive to cdk4/6 inhibition, while those expressing pRb are arrested by such inhibition. The most likely additional substrates for cdk4/6 are the pRb-related proteins p107 and p130, which can also critically regulate E2F function. Each of these has been shown to be a likely substrate for cdk4/6, and, most importantly, loss of both p107 and p130 renders MEFs insensitive to cdk4/6 inhibition, despite continued expression of pRb (Bruce *et al*., 2000). It is thus likely that the loss of pRb (or of p107 and p130) changes the cellular milieu in such a way that other targets of cdk4/6 become 'transparent' in cellular proliferation control, and thus are not able to cause overt proliferative changes when cdk4/6 are inhibited. Nevertheless, in cancer cells that express high levels of cdk4/6 activity (and these are the majority), phosphorylation of these multiple targets may each contribute to the phenotype of the tumor, and may each serve as potential targets for therapeutic intervention. In keeping with this, Smad3 has recently been shown to be a substrate for cdk4, and it was proposed that this activity of cdk4 (and, in a similar manner, cdk2) could act to limit the TGF-β responsiveness of tumor cells (Matsuura *et al*., 2004). It remains to be seen if phosphorylation of this target impacts tumor growth in situations where pRb is absent, or if pRb loss precludes the need for this type of Smad3 regulation. Thus, the notion that the pocket proteins represent the only targets for cyclin D–CDK4(6) complexes in tumor cells may be simplistic.

A second, critical set of observations that throws the strict linearity of the pRb pathway into doubt is the finding that within sets of tumors arising from specific tissue types, there are preferential target choices in the pRb pathway. For example, pRb itself is almost always mutated or lost in retinoblastomas and osteosarcomas, cyclin D1 is highly expressed in the majority of breast cancers and p16INK4a is lost at very high frequency in melanomas. Indeed, in most common tumors, as in breast cancer, pRb loss is quite rare and cancer cells seem to 'prefer' cdk4/6 activation by one or another mechanism. Thus, on a tissue-specific basis, a particular alteration of the pRb pathway may be 'better' than another, implying, perhaps, a multifunctional consequence of a particular mutation in a particular cell type.

The preferential loss of pRb in certain cell types may be explained by roles of pRb that extend beyond E2F regulation. For example, we have shown that pRb can bind to and activate the bone-specific transcription factor RUNX2 (Thomas *et al*., 2001). Loss of pRb appears to inhibit expression of certain RUNX2 target genes, particularly those acting late in differentiation (Thomas *et al*., 2001, 2004). Thus, loss of pRb may alter not only E2F function but also impact other aspects of differentiation in certain tissues, and these changes together may promote inappropriate proliferation of progenitor cells blocked from proper differentiation. This hypothesis additionally surmises that regulation of these disparate functions of pRb by cdks may not be equivalent. That is, cdk4/6- and cdk2-dependent phosphorylation events that inactivate pRb's E2F regulatory ability may not be identical to those that regulate RUNX2, for example, rendering pRb loss distinct from kinase hyperactivation. However, a definitive understanding of this process requires further investigation. Finally, pRb has been reported to influence the activity of a variety of other differentiation-specific transcription factors such as myoD and PPARγ, raising the possibility that pRb loss may impact tumor phenotype more broadly than is presently apparent, and suggesting that pRb-null tumors may, in general, be phenotypically distinct from those resulting from hyperactive cdks (Fajas *et al*., 2003; Thomas *et al*., 2003).

Hyperactivation of cdk4/6 may have multiple impacts on tumor phenotype as well. As discussed above, p107 and p130 are likely regulated by cdk4/6 activity, and at least one additional substrate, Smad 3, has been reported. Coregulation of these factors may contribute to tumor fitness *in vivo*, even though evidence for their role in tumorigenesis remains rather weak. Indeed, in mice, loss of pRb alone does not result in retinoblastoma or osteosarcoma, but does so when combined with loss of other pocket proteins (Robanus-Maandag *et al*., 1998; Dannenberg *et al*., 2004). One prediction arising from this argument is that hyperactive cdk4 or cdk6 in animal models might be a more potent inducer of tumors than is pRb loss alone, and indeed this appears to be the case. Animals expressing a p16INK4a-insensitive allele of cdk4 suffer tumors in many tissues (Sotillo *et al*., 2001), whereas the spectrum of tumors in pRb-null heterozygotes or chimeric null animals is more restricted. Although some of this difference may be due to increased levels of p107 expression in pRb-null cells, it remains likely that cdk4/6 hyperactivation is more potently tumorigenic than pRb loss in many tissues, based on both animal models and human genetics, supporting the idea that other targets of cdk4/6, and in particular p107 and p130, can act as tumor suppressors or modifiers even in the absence of pRb.

An additional example of potential multifunctionality in the role of pRb-pathway components in cancer is provided by overexpression of cyclin D1. This is common in several tumor types and, particularly, in breast tumors. The selection of cyclin D1 as a favored oncogene in breast and other tumors may arise from its multifunctionality in influencing proliferation. In addition to activating directly cdk4/6, cyclin D1/cdk complexes have been clearly established to bind and sequester p21CIP1 and p27KIP1, which act as potent inhibitors of cyclin E/cdk2 complexes, among others (Polyak *et al*., 1994; Reynisdottir *et al*., 1995; Cheng *et al*., 1998). High levels of cyclin D1 might then be able to lead to ectopic activation of cdk2 and/or other cdks acting downstream of cdk4/6. Indeed, such a role for cyclin D1/cdk4(6) complexes supports a model in which the major tumor-promoting function of pRb inactivation is deregulated expression of cyclin E, which in turn leads to unchecked S-phase entry and replication origin firing. This model is supported in concept by the observation that mice expressing cyclin E1 from the cyclin D1 locus in mice are largely protected from the phenotypic consequences of cyclin D1 loss (Geng *et al*., 1999). Likewise, expression of cyclin E1 in place of cyclin D1 'corrected' the resistance of cyclin D1-null mice to c-Erb-2-driven mammary tumor formation (Yu *et al*., 2001). Further, loss of p27KIP1 can partly compensate for cyclin D1 loss in animal models (Geng *et al*., 2001a; Tong and Pollard, 2001) and reduced expression of this cdk2 inhibitor is common in breast cancer, among others (Alkarain *et al*., 2004). However, the observation that altered timing and level of cyclin E1 expression is sufficient for development and tumorigenesis in the mammary gland does not explain why cyclin D1 is a common oncogene in human mammary tumors. Presumably, high levels of cyclin D1 and deregulated cyclin E1 expression collaborate to increase tumor fitness.

A third function of cyclin D1 that may contribute to tumorigenesis is the reported ability of cyclin D1 to alter the function of a variety of transcription factors in a noncatalytic manner. Cyclin D1 has been reported to augment the transcription activation function of the estrogen receptor as well as that of C/EBPβ, and to repress ligand-dependent androgen receptor activity, thyroid hormone receptor and the transcription factors DMP1, STAT3, BETA2/neuroD, v-myb, and PPARγ (Hirai and Sherr, 1996; Neuman *et al*., 1997; Zwijsen *et al*., 1997; Inoue and Sherr, 1998; Knudsen *et al*., 1999; Bienvenu *et al*., 2001; Reutens *et al*., 2001; Lin *et al*., 2002; Ratineau *et al*., 2002; Lamb *et al*., 2003; Wang *et al*., 2003). Although the biological importance of each of these activities remains to be clearly demonstrated *in vivo*, it is obvious in concept that such a noncatalytic alteration of transcriptional programs could have variable impact on tumor phenotype, and these effects could be of quite different levels of importance in different tissues. In support of a role for these noncatalytic functions of cyclin D1 in tumorigenesis, we and others have found that cyclin D1 mutants incapable of phosphorylating pRb can interfere with certain growth-inhibitory functions of pRb in cell culture models (Zwicker *et al*., 1999; Baker *et al*., 2005). Resolution of the relative importance of each of these functions of cyclin D1 on development and tumorigenesis awaits further genetic dissection of cyclin D1 in mouse models and cultured cell lines.

New models and new challenges to the 'master regulators' of the cell cycle

The central role for cyclins and cdks within and downstream of the pRb pathway as depicted in Figure 1 and described above presents a compelling and straightforward view of the relationship between normal cell cycles, tissue development (through regulation of cyclin/cdk function as differentiating cells become postmitotic) and cancer. In general, despite the potential for multifunctionality of some elements of the pRb pathway as described above, this model underscores the critical nature of pRb inactivation and consequent cyclin/cdk2 regulation in S-phase entry and DNA replication, a function clearly key to both normal cell proliferation and cancer. However, recent experiments both in cell culture and animal models have thrown into doubt the apparent central, indispensable nature of cdk functions in G1–S control, and suggest that our notions of the role of cdks in tumor formation needs careful rethinking.

The first blow to the commonly held view that cdk2 activity is critical for cell cycle and tumorigenesis was delivered by the McCormick lab with the demonstration that severe reduction of cdk2 levels by siRNA methods in human tumor cell lines had no impact on proliferation (Tetsu and McCormick, 2003). In contrast, inhibition of cdk4 had a profound effect on tumor cell proliferation. Although no cyclin E-associated kinase activity could be detected in such cells, this observation leaves open the possibility that cyclin E or A in association with other kinases can drive cell cycles. Indeed, cdk2 has been shown to be important for melanoma growth, so cdk2 functions, like those postulated for pRb above, may well be tissue specific (Du *et al*., 2004). Nevertheless, the observation that cdk2 is dispensable in many cell types in culture was soon supported by genetic ablation of cdk2 in the mouse, since such animals are viable (Berthet *et al*., 2003; Ortega *et al*., 2003). In both studies, it is possible that plasticity in substrate recognition allows cdk4/6 or cdk1, or some combination thereof, to fill in for the missing cdk2 and allow proliferation.

Perhaps more disconcerting are similar observations that mice lacking all three D-type cyclins, or both cdk4 and cdk6, survive until relatively late stages of embryogenesis or even until birth, respectively (Kozar *et al*., 2004; Malumbres *et al*., 2004). As with cdk2- or cyclin E-null mice, it is possible that plasticity in cyclin/cdk assembly contributes to developmental cell cycles. For example, D cyclins may be able to utilize cdk2 in animals lacking cdk4 and cdk6. Most interesting from the point of view of tumorigenesis, however, is the proliferative consequence of losing one or another of these cyclin or cdk classes in MEFs. While cyclin- or cdk-deficient MEFs can proliferate relatively normally *in vitro*, they show deficiencies in cell cycle re-entry and reduced susceptibility to transformation. For instance, cyclin E-deficient cells are unable to normally re-enter the cell cycle from quiescence, while cyclin D-deficient cells require higher mitogenic stimulation than their wild-type counterparts to exit G0. Likewise, MEFs lacking E cyclins, D cyclins or cdk4 show reduced susceptibility to oncogenic transformation (Zou *et al*., 2002; Berthet *et al*., 2003; Geng *et al*., 2003; Ortega *et al*., 2003; Kozar *et al*., 2004; Malumbres *et al*., 2004). Of relevance here is the information that double-knockout MEFs expressing a single remaining D-type cyclin remain susceptible to oncogenic transformation (Yu *et al*., 2005, submitted), underscoring a redundant role for the D cyclins in oncogenic transformation of fibroblasts. Thus, the general (albeit not complete) developmental indifference to loss of various cyclins and cdks in their entirety is not recapitulated in transformation, which seems to show profound sensitivity to loss of D- and E-type cyclins and cdk4/6 in particular.

Recently, the phenotypic consequences of each of these model systems have been elegantly analysed and compared, with the conclusion that although we may understand the nuts-and-bolts of the cdk engine that drives cycling cells, we still have much to learn about recruitment of cells into the cycle from a resting state, and the consequences of this process for tumor formation (Pagano and Jackson, 2004; Sherr and Roberts, 2004). Indeed, while it remains unclear why aberrant cell cycles are so acutely sensitive to cyclin/cdk loss, these observations parallel the accepted, highly penetrant role for these molecules in tumorigenesis described above. It is tempting to speculate that the critical role for G1 cyclins and cdks in tumor cells reflects their role in a rare progenitor cell that is crucial for cancer cell immortality, but less so for normal tissue development and homeostasis. Consistent with this hypothesis is the observation that mice lacking all three D-type cyclins display severe defects of the hematopoietic stem cells (Kozar *et al*., 2004). Clearly, this hypothesis is attractive from a therapeutic point of view, but considerable work in cell culture and animal models will be required to determine the exact and discrete roles of G1 cyclin and cdk functions in development and cancer.

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