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Cyclins and CDKS in development and cancer: lessons from genetically modified mice

David Santamaria [1] and Sagrario Ortega [2]

[1] Molecular Oncology Program and [2] Biotechnology Program, Spanish National Cancer Centre (CNIO),28029- Madrid, Spain

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1. ABSTRACT

From yeast to humans, cell cycle progression and cell division are driven by the sequential activation of a group of serine-threonine kinases called cyclin-dependent kinases (Cdks). Multiple Cdks control the cell cycle in mammals and have been long considered essential for normal proliferation, development and homeostasis. The importance of the Cdk-cyclin complexes in cell proliferation is underscored by the finding that deregulation of the Cdk activity is found in virtually the whole spectrum of human tumors. Recent information from gene-targeted mouse models for the various cyclins and Cdks have made some of the generally accepted concepts of cell cycle regulation to be revised and new and exciting questions to be investigated. Unexpectedly, most of the canonical Cdk-cyclin complexes have turned out to be dispensable for cell proliferation due to a high level of functional redundancy, promiscuity and compensatory mechanisms. As a consequence, a "yeast-like" model where only one Cdk is essential to drive all stages of cell cycle progression is starting to be envisioned for mammalian cells. Moreover, the specific molecular players that drive the cell cycle in mammals seem to be cell-type-specific, and new, non-canonical functions of cyclins and Cdks have been revealed. This review will discuss these new findings and their implications for cancer therapy.

2. INTRODUCTION

From early studies in yeast it has been widely accepted that, in all eukaryotic cells, periodic and sequential activation of one or more Cdks, achieved by controlled oscillatory levels of their regulatory subunits, the cyclins, promotes cell cycle entrance from quiescence (G0) and subsequent DNA synthesis and cell division. Unlike yeast where only one Cdk (Cdc28 in *S. cerevisiae* or Cdc2 in *S. pombe*) drives all the stages of the cell cycle, sequentially activated by different cyclin partners, in mammalian cells at least four different Cdks (Cdk1= Cdc2, Cdk2, Cdk4, and Cdk6) are associated to cell cycle control and cell division. Four cyclin families are responsible for the activation of the Cdks in different stages of the cell cycle, cyclin D (D1, D2 and D3), E (E1 and E2), A (A1 and A2) and B (B1, B2 and B3). The basic molecular mechanism of cell cycle control has been thoroughly revised elsewhere (1-6).

Cdk activity is positively and negatively regulated at multiple levels that, besides activation by binding to the cyclin partner, include phosphorylation and dephosphorylation of specific amino acid residues, subcellular localization, and inhibition mediated by protein-protein interaction. The complex molecular network that regulates Cdk activity underscores the exquisite control of

proliferation that each cell entails to fit into the development program and eventually produce a normal individual. The regulation of Cdk activity has been extensively reviewed (6, 7).

According to the long prevailing model of cell cycle control in mammalian cells, Cdk4/6-cyclin D and -Cdk2-cyclin E complexes are sequentially required to promote cell cycle entrance from quiescence, progression through the G1 phase and transition from G1 into S phase in response to mitogenic stimulation. Cell culture and biochemical studies have indicated that both Cdk4/6-cyclin D and Cdk2-cyclin E complexes are essential and rate limiting for the phosphorylation and inactivation of the tumor suppressor protein retinoblastoma (pRb) and subsequent induction of the E2F-dependent transcriptional program required for entering S-phase (8). More recently another Cdk, Cdk3, activated by binding to cyclin C, has been shown to be potentially involved in exit from quiescence by contributing to early pRb phosphorylation (9). However the role of Cdk3 in cell cycle control is still not completely understood as this gene is not expressed in many cell types and is mutationally inactivated in the mouse genome (10).

Progression through S-phase seems to be also dependent on Cdk2-cyclin E complexes as well as on cyclin A that binds and activates both Cdk2 and Cdk1 (during S phase and in G2 respectively). Finally, activation of Cdk1 by cyclin B promotes mitosis and cell division. Inactivation of Cdk1 late in mitosis by proteasome-mediated cyclin B degradation contributes to exit mitosis and re-enter G1. This model has been widely accepted as an archetype of cell cycle regulation for most if not all mammalian cell types.

Interestingly, in humans the Cdk protein family includes 20 members, 11 of which are homologous to the originally discovered Cdc28 protein of *S. cerevisiae* (reviewed in 11). The function of most of these other Cdks is still poorly understood. Whether some of them are involved in some aspects of cell cycle regulation or could functionally replace some of the canonical cell cycle Cdks needs further investigation (12).

Recently the generation of mouse strains, in which individual or multiple *cyclin* or *cdk* genes have been inactivated by gene targeting, has provided data that challenge several aspects of this canonical model leading to a more accurate view of cell cycle control in mammals .

3. DISCUSSION

3.1. G1/S control in mammalian cells: the basic model

Cells make the commitment to proliferate or not during the G1 phase of the cell cycle. The extracellular environment, by means of nutrient availability, cell-cell interaction, survival factors and mitogenic signalling dictates whether a resting (G0) cell eventually resumes proliferation. This is a very complex and still poorly understood process which impinges onto the cell cycle apparatus at many levels, one of them being the activation of the Cdk4/6-cyclin D complexes.

Unlike other cyclins, the early G1 D-type cyclin family is mostly regulated by mitogen induced signal transduction pathways, which not only promote their synthesis but subsequently control: a) their activity by modulating cyclin D translation and stability, b) their assembly with the Cdk partners Cdk4 and Cdk6, and c) the subcellular localization of the resulting heterodimers (13). Given the reduced half-life of the three D-type cyclins (14), mitogen withdrawal is rapidly followed by a reduction in the net cyclin D-dependent kinase activity, which becomes a simple and yet finely tuned oscillator mechanism that governs early G1 progression. Cyclin D turnover is also inhibited by growth factors since cyclin D degradation by the proteasome depends on the phosphorylation of threonine 286 by glycogen synthase kinase-3beta, a process that can be repressed by the PI3K/Akt signalling pathway (15). Therefore, growth factors not only induce cyclin D synthesis but also prevent its degradation.

Besides cyclin D levels, the cell cycle inhibitors of the INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$) also contribute to regulate cell cycle entrance from quiescence in response to the cellular environment. These proteins are allosteric competitors of the D-cyclins for binding to Cdk4 and Cdk6. They act as brakes for G1 progression in response to different factors such as mitogen withdrawal, proliferation inhibitors like TGF-beta, differentiation signals, oncogenic stress or senescence (16).

If mitogenic signalling prevails, the newly synthesized cyclin D pool assembles into Cdk4 and Cdk6 holoenzymes and promotes cell cycle progression by negatively regulating the so-called pocket proteins (pRb, p107 and p130). The unphosphorylated forms of these three pRb family members repress transcription from E2F-responsive promoters at different levels (17). This inhibitory effect is first mediated by binding to (and thus blocking) the activation domain of the transcription factor. Additionally this interaction impedes the formation of the pre-initiation complex within the promoter region. The pRb protein also functions as a landing pad for the recruitment of chromatin-modifying enzymes resulting in a hostile environment for active transcription (18). Conceptually, the relevance of this mechanism for G1 phase control was recently underscored by the characterization of Whi5, the so far elusive pRb orthologue in *S. cerevisiae*, describing a functionally equivalent pathway in yeast (19).

The phosphorylation of pRb family members by Cdk4/6-cyclin D complexes partially alleviates their transcriptional repressor activities. This occurs in a subset of E2F responsive promoters at mid-G1 and results in the transcription of *cyclin E* and also *cyclin A2* among many other genes. The mechanism that underlies the differential response of E2F promoters to Cdk4/6-cyclin D, and the factors that mediate it, are still unresolved issues. In any case, this is a key event in the progression through the G1 phase as the accumulation of cyclin E-dependent kinase activity above a certain threshold marks out a point of no return from which a cell is committed to division and proceeds towards DNA synthesis.

This stage of the cell cycle was initially described as the restriction point, defining a G1 step when growth factor dependency ends and cell proliferation irreversibly switches to a mitogen independent fashion (20). In molecular terms it is accomplished by the formation of Cdk2-cyclin E complexes, which conclude the inhibitory phosphorylation of pRb previously initiated by cyclin D-dependent kinases. The linearity of this pathway has been experimentally proven and it is further supported by genetic evidence. For instance, cyclin E overexpression renders proliferation of rat fibroblasts independent of both cyclin D and the phosphorylation state of pRb (21). More importantly, all defects associated to cyclin D1 deficiency are rescued by *cyclin E* cDNA when knocked-in into the *cyclin D1* genomic locus (22) or by ablation of the Cdk2 inhibitor $p27^{Kip1}$ (23). As a consequence, cyclin E expression increases from the restriction point reaching a maximal peak concomitantly to S phase entry.

However, the activation of Cdk2-cyclin E complexes does not merely rely on the increased transcriptional rate of the cyclin counterpart. In this scenario, Cdk-cyclin D complexes play an additional and non-catalytic role in G1 progression that also contributes to the activation of Cdk2-cyclin E. The Cip/Kip family of proteins ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$), true inhibitors of the Cdk2-cyclin E complex, keeps the newly formed Cdk2-cyclin E inactive by forming heterotrimeric complexes and interfering with the kinase activity. These inhibitors also bind to Cdk4/6-cyclin D complexes without causing a reduction in their enzymatic activity, at least at stoichiometrical levels. Several lines of evidence suggest that $p21^{Cip1}$ and $p27^{Kip1}$ actually play a positive role by stabilizing and facilitating the nuclear import of Cdk-cyclin D complexes (24). Although the dependency on Cip/Kip proteins for the Cdk-cyclin D activation is a controversial issue (25), it is generally believed that by titrating these inhibitors away from Cdk2-cyclin E, cyclin D- dependent kinases promote cell cycle progression independently of their enzymatic activity (6).

Completion of pRb phosphorylation by Cdk2-cyclin E allows for the full activation of the E2F transcriptional program and the synthesis of a plethora of factors required for the successful completion of subsequent phases of the cell cycle (26).

It is now becoming evident that the straight and linear input of the different Cdk-cyclin complexes on G1 progression depicted above is clearly an oversimplification. Cyclins and Cdks also function in other parallel pathways with a direct influence on cell proliferation. Until recently, the only documented substrates of the cyclin D-dependent kinases were pRb and the related proteins p107 and p130. However, recently, both Cdk4 and Cdk2 have been shown to antagonize TGF-beta- mediated growth inhibition by phosphorylating Smad3 (27). This means that G1 Cdks directly promote proliferation by revoking the activities of at least two families of transcriptional repressors. In this respect, it will be interesting to address whether pRb ablation or silencing may eliminate the need for Smad3 phosphorylation.

What is more, D-type cyclins play additional Cdk-independent functions and are involved in a complex network of interactions that modulates transcription of many genes. They do so by direct interaction with transcription factors and components of the transcriptional machinery such as the estrogen receptor or Myo D (28).

Unlike Cdk4/6-cyclin D complexes for which a relatively short number of substrates have been so far identified, many proteins have been found to be phosphorylated by Cdk2-cyclin E, including itself (cyclin E is phosphorylated by the Cdk2-cyclin E complex) and its inhibitor $p27^{Kip1}$. Phosphorylation by Cdk2 contributes to $p27^{Kip1}$ downregulation at the G1/S transition, by inducing its proteosome-mediated degradation, and therefore contributes to increasing Cdk2 activity at this crucial stage of the cell cyle by a self-activating mechanism (29, 30). On the other hand, phosphorylation of cyclin E by the Cdk2-cyclin E complex also contributes to the degradation of cyclin E in a proteasome-dependent fashion once S phase is initiated, favoring the switch of Cdk2 activity to that of Cdk2 complexed with cyclin A (31).

The Cdk2-cyclin E complex also phosphorylates other proteins involved in centrosome duplication, initiation of DNA synthesis and induction of histone gene transcription (32). The physiological relevance of these phosphorylation events, whether or not they actually take place *in vivo*, and their connection with cell cycle progression are still poorly understood. Nevertheless, taken altogether, these data have supported the concept of a non-redundant and essential function of Cdk2-cyclin E as the master key for entering S-phase, coordinating cell cycle progression with other critical cellular processes such as initiation of DNA synthesis, centrosome duplication and chromatin assembly at the G1/S transition.

Our understanding of cell cycle regulation in mammalian cells for many years has relied on tissue culture studies and biochemical *in vitro* approaches. These studies have been historically hampered by the high level of functional redundancy among different family members, and the technical difficulty for interfering specifically with a particular protein without disturbing the normal function of other family members or even other functionally unrelated proteins. The recent generation of gene targeted loss of function mouse models for individual cyclins and Cdks, or different combinations of them, has provided information about the physiological function of each one of these proteins in development and cell homeostasis.

3.2. CDK4/6-cyclin D signalling in mouse development and cell proliferation

All three D-cyclins (D1, D2, D3) have been individually eliminated in the mouse by gene targeting. The three combinations of double knock-out mice, expressing only one of the D-cyclins, and the triple knock-out mice lacking cyclin D-dependent signalling, have also been generated by intercrossing the single knock-out mice (Table 1). In parallel the cyclin D catalytic subunits, Cdk4 and Cdk6 have been ablated in the mouse by gene targeting, and the double knock-out mice have also been generated (Table 2).

Table 1. Major phenotypes of gene targeted mice lacking one or more cyclins

Targeted gene	Functional effect	Life span	Major phenotype	References
Cyclin D1	Knock-out	Viable	Neurological abnormalities. Impaired mammary epithelial proliferation during pregnancy. Retinal hypoplasia. Reduced body size.	34, 35, 38,139
Cyclin D2	Knock-out	Viable	Impaired pancreatic beta cell proliferation. Impaired granulosa cell proliferation in response to FSH. Female sterility. Hypoplastic testes, decreased sperm counts. Impaired proliferation of B-lymphocytes. Impaired cerebellar cell development.	36, 47, 140-143
Cyclin D3	Knock-out	Viable	Hypoplastic thymus.	37
Cyclins D1 and *D2*	Double knock-out	Viable. Die in the first three weeks	Hypoplastic cerebellum. Reduced body size	40
Cyclins D1 and *D3*	Double knock-out	Neonatal death. Some survive up to two months	Neurological defects.Respiratory failure.	40
Cyclins D2 and *D3*	Double knock-out	Embryonic lethality at E17,5-E18,5	Megaloblastic anemia	40
Cyclins D1, D2 and *D3*	Triple knock-out	Embryonic lethality at E16,5	Megaloblastic anemia. Defective fetal hematopoiesis.	51
Cyclin E in *cyclin D1*	Knock-in. Gene replacement	Viable	Rescue of the cyclin D1 knock-out Phenotypes.	22
Cyclin D2 in *cyclin D1*	Knock-in. Gene replacement	Viable	Rescue of the cyclin D1 knock-out phenotype in breast.	41
Cyclin E1	Knock-out	Viable	No detectable abnormalities.	76, 77
Cyclin E2	Knock-out	Viable	Reduced male fertility.	76, 77
Cyclin E1 and *E2*	Double knock-out	Embryonic lethality at E.11,5	Lack of trophoblast-derived polyploid giant cells of the placenta: placental failure. Tetraploid rescue: normal embryo development. Perinatal death: cardiac abnormalities.	76, 77
Cyclin A1	Knock-out	Viable	Male sterility. Meiotic arrest at the prophase-metaphase transition in spermatocytes.	71, 72
Cyclin A2	Knock-out	Embryonic lethal at implantation	?	92
Cyclin B	Knock-out	Embryonic lethal at E10,5	?	96
Cyclin B2	Knock-out	Viable	No detectable abnormalities.	96

Table 2. Major phenotypes of gene targeted or spontaneous mutant mice lacking one or more Cdks

Targeted gene	Functional effect	Life span	Major phenotype	References
Cdk4	Knock-out	Viable. Reduced life span due to diabetes	Pancreatic beta cell proliferation impaired: diabetes. Anterior pituitary cell proliferation impaired, particularly lactotrophs. Leydig cell numbers reduced. Defective spermatogenesis. Reduced body size.	42, 43
Cdk6	Knock-out	Viable. Normal life span	Hypoplastic thymus and spleen. Reduced erythrocyte and megakaryocyte numbers. Reduced body size only in females.	50
Cdk2	Knock-out	Viable. Normal life span	Spermatocytes die in pachytene. Oocites die in diplotene. 100% esterility in males and females. Strain dependent reduced body size.	60, 61
Cdk3	Natural mutation. Premature translation termination. Lack of Cdk3.	Viable	Most laboratory mouse strains carry this mutation. Therefore all the other models are also null for Cdk3.	10
Cdk4 and *Cdk6*	Only one allele of *Cdk4* is expressed. *Cdk6* knock-out.	Viable	?	50
Cdk4 and *Cdk6*	Only one allele of *Cdk6* is expressed *Cdk4* knock-out	Partial embryonic lethality	?	50
Cdk4 and *Cdk6*	Double knock-out	Embryonic lethality at E14,5-E18,5	Defective fetal hematopoiesis. Severe anemia.	50
Cdk2 and *Cdk6*	Double knock-out	Viable. Normal life span.	Addition of individual knock-out phenotypes.	50

In this section we will summarize the most important findings related to the function of Cdk4/6-cyclin D in the control of mouse development based on the information obtained from the phenotypical characterization of these gene targeted mouse strains.

3.2.1. Individual D-cyclins are essential in specific cell types

Throughout most of the embryonic development and in the adult, expression of each of the D-type cyclins occurs in a tissue-specific although greatly overlapping manner (33). Thus, the consequences derived from the ablation of each one of the three D-cyclins in the mouse may be alleviated by any of the two remaining family members. Indeed, that would seem to be the case in light of the very limited developmental abnormalities found in single D-type cyclin knock-out mice (34-39). All three single knock-out strains are viable with only tissue specific defects often circumscribed to very particular cell types (Table 1).

However, as the three single *cyclin D* knock-out models are germ line knock-outs, where the genes are constitutively inactivated, compensatory mechanisms during embryonic development may also contribute to explain the mild phenotypes of these mice. The existence of such compensatory mechanisms, often vaguely defined as embryonic plasticity or developmental compensation, was unambiguously demonstrated for the D-cyclin gene family by Ciemerych and co-workers by generating "single-cyclin" mice, where all the different combinations of two out of the three D-cyclins are constitutively inactivated in mice (40), and proliferation of specific cell types becomes critically dependent on the remaining intact D-cyclin. The analysis of the embryonic development of such single-cyclin mice showed that in these animals the typical tissue-specific pattern of D-cyclin expression was not maintained and the knock-out embryos displayed ubiquitous expression of the remaining intact cyclin. Interestingly this "compensation" is somehow achieved by different molecular mechanisms in different embryonic tissues. In some of them compensation relies on altered transcriptional regulation, such that an increase in the mRNA level of the remaining cyclin is observed, whereas in other tissues it depends on posttranslational pathways (40). In any case these results clearly show that during embryogenesis, cellular proliferation relays on net cyclin D activity and the three family members are essentially functionally exchangeable in most, if not all, cell types.

Conclusions from the analysis of adult single-cyclin mice are in essence equivalent. Whereas cyclin D1-only animals die during late embryogenesis, cyclin D2-only and D3-only mice are viable and some survive for several weeks with the majority of organs showing no apparent defects (40). Interestingly, the double knock-out mice do not show new phenotypes, but the sum of the ones observed in mice lacking the individual cyclins. Just like the embryonic cycles, proliferation of adult cells not only requires the remaining D-type cyclin but also the ability to up-regulate its expression in tissues where, under normal circumstances, it would be absent or expressed at reduced levels. Failure to achieve enough expression of the remaining cyclin D results in tissue-specific defects with variable penetrance depending on the nature of the remaining cyclin. Remarkably, these defects were traced to the inability of specific transcription factors to specifically up-regulate the remaining cyclin in a particular tissue. Still, whether at least in these defective tissues there are inherent properties unique to each specific cyclin is still a possibility that cannot be totally ruled out.

However, recent work from the Sicinski´s laboratory has provided further insight in this direction. Cyclin D1 deficient mice showed the most severe phenotype of the three single knock-out strains and displayed postnatal lethality, reduced body size, hypoplastic retina, neuropathy and defective breast development during pregnancy (34, 35). Carthon and coworkers have now generated a knock-in mouse strain in which cyclin D2 is expressed in place of D1 and shown that, with the exception of the mammary glands where cyclin D2 fully corrects the *cyclin D1* null phenotype, the rescue of other phenotypes is incomplete (41). Thus, while it seems that the distinction between these two cyclins (and possibly between all three family members) relies mostly on their tissue-specific expression pattern, still subtle functional differences exist. This, in turn, may be essential for the delicate temporal and spatial regulation of proliferation and development in such a variety of coexisting cell types.

3.2.2. Cdk4 or Cdk6 ablation in the mouse: the other side of the coin

Concurrently with the above findings, mouse strains devoid of the catalytic partners of the D-type cyclins have been generated. Cdk4 and Cdk6 are considered ubiquitous enzymes, co-expressed in most cells along all stages of development. However, lack of reliable antibodies for these proteins impairs a thorough characterization of their expression pattern by immunohistochemical techniques. Individual Cdk4 and Cdk6 defective mice are viable and only show tissue-specific defects similar to those observed in the single *cyclin D* knock-outs, underscoring the robust functional correlation between these two protein families (Table 2).

Cdk4 knock-out mice are viable albeit with reduced body size, resembling in this aspect the phenotype of *cyclin D1* null mice (42, 43). They exhibit defects in specific subsets of endocrine cells. Males have reduced numbers of Leydig cells and decreased fertility. Females are sterile as a consequence of low counts of anterior pituitary cells that results in prolactin deficiency, abnormal estrus cycle and impaired pregnancy (44, 45).

But the most striking phenotype in these mice is a severe defect in another endocrine cell type. *Cdk4* knock-out animals show a dramatic reduction in the numbers of insulin-producing beta cells resulting in the early onset of insulin-dependent diabetes and therefore limited lifespan (42, 43). Remarkably, Cdk4 deficiency does not affect neogenesis of beta cells during embryonic development but becomes essential for a proliferative burst of this cell type

in late gestation and postnatal development (45). Interestingly, a knock-in strain that expresses endogenous levels of a mutant form of Cdk4 that is resistant to inhibition by INK4 proteins (Cdk4R24C), develops beta cell hyperplasia and insulinomas with age (42, 46) underscoring the importance of the regulation of the Cdk4 activity for beta cell proliferation. This knock-in strain develops additional phenotypes that are discussed in a latter section of this article.

The strong dependence on Cdk4 for postnatal beta-cell proliferation is probably mostly attributable to the virtually lack of Cdk6 expression in pancreatic beta cells (45) and therefore the complete reliance of this cell type on Cdk4 activation to enter the cell cycle. Alternatively, the existence of a specific function of Cdk4 in postnatal beta cells can not be totally ruled out. An attempt to rescue the beta cell phenotype by expressing a Cdk6 transgene specifically in beta cells of mice lacking Cdk4 is in progress in our laboratory and will help to resolve that issue.

Among the D-cyclins, cyclin D2 is the one that is more abundantly expressed in beta cells (45), and it is reassuring to learn that mice lacking cyclin D2 also have impaired beta cell proliferation and develop diabetes, albeit less severe than *Cdk4* null mice (47). This is likely due to functional redundancy of cyclin D2 with cyclin D1, as the loss of a functional *cyclin D1* allele, in mice lacking cyclin D2, exacerbates the diabetic phenotype (48). Therefore, it seems that beta cells depend mostly on the Cdk4-cyclin D2 complex to proliferate in response to metabolic needs. The implications that this may have in terms of developing new therapies for beta cell regeneration of diabetic patients are starting to be explored.

The reduced body size phenotype in Cdk4 deficient mice deserves additional attention. This phenotype is caused by a reduction in the total number of cells rather than to an overall decrease in cell size. In an attempt to rescue the *Cdk4* null phenotype, *Cdk4* knock-out mice, that have a targeted inactivation of the *Cdk4* gene by insertion of a floxed *PGK-neo* cassette into the first intron and in opposite transcriptional orientation to the one of the *Cdk4* gene, were crossed with a transgenic mouse line in which the Cre recombinase is expressed under the control of the rat insulin gene promoter (RIP) (45). This line expresses Cre in beta cells and in the primordial pituitary (the Rathke pouch) during embryonic development. The Cre-mediated excision of the *neo* cassette, restored the endogenous expression of Cdk4 specifically in beta-cells and in the pituitary of Cdk4-deficient mice. This genetic approach effectively rescued the diabetes and female sterility phenotypes but the animals remained small, with identical body size to that of *Cdk4* knock-out mice (45). Therefore, the reduced body size phenotype is not a secondary effect of the endocrine malfunction, but rather a defect caused by a reduced proliferation rate of most cell types in the body in the absence of Cdk4 (45). It is then tempting to speculate that this is a cell autonomous phenomenon and that Cdk4 may play a role in establishing homeostatic cell numbers. Remarkably, ablation of the *Cdk4* locus in *Drosophila* results in a very similar outcome. Cdk4 deficient flies develop and eclose normally but are smaller due to reduced cell numbers (49). As *cyclin D1* null mice exhibit the same small size phenotype, it is likely that control of cell numbers in mammals relies mostly on Cdk4-cyclin D1 activation by mitogenic signalling.

Animals devoid of Cdk6 have also been generated. They show the least compromised phenotype of all the single D-cyclin and D-cyclin-dependent-Cdk knock-out models. Intriguingly, only females have marginally reduced body size but are fertile and show a normal life span. Males have normal body size and are fully fertile. Animals of both sexes exhibit mild defects in some haematopoietic compartments. For instance, thymus and spleen are hypoplastic, showing a specific dependence of lymphatic tissues on Cdk6 for normal development (50).

Perhaps the most logical explanation for these narrow phenotypes in Cdk4 and Cdk6 deficient mice, again comes from the high structural and functional homology between these two kinases that would result in their *in vivo* functional redundancy, at least in those tissues where both proteins are expressed. Although this may be at least partially true in some cell types, an alternative explanation to account for the absence of extreme phenotypes in the cyclin and Cdk deficient strains described above is that proliferation and differentiation of certain (if not the majority) of cell types can be brought about in a Cdk4/6-cyclin D-independent manner.

3.2.3. Most of the embryonic tissues are Cdk4/6-cyclin-D independent

According to our current understanding of the core cell cycle machinery, D-type cyclins are regarded as essential mediators of extracellular signalling. Therefore, the hypothesis stemming from the results depicted above, that at least some cell types might proliferate in a cyclin D autonomous fashion, required unambiguous genetic support. This has now taken form in two recent studies from the Barbacid and Sicinski groups providing genetic evidence that many cell types of the developing embryo indeed proliferate normally in the absence of Cdk4/6-cyclin D complexes.

Kozar and coworkers inter-crossed cyclin D1, D2 and D3 deficient mice in an attempt to generate triple knock-out embryos. Surprisingly, conceptuses lacking all D-cyclins developed until mid/late gestation without any overt evidence of pathology (51). In a parallel set of experiments, Malumbres et al. reported the effect of eliminating both *Cdk4* and *Cdk6* genes in the mouse (50). On balance, the phenotype of the double *Cdk4/6* null mice reassuringly mirrors that of cyclin D deficient animals and viceversa. In both cases embryos begin to die in utero at E14.5, i.e., a time when many organs are well developed. Although none of the *D-cyclin* null embryos progress further than E17.5, a number of Cdk4/6 deficient embryos do, and actually some even complete the gestational period only to die soon after birth. In both cases the cause of death is attributed to multilineage haematopoietic abnormalities resulting in a severe megaloblastic anemia. In accordance with this finding, the numbers and the proliferative capacity of all haematopoietic stem cells from fetal livers, are drastically reduced both *in vivo* and *in vitro*.

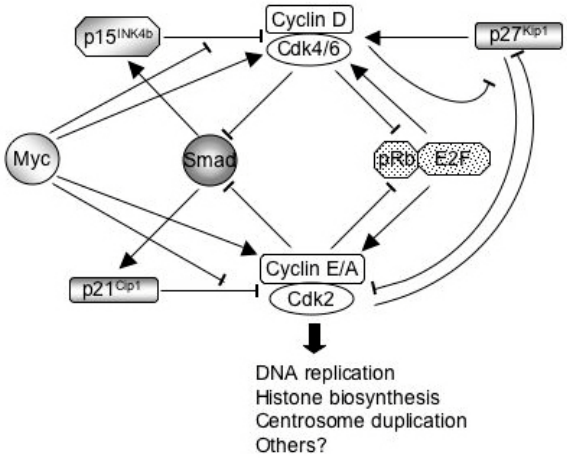

Figure 1. Schematic representation of Cdk substrates and built-in regulators at the G1 phase of the cell cycle. The diagram portrays the functional parallelism between Cdk4/6 and Cdk2, supporting the existence of compensatory mechanisms and functional redundancy that may emerge upon inactivation of one of the two kinase-pathways.

With the only exception of a ventricular septal defect, not present in the Cdk4/6 double mutant mice, the fact that the very same cellular compartment, the hematopoietic tissue, is affected in both groups of mice provides genetic evidence that the main function of the D-type cyclins in development is to activate Cdk4 and Cdk6. It is important to note though, that knock-out embryos of both strains show reduced body size, highlighting the significance of these Cdk-cyclin complexes in controlling cell growth, cell proliferation or both. However, the tantalizing finding that the majority of cell types can arise in the absence of such complexes has challenged our understanding of cell cycle regulation. Or has it not?

In this respect, it is important to keep in mind that the most drastic consequences of the absence of Cdk4/6-cyclin D complexes arise in tissues with a high proliferative demand like the fetal haematopoietic compartment. Some precedent for this finding exists; mice devoid of Bmi-1, a repressor of the Cdk4/6 inhibitor $p16^{INK4a}$, display impairment of haematopoietic proliferation and T-cell development (52). Also *cyclin D3* null T-cell progenitors are severely crippled in their ability to undergo pre-TCR expansion (37). Finally, *Cdk4* null animals fail to undergo the proliferative burst of pancreatic beta cells during late embryogenesis (45). The characterization of the cell autonomous differences between Cdk4/6-cyclin D-dependent and -independent tissues will obviously help to better understand how mammalian cell cycle is regulated, but *a priori* it seems that different cell types may require different thresholds of cyclin-dependent kinase activity for efficient proliferation. "Turbo-proliferative" tissues, such as the fetal hematopoietic compartment, may have a very high threshold, that can not be reached in the absence of cyclin D-dependent signalling.

Altogether, one synthesis of these findings is that the so-called plasticity or developmental compensation may comprise a plethora of different but not mutually exclusive mechanisms that cooperate to promote proliferation. At the end of the day, the exact combination of these redundant pathways that is activated in each particular cell type will likely reflect their different proliferative requirements (Figure 1). This, of course, underlies a critical issue in cancer biology and may be related to the exquisite tissue selectivity for specific mutations (and combinations of them) during tumor progression, a process which we are only beginning to unravel (53).

3.2.4. Mouse embryonic fibroblasts (MEFs) lacking cyclin D-dependent signalling can cycle

The vast majority of the experiments that support the central role of Cdk4/6-cyclin D complexes in the response to the extracellular mitogenic environment involve interfering with Cdk activity *in vitro* by a variety of methods which include: chemical inhibitors, expression of dominant negative Cdk mutants, microinjection of neutralizing antibodies, overexpression of Cdk negative regulators and the use of antisense oligonucleotides (reviewed in 13). While having in mind the technical limitations of these experimental approaches, mounting evidence has accumulated to substantiate an indisputable function of Cdk4/6-cyclin D in promoting G1 progression. Experiments performed *in vitro* with primary cultures derived from the compound mutant mice are illuminating as allow for the direct testing of this function of D-cyclin signalling with much less technical caveats.

Cdk4/6 double knock-out and *D-cyclins* null primary MEFs display a decreased rate of proliferation in culture, which is aggravated with time, resulting in a premature senescent phenotype. Even when these cells, as expected, are resistant to the Cdk4/6-cyclin D inhibitor $p16^{INK4a}$ (one of the major mediators of the cell cycle arrest experienced by senescent cells) their life span *in vitro* is compromised by the lack of D-cyclin dependent signalling. In any case, the proliferation of asynchronous fibroblasts of both genotypes, under continuous mitogenic stimulation, proceeds reasonably unaffected (50, 51).

Considering the previously ascribed functions for the ablated proteins as major intracellular sensors for mitogenic signalling, the analysis of G0 exit in response to growth factors in MEFs lacking D-cyclins or Cdk4 and Cdk6 was an absolute requisite. In this approach cells are rendered quiescent by serum deprivation and re-entry into the cell cycle is analyzed after arrested cultures are re-stimulated with 10% serum. In every experiment mutant cells failed to respond as efficiently as the controls to the mitogenic stimuli and only a subset of cells resumed proliferation. This phenotype was exacerbated when cells were stimulated with lower concentrations of serum causing the appearance of more severe cell cycle re-entry defects. Remarkably, in these assays the cells that did respond to the proliferative stimuli appeared to complete cell division with normal kinetics indicating that Cdk4/6-cyclin D complexes are not essential for mitogen induced cell cycle entrance from G0, although they do play a role in this process (50, 51)

At a molecular level, the proliferation defects in both groups of mutant cells correlated with a significant

reduction in the overall levels of pRb phosphorylation during cell cycle re-entry and a concomitant delay in the activation of E2F targets. Several consensus Cdk target sites exist on pRb but their kinase specificity and, specially, their functional implications still require more in depth characterization. The prevailing model in the field assumed that phosphorylation of Cdk4/6 specific sites would precede pRb phosphorylation by Cdk2 (54). However, while phosphorylation of a range of Cdk4/6 specific sites was significantly diminished in the knock-out cells this was not the case for T-821, a known Cdk2-cyclin E and -cyclin A specific residue (50, 51). Altogether these results suggest that pRb phosphorylation on the cyclin E- and A-specific sites, not only is independent of Cdk-cyclin D complexes but it is also sufficient to functionally inactivate its repressor activities, allowing for the activation of E2F targets and cell cycle progression. In fact, shRNAs directed to Cdk2 in both double-Cdk4/6, and triple-cyclin D deficient MEFs strongly inhibited cell proliferation while having a marginal effect on wild-type cells. Interestingly, in these cells the activity of Cdk2 was not diminished in spite of the fact that free $p27^{Kip1}$ can not be sequestered by Cdk-cyclin D complexes. This may be in part due to the fact that overall levels of $p27^{Kip1}$, and also of $p21^{Cip1}$ at least in Cdk4 and 6 deficient cells, happen to be reduced compared to wild-type counterparts (50).

On balance, although still lacking genetic confirmation, in the absence of cyclin D-dependent kinase activity, cell proliferation seems to become absolutely dependent on Cdk2. In this respect it is also interesting to note that in the absence of Cdk4 and 6 there is an increased association of cyclin D to Cdk2, and in agreement with previous reports (55) these complexes show pRb kinase activity (50). This in turn may partially compensate for the absence of Cdk4 and 6 and explain the slightly delayed embryonic lethality and milder haematopoietic defects shown in Cdk4/6 deficient mice compared to *cyclin D*-null animals.

An interesting argument raised by the above mentioned experiments is the existence of Cdk4/6-cyclin D-independent mechanisms that connect the activation of cyclins E and A to the extracellular environment. Earlier experiments showed that overexpression of a non-phosphorylatable form of pRb only induced a transient G1 arrest, and in this scenario induction of cyclin E was achieved via a c-Myc-dependent pathway (56). It is currently unknown whether other parallel pathways functionally coexist in mammalian cells, but *Cdk4/6* double knock-out- and *cyclin D*- null cells will help to address this issue.

3.3. CDK2-cyclin E signalling in mouse development and cell proliferation

Elucidation of the importance of Cdk2-cyclin E signalling in cell cycle control *in vivo* has been made possible by the generation of individual knock-out mice lacking either cyclin E1 or E2, and the double knock-out mice lacking both E-type cyclins. In parallel, Cdk2, the only so far known catalytic partner of the E-cyclins, has been also inactivated in mice by gene targeting (Tables 1 and 2). The phenotypical characterization of these mice has also provided unexpected results that will be discussed in this section.

3.3.1. Cdk2 is dispensable for somatic cell proliferation and development

As discussed above, Cdk2 and its activators the E-type cyclins have been long considered essential regulators of the mammalian somatic cell cycle. The general thinking was that these proteins were required for promoting and coordinating not only the G1/S transition but also chromosome and centrosome duplication, histone synthesis and chromatin assembly. Recent work from different laboratories, including ours, has utterly challenged this model and showed that these seemingly essential molecules are dispensable for mouse development and cell proliferation.

The first surprising setback to the up to then prevailing model took form when depletion of Cdk2 through antisense oligonucleotides or siRNA, opposite to what had been previously reported and generally accepted from very similar experimental settings (57), was shown to have a negligible effect on the proliferation of colon cancer cell lines (58). However, a note of caution was raised to the generalization of Cdk2 dispensability as cancer cells often have anomalous cell cycle regulation that could result in compensatory mechanisms by the action of other misregulated pathways (59). This uncertainty was erased immediately afterwards when Ortega and co-workers reported the targeted gene inactivation of Cdk2 in the mouse. Unexpectedly, *Cdk2* null mice are fully viable and, with the exception of a dramatic meiotic failure, show no sign of developmental abnormalities indicating that this kinase was truly dispensable for the majority, if not all, of somatic cells (60, 61).

In fact, unexpectedly, *Cdk2* knock-out mice are the least affected of all the cyclin and Cdk deficient strains generated so far in terms of somatic cell phenotype, and have a lifespan equal to that of wild-type littermates. Cdk2 dispensability, however, does not necessarily imply that this kinase does not perform central (although not essential) functions in cell proliferation and embryonic development. Likewise, compensatory mechanisms could play a role in substituting Cdk2 function raising the question of how its absence may be balanced in such an immense variety of cellular settings. However, the *Cdk2* knock-out allele generated by Ortega and co-workers is a conditional allele ($Cdk2^{fl}$). Cre-mediated inactivation of Cdk2 in primary cultures of $Cdk2^{fl/fl}$ mouse embryonic fibroblast has no effect on cell proliferation, therefore arguing against compensatory mechanisms activated by developmental plasticity during embryonic development (60).

It is tempting to speculate that in the absence of Cdk2 cell cycle progression might be driven by other G1 kinase, mainly Cdk4. For instance, upon Cdk2 depletion Cdk4 phosphorylates some of the "Cdk2-specific" sites on pRb (58). The converse situation is also true. As previously mentioned, in the absence of Cdk4/6-cyclin D, pRb phosphorylation is reduced but not eliminated (50, 51) and

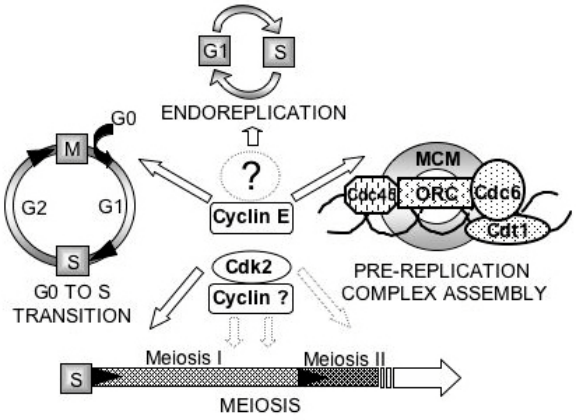

Figure 2. New functions of cyclin E and Cdk2 revealed by gene targeted mouse models. Cyclin E promotes entrance in S-phase from quiescence (G0) and DNA endoreplication in trophoblast-derived placental giant cells and megakaryocytes in a Cdk2-independent manner. At the G0 to S transition cyclin E is required for the recruitment of the MCM complex to the PRCs at the origins of DNA replication, while Cdk2 is not. The catalytic partner of cyclin E in these processes is unknown. Cdk2 is essential in spermatogenesis for progression of prophase I beyond pachytene, and for survival of oocytes beyond diplotene, also at prophase I. The cyclin(s) that activate Cdk2 at different stages of meiosis are still unknown. Cdk2 may have other functions at later stages of meiosis.

proliferation becomes dependent on Cdk2 activity. Additionally, just like Cdk2, Cdk4-cyclin D1 associates to the pre-replicative complex at origins of DNA replication (62). Therefore, the degree of built-in redundancy of the system may be much higher than anticipated to the extent that a single G1-Cdk might suffice to promote cell proliferation (Figure 1). Indeed, *Cdk2/6* double knock-outs are perfectly viable and show no sign of pathology throughout their entire lifespan (50), indicating that Cdk4 by itself is able to drive normal cell proliferation, development and homeostasis. Genetic linkage between *Cdk2* and *Cdk4* has so far precluded the generation of *Cdk2/4* double knock-out mice, but in the light of the above mentioned results it will be very interesting to asses whether Cdk6 alone can sustain G1 progression and promote exit from quiescence.

Considering that D-cyclins, Cdk4, Cdk6, Cdk2 and also cyclin E (see below) are all dispensable for cell proliferation another conceivable scenario would be one in which concatenation of unperturbed mitotic cycles could be driven by Cdk1 alone associated to A- or B-cyclins (63), or stretching the dogma a bit further, Cdk1-cyclin B *in solo* would just do the job. In this respect, cyclin B1 is sequestered in the cytoplasm and translocates into the nucleus in prophase (64). However, using a *Xenopus* cell free system Moore and co-workers have recently shown that if cyclin B1 is prematurely relocated into the nucleus it can then successfully promote DNA replication (65). This is reminiscent of the cell cycle in yeast where a single mitotic cyclin allows ordered progression through the cell cycle (66). However, the simplicity of alternating rapid S and M phases during the syncitial phase of early development in *Xenopus* or *Drosophila* is difficult to reconcile with a far more complex setting such as the developing mammalian embryo. Even in budding yeast, cell cycle regulation is not that undemanding and S and M cyclins are not equally interchangeable due to subtle substrate specificity (67). Maybe other Cdks, not directly implicated in cell proliferation control, may come into play in a situation where "canonical" cell cycle Cdks are absent. As an example Cdk9 is able to bind and phosphrylate pRb (68) and Cdk3, although inactive in all mice laboratory strains due to a point mutation, has been recently found to play a role in promoting G0 exit (9).

Finally, there is a substantial degree of homology between the Cdks and other kinases (69) of which only a minority have been sufficiently studied, and thus could also cooperate in cell cycle regulation (70). Whether any of these kinases may account for a Cdk-independent mechanism promoting G1 phase progression and to what extent they are related to the still undefined mechanisms of the so called "functional compensation" still requires much more effort to elucidate.

3.3.2. Cdk2 is essential in meiosis

The meiotic defect of the *Cdk2* knock-out mice deserves additional comment. Although the male and female phenotypes differ, both sexes are completely infertile with a 100% penetrance.

Male germ cells do not progress beyond the pachytene stage of prophase I. At this stage primary spermatocytes undergo apoptosis probably as a consequence of defective synaptonemal complex formation both at the level of axial/lateral and central elements (60). Oocytes however, progress normally through pachytene where they show normal synapsis and continue further to the late diplotene or dictyate stage where they die by apoptosis, such that in the first week of age the ovaries of *Cdk2* null females are devoid of oocytes.

Heterodymorphic meiotic phenotypes are common in genetic modified mice demonstrating sexual divergence in the molecular control of the meiotic cycle. Nevertheless the phenotype of *Cdk2* null mice indicates that Cdk2 is essential for germ cell development beyond meiotic prophase I, both in males and in females (Figure 2). Interestingly, although some kind of meiotic collapse is a common phenotype in most mouse models missing cell cycle regulators (39), this is the first cell cycle regulatory gene shown to be essential for both spermatogenesis and oogenesis.

The male germ-cell specific and Cdk2 regulatory partner cyclin A1 is also essential for spermatogenesis during prophase I (71). However *cyclin A1* knock-out spermatocytes progress normally up to the transition from prophase to metaphase. They stop just before metaphase I due to their inability to activate the Cdk1-cyclin B complex (72). Nevertheless, unlike in *Cdk2* knock-out mice, the chromosomes undergo normal synapsis in the absence of

cyclin A1. Therefore, Cdk2 must have a function in spermatogenesis, earlier in the meiotic prophase I, that is independent of cyclin A1.

A search for meiotic substrates of the Cdk2-cyclin A1 complex has provided a new clue of what a function of Cdk2 may be in spermatogenesis and perhaps a more general function of Cdk2 in DNA double strand break repair. A triple hybrid screening in yeast has shown that the Ku70 protein, one of the subunits of the DNA-PK complex involved in DNA repair, interacts with and is phosphorylated by Cdk2–cyclin A1 (73, 74). Similarly, recent data indicate that BRCA2, a protein involved in DNA repair by homologous end joining is phosphorylated by either Cdk1 or Cdk2 and this phosphorylation modulates the binding of BRCA2 to Rad51 (75). Massive double strand brake activity and repair by homologous recombination are some of the hallmarks of the prophase I of meiocytes. Whether Cdk2 is involved in double strand brake repair in meiosis requires further investigation.

3.3.3. E-cyclins are dispensable for most, if not all, embryonic develoment but required for placental function

The unexpected finding that murine Cdk2 was overtly dispensable for cell cycle progression and embryonic development was accompanied by another equally surprising result coming from the generation of mice devoid of the E-type cyclins (76, 77). The comparison of the phenotypes of these two models has brought to light very intriguing Cdk2-independent functions of the E-type cyclins with profound implications for cell cycle regulation.

Both cyclin E1 and E2 share significant amino acid homology and are expressed in an overlapping manner in virtually all proliferating cells (78). With the exception of a 50% male infertility in *cyclin E2* null mice, individual deletion of either gene has no significant phenotypical consequences. Actually, mice carrying a single wild-type allele encoding cyclin E1 and null for *cylin E2* also develop to term and are born at Mendelian ratios with males showing a more pronounced infertility phenotype compared to *cyclin E2* single null males. Therefore, either the two E-type cyclins perform overlapping functions and a single *cyclin E* allele is sufficient for proliferation and normal embryonic development, or alternatively, cell proliferation and development of most embryonic tissues may not require E-type cyclins.

A preliminary analysis rejected the latter explanation as no *cyclin E1* and *E2* double knock-out mice were born alive and embryonic lethality was estimated to occur at around E11.5. However, at this stage double knock-out embryos, albeit showing some growth retardation, displayed normal organogenesis and morphogenesis and even their proliferative rate mirrored that of wild-type littermates as shown by BrdU incorporation (76, 77). However mutant embryos did show a dramatic defect in their placental structures that resulted in reduced vascularization of the yolk sac and the embryonic tissues. In particular the layer of trophoblast giant cells was nearly absent in the placentas of mutant conceptuses which instead contained a layer of underdeveloped trophoblastic cells. Given the pivotal role of this cell type in placental physiology this defect was likely the cause of death of cyclin E deficient mice.

But should this hypothesis be valid the embryonic development of mutant mice could in principle be recovered by tetraploid complementation rescue. In this method wild-type two-cell embryos are fused by an electric shock giving rise to a tetraploid embryo that eventually develops into a blastocyst. Mutant embryonic stem (ES) cells are injected into these tetraploid blastocysts that are then implanted into foster females. Importantly, tetraploid blastocysts fail to contribute to the embryo proper but yet form normal placentas and other extraembryonic tissues (79, 80). Geng and co-workers applied this method with several ES cell lines established from double mutant *cyclin E1* and *E2* null blastocysts and managed to fully rescue the embryonic lethality of cyclin E deficient mice (76). This approach not only proved that the embryonic lethality was indeed due to a placental dysfunction but remarkably, that the entire embryonic development can occur in a cyclin E independent manner.

This, however, does not apply to the cardiovascular system. Up to 50% of the rescued mutant embryos died perinatally due to multiple cardiac abnormalities, preventing the characterization of cyclin E deficiency in adult mice. As all embryos generated by tetraploid rescue die soon after birth unless they are originated from hybrid ES cells (81), the question remains of whether the lethality in tetraploid rescued *cyclin E* null fetuses and newborns is due just to the lack of E-cyclins or the consequence of other genetic or epigenetic abnormalities of the established ES cells. Nevertheless this result demonstrated that cyclin E plays a role in placental development that is independent of Cdk2, as no abnormalities are found in placentas of *Cdk2* null mice.

The placental dysfunction in *cyclin E* null mice is itself very interesting. It is caused by a defect in the trophoblast giant cells present in this organ (76). These cells normally undergo a process known as endoreplication: repeated rounds of DNA synthesis without intervening mitoses leading to increases in DNA content up to 1000N. In this particular cell type the process of endoreplicaton has been postulated to rely on the inhibition of cyclin B1 translation (82). Remarkably, the increment on DNA content fails to occur in the absence of cyclin E suggesting that cyclin E is essential for endoreplication (76, 77).

In addition to trophoblast giant cells, megakaryocytes also undergo endoreplicative cycles. Rescued cyclin E deficient embryos permitted the analysis of this cell type and revealed that the absence of E-type cyclins also impaired the increment of DNA content in this cellular context (76). Importantly, cyclin E has been previously implicated in endoreplicative cycles of a very different cellular setting, namely that of *Drosophila* salivary glands (83). The phenotype of *cyclin E*-null mice clearly demonstrates that in mammals cyclin E is essential for endoreplication and that this function is independent of Cdk2 (Figure 2).

3.3.4. E-cyclins but not Cdk2 are required for entering S-phase from quiescence

Importantly, the tetraploid complementation strategy also makes possible the isolation of primary MEFs derived from rescued *cyclin E* null embryos. MEFs lacking E-cyclins proliferate normally in continuous culture and only show a negligible increase in their population doubling time together with a precocious entry in senescence. This behaviour very much parallels that of *Cdk2* knock-out primary MEFs which also proliferate normally, as expected from the phenotype of *Cdk2* null mice (60, 61).

However, the comparison between these two types of MEFs produced a perplexing finding when their ability to exit quiescence was analysed. While *Cdk2* null MEFs, and only in the case of late passage cells, barely showed a slight impairment to exit G0 and enter S-phase, cyclin E deficient cells completely failed to do so, despite of the fact that they showed normal levels of cyclin A-associated kinase activity and pRb phosphorylation. Indeed these cells responded normally to mitogenic stimulation activating the E2F transcriptional program but, unlike *Cdk2* null MEFs, they remarkably failed to progress into S phase (76). This finding clearly demonstrated that E-cyclins play an additional and essential role in the G0 to S transition that is Cdk2-independent, and that seems to be related to a specific activity of the E-cyclins directly in the initiation of DNA replication (Figure 2). This role can not be assumed by other cyclins, or other unrelated proteins, in the absence of cyclin E.

The competence of eukaryotic chromosomes to replicate is established by the ordered assembly of a multi-protein complex (pre-replicative complex or pre-RC) at the still ill defined origins of DNA replication (for a detailed review see 84). The process begins with the binding to the replication origins of a group of proteins known as the origin recognition complex (ORC) that subsequently allow recruitment of the initiation factors Cdc6 and Cdt1. These, in turn, are both required for the loading of the heterohexameric MCM2-7 complex. This is a crucial event as once the MCM complexes are loaded onto chromatin the other pre-RC components are dispensable for replication initiation during S phase entry (85). In quiescent mammalian cells, that are serum stimulated to re-enter the cell cycle, pre-RC assembly occurs in late G1 phase (86). And it is here where puzzling differences first appear between Cdk2 and cyclin E deficient cells.

Geng and co-workers have shown that *cyclin E* null MEFs fail to load the MCM proteins onto chromatin despite having normal levels (and timing) of ORC and Cdc6 binding (76). This observation is not without precedent: in quiescent 3T3 nuclei induced to replicate in a cell-free system, cyclin E is required for loading MCM proteins onto chromatin (87). In these experiments the positive role exerted by cyclin E was believed to depend on Cdk2 (cyclin E alone was not tested nor the experiments performed in the presence of Cdk inhibitors). Consequently Cdk2-cyclin E was assumed to be required to phosphorylate MCM proteins and/or to inactivate an inhibitor of this process. However, it has now become clear that this is not the case and that cyclin E plays a critical, Cdk2-independent function. Interestingly, DNA replication occurs normally in continuously proliferating cyclin E deficient cells suggesting that the molecular aspects of pre-RC formation differ depending on whether cells emerge from M or G0 phases when they enter S-phase.

It is then tempting to assume that both the inability of cyclin E deficient MEFs to enter S phase from G0 and the endoreplicative defect seen in *cyclin E* null mice are due to the same cause: a defective binding of MCM proteins to the replication origins. In support of this hypothesis is the finding that, during the endoreplication cycle of *Drosophila´*s salivary gland cells, cyclin E is implicated in promoting the relocalization of the MCM proteins to DNA replication origins (83).

On balance, the differences between *cyclin E* and *Cdk2* knock-out models bring up an intriguing query. E-type cyclins have no known catalytic partners in addition to Cdk2 and Cdk3, and the latter is non-functional in all laboratory mouse strains (10). Indeed, immunoprecipitates from *Cdk2* knock-out cell extracts show no cyclin E - associated kinase activity, at least when pRb or histone H1 are used as the *in vitro* substrates (60, 61). Therefore, either cyclin E has a yet undiscovered catalytic partner or, alternatively, it plays some essential functions that do not involve a kinase activity. Both possibilities are obviously not mutually exclusive and further work needs to be done to learn more about this very intriguing and important question.

Finally, it is important to mention other data in support of Cdk2-independent activities of cyclin E. Matsumoto and Maller have recently described a centrosomal targeting domain in cyclin E, essential for promoting S phase entry in a Cdk2-independent manner (88). Additionaly, the oncogenic activity of cyclin E relies on certain domains of the protein, which are distinct in their function to those mediating binding and activation of Cdk2 (89). In this respect, as we discuss below, it is of interest to note that the absence of E-type cyclins results in a greatly reduced susceptibility to oncogenic transformation while, significantly, this is not the case with *Cdk2* knock-out cells (60, 76).

3.4. G2/M cyclins: essential for proliferation?

Two A-type cyclins, A1 and A2, have been described in mammalian cells and both have been succesfully deleted in mice. While cyclin A1 expression seems to be restricted almost exclusively to the male germ cell lineage in physiological conditions, cyclin A2 is ubiquitously expressed in all cell types from the the very early (four-cell stage) embryo onwards (90). And, almost as an exception, the phenotype of the individual knock-out mouse strains of these two genes reflect the outcome expected *a priori* based on their expression pattern.

Cyclin A1 null animals are viable and females are completely normal and fertile. Males, however, display a complete disruption of spermatogenesis due to an arrest

before the first meiotic division (71). Although no other phenotypic abnormalities have been associated to cyclin A1 deficiency, a recent report postulates o role for cyclin A1 in driving cell cycle progression of primary MEFs and also leukemic cell lines where cyclin A1 is expressed (91).

In contrast to the mild phenotype produced by cyclin A1 disruption in somatic cells, ablation of cyclin A2 in mice results in early embryonic lethality (92). Cyclin A2 deficient embryos reach the blastocyst stage only to die soon after implantation. Interestingly, both cyclin A2 protein and mRNA of maternal origin are degraded before the embryos reach the four-cell stage, implying that at least early embryonic cycles can occur in the absence of cyclin A2 (92). This is nonetheless not the case in cultured somatic cells where this cyclin plays a critical and non-redundant role during S and M phase progression (93) underscoring the different molecular requirements for embryonic and adult cell cycles (39). Generation of a conditional *cyclin A2* knock-out mouse strain will undoubtedly help to better characterize the requirement of cyclin A2 for adult cell proliferation.

In addition to the prophase functions ascribed to cyclin A2 (93), mitotic control primarily relies on B type cyclins complexed to Cdk1. There are three mammalian B type cyclins with quite divergent attributes. Cyclin B1 and B2 show almost ubiquitous expression, but that is where their similarities end. Both proteins differ in their function and this may be partly due to their distinct subcellular localization. Cyclin B1 is a microtubule-associated protein that swiftly translocates into the nucleus prior to nuclear-envelope breakdown (94). In contrast, cyclin B2 associates with intracellular membranes and is implicated in the reorganization of the Golgi apparatus that takes place during mitosis (95). Cyclin B1 is an essential gene and knock-out embryos die before mid-gestation while B2-deficient animals display no obvious phenotype and normal lifespan (96). This, once again, may be due to functional redundancy because cyclin B1 is much more abundant and able to phosphorylate the same Golgi substrates as B2.

The third family member, cyclin B3, is by far less studied. It is a Cdk2-interacting nuclear protein expressed in testes and also fetal ovaries (97). Although there is speculation that it may be implicated in meiosis this still requires further characterization and so far no *cyclin B3*-null mice have been reported.

3.5. Tumorigenic properties of cyclins and CDKs

Cancer is often referred to as a cell cycle disease. Uncontrolled proliferation, genetic instability and aberrant chromosome segregation, blueprints of cancer cells, are often associated to deregulation of genes involved in cell cycle control. Not only cyclins and Cdks are often target of genetic alterations in cancer themselves but mutations that affect a wide variety of signal transduction pathways ultimately lead to a deregulation of the core machinery that drives cell cycle progression and therefore to deregulation of the Cdk activity.

It is not a surprise then that the pRb pathway, the major regulator of cell cycle entry from quiescence, is one of the most frequent targets of genetic alterations in tumors (98), and therefore the Cdks that control pRb function at the G0 to S transition, mainly Cdk4, Cdk6 and Cdk2 have been long considered potential targets for therapeutic intervention in cancer. Moreover, the existence of natural inhibitors of these Cdks, the INK4 and Cip/Kip proteins, that have a negative effect on cell proliferation, has encouraged the pharmaceutical industry to search for small compounds that exert the same effect on cell proliferation by inhibiting Cdk activity.

The implication of the individual cyclins and Cdks in tumor development and their therapeutic potential as targets for anti-cancer drugs needs to be revised according to recent findings in transgenic and gene targeted mice.

3.5.1. Lessons from transgenic mouse models of cyclins and Cdks

Molecular analysis of human tumors has shown that cyclins and Cdks are often overexpressed in tumor cells. However, it has been hard to establish a causal relationship between overexpression of these genes and tumor development. Transgenic mice have been generated to study the tumorigenic potential of the overexpressed cyclins and Cdks *in vivo* using different tissue-specific promoters.

Although these models are informative, intrinsic limitations are always associated to them. For instance, overexpression of a certain gene may interfere with the normal regulation of another gene of the same family; unrelated pathways may be also affected by transgene overexpression; ectopic expression of the transgene is a common phenomenon in these models and can lead to data missinterpretation; comparison between different models is not straight forward as phenotypes may depend on promoter specificity, expression levels etc. In spite of these drawbacks, and even when transgenic models are just an aproximation to the real pathology found in human tumors, these models are valid for testing the transforming potential of *cyclin* and *cdk* genes *in vivo* and for the development of new therapies targeting cell cycle genes to interfere with cell proliferation.

D-cyclins are commonly found overexpressed in tumors, often associated to chromosomal alterations including translocations and gene amplifications. Breast tumors show a particularly high incidency of cyclin D1 overexpression, approximately 50%. *Cyclin D2* and *D3* have also been found amplified and overexpressed in different types of human cancer (reviewed in 99).

Overexpression of cyclin D1 in the mammary gland of transgenic mice under the control of the MMTV promoter causes constitutive mammary hyperplasia. Multifocal lessions and metastasis, albeit with low incidency, are observed in older mice. These results suggest that cyclin D1 overexpression contributes to breast cell transformation but other genetic events are also required (100).

The tumorigenic properties of D-cyclins in breast are not restricted to cyclin D1. Overexpression of cyclin D2

in transgenic mice under the control of the same promoter (MMTV) results in a similar phenotype. Thus, the long term expression of cyclin D1 or cyclin D2, together with other genetic events, induces mammary tumorigenesis in mice. However, interestingly, the earlier phenotypic consequences of cyclin D2 overexpression in the mammary gland are similar to those found in *cyclin D1* knock-out mice: inhibition of normal alveologenesis and nursing in pregnant females, without associated developmental alterations (101). In fact, this phenotype seems to be, at least partially, due to a "dominant negative-like" effect of cyclin D2 over the normal cyclin D1 activity. This is in sharp contrast with the complete rescue of the *cyclin D1* knock-out breast phenotype by expressing the *cyclin D2* cDNA in place of *cyclin D1* using a knock-in strategy (41). This model differs from the cyclin D2 transgenic model in that cyclin D2 expression replaces the endogenous expression of cyclin D1. This is an example of how in tissues highly dependent on a particular cyclin activity, as the mammary gland is on cyclin D1, any distortion from the physiological regulation of this pathway interferes with normal development. As discussed below, the importance of cyclin D1 in breast tissue proliferation is underscored by the observation that lack of cyclin D1 selectively prevents tumor development originated by alterations in the Ras and Wnt pathways, both of them involved in cyclin D1 induction.

However, not all tissues are equally sensitive to overexpression of cyclin D1. Transgenic mice overexpressing cyclin D1 in lymphocytes under the control of the Eμ promoter did not exhibit lymphocyte hyperplasia (102). Therefore, cyclin D1 overexpression *per se* is not enough to induce lymphocyte proliferation. Nevertheless, overexpression of cyclin D1 in mice doubly transgenic for cyclin D1 and Eμ-*myc* leads to accelerated development of lymphoma. This result further supports the conclusion that activation of the cyclin D1 cooperates with other oncogenic pathways to promote tumorigenesis.

Other transgenic mouse models for D-cyclins have also been generated (103, 104), but they are not within the scope of this discussion.

Like their regulatory partners, Cdk4 and Cdk6 are also target of genetic alterations in cancer. Cdk4 is amplified and overexpressed in a variety of tumors from different cellular origins. Cdk6 is also upregulated by gene amplification in squamous cell carcinomas and gliomas and is frequently found overexpressed in lymphoid tumors often associated with chromosomal translocations (reviewed in 105).

Cdk4 has been overexpressed in transgenic mice in the basal cell layer of the skin under the control of the K5 promoter (106). The epidermis of these mice shows a normal pattern of epidermal differentiation. Although the mice develop epidermal hyperplasia, hipertrophy and dermal fibrosis with age, they do not develop skin tumors. However, these mice are more susceptible to tumor development when subjected to the classical skin carcinogenesis protocol (DMBA initiation followed by TPA promotion) that normally leads to oncogenic *H-ras* activation. Overexpression of Cdk4 increases the rate of malignant progression from papillomas to squamous cell carcinomas in DMBA/TPA treated transgenic mice, which indicates that Cdk4 upregulation cooperates with *ras* oncogenes in skin tumorigenesis.

Interestingly however, double transgenic mice for both Cdk4 and cyclin D1 did not show increased malignant tumor formation compared to Cdk4 single transgenics, suggesting that either the oncogenic properties of Cdk4 are not entirely dependent on cyclin D1 (another D-cyclin could be activated by Cdk4 in this model) or the expression level of cyclin D1 in the epidermal cells is not rate limiting to titrate all the exogenous Cdk4. In support of the latter is the finding that cyclin D1 overexpression under the control of the same promoter does not affect tumor development induced by DMBA/TPA in the skin (107).

Cyclin E1 is also amplified and overexpressed in human tumors (reviewed in 32), particularly, in breast tumors and breast cancer cell lines. Interestingly, in addition to overexpression of full length cyclin E1 some of these tumors express up to five different short isoforms of cyclin E, generated by elastase-mediated proteolysis of the N-terminal portion of the protein followed by post-translational modifications (108, 109). These short forms are hyperactive compared to the long form, and are resistant to $p27^{Kip1}$ and $p21^{Cip1}$ inhibition (110). Overexpression of the short forms of cyclin E in breast tumor cell lines such as MCF7 induced increased polyploidy and chromosome instability (109), and their presence is considered a significant predictor of poor prognosis in breast cancer (111).

Transgenic mouse models have been generated to study the implication of cyclin E in breast tumors. Overexpression of human full length cyclin E in the epithelial cells of the mammary glands of pregnant and lactating mice under the control of the ovine *beta-lactoglobulin* gene promoter leads to epithelial cell hyperplasia after the first lactation cycle. In most transgenic mice the hyperplastic structures were eliminated after mammary gland regression. Yet, aproximately 10-15% of mice developed mammary gland carcinomas (112). Tumor development in this model, however, is strain dependent. In a C57BL/6 background no tumor development was observed indicating the existence of modifier genes for the tumorigenic phenotype.

Overexpression of either cyclin D1 or cyclin E in the mammary gland of mice, therefore, results in hyperplasia and tumor formation. This is consistent with a linear functional pathway for these two cyclins as shown by the rescue of the breast phenotype of *cyclin D1* knock-out mice by knocking-in the *cyclin E* cDNA in the locus encoding cyclin D1 (22). However, the phenotypes of both transgenic models are significantly different. In MMTV-*cyclin D1* transgenic mice, hyperplasia is detected after 2 months of age, before pregnancy, while in cyclin E transgenics hyperplasia is dependent on pregnancy and lactation and the incidency of tumors is lower. This

probably reflects different promoter activity and/or specificity. Alternatively, cyclin D1 may have other activities in breast independent of cyclin E activation. In this line, Cdk4-independent activation of estrogen receptor by cyclin D1 has been described (113) and could contribute to the tumorigenic properties of cyclin D1 in the breast.

As in the case of D-cyclins, tumorigenesis induced by cyclin E overexpression is cell type-dependent. Transgenic mice that express high levels of cyclin E in T cells under the control of the CD2 promoter did not develop lymphoid neoplasia, but were more susceptible to tumor development after treatment with methyl-nitroso-urea (114). Moreover, in the absence of $p27^{Kip1}$ these mice developed monoclonal T-cell lymphoma after a latency period of about seven months. This result indicates that both events cooperate in lymphomagenesis (115) and correlates with the presence of high levels of cyclin E and low levels of the $p27^{Kip1}$ protein often found in human T-cell lymphomas.

Cyclin A1 has been found overexpressed in leukemias and testicular cancers. Cyclin A1 transgenic models have also been produced to test the capacity of this cyclin to act as an oncogene. The wild-type and a proteasome resistant mutant form of cyclin A1 have been expressed in the mammary gland of mice under the control of the ovine *beta-lactoglobulin* gene promoter (112). Transgenic mice showed nuclear abnormalities such as multinucleation and karyomegaly suggestive of preneoplastic alterations. These abnormalities were more severe when a proteolysis resistant mutant form of cyclin A1 was expressed. Co-expression of Cdk2 and cyclin A1 in double transgenic mice exacerbated the phenotype suggesting that these two genes cooperate in cellular transformation or that, in this case, cellular cyclin A levels are indeed rate limiting for cell proliferation in the mammary gland.

3.5.2. Tumor suppressor properties of INK4 and Cip/Kip cell cycle inhibitors

The relatively mild tumorigenic phenotypes induced by overexpression of cyclins and Cdks in transgenic mice are in contrast with the more severe phenotypes observed in models where the control of Cdk activity by INK4 or Cip/Kip proteins is partially or completely lost, underscoring the importance of this control in cell proliferation and cell transformation.

INK4 proteins inhibit Cdk-cyclin D complexes by competing with cyclin D for binding to Cdk4 and 6, therefore its inactivation leads to up-regulation of the Cdk-cyclin D activity. With the exception of $p19^{INK4d}$, inactivation of either one of the *INK4* genes (*$p15^{INK4b}$, $p16^{INK4a}$ or $p18^{INK4c}$*) by deletion, promoter methylation and/or point mutations has been found in a wide variety of human tumors (117). All the *INK4* genes have been knocked-out in mice, and in parallel with what is found in humans, with the exception of $p19^{INK4d}$, all these proteins act as tumor suppressors in mice (Table 3).

The relevance of the cell cycle control by the INK4 family of cell cycle inhibitors in tumor suppression is best illustrated by the phenotype of the *Cdk4R24C* knock-in mouse strain described above. Biochemically this mutation makes Cdk4 unable to interact with cell cycle inhibitors of the INK4 family, and therefore yields it resistant to negative control by these proteins. Importantly this mutation does not seem to affect either the ability of Cdk4 to interact with D-cyclins or its kinase catalytic domain. (118, 119). Knock-in mice were generated to test the ability of this mutant form of Cdk4 to function as an oncogene *in vivo* (Rane et al. 1999). A floxed *neo* cassette was inserted into the first intron in opposite transcriptional orientation to that of the *Cdk4* gene (*$Cdk4^{neo}$* allele). This resulted in the abolition of Cdk4 expression. When the *neo* cassette is removed by the Cre recombinase the resultant allele expresses the mutated form of the Cdk4 protein, Cdk4R24C. Mice heterozygous or homozygous for the knock-in allele, expressing endogenous Cdk4R24C in the germ line, develop multiple tumors of diverse origin, including pituitary tumors, with 100% penetrance (46, 120), demonstrating that loss of INK4 inhibition of Cdk4 activity leads to tumor development. Although this mutation of Cdk4 was first identified in human melanoma, *Cdk4R24C* knock-in mice do not develop this type of tumor spontaneously, but they do after treatment with the skin carcinogenic protocol DMBA/TPA (121), showing that loss of Cdk4 inhibitors cooperates with *H-ras* mutations in melanoma development. This correlates with the observation that transgenic *H-ras* expression in melanocytes of mice lacking the locus *INK4a* also leads to melanoma development (122).

Within the Cip/Kip protein family, $p21^{Cip1}$ and $p57^{Kip2}$ are rarely found altered in human cancer. On the other hand, although not a genetic target in cancer itself, $p27^{Kip1}$ protein levels are frequently reduced in human tumors and tumor cell lines. Reduced level of $p27^{Kip1}$ protein is related to bad prognosis of a wide variety of tumors including breast, prostate and gastric carcinomas, among others (123, 124).

The phenotypes of single *INK4* or *Cip/Kip* gene knock-outs or combinations of them, as well as the implication of these genes in human tumors has been reviewed elsewhere (38, 105) and we will not discuss them here. However, the major phenotypes of these models in development and cancer are summarized in Table 3.

3.5.3. Mice lacking D-cyclin-dependent signalling are resistant to cancer

Up-regulation of cyclins and Cdks predisposes to tumor development as described above. However, the question remains as to whether inhibition of Cdk activity is an effective therapy for cancer. In the adult organism, most cells are in a quiescent, non proliferative, state. Tumor cells, however, proliferate very actively. So, in principle, tumor cells will be more dependent than normal mature cells on Cdk-cyclin activity as they have to efficiently progress through the cell cycle, replicate their DNA and ultimately divide. Cdk activity, therefore, would be an ideal target for cancer treatment as it would be *a priori* dispensable for normal quiescent adult cells but essential for tumor cells. Different experimental approaches, using

Table 3. Major phenotypes of gene targeted mice lacking one or more INK4 or Cip/Kip cell cycle inhibitors.

Protein	Functional effect	Life span / tumors	Major phenotype	References
$p16^{INK4a}$	Knock-out	Viable /Some spontaneous tumors with ageing	Thymic hyperplasia. Tumors: mostly sarcomas and lymphomas. More sensitive to carcinogen induced cancers.	144, 145
$p15^{INK4b}$	Knock-out	Viable /Some spontaneous tumors with ageing	Extramedullary hematopoiesis and lymphoproliferative disorders. Tumors : angiosarcomas and lymphomas.	146
$p18^{INK4c}$	Knock-out	Viable /Some spontaneous tumors with ageing	Increased body size. Multiple cysts in the kidney and mammary glands. Leydig cell hyperplasia. Tumors: mainly pituitary adenomas. Also adrenal medulla and thyroid tumors. Haplo–insufficiency for carcinogen induced tumorigenesis.	146-149
$p19^{INK4d}$	Knock-out	Viable/No tumors	Testicular atrophy but preserved fertility. Progressive hearing loss.	150, 151
$p15^{INK4b}$ and $p18^{INK4c}$	Double knock-out	Viable/Some spontaneous tumors with ageing	Addition of single knock-out phenotypes. Multiple cysts in pancreas and testis.	146
$p18^{INK4c}$ and $p19^{INK4d}$	Double knock-out	Viable/Some spontaneous tumors with ageing	Addition of the single knock-out phenotypes.	148
$p16^{INK4a}$ and $p19^{ARF}$	Double knock-out	Viable/High level of spontaneous tumors with short latency	Tumors: mostly lymphomas and sarcomas.	152
$p21^{Cip1}$	Knock-out	Viable / Some spontaneous tumors with ageing	Normal development. Tumors: histiocytic sarcomas, hemangiomas, B-cell lymphomas, lung carcinomas.	156-161
$p27^{Kip1}$	Knock-out	Viable /Some spontaneous tumors with ageing	Increased body size and organomegaly. Female sterility. Retinal dysplasia. Pituitary hyperplasia and adenomas of the intermediate lobe. Intestinal adenocarcinomas Haplo–insufficiency for tumor supression	162-169
$p57^{Kip2}$ (imprinted gene)	Knock-out	Neonatal lethality	Several developmental defects in the gastrointestinal tract. Cleft palate. Abnormal cell proliferation in placenta, cartilage and lens.	170, 171
$p21^{Cip1}$ and $p27^{Kip1}$	Double knock-out	Viable/Some spontaneous tumors with ageing	Similar phenotypes to those of $p27^{Kip1}$ knock-out with more pronounced hyperplasia of the ovaries (granulosa cells)	172, 173
$p21^{Cip1}$ and $p57^{Kip2}$	Double knock-out	Late embryonic lethality (E16.5-E18.5)	Abnormal skeletal musculature (failure to form myotubes). Abnormal development of the lung alveoli. Abnormal skeletal development.	174
$p27^{Kip1}$ and $p57^{Kip2}$	Double knock-out	Embryonic lethality (E12-E16.5)	Abnormal placenta and lens development due to increased rate of proliferation.	175
$p18^{INK4c}$ and $p27^{Kip1}$	Double knock-out	Viable/Spontaneous tumors	Accelerated development of pituitary tumors. Tumors in the thyroid, parathyroid, adrenal gland, endocrine pancreas, testis and duodenum.	147, 176
$p19^{INK4d}$ and $p27^{Kip1}$	Double knock-out	Postnatal lethality (3 weeks)	Neurological disorders. Abnormal proliferation of some neuronal populations in the central nervous system.	177
$p18^{INK4c}$ and Cdk4	Double knock-out	Viable /No tumors	Similar to Cdk4 knockout phenotype. Rescue of the tumor phenotype of $p18^{INK4c}$ knock-out.	178
$p18^{INK4c}$ and CdkR24C//R24C	$p18^{INK4c}$ knock-out and endogenous expression of Cdk4R24C mutant	Viable/Spontaneous tumors with ageing	Similar phenoype to that of the Cdk4R24C/R24C knock-in mice.	179
$p27^{Kip1}$ and Cdk4	Double knock-out	Viable	Increased body size with respect to Cdk4 knockout but smaller than $p27^{Kip1}$ individual knock-out.	178
$p27^{Kip1}$ and cyclin D1	Double knock-out	Viable	Rescues the abnormal development of cyclin D1 knock-out mice. Does not rescue the phenotype of $p27^{Kip1}$ knock-out.	23, 180

loss of function mouse models of cyclins and Cdks, have provided some genetic clues to help validating these proteins as targets for cancer therapy.

A relatively simple and widely used strategy is to test the sensitivity of primary cells, obtained from knock-out mice, to oncogene-induced transformation. The standard primary cell-type used for transformation assays is MEFs. Wild-type MEFs can be transformed by *ras* in combination with other oncogenes such as *myc*, adenovirus *E1A* or dominant negative (DN) *p53*. Transformed MEFs loose contact inhibition and anchorage dependent growth. They become able to form colonies when grown in soft agar, and tumors when injected in immunodeficient mice.

MEFs lacking all the three D-type cyclins are resistant to transformation induced by any of the oncogene combinations that transform wild-type cells while MEFs lacking only cyclin D1 are not (51), probably due to functional redundancy with cyclin D2 or D3. This is an intriguing finding, as MEFs lacking D-type cyclins can proliferate and enter cell cycle from quiescence, although less efficiently than wild-type cells. A possible explanation is that D-cyclins are required for cells to respond effectively to the strong and constitutive mitogenic stimulation required for cell transformation *in vitro*, although they can reach the threshold of kinase activation required for normal proliferation in the absence of D-cyclins. This theory is supported by the finding that highly

proliferative tissues such as the fetal haematopoietic system, but not others, are those affected by the lack of D-cyclins or cyclin D-dependent Cdks, as previously discussed.

MEFs lacking Cdk4, although they still express Cdk6, are also resistant to transformation by *ras* and DN *p53*, (125) indicating that Cdk6 by itself is not enough to sustain cell transformation. Cdk4 is also required for long term proliferation of MEFs. MEFs without Cdk4 senesce in culture more rapidly and become immortalized less frequently than wild-type MEFs. This phenotype becomes more severe in the absence of both Cdk4 and Cdk6 (50). It could be anticipated that MEFs lacking both Cdks will be also resistant, as D-cyclin deficiente MEFs are, to oncogenic transformation, but this still remains to be determined.

How do the results in MEFs translate to tumor development *in vivo*? First, the absence of D-cyclins makes mice more resistant to tumor development. For instance, *cyclin D1* knock-out mice become resistant to intestinal tumors induced by beta-Catenin or Apc (adenomatous polyposis coli) loss (126), to skin papillomas induced by H-Ras activation (127) and to breast tumors induced by Ras or Neu activation (128). However the response of tumors to cyclin D1 inactivation is dependent on the oncogenic pathway by which tumors are driven. Thus, *cyclin D1* knock-out mice are as sensitive as wild-type mice to breast tumors driven by c-Myc or Wnt-1 (128). This result indicates that the mitogenic Neu-Ras pathway connects with the cell cycle through cyclin D1 activation in the mammary epithelium and therefore cyclin D1 is absolutely required for cell transformation in tumors driven by constitutive activation of this pathway.

It would be interesting to test whether or not mice lacking Cdk4 are as resistant to tumors as those lacking cyclin D1 are, or alternatively, other Cdk4-independent activities of cyclin D1 contribute to the tumorigenic phenotype. Against the latter is the finding that a gene replacement knock-in model expressing cyclin E in the *cyclin D1* locus is no longer resistant to Ras induced breast tumors, demonstrating the linearity of the cyclin D1-cyclin E pathway to drive cell proliferation and cell transformation induced by Ras activation in the mammary epithelium.

Cyclin D2 and D3 ablation also protects mice against certain types of tumors. Mice lacking cyclin D2 are more resistant to gonadal tumors (129) and mice lacking cyclin D3 are more resistant to leukemias driven by Notch and thymomas induced by p56LCK (130).

Cdk4 ablation also confers resistance to certain tumors in mice. For instance it interferes with skin carcinogenesis induced by Ras activation (131) and to skin tumors driven by a K5-*c-myc* transgene (132).

These results suggest that, at least in certain types of tumors, for instance those driven by *ras* oncogene activation in breast or skin, interfering with Cdk4-cyclin D activity may be effective as an anti-cancer therapy. However this needs to be taken with caution. Ablation of cyclin D or Cdk4 in the germ line may not have the same consequences for tumor development than their inactivation in tumor cells. In this respect, we have already mentioned that lack of D-cyclins or Cdk4 activity reduces the life span of primary MEFs, thus it is possible that the tissue stem cell proliferative potential in germ line knock-out models may be limited by the lack of D-cyclins or Cdk4 from early development, or even that a subset of those stem cells be absent. If this is the case, and this is the major reason why tumors do not develop in the knock-out mice, inhibiting cyclin D or Cdk4 in the tumor could not effectively ablate tumor progression. Conditional models of cyclin D and Cdk4 inactivation that allow to inhibit this pathway in adult mice will help to address these issues.

3.5.4. Lack of E-cyclins but not Cdk2 confers resistance to oncogenic transformation

MEFs lacking E-cyclins (double null for *cyclin E1* and *E2*) are also resistant to oncogenic transformation by a combination of *ras* plus an immortalizing oncogene (76). In principle, the resistance to oncogenic *ras* activation in these cells could be potentially explained, as in the case of MEFs lacking D-cyclins, by the inability of these MEFs to reach the threshold of kinase activation required for cell transformation in the absence of E-cyclins. However, in this case the complete resistance to oncogenic tranformation of *E-cyclin* knock-out MEFs contrast sharply with the mild resitance observed in MEFs lacking Cdk2 (60). *Cdk2* null MEFs can be transformed by *ras* and adenovirus *E1A* although with about 30% less efficiency than wild-type MEFs. These results indicate that cyclin E plays roles in cell transformation that are independent of Cdk2, and as Cdk2 is the only known partner of cyclin E, a lower threshold of kinase activity may not to be a valid explanation for the resistance to transformation found in the absence of E-cyclins.

Previous data had shown that mutant forms of cyclin E that fail to form an active kinase complex with Cdk2, are able to transform rat embryo fibroblasts in cooperation with H-*ras* (89). Therefore it had been proposed that cyclin E harbors other functions, independent of Cdk2 activation and $p27^{Kip1}$ binding, that contribute to its oncogenic activity. Comparison of the phenotypes of *cyclin E* and *Cdk2* knock-out mice has in fact revealed Cdk2-independent functions of cyclin E in DNA endoreplication and in entering S-phase from quiescence. In the latter process, cyclin E is essential for the incorporation of MCM proteins to the PR-complexes at the origins of replication. Whether this Cdk2-independent activity of cyclin E is required for cellular transformation remains to de determined.

The results in MEFs suggest that interfering with E-cyclin activity, somehow, may be a good strategy for cancer intervention. This needs to be further supported by studies *in vivo*, similar to the ones described for D-cyclins. However, these studies are precluded by the embryonic lethality of mice lacking E-cyclins, and require the generation of conditional knock-out models.

Cdk2 had itself long been considered a good target for cancer therapy and a long list of Cdk2 inhibitors developed by the pharmaceutical industry already exist. The concept of Cdk2 being essential for proliferation was originated by early experiments in tumor cell lines showing that inhibition of Cdk2 by different strategies blocked cell proliferation while inhibition of Cdk4 activity in the same conditions did not (57). However, the fact that *Cdk2* knock-out MEFs can be transformed by oncogenes suggests that interfering with Cdk2 activity by itself is not efective to inhibit tumor development. Although more studies need to be undertaken to better understand the implication of Cdk2 in tumor growth, in the next section we revise recent *in vivo* data that further support the concept of Cdk2 activity being dispensable for tumor development.

3.5.5. The tumor suppressor actvities of $p21^{Cip1}$ and $p27^{Kip1}$ are Cdk2-independent

The dispensability of Cdk2 for cell proliferation and oncogenic transformation, demonstrated by gene targeting of Cdk2 in the mouse, rises the important question of whether in fact the molecular mechanisms involved in cell cycle inhibition and tumor suppressor activities of $p21^{Cip1}$ and $p27^{Kip1}$ rely on the inhibition of Cdk2. Recently this question has been addressed by Barbacid´s group (133). Double mutant mice, lacking either $p21^{Cip1}$ and Cdk2 or $p27^{Kip1}$ and Cdk2, have been generated by intercrossing the individual null strains and MEFs have been isolated from these mice. MEFs lacking $p21^{Cip1}$ or $p27^{Kip1}$ have the same proliferative advantages independently of whether or not Cdk2 is present. Similarly, retroviral overexpression of $p21^{Cip1}$ or $p27^{Kip1}$ in MEFs exerts the same antiproliferative effect in the presence than in the absence of Cdk2. Moreover, $p21^{Cip1}$-mediated cell cycle arrest in response to DNA damage in MEFs is also independent of Cdk2 (133). Therefore, these results clearly establish that at least in MEFs, Cdk2 is not required for cell cycle arrest induced by $p21^{Cip1}$ or $p27^{Kip1}$.

The same conclusion has been reached from *in vivo* experiments. Mice lacking $p27^{Kip1}$ and Cdk2 exhibit organomegaly and develop pituitary tumors at the same rate and with the same incidency as mice lacking only $p27^{Kip1}$ (133). These results indicate that a Cdk2-independent mechanism is responsible for the *in vivo* growth inhibitory and tumor suppressor activities of $p27^{Kip1}$. As mentioned above, many human tumors show reduced levels of $p21^{Cip1}$ and $p27^{Kip1}$. These results argue against the adequacy of Cdk2 inhibition as a strategy for therapeutical intervention in these tumors.

The mechanism by which $p21^{Cip1}$ and $p27^{Kip1}$ inhibit cell growth in the absence of Cdk2 remains obscure. Both proteins bind to Cdk1 and inhibit its kinase activity (134, 135). Therefore, Cdk1 inhibition could mediate some of the effects of these proteins on cell proliferation. In support of this hypothesis, expression of a $p27^{Kip1}$ mutant form, lacking a functional Cdk binding domain, is no longer able to suppress cell proliferation in the same experimental setting, in the presence or in the absence of Cdk2 (133). However Cdk-independent activities of these proteins or their implication in processes other than cell cycle regulation can not be completely ruled out. In fact, the concept of Cdk-independent activities is now emerging for some of the Cdk activators and inhibitors: cyclin D1 binds and activates estrogen receptor independently of Cdk4; similarly, cyclin D1 can modulate the activity of Myb transcription factors in the absence of Cdk4 binding (136, 137); comparison of the phenotypes of Cdk2 and cyclin E lacking mice suggests that cyclin E may participate in the initiation of DNA replication as well as in cell transformation, molecular mechanisms that are independent of its catalytic partner Cdk2 (76, 77). Similarly there may be other targets responsible of mediating the cell proliferation block and tumor suppression activities of $p27^{Kip1}$ and $p21^{Cip1}$.

4. SUMMARY AND PERSPECTIVES

Mouse models are a powerful tool to study gene function *in vivo*. Recently the analysis of gene targeted and transgenic mice of cyclins and Cdks and of their inhibitors the Cip/Kip or INK proteins, has provided new information about cell cycle regulation and the control of cell proliferation in mammalian cells.

Most of the cyclins and Cdks, previously considered essential for cell proliferation, have turned out to be dispensable. The case of Cdk2 was particularly unexpected, as it results to be dispensable for most, if not all, somatic cells in the mouse. The surprise promoted by this finding could possibly be atributed to the over-generalization of previous data obtained from experiments in yeast and in tissue culture cells. Potential reasons for the lack of more severe phenotypes in individual gene knock-out mice include lack of expression of some genes in certain cell types, functional redundancy among family members and the activation of compensatory mechanisms, for instance ablation of two of the three D-cyclins leads to ectopic expression of the third one in some cell types. Nevertheless, individual cyclins and Cdks are essential in specific cell-types; even Cdk2 is essential in meiocytes, both in males and females.

In some cases, the results obtained from mouse models confirm previous biochemical and tissue culture data. For instance, ablation of D-type cyclins leads to the same phenotype as ablation of their catalytic partners Cdk4 and Cdk6. In other cases, the opposite is found: ablation of the E-cyclins has different phenotypical consequences than ablation of their only known partner Cdk2, demonstrating that cyclin E may have other interacting partners or other non-catalytic functions, that are still to be revealed.

Analysis of the mouse mutant phenotypes has also provided information about the cell-type specific molecular interactions that take place *in vivo*. For instance, beta cell proliferation seems to be mediated mostly by Cdk4-cyclin D2 complexes, as mice lacking either of them show similar phenotypes in beta cells; similarly, body size, in terms of cell numbers, is likely to be controlled primarily by Cdk4 and cyclin D1; ablation of $p27^{Kip1}$ rescues some of the phenotypes of *cyclin D1* knock-out mice, demonstrating the genetic interaction between these proteins *in vivo*.

Linear pathways of proliferation control, for instance Neu-Ras-cyclin D1-cyclin E in the mammary epithelium, Ras-Cdk4-cyclin D1 in the skin or Apc-cyclin D1 in the intestine, among others, can also be demonstrated by genetic analysis in mouse models.

Most of the cyclin and Cdk knock-out models that have been generated so far are constitutive knock-outs, resulting in the complete absence of a particular protein, from embryonic day one. As powerful as they are, these models have also some limitations. One of them, already mentioned, is the potential activation of compensatory mechanisms during embryonic development. Moreover, the phenotype observed in each particular case corresponds to the first non redundant and uncompensated function of the gene, obscuring other phenotypes that may appear later during development or producing secondary effects that sometimes are dificult to characterise as such. But in terms of elucidating the implication of cyclins and Cdks in tumor development the limitation of these models is more evident. Even in those cases where the constitutive inactivation of the gene is viable, inhibiting a particular protein in tumor cells is not equivalent to it being absent from the beginning of development. Models in which Cdk-kinase activity is abolished by point mutations, or Cdk/cyclin interactions with cell cycle inhibitors modified, or conditional knock-out models in which a particular cyclin, Cdk or cell cycle inhibitor can be especifically ablated in tumors will contribute to improve our knowledge of how the cell cycle control works and how to modify it for therapeutical intervention.

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6. REFERENCES

1. Norbury C & P. Nurse: Animal cell cycles and their control. *Annu Rev Biochem* 61, 441-70 (1992)

2. Weinberg RA: The retinoblastoma protein and cell cycle control. *Cell* 81: 323-30 (1995)

3. Sherr CJ & J.M. Roberts: Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev* 9, 1149-63 (1995)

4. Sherr CJ: Cancer cell cycles. *Science* 274, 1672- 77 (1996)

5. Murray AW: Recycling the cell cycle: cyclins revisited. *Cell* 116: 221-234 (2004)5.

6. Sherr CJ & J.M. Roberts: CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & Dev* 13, 1501-12 (1999)

7. Morgan DO: Cyclin-dependent kinases: engines, clocks and microprocessors. *Annu Rev Cell Dev Biol* 13, 261-91 (1997)

8. Lundberg AS, & R.A. Weinberg: Functional inactivation of the retinoblastoma protein requires sequential modification by at least two cyclin-cdk complexes. *Mol Cell Biol* 18, 753-61 (1998)

9. Ren S & B.J. Rollins: Cyclin C / Cdk3 promotes RB-dependent G_0 exit. *Cell* 117, 239-51 (2004)

10. Ye X, C. Zhu & J.W. Harper: A premature termination mutation in the Mus musculus cyclin dependent kinase 3 gene. *Proc Natl Acad Sci USA* 98:1682-6 (2001)

11. Malumbres M: Revisiting the "Cdk-centric" view of the mammalian cell cycle. Cell Cycle 4, e19-e23 (2005)

12. Tezlaff MT, C. Bai, M. Finegold, J. Wilson, J.W. Harper, K.A. Mahon & S.J. Elledge: Cyclin F disruption compromises placental development and affects normal cell cycle progression. *Mol Cell Biol* 24, 2487-98 (2004)

13. Sherr CJ & J.M. Roberts: Living with or without cyclins and cyclin dependent kinases. *Genes & Dev* 18, 2699-711 (2004)

14. Koepp DM, J.W. Harper & S.J. Elledge: How the cyclin became a cyclin: regulated proteolysis in the cell cycle. *Cell* 97, 431-4 (1999)

15. Jones SM & A. Kazlauskas: Growth-factor-dependent mitogenesis requires two distinct phases of signalling. *Nat Cell Biol* 3, 165-72 (2001)

16. Pavletich NP: Mechanisms of cyclin-dependent kinase regulation: structures of cdks, their cyclin activators and Cip and INK inhibitors. *J Mol Biol* 287, 821-8 (1999)

17. Frolov MV & N.J. Dyson: Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. *J Cell Sci* 117: 2173-81 (2004)

18. Brehm A & T. Kouzarides: Retinoblastoma protein meets chromatin. *Trends Biochem Sci* 24, 142-5 (1999)

19. Constanzo M, J.L. Nishikawa, X. Tang, J.S. Millman, O. Schub, K. Breitkreuz, D. Dewar, I. Rupes, B. Andrews & M. Tyers: CDK activity antagonizes Whi5, an inhibitor of G1/S transition in yeast. *Cell* 117, 849-50 (2004)

20. Pardee AB: A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci USA* 71, 1286-90 (1974)

21. Lukas J, T. Herzinger, K. Hansen, M.C. Moroni, D. Resnitzky, K. Helin, S.I. Reed & J. Bartek: Cyclin E-induced S phase without activation of the pRB/E2F pathway. *Genes Dev* 11, 1479-92 (1997)

22. Geng Y, W. Whoriskey, M.Y. Park, R.T. Bronson, R.H. Medema, T. Li, R.A. Weinberg & P. Sicinski: Rescue of cyclin D1 deficiency by knockin cyclin E. *Cell* 97, 767-77 (1999)

23. Geng Y, Q. Yu, E. Sicinska, M. Das, R.T. Bronson & P. Sicinski: Deletion of the p27Kip1 gene restores normal development in cyclin D1-deficient mice. *Proc Natl Acad Sci USA* 98, 194-9 (2001)

24. Cheng M, P. Olivier, J.A. Diehl, M. Fero, M.F. Roussel, J.M. Roberts & C.J. Sherr: The p21Cip1 and p27Kip1 CDK inhibitors are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J* 18, 1571-83 (1999)

25. Olashaw N, T.K. Bagui & W.J. Pledger: Cell cycle control: a complex issue. *Cell Cycle* 3, 263-4 (2004)

26. Bracken AP, M. Ciro, A. Cocito. & K. Helin: E2F target genes: unravelling the biology. *Trends Biochem Sci* 29, 409-17 (2004)

27. Matsuura I, N.G. Denissova, G. Wang, D. He, J. Long. & F. Liu: Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* 430, 226-31 (2004)

28. Coqueret O: Linking cyclins to transcriptional control. *Gene* 299, 35-55 (2002)

29. Sheaff RJ, M. Groudine, M. Gordon, J.M. Roberts & B.E. Clurman: Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev* 11, 1464-78 (1997)

30. Vlach J, S. Henneke & B. Amati: Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. *EMBO J* 16, 5334-44 (1997)

31. Koepp DM, L.K. Schaeffer, X. Ye, K. Keyomarsi, C. Chu, J.W. Harper & S.J. Elledge: Phosphorylation dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science* 294, 173-7 (2001)

32. Möröy T, and C. Geisen: Cyclin E. *Int J Biochem Cell Biol* 36, 1424-39 (2004)

33. Wianny F, F.X. Real, C.L. Mummery, M. Van Rooijen, J. Lahti, J. Samarut. & P. Savatier: G1-phase regulators, cyclin D1, cyclin D2 and cyclin D3: up-regulation at gastrulation and dynamic expression during neurulation. *Dev. Dyn* 212, 49-62 (1998)

34. Fantl V, G. Stamp, A. Andrews, I. Rosewell & C. Dickson: Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes & Dev* 9, 2364-72 (1995)

35. Sicinski P, J.L. Donaher, S.B. Parker, H. Gardner, S.Z. Haslam, R.T. Bronson, S.J. Elledge & R.J. Weinberg: Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* 82, 621-30 (1995)

36. Sicinski P, J.L. Donaher, Y. Geng, S.B. Parker, H. Gardner, M.Y. Park, R.L. Robker, J.S. Richards, L.K. McGinnis, J.D. Biggers, J.J. Eppig, R.T. Bronson, S.J. Elledge. and R.A. Weinberg: Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 384, 470-474 (1996)

37. Sicinska E, I. Aifantis, L. Le Cam, W. Swat, C. Borowski, Q. Yu, A.A. Ferrando, S.D. Levin, Y. Geng, H. von Boehmer & P. Sicinski: Requirement for Cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* 4, 451-61 (2003)

38. Ciemerych MA & P. Sicinski: Cell cycle in mouse development. *Oncogene* 24, 2877-2898 (2005)

39. Pagano M & P.K. Jackson: Wagging the dogma: tissue-specific cell cycle control in the mouse embryo. *Cell* 118, 535-8 (2004)

40. Ciemerych MA, A.M. Kenney, E. Sicinska, I. Kalaszcynska, R.T. Bronson, D.H. Rowitch, H. Gardner & P. Sicinski: Development of mice expressing a single D-type cyclin. *Genes & Dev* 16, 3277-89 (2002)

41. Carthon BC, C.A. Neumann, M. Das, B. Pawlik, T. Li, Y. Geng. & P. Sicinski: Genetic replacement of Cyclin D1 function in mouse development by cyclin D2. *Mol Cell Biol* 25, 1081-8 (2005)

42. Rane SG, P. Dubus, R.V. Mettus, E.J. Galbreath, G. Boden, E.P. Reddy & M. Barbacid: Loss of Cdk4 expression causes insulin-deficiente diabetes and Cdk4 activation results in beta-islet cell hyperplasia. *Nat Genet* 22, 44-52 (1999)

43. Tsutsui T, B. Hesabi, D.S. Moons, P.P. Pandolfi, K.S. Hansel, A. Koff & H. Kiyokawa: Targeted disruption of Cdk4 delays cell cycle entry with enhanced p27Kip1 activity. *Mol Cell Biol* 19, 7011-9 (1999)

44. Moons DS, S. Jirawatnotai, A.F. Parlow, G. Gibori, R.D. Kineman & H. Kiyokawa: Pituitary hypoplasia and lactotroph dysfuntcion in mice deficient for cyclin-dependent kinase 4. *Endocrinology* 143, 3001-8 (2002)

45. Martín J, S. Hunt, P. Dubus, R. Sotillo, F. Néhmé-Pélluard, M.A. Magnuson, A.F. Parlow, M. Malumbres, S. Ortega & M. Barbacid: Genetic rescue of Cdk4 null mice restores pancreatic beta-cell proliferation but not homeostatic cell number. *Oncogene* 22, 5261-9 (2003)

46. Sotillo R, P. Dubus, J. Martín , E. de la Cueva, S. Ortega, M. Malumbres & M. Barbacid: Wide spectrum of tumors in knockin mice carrying a Cdk4 protein insensitive to INK4 inhibitors. *EMBO J* 20, 6637-47 (2001)

47. Georgia S & A. Bhushan: Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest* 114, 963-8 (2004)

48. Kushner JA, M.A. Ciemerych, E. Sicinska, L.M. Wartschow, M. Teta, S.Y. Long, Sicinski, P. & M.F.

White: Cyclins D2 and D1 are essential for postnatal pancreatic beta-cell growth. *Mol Cell Biol* 25, 3752-62 (2005)

49. Meyer CA, H.W. Jacobs & C.F. Lehner: Cyclin D-cdk4 is not a master regulator of cell multiplication in Drosophila embryos. *Curr Biol* 12, 661-6 (2002)

50. Malumbres M, R. Sotillo, D. Santamaría, J. Galán, A. Cerezo, S. Ortega, P. Dubus & M. Barbacid: Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. *Cell* 118, 493-504 (2004)

51. Kozar K, M.A. Ciemerych, V.I. Rebel, H. Shigematsu, A. Zagozdzon, E. Sicinska, Y. Geng, Q. Yu, S. Bhattacharya, R.T. Bronson, K. Akashi & P. Sicinski: Mouse development and cell proliferation in the absence of D-cyclins. *Cell* 118, 477-91 (2004)

52. Park I, D. Qian, M. Kiel, M. Becker, M. Pihalja, I. Weissman, S. Morrison & M. Clarke: Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 423, 302-5 (2003)

53. Sherr C J: Principles of tumor suppression. *Cell* 116, 235-46 (2004)

54. Zarkowska T & S. Mittnacht: Differential phosphorylation of the retinoblastoms protein by G1/S cyclin-dependent kinases. *J Biol Chem* 272, 12738-46 (1997)

55. Ewen ME, B. Faha, E. Harlow & D.M. Livingston: Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. *Science* 255, 85-7 (1992)

56. Santoni-Rugiu E, J. Falck, N. Mailand, J. Bartek & J. Lukas: Involvement of Myc activity in a G(1)/S-promoting mechanism parallel to the pRb/E2F pathway. *Mol Cell Biol* 20, 3497-509 (2000)

57. van den Heuvel SJL & E. Harlow: Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* 262, 2050-4 (1993)

58. Tetsu O & F. McCormick: Proliferation of cancer cells despite CDK2 inhibition. *Cancer Cell* 3, 233-45 (2003)

59. Roberts JM & C.J. Sherr: Bared essentials of CDK2 and cyclin E. *Nat Genet* 35, 9-10 (2003)

60. Ortega S, I. Prieto, J. Odajima, A. Martín, P. Dubus, R. Sotillo, J.L. Barbero, M. Malumbres & M. Barbacid: Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. *Nat Genet* 35, 25-31 (2003)

61. Berthet C, E. Aleem, V. Coppola, L. Tessarollo & P. Kaldis: Cdk2 knockout mice are viable. *Curr Biol* 13, 1775-85 (2003)

62. Gladden AB & J.A. Diehl: Cell cycle progression without cyclin E/CDK2: breaking down the walls of dogma. *Cancer Cell* 4, 160-2 (2003)

63. Pacek M, T.A. Prokhorova & J.C. Walter: Cdk1: Unsung hero of S phase?. *Cell Cycle* 3, 401-3 (2004)

64. Jackman M, C. Lindon, E.A. Nigg & J. Pines: Active cyclin B1-Cdk1 first appears on centrosomes in prophase. *Nat Cell Biol* 5, 143-8 (2003)

65. Moore JD, J.A. Kirk & T. Hunt: Unmasking the S-phase-promoting potential of cyclin B1. *Science* 300, 987-90 (2003)

66. Fisher DL & P. Nurse: A single fission yeast mitotic cyclin B-p34cdc2 kinase promotes both S-phase and mitosis in the absence of G1 cyclins. *EMBO J* 15, 850-60 (1996)

67. Donaldson AD: The yeast mitotic cyclin Clb2 cannot substitute for S phase cyclins in replication origin firing. *EMBO Rep* 1, 507-12 (2000)

68. Simone C, L. Bagella, C. Bellan & A. Giordano: Physical interaction between pRb and cdk9/cyclin T2 complex. *Oncogene* 21, 4158-65 (2002)

69. Manning G, G.D. Plowman, T. Hunter & S. Sudarsanam: Evolution of protein kinase signalling from yeast to man. *Trends Biochem Sci* 27, 514-20.

70. Caenepeel S, G. Charydczak, S. Sudarsanam, T. Hunter & G. Manning: The mouse kinome: discovery and comparative genomics of all mouse protein kinases. *Proc Natl Acad Sci USA* 101, 11707-12 (2004)

71. Liu D, M.M. Matzuk, W.K. Sung, Q. Guo, P. Wang & D.J. Wolgemuth: Cyclin A1 is required for meiosis in the male mouse. *Nat Genet* 20, 377-80 (1998)

72. Liu D, C. Liao & D.J. Wolgemuth: A role for Cyclin A1 in the activation of MPF and G2-M transition during meiosis of male germ cells in mice. *Dev Biol* 224, 388-400 (2000)

73. Müller-Tidow C, P. Ji, S. Diederichs, J. Potrazt, N. Bäumer, G. Kohler, T. Cauvet et al.: The Cyclin A1-CDK2 complex regulates DNA double-strand break repair. *Mol Cell Biol* 24, 8917-28 (2004)

74. Diederichs S, N. Bäumer, P. Ji, S.K. Metzelder, G.E. Idos, T. Cauvet et al.: Identification of interaction partners and substrates of the Cyclin A1-CDK2 complex. *J Biol Chem* 279, 33727-41 (2004)

75. Esashi F, N. Christ, J. Gannon, Y. Liu, T. Hunt, M. Jasin & S. West: CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombination and repair. *Nature* 434, 598-604 (2005)

76. Geng Y, Q. Yu, E. Sicinska, M. Das, J.E. Schneider, S. Bhattacharya, W.M. Rideout III, R.T. Bronson, H. Gardner & P. Sicinski: Cyclin E ablation in the mouse. *Cell* 114, 431-43 (2003)

77. Parisi T, A.R. Beck, N. Rougier, T. McNeil, L. Lucian, Z. Werb & B. Amati: Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells. *EMBO J* 18, 4794-803 (2003)

78. Geng, Y, Q. Yu, W. Worishek, F. Dick, K.Y. Tsai, H.L. Ford, D.K. Biswas, A.B. Pardee, B. Amati, T. Jacks, A, Richardson, N. Dyson & P. Sicinski: Expression of cyclins E1 and E2 during mouse development and in neoplasia. *Proc Natl Acad Sci USA* 98, 13138-43 (2001)

79. Tanaka M, M. Gertsenstein, J. Rossant, & A. Nagy: Mash2 acts cell autonomously in mouse spongiotrophoblast development. *Dev Biol* 190, 55-65 (1997)

80. Eggan K, , H. Akutsu, J. Loring, L. Jackson-Grusby, M. Klemm, W.M. 3rd Rideout, R. Yanagimachi, & R. Jaenisch: Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc Natl Acad Sci USA* 98, 6209-6214 (2001)

81. Schwenk F, B. Zevnik, J. Bruning, M. Rohl, A. Willuweit, A. Rode, T. Hennek, G. Kauselmann, R. Jaenisch & R. Kuhn: Hybrid embryonic stem cell-derived tetraploid mice show apparently normal morphological, physiological and neurological characteristics. *Mol Biol Cell* 23, 3982-9 (2003)

82. Palazón LS, T.J. Davies & R.L. Gardner: Translational inhibition of cyclin B1 and appearance of cyclin D1 very early in the differentiation of mouse trophoblast giant cells. *Mol Hum Reprod* 4, 1013-20 (1998)

83. Su TT & P.H. O´Farrel: Chromosome association of minichromosome maintenance proteins in Drosophila endoreplicative cycles. *J Cell Biol* 140, 451-60 (1998)

84. Diffley JF: Regulation of early events in chromosome replication. *Curr Biol* 21, 778-86 (2004)

85. Harvey KJ & J. Newport: Metazoan origin selection: origin recognition complex chromatin binding is regulated by CDC6 recruitment and ATP hydrolysis. *J Biol Chem* 278, 48524-8 (2003)

86. Madine MA, M. Swietlik, C. Pelizon, P. Romanowski, A.D. Mills & R.A. Laskey: The roles of the MCM, ORC, and Cdc6 proteins in determining the replication competence of chromatin in quiescent cells. *J Struc Biol* 129, 198-210 (2000)

87. Coverley D, H. Laman & R.A. Laskey: Distinct roles for cyclins E and A during DNA replication complex assembly and activation. *Nat Cell Biol* 4, 523-8 (2002)

88. Matsumoto Y & J.L. Maller: A centrosomal localization signal in cyclin E is required for Cdk2-independent S phase entry. *Nature* 306, 885-8 (2004)

89. Geisen G and T. Möröy: The oncogenic activity of cyclin E is not confined to Cdk2 activation alone but relies on several other, distinct functions of the protein. *J Biol Chem* 277, 39909-18 (2002)

90. Sweeney C, M. Murphy, M. Kubelka, S.E. Ravnik, C.F. Hawkins, D.J. Wolgemuth & M. Carrington: A distinct cyclin A is expressed in germ cells in the mouse. *Development* 122, 53-64 (1996)

91. Ji P, S. Agrawal, S. Diederichs, N. Bäumer, A. Becker, T. Cauvet, S. Kowski, C. Beger, K. Welte, W.E. Berdel, H. Serve & C. Müller-Tidow: Cyclin A1, the alternative A-type cyclin, contributes to G1/S cell cycle progression in somatic cells. *Oncogene* 24, 2739-44 (2005)

92. Murphy M, M.G. Stinnakre, C. Senamaud-Beaufort, N.J. Winston, C. Sweeney, M. Kubelka, M. Carrington, C. Brechot & J. Sobzack-Thepot: Delayed early embryonic lethality following disruption of the murine cyclin A2 gene. *Nat Genet* 15, 83-86 (1997)

93. Furuno N, N. den Elzen & J. Pines: Human cyclin A is required for mitosis until mid prophase. *J Cell Sci* 147, 295-306 (1999)

94. Clute P & J. Pines: Temporal and spatial control of Cyclin B1 destruction in metaphase. *Nat Cell Biol* 1, 82-7 (1999)

95. Draviam VM, S. Orrechia, M. Lowe, R. Pardi & J. Pines: The localization of human cyclins B1 and B2 determines CDK1 substrate specificity and neither enzyme requires MEK to disassemble the Golgi apparatus. *J Cell Biol* 152, 945-58 (2001)

96. Brandeis M, I. Rosewell, M. Carrington, T. Crompton, M.A. Jacobs, J. Kirk, J. Gannon & T. Hunt: Cyclin B2-null mice develop normally and are fertile whereas cyclin B1-null mice die in utero. *Proc Natl Acad Sci USA* 95, 4344-9 (1998)

97. Nguyen TB, K. Manova, P. Capodieci, C. Lindon, S. Bottega, X.Y. Wang, J. Refik-Rogers, J. Pines, D. Wolgemuth & A. Koff: Characterization and expression of mammalian Cyclin B3, a prepachytene meiotic cyclin. *J Biol Chem* 277, 41960-9 (2002)

98. Malumbres M & M. Barbacid: To cycle or not to cycle: a critical decision in cancer. *Nat Rev Cancer* 1, 222-31 (2001)

99. Deshpande A, P. Sicinski & P.W. Hinds: Cyclins and cdks in development and cancer: a perspective. *Oncogene* 24, 2909-15 (2005)

100. Wang TC, R.D. Cardiff, L. Zukergerg, E. Lees, A. Arnold & E.V. Schmidt: Mammary hyperplasia and

carcinoma in MMTV-Cyclin D1 transgenic mice. *Nature* 369, 669-71 (1994)

101. Kong G, S.S. Chua, Y. Yijun, F. Kittrell, R.C. Moraes, D. Medina & T.K. Said: Functional analysis of cyclin D2 and p27Kip1 in cyclin D2 transgenic mouse mammary gland during development. *Oncogene* 21, 7214-25 (2002)

102. Bodrug SE, B.J. Warner, M.L. Bath, G.J. Lindeman, A.W. Harris & J.M. Adams: Cyclin D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the myc gene. *EMBO J* 13, 2124-30 (1994)

103. Soonpaa MH, G.Y. Koh, L. Pajak, S. Jing, H. Wang, M.T. Franklin, K.K. Kim & L.J. Field: Cyclin D1 overexpression promotes cardyomyocite DNA synthesis and multinucleation in transgenic mice. *J Clin Invest* 99, 2644-54 (1997)

104. Gómez LaHoz E, N.J. Liegeois, P. Zhang, J.A. Engelman, J. Horner, A. Silverman, R. Burde, M.F. Roussel, C.J. Sherr, S.J. Elledge and R. DePinho: CyclinD- and E- dependent kinases and the p57Kip2 inhibitor: cooperative interactions in vivo. *Mol Cell Biol* 19, 353-63 (1999)

105. Ortega S, M. Malumbres & M. Barbacid: Cell Cycle and cancer: the G1 restriction point and the G1/S transition. *Current Genomics* 3, 245-263 (2002)

106. Miliani de Marval P, I. Giménez-Conti, M. La Cava, L. Martínez, C. Conti & M. Rodríguez-Puebla: Transgenic expression of Cyclin-dependent kinase 4 results in epidermal hiperplasya, hipertrophy and severe dermal fibrosis. *Am J Pathol* 159, 369-79 (2001)

107. Rodríguez-Puebla ML, M. LaCava & C. Conti: Cyclin D1 overexpression in mouse epidermis increases Cyclin-dependent kinase activity and cell proliferation in vivo but does not affect skin tumor development. *Cell Growth Diff* 10, 467-472 (1999)

108. Keyomarsi K & A.B. Pardee: Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc Natl Acad Sci USA* 90, 1112-16 (1993)

109. Porter DC, N. Zhang, C. Danes, M.J. McGrehan, R.M. Harwell, S. Faruki & K. Keyomarsi: Tumor-specific proteolytic processing of Cyclin E generates hyperactive lower-molecular-weight forms. *Mol Cell Biol* 21, 6254-69 (2001)

110. Wingate H, N. Zhang, M.J. McGarhen, I. Bedrosian, J.W. Harper & K. Keyomarsi: The tumor-specific hyperactive forms of cyclin E are resistant to inhibition by p21 and p27. *J Biol Chem* 280, 15148-57 (2005)

111. Keyomarsi K, S.L. Tucker, T.A. Buchholz, M. Callister, Y. Ding, I. Bedrosian, G.N. Hortobagyi, C. Knickerbucker, W. Toyofuku, M. Lowe, T.W. Herliczec & S.S. Bacus: Cyclin E and survival in patients with breast cancer. *New England J Med* 347, 1566-75 (2002)

112. Bortner DM & M.P. Rosenberg: Induction of mammary gland hyperplasia and carcinomas in transgenic mice expressing human Cyclin E. *Mol Cell Biol* 17, 453-59 (1997)

113. Zwijsen RM, E. Wientjens, R. Klompmaker, J. van der Sman, R. Bernards & R.J. Michaelide: CDK-independent activation of estrogen receptor by Cyclin D1. *Cell* 88, 405-15 (1997)

114. Karsunky H, C. Geisen, T. Schmidt, K. Haas, B. Zevnik, E. Gau et al.: Oncogenic potential of Cyclin E in T-cell lymphomagenesis in transgenic mice: evidence for the cooperation between Cyclin E and Ras but not Myc. *Oncogene* 18, 7816-24 (1999)

115. Geisen C, H. Karsunky, R. Yucel & T. Möröy: Loss of p27(kip1) cooperates with Cyclin E in T-cell lymphomagenesis. *Oncogene* 22, 1724-29 (2003)

116. Bortner DM & M.P. Rosenberg: Overexpression of cyclin A in the mammary glands of transgenic mice results in the induction of nuclear abnormalities and increased apoptosis. *Cell Growth Differ* 6, 1579-89 (1995)

117. Ruas M & G. Peters: The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1378, F115-77 (1998)

118. Wolfel T, M. Hauer, J. Schneider, M. Serrano, C. Wolfel, E. Klehmann-Hieb, E. De Plaen, T. Hankeln, K.H. Buschenfelde & D. Beach: A p16INK4a-insensitive Cdk4 mutant targeted by cytolitic lymphocytes in a human melanoma. *Science* 269, 1281-4 (1995)

119. Zuo L, J. Weger, Q. Yang, A.M. Goldstein, M.A. Tucker, G.J. Walker, N. Hayward & N.C. Dracopoli: Germline mutations in the p16INK4a binding domain of Cdk4 in familial melanoma. *Nat Genet* 12, 97-9 (1996)

120. Rane SG, S.C. Cosenza, R.V. Mettus & E.P. Reddy: Germ line transmission of the $Cdk4^{R24C}$ mutation facilitates tumorigenesis and escape from cellular senescence. *Mol Cell Biol* 22, 644-56 (2002)

121. Sotillo R, J.F. García, S. Ortega, J. Martín, P. Dubus, M. Barbacid & M. Malumbres: Invasive melanoma in Cdk4 targeted mice. *Proc Natl Acad Sci USA* 98, 13312-7 (2001)

122. Chin L, A. Tam, J. Pomerantz, M. Wong, J. Holash, N. Bardeesy, Q. Shen, R. O´Hagan, J. Pantginis, H. Zhou, J.W. Horner. 2nd, C. Cordón-Cardo, G.D. Yancopoulos & R.A. DePinho: Essential role for oncogenic Ras in tumour maintenance. *Nature* 400, 468-72 (1999)

123. Lloyd RV, L.A. Erickson, L. Jin, E. Kulig, X. Qian, J.C. Cheville & B.W. Scheithauer: p27Kip1: a multifunctional cyclin-dependent kinase inhibitor with

prognostic significance in human cancers. *Am J Pathol* 154, 313-23 (1999)

124. Tsihlias J, L. Kapusta & J. Slingerland: The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer. *Annu Rev Med* 50, 401-23 (1999)

125. Zou X, D. Ray, A. Aziyu, K. Christov, A.D. Boiko, A.V. Gudkov & H. Kiyokawa: Cdk4 disruption renders primary mouse cells resistant to oncogenic transformation, leading to Arf/p53-independent senescence. *Genes Dev* 16, 2923-34 (2002)

126. Hulit J, C. Wang, Z. Li, C. Albanese, M. Rao, D. Di Vizio, S. Shah, S.E.W. Byers, R. Mahmood, L.H. Augenlicht, R. Russell & R.G. Pestell: Cyclin D1 genetic heterozygosity regulates colonic epithelial cell differentiation and tumor number in ApcMin mice. *Mol Cell Biol* 24, 7598-611 (2004)

127. Robles AI, M.L. Rodríguez-Puebla, A.B. Glick, C. Trempus, L. Hansen, P. Sicinski, R.W. Tennant, R.A. Weinberg, S.H. Yuspa & C.J. Conti: Reduced skin tumor development in Cyclin-D1 deficient mice highlights the oncogenic ras pathway in vivo. *Genes Dev* 12, 2469-74 (1998)

128. Yu Q & P. Sicinski: Specific protection against breast cancers by cyclin D1 ablation. *Nature* 411, 1017-21 (2001)

129. Burns KH, J.E. Agno, P. Sicinski & M.M. Matzuk: Cyclin D2 and p27 are tissue-specific regulators of tumorigenesis in inhibin alpha knockout mice. *Mol Endocrinol* 10, 2053-69 (2003)

130. Sicinska E, I. Aifantis, L. Le Cam, W. Swat, C. Borowski, Q. Yu, A. Ferrando, S.D. Levin, Y. Geng, H. von Boehmer & P. Sicinski: Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* 4, 451-61 (2003)

131. Rodríguez-Puebla ML, P.L. Miliani de Marval, M. LaCava, D.S. Moons, H. Kiyokawa & C.J. Conti: Cdk4 deficiency inhibits skin tumor development but does not affect keratynocite proliferation. *Am J Pathol* 161, 405-11 (2002)

132. Miliani de Marval PL, E. Macias, R. Rounbehler, P. Sicinski, H. Kiyokawa, D.G. Johnson, C.J. Conti & M.L. Rodríguez-Puebla: Lack of Cyclin-dependent kinase 4 inhibits c-myc tumorigenic activities in epithelial tissues. *Mol Cell Biol* 24, 7538-47 (2004)

133. Martín A, J. Odajima, S.L. Hunt, P. Dubus, S. Ortega, M. Malumbres, & M. Barbacid: Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by p27Kip1 and p21Cip1. *Cancer Cell* 7, 1-8 (2005)

134. Toyoshima H & T. Hunter: p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. *Cell* 78, 67-74 (1994)

135. Harper JW, S.J. Elledge, K. Keyomarsi, B. Dynlacht, L.H. Tsai, P. Zhang, S. Dobrowolski, C. Bai, L. Connell-Crowley, E. Swindell et al.: Inhibition of Cyclin-dependent kinases by p21. *Mol Biol Cell* 6, 387-400 (1995)

136. Bernards R: CDK-independent activities of D type cyclins. *Biochim Biophys Acta* 1424, M17-22 (1999)

137. Ewen ME & J. Lamb: The activities of cyclin D1 that drive tumorigenesis. *TRENDS Mol Med* 10, 158-62 (2004)

138. Kim HA, S.L. Pomeroy, W. Whoriskey, I. Pawlitzky, L.I. Benowitz, P. Sicinski, C.D. Stiles & T.M. Roberts: A developmentally regulated switch directs regenerative growth of Schwann cells through cyclin D1. *Neuron* 26, 405-16 (2000)

139. Atanasoski S, S. Shumas, C. Dickson, S.S. Scherer & U. Suter: Differential cyclin D1 requirements of proliferating Schwann cells during development and after injury. *Mol Cell Neurosci* 18, 581-92 (2001)

140. Huard JM, C.C. Foster, M.L. Carter, P. Sicinski & M.E. Ross: Cerebellar histogenesis is disturbed in mice lacking cyclin D2. *Development* 126, 1927-35 (1999)

141. Lam EW, J. Glassford, L. Banerji, N.S. Thomas, P. Sicinski & G.G. Klaus: Cyclin D3 compensates for loss of cyclin D2 in mouse B-lymphocytes activated via the antigen receptor and CD40. *J Biol Chem* 275, 3479-84 (2000)

142. Solvason N, W.W. Wu, D. Parry, D. Mahony, E.W. Lam, J. Glassford, G.G. Klaus, P. Sicinski, R. Weinberg, Y.J. Liu, M. Howard & E. Lees: Cyclin D2 is essential for BCR-mediated proliferation and CD5 B cell development. *Int Immunol* 12, 631-8 (2000)

143. Kowalczyk A, R.K. Filipkowski, M. Rylski, G.M. Wilczynski, F.A. Jaworski, M.A. Ciemerych. P. Sicinski & L. Kaczmarek: The critical role of cyclin D2 in adult neurogenesis. *J Cell Biol* 167, 209-13, (2004)

144. Krimpenfort P, K.C. Quon, W.J. Mooi, A. Loonstra & A. Berns: Loss of $p16^{Ink4a}$ confers susceptibility to metastatic melanoma in mice. *Nature* 413, 83-6 (2001)

145. Sharpless NE, N. Bardeesy, K.H. Lee, D. Carrasco, D.H. Castrillon, A.J. Aguirre, E.A. Wu, J.W. Horner & R.A. DePinho: Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* 413, 86-91 (2001)

146. Latres E, M. Malumbres, R. Sotillo, J. Martín, S. Ortega, J. Martín-Caballero, J.M. Flores, C. Cordón-Cardo & M. Barbacid: Limited overlapping roles of p15INK4b and p18INK4c cell cycle inhibitors in proliferation and tumorigenesis. *EMBO J* 19, 3496-506 (2000)

147. Franklin DS, V.L. Godfrey, H. Lee, G.I. Kovalev, R. Schoonhoven, S. Chen-Kiang, L. Su & Y. Xiong: Cdk inhibitors p18INK4c and p27Kip1 mediate two separate

pathways to collaboratively suppress pituitary tumorigenesis. *Genes Dev* 12, 2899-911 (1998)

148. Zindy F, W. den Besten, B. Chen, J.E. Reg, E. Latres, M. Barbacid, J.W. Pollard, C.J. Sherr, P.E. Cohen & M.F. Roussel: Control of spermatogenesis in mice by the Cyclin D-dependent kinase inhibitors p18 (Ink4c) and p19 (Ink4d). *Mol Cell Biol* 21, 3244-55 (2001)

149. Bai F, X.H. Pei, V.L. Godfrey & Y. Xiong: Haploinsufficiency of p18(INK4c) sensitizes mice to carcinogen-induced tumorigenesis. *Mol Cell Biol* 23, 1269-77 (2003)

150. Zindy F, J. van Deursen, G. Grosveld, C.J. Sherr & M.F. Roussel: INK4d-deficient mice are fertile despite testicular atrophy. *Mol Cell Biol* 20, 372-8 (2000)

151. Chen P, F. Zindy, C. Abdala, F. Liu, X. Li, M.F. Roussel & N. Segil: Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. *Nat Cell Biol* 5, 385-7 (2003)

152. Serrano M, H.-W. Lee., L. Chin, C. Cordon-Cardo, D. Beach & R.A. DePinho: Role of the INK4a locus in tumor supression. *Cell* 85, 27-37 (1996)

153. Deng C, P. Zhang, J.W. Harper, S.J. Elledge & P. Leder: Mice lacking $p21^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. *Cell* 82, 675-84 (1995)

154. Brugarolas J, C. Chandrasekaran, J.I. Gordon, D. Beach, T. Jacks. & G.J. Hannon: Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* 377, 552-7 (1995)

155. Martín-Caballero J, J.M. Flores, P. García-Palencia & M. Serrano: Tumor susceptibility of *p21*$^{Waf1/Cip1}$-deficient mice. *Cancer Res* 61, 6234-38 (2001)

156. Yang K, J. Mathew, A. Velcich, W. Edelmann, R. Kucherlapati, M. Lipkin, K. Yang & L.H. Augenlicht: Targeted inactivation of $p21^{WAF1/CIP1}$ enhances Apc-initiated tumor formation and the tumor-promoting activity of a Western-style high-risk diet by altering cell maturation in the intestinal mucosa. *Cancer Res* 61, 565-9 (2001)

157. Brugarolas J, R.T. Bronson & T. Jacks: p21 is a critical CDK2 regulator essential for proliferation control in Rb-deficient cells. *J Cell Biol* 141, 503-14 (1998)

158. Adnane J, R.J. Jackson, S.V. Nicosia, A.B. Cantor, W.J. Pledger & S.M. Sebti: Loss of $p21^{WAF1/CIP1}$ accelerates Ras oncogenesis in a transgenic/knockout mammary cancer model. *Oncogene* 19, 5338-47 (2000)

159. Bearss DJ, R.J. Lee, D.A. Troyer, R.G. Pestell & J.J. Windle: Differential effects of p21(WAF1/CIP1) deficiency on MMTV-ras and MMTV-myc mammary tumor properties. *Cancer Res* 62, 2077-84 (2002)

160. Yang WC, J. Mathew, A. Velcich, W. Edelmann, R. Kucherlapati, M. Lipkin, Y.A. Wang, A. Elson & P. Leder: Loss of p21 increases sensitivity to ionizing radiation and delays the onset of lymphoma in atm-deficient mice. *Proc Natl Acad Sci USA* 94, 14590-5 (1997)

161. Fotedar R, H. Brickner, N. Saadatmandi, T. Rousselle, L. Diederich, A. Munshi, B. Jung, J.C. Reed & A. Fotedar: Effect of p21waf1/cip1 transgene on radiation induced apoptosis in T cells. *Oncogene* 18, 3652-8 (1999)

162. Kiyokawa H, R.D. Kineman, K.O. Manova-Todorova, V.C. Soares, E.S. Hoffamn, M. Ono, D. Khanam, A.C. Hayday, L.A. Forman & A. Koff: Enhanced growth of mice lacking the cyclin-dependen kinase inhibitor function of p27Kip1. *Cell* 85, 721-32 (1996)

163. Nakayama K, N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii & D. Y. Loh: Mice lacking p27Kip1 display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85, 707-20 (1996)

164. Fero ML, -M. Rivkin, M. Tasch, P. Porter, C.E. Carow, E. Firpo, K. Polyak, L.H. Tsai, V. Broudy, R.M. Perlmutter, K. Kaushansky & J.M. Roberts: A syndrome of multi-organ hyperplasia with features of gigantism, tumorigenesis and females esterility in p27Kip1-deficient mice. *Cell* 85, 733-744 (1996)

165. Fero ML, E. Randel, K.E. Gurley, J.M. Roberts & C.J. Kemp: The murine gene $p27^{Kip1}$ is haplo-insufficient for tumour supresión. *Nature* 396, 177-80 (1998)

166. Muraoka RS, A.E. Lenferink, B. Law, E. Hamilton, D.M. Brantley, L.R. Roebuck & C.L. Arteaga: ErbB2/Neu-induced, Cyclin D1-dependent transformation is accelerated in p27-haploinsufficient mammary epithelial cells but impaired in p27-null cells. *Mol Cell Biol* 22, 2204-19 (2002)

167. Geisen C, H. Karsunky, R. Yucel & T. Moroy: Loos of p27Kip1 cooperates with Cyclin E in T-cell lymphomagenesis. *Oncogene* 22, 1724-9 (2003)

168. Martín-Caballero J, J.M. Flores, P. García-Palencia, M. Collado & M. Serrano: Different cooperating effect of p21 or p27 deficiency in combination with INK4a/ARF deletion in mice. *Oncogene* 23, 8231-7 (2004)

169. Martins CP & A. Berns: Loss of p27(Kip1) but not p21(Cip1) decreases survival and synergizes with MYC in murine lymphomagenesis. *EMBO J* 21, 3739-48 (2002)

170. Yan Y, J. Frisen, M.H. Lee, J. Massagué & M. Barbacid: Ablation of the Cdk inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev* 11, 973-83 (1997)

171. Zhang P, N. Liegeois, C. Wong, M. Finegold, H. Hou, J.C. Thompson, A. Silverman, J.W. Harper, R.A. DePinho & S.J. Elledge: Altered cell differentiation and proliferation

in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann síndrome. *Nature* 387, 151-158 (1997)

172. Jirawatnotai S, D.S. Moons, C.O. Stocco, R. Franks, D.B. Hales, G. Gibori & H. Kiyokawa: The cyclin-dependent kinase inhibitors p27Kip1 and p21Cip1 cooperate to restrict proliferative life span in differentiating ovarian cells. *J Biol Chem* 278, 17021-7 (2003)

173. Wolfraim LA, T.M. Walz, Z. James, T. Fernández & J. Letterio: p21Cip1 and p27Kip1 act in synergy to alter the sensitivity of naïve T cells to TGF-beta-mediated G1 arrest through modulation of IL-2 responsiveness. *J Immunol* 3093-102 (2004)

174. Zhang P, C. Wong, D. Liu, M. Finegold, J.W. Harper & S.J. Elledge: $p21^{Cip1}$ and $p57^{Kip2}$ control muscle differentiation at the myogenin step. *Genes Dev* 13, 213-24 (1999)

175. Zhang P, C. Wong, R.A. DePinho, J.W. Harper & S.J. Elledge: Cooperation between the Cdk inhibitors $p27^{Kip1}$ and $p57^{Kip2}$ in the control of tissue growth and development. *Genes Dev* 12, 3162-7 (1998)

176. Franklin DS, V.L. Godfrey, D.A. O´Brien, C. Deng, & Y. Xiong: Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity. *Mol Cell Biol* 20, 6147-58 (2000)

177. Zindy F, J.J. Cunningham, C.J. Sherr, S. Jogal, R.J. Smeyne & M.F. Roussel: Postnatal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. *Proc Natl Acad Sci U S A* 96, 13462- 7 (1999)

178. Pei XH, F. Bai, T. Tsutsui, H. Kiyokawa & Y. Xiong: Genetic evidence for functional dependency of p18Ink4c on Cdk4. *Mol Cell Biol* 24, 6653-64 (2004)

179. Sotillo R, O. Renner, P. Dubus, J. Ruiz-Cabello, J. Martín-Caballero, M. Barbacid, A. Carnero & M. Malumbres: Cooperation between Cdk4 and p27Kip1 in tumor development: a preclinical model to evaluate cell cycle inhibitors with therapeutic activity. *Cancer Res* 65, 3846-52 (2005)

180. Tong W & J.W. Pollard: Genetic evidence for the interactions of Cyclin D1 and p27 (Kip1) in mice. *Mol Cell Biol* 21, 1319-28 (2001)

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Send correspondence to: Sagrario Ortega Jimenez, Programa de Biotecnología, Centro Nacional de Investigaciones Oncológicas, C/Melchor Fernández Almagro 3, 28029 Madrid, Spain, Tel.: 34-91-224-6987, Fax: 34-91-224-6972, E-mail: s.ortega@cnio.es