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Perspective

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The G_2 Barrier to Cancer

Floris Foijer

Hein te Riele*

Division of Molecular Biology; The Netherlands Cancer Institute; Amsterdam, The Netherlands

*Correspondence to: Hein te Riele; Division of Molecular Biology; The Netherlands Cancer Institute; Plesmanlaan 121; 1066CX Amsterdam, The Netherlands; Tel.: +31.20.512.20.84; Fax: +31.20.669.13.83; Email: h.t.riele@nki.nl

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ABSTRACT

Loss of the G_1/S checkpoint is recognized as a mandatory step in the development of cancer. Nevertheless, both in vivo and in vitro experiments have indicated that this condition highly sensitizes cells to apoptosis, e.g., primary mouse embryonic fibroblasts that lack the complete retinoblastoma suppressor gene family (*TKO* MEFs) massively die under mitogen-deprived conditions. The prevailing hypothesis therefore is that the increased proliferative capacity of cells that have lost the G_1/S checkpoint becomes apparent by suppression of apoptosis. However, this view was recently challenged by the finding that suppression of apoptotic cell death in *TKO* MEFs did not allow unconstrained proliferation; instead, cells became arrested in G_2. This mechanism, which is dependent on p53, provides yet another barrier to oncogenic transformation. Thus, progression to malignancy of *Rb*-deficient lesions by alleviation of G_2 arrest may offer an alternative explanation for the synergism between loss of *Rb* and *p53* in tumorigenesis.

INTRODUCTION

Cancer is the result of a multi-step process in which cells have acquired genetic or epigenetic alterations that critically affect the functioning of a handful of proto-oncogenes and tumor suppressor genes. These alterations define a number of defense mechanisms against cancer of which some lay in the heart of the cell cycle machinery and protect cells from uncontrolled proliferation.

The mammalian cell cycle consists of four phases, designated G_1, S, G_2 and M.[1] During each cell cycle, the complete genome is duplicated in S phase (the synthetic phase), and subsequently the two copies are divided over two daughter cells in M phase (mitosis). Authorization for DNA synthesis and chromosome segregation is tightly controlled by cyclins and their associated kinase activities that become active during the gap-phases G_1 and G_2, preceding S and M phase, respectively. D- and E-type cyclins control the G_1/S transition, while Cyclin A and Cyclin B1 activity is essential for entry from G_2 into M phase. These cyclins are the targets through which mitogenic and anti-mitogenic stimuli can stimulate or block cell cycle progression, respectively.

G_1/S CONTROL

An important barrier to limitless proliferation is raised by a cell cycle control mechanism that prevents G_1/S transition in response to growth-inhibiting stimuli like differentiation, DNA damage, oncogene activation, loss of anchorage, confluency and mitogen deprivation. Essential actors in this machinery are the retinoblastoma suppressor protein pRB and its homologues p107 and p130. These so-called 'pocket proteins' are direct targets of the G_1 cyclins. Hypophosphorylated pocket proteins can bind to and inactivate E2F transcription factors, whose activity is required for the transcription of genes that are essential for DNA synthesis. Mitogenic signaling induces the G_1 cyclins CYCLIN D-CDK4/CDK6 and CYCLIN E-CDK2 to phosphorylate pocket proteins leading to the release of E2Fs. Derepression and activation of E2F target genes then allows progression from G_1 into S-phase.[2-5] Conversely, growth-inhibitory signals exert their effect by direct down regulation of Cyclin protein levels or by inducing members of the INK4A and/or CIP/KIP family of cyclin dependent kinase inhibitors (CKI). E.g., mitogen deprivation results in reduced transcription of *Cyclin D1*, destabilization of CYCLIN D1 protein and induction of the CYCLIN E-CDK2 inhibitor $p27^{KIP1}$. This promotes the unphosphorylated state of pocket proteins resulting in sequestration of E2Fs, repression of E2F target genes and ultimately

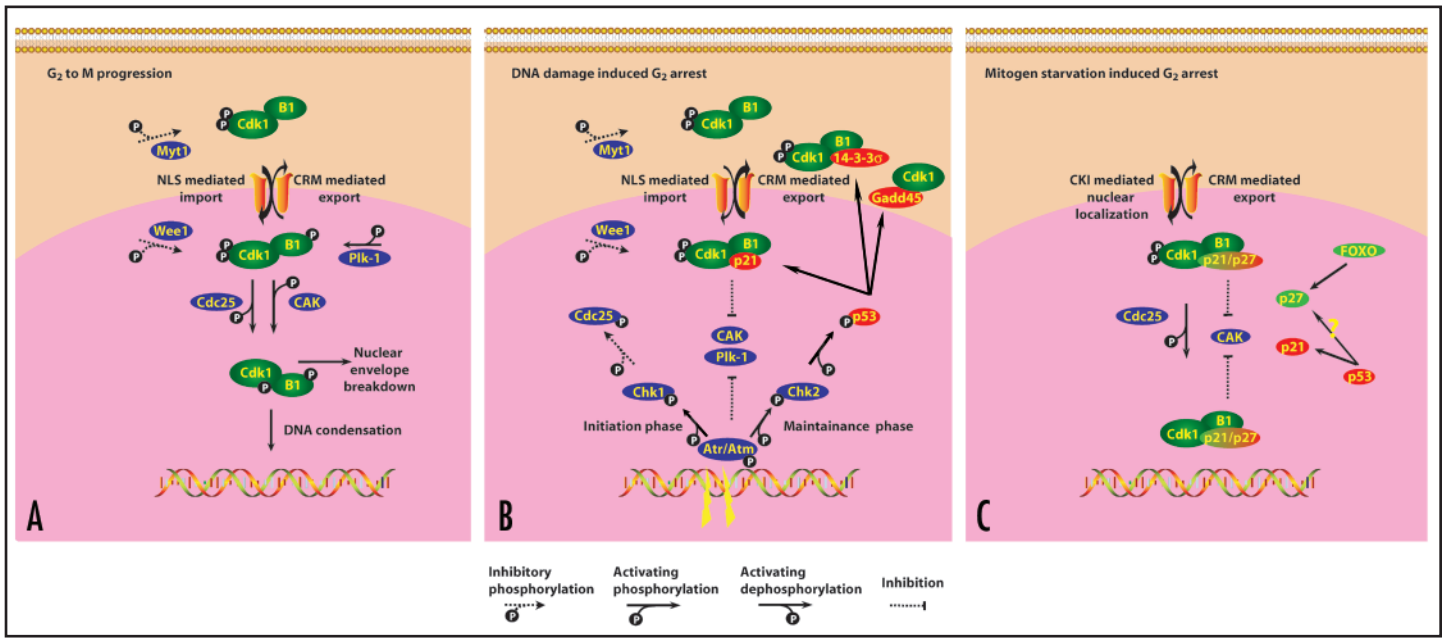

Figure 1. Schematical representation of G_2/M progression (A) and G_2 arrest by DNA damage (B) and mitogen starvation (C).

cell cycle arrest or exit from the cell cycle.[6-8] Thus, pocket proteins can be viewed as molecular cell cycle switches that are turned -on- by growth-inhibiting signals or turned -off- by growth-promoting signals resulting in cell cycle arrest or cell cycle progression, respectively.

The importance of this G_1/S control mechanism is underscored by the finding that the majority of human cancers suffers from genetic alterations that favor the pocket protein -off- state. Examples include genetic loss of *RB* in hereditary retinoblastoma and sporadic breast, bladder, prostate and small cell lung carcinomas[9-14] loss of *p16*^{INK4A} in melanoma, T-cell leukemia, pancreatic and bladder carcinomas, amplification of *CYCLIN D1* in breast, esophagus and head-and-neck cancer, *CDK4* amplification or mutational activation in melanoma.[15,16] Consistently, mice with defects in pocket proteins or CKIs are highly cancer prone.[17,18]

Although loss of G_1/S control clearly is a mandatory step in tumor development, evidence is accumulating that it is not sufficient to fully upset the cell cycle machinery. Also the G_2/M cyclins appear subject to inhibition by growth-restricting conditions, preventing entry into M phase. The best-known example is activation of a G_2 checkpoint by DNA damage. Furthermore, we recently found that complete abrogation of G_1/S control by ablation of the retinoblastoma gene family in primary mouse embryonic fibroblasts (MEFs) does not allow completion of the cell cycle in the absence of mitogenic stimuli. Instead, such cells can enter and complete S phase, but then activate a mechanism that fully arrests them in G_2.[19]

G_2/M CONTROL

Cyclin-B1-associated kinase activity is essential for entry into mitosis,[20] dictating chromatin condensation and nuclear envelope breakdown. During G_2, the Cyclin B1-Cdk1 complex is kept inactive by two mechanisms: phosphorylation of threonine 14 and tyrosine 15 of Cdk1 by the kinases Wee1 in the nucleus and Myt1 in the cytoplasm, and accumulation of Cyclin B1-Cdk1 in the cytoplasm by CRM mediated transport from the nuclear compartment.[21] When cells enter mitosis, just before nuclear envelope breakdown, Cyclin B1-Cdk1 accumulates in the nucleus as a result of phosphorylation of the cytoplasmatic retention signal in Cyclin B1 by Plk-1 or by autophosphorylation.[22,23] However, nuclear entry is not sufficient for initiation of mitosis.[24] In addition to Cyclin B1 phosphorylation, Cdk1 needs to be phosphorylated by Cyclin Activating Kinase (CAK) on threonine 161. Furthermore, the activity of Cdk1 is regulated by the balance between the inhibitory kinases Wee1 and Myt1 and the activating Cdc25 phosphatases that remove phosphates from threonine 14 and tyrosine 15. Together these regulatory mechanisms allow accurate tuning of Cyclin B1-Cdk1 activity ensuring timely mitotic entry.[25] Figure 1A shows a schematic overview of the phosphorylation and dephosphorylation steps that activate Cyclin B1-Cdk1 and drive cells into mitosis.

DNA DAMAGE INDUCED G_2 ARREST

DNA damage can elicit a G_2 arrest basically by counteracting the activating dephosphorylation and phosphorylation of Cyclin B1-Cdk1. Briefly, DNA damage results in the activation of the ATM and ATR kinases. These kinases phosphorylate the kinase Chk1, which in turn inactivates the Cdc25 phosphatases. This abrogates the activity of Cyclin B1-Cdk1, which initiates G_2 arrest in response to DNA damage.[26] Additionally, ATM/ATR can either directly, or via phosphorylation-dependent activation of Chk2, phosphorylate p53, leading to increased transcription of p21^{CIP1}, GADD45 and 14-3-3σ.[25] These proteins are thought to be responsible for the maintenance of the G_2 arrest. p21^{CIP1} can interact with Cyclin B1-Cdk1 and prevent CAK mediated phosphoryation of Cdk1 threonine 161.[27,28] Gadd45 is thought to dissociate Cyclin B1-Cdk1 complexes by binding to the Cdk1 subunit.[25] 14-3-3σ has been suggested to sequester Cyclin B1-Cdk1 into the cytoplasm,[29] although it is still unclear whether this occurs through direct interaction.[29,30] Additionally, ATM/ATR activation leads to inhibition of Plk-1 kinase activity.[31] As a consequence of these regulatory mechanisms, Cyclin B1-Cdk1 localization is mostly cytoplasmatic upon activation of the G_2 DNA damage checkpoint. However,

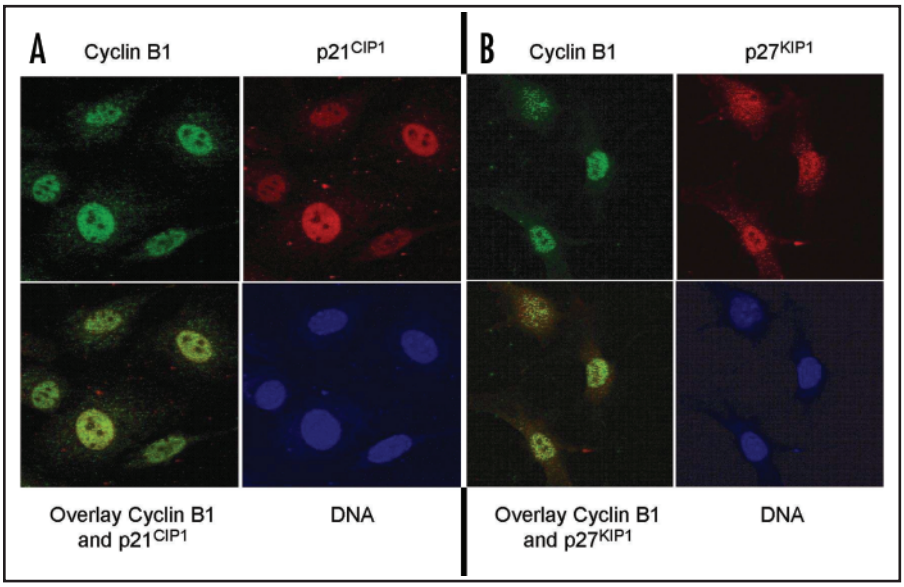

Figure 2. Nuclear colocalization of the Cyclin dependent kinase inhibitors $p21^{CIP1}$ (red) (A) or $p27^{KIP1}$ (red)(B) and Cyclin B1 (green) in mitogen starved pocket protein deficient MEFs.

nuclear accumulation of Cyclin B1-Cdk1 upon DNA damage has also been reported.[28] This has been attributed to nuclear localization of $p21^{CIP1}$. Figure 1B summarizes how DNA damage can result in a G_2 arrest dependently and independently of p53.

MITOGEN STARVATION INDUCED G_2 ARREST

We recently found that besides DNA damage, also mitogen deprivation can impinge on the activity of Cyclin B1-Cdk1 and impose a G_2 arrest. In normal cells, this response is largely masked by strong accumulation of cells in G_1 due to activation of the G_1/S checkpoint. Mitogen-deprivation-induced G_1 arrest is critically dependent on the retinoblastoma gene family as mouse embryonic fibroblasts lacking the complete retinoblastoma gene family (*TKO* MEFs) fail to arrest in G_1.[32,33] Nonetheless, while these cells do complete S phase, they are subsequently blocked from finalizing the cell cycle by two mechanisms: (1) the majority of cells undergoes massive apoptosis;[19,32,33] (2) surviving cells arrest in the G_2 phase of the cell cycle.[19] Strikingly, when apoptosis was suppressed by ectopic expression of Bcl2, virtually all cells arrest in G_2.

The mitogen-starvation-induced G_2 arrest is mediated by upregulation of the cell cycle inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ that act as potent inhibitors of Cyclin A- and Cyclin B1-associated kinase activities through direct interaction in the nuclear compartment (Fig. 2A and B). Previous work has demonstrated that ectopic expression of $p21^{CIP1}$ or $p27^{KIP1}$ can block progression through the cell cycle in both G_1 and G_2, the latter by direct interaction with Cyclins A and B1.[34,35] The mitogen-starvation-induced G_2 arrest was fully reversible and may thus be considered as a true restriction point: readdition of serum abrogated the interaction of Cyclin B1 and $p27^{KIP1}$ and (to a lesser extent) $p21^{CIP1}$, induced Cyclin A- and B1-associated kinase activities and allowed mitotic entry immediately thereafter.

Both apoptosis and G_2 arrest in serum-starved *TKO* MEFs are dependent on the presence of functional p53. Down regulation of p53 protein level by RNA interference suppressed apoptosis and allowed *TKO* MEFs to proliferate for at least three generations in the absence of mitogens.[19] As p53 is also a central player in DNA-damage-induced G_2 arrest,[36] one may wonder to which extent the G_2 restriction point differs from a DNA damage response. E.g., is it possible that the absence of appropriate mitogenic signaling leads to a high level of stalled or collapsed replication forks that activate the G_2 checkpoint? Some support for this scenario may come from the observation that caffeine treatment of serum-starved G_2 cells to inhibit ATM/ATR kinases leads to a marked drop in $p21^{CIP1}$ levels. However, caffeine treatment is not sufficient to force the cells into mitosis. This can be attributed to the unaltered high levels of $p27^{KIP1}$ that remains associated with Cyclin B1 and is apparently sufficient to suppress the activities of Cyclin A and B1 kinase complexes.[19] The critical role of $p27^{KIP1}$ is also underscored by the observation that mitogen deprivation still induced G_2 arrest in *TKO* cells in which $p21^{CIP1}$ was down regulated by RNAi, albeit with some delay. $p27^{KIP1}$ can be induced via the Forkhead transcription factors,[37] which are activated upon several forms of stress like hypoxia or mitogen deprivation, without a known role of p53. However, we found that in p53 and retinoblastoma family ablated cells, $p27^{KIP1}$ is not induced upon mitogen deprivation, suggesting that it is subject to p53-mediated regulation, either directly or indirectly.[19] Future work will be required to determine whether p53 plays an active role in the G_2 restriction point or acts as a mere bystander that is required to ensure sufficiently high levels of $p21^{CIP1}$ and possibly $p27^{KIP1}$.

There are several other features of the G_2 restriction point that suggest it to be mechanistically different from the DNA damage checkpoint. Induction of G_2 arrest by mitogen starvation is highly robust. Apoptosis-resistant *TKO* MEFs can be kept arrested under serum-deprived conditions for at least 8 weeks (Foijer F, te Riele H, unpublished data). Another contrasting aspect of the G_2 restriction point is the nuclear Cyclin B1 localization, which could very well be the consequence of the localization of $p21^{CIP1}$ and $p27^{KIP1}$. PKB activity, which is induced by mitogenic signaling, can result in a redistribution of $p21^{CIP1}$ and $p27^{KIP1}$ from the nuclear to the cytoplasmatic compartment by phosphorylation of the CKIs.[38,39] Conversely, low PKB activity by mitogen starvation, may promote nuclear accumulation of CKIs and hence nuclear sequestration of Cyclin B1-Cdk1.[19] A schematic overview of the mitogen starvation induced G_2 arrest is presented in Fig. 1C. We speculate that besides mitogen deprivation, other extracellular signals may also induce a G_2 arrest in the absence of pocket proteins. E.g., experiments in which primary mouse embryonic fibroblasts were induced to differentiate into myoblasts, showed that a subset of $Rb^{-/-}$ myoblasts failed to arrest in G_0, entered S-phase and subsequently arrested in the G_2 phase of the cell cycle. Interestingly, these differentiated cells showed elevated levels of $p21^{CIP1}$ and $p27^{KIP1}$.[40]

CELL CYCLE ARREST OF POCKET-PROTEIN-DEFECTIVE CELLS IN VIVO

These results disclose a cell cycle arrest mechanism in G_2 that may serve as an emergency brake to prevent unconstrained proliferation of mitogen-deprived cells that have lost proper G_1/S control.

An important question now is: does this mechanism also operate in vivo, i.e., is there evidence for cells arrested in G_2 in early tumor lesions in which the G_1/S transition is affected?

Developmental defects in Rb-deficient mice. Homozygous disruption of *Rb* in mice is incompatible with embryonic development: it is known for a long time that $Rb^{-/-}$ embryos die around day 12–14 postcoitum, showing massive cell death in the central and peripheral nervous systems (CNS and PNS) and defects in erythropoiesis and lens development.[41-44] More recently, it was shown that many of these defects do not indicate a cell-autonomous requirement for pRB in the affected tissues, but rather appear to be secondary to severe anemia, which has been attributed to a placental defect.[45] Indeed, when $Rb^{-/-}$ embryos were supplied with a wild type placenta, apoptosis in the CNS was virtually absent. Now, $Rb^{-/-}$ embryos developed to term although they died soon after birth due to skeletal muscle defects.[45,46] This may also explain previous observations in chimeric mice showing that $Rb^{-/-}$ ES cells could contribute to almost all tissues including the CNS without overt abnormalities.[47,48] However, careful analyses of chimeric embryos revealed that *Rb*-deficient neuronal progenitors in the developing CNS showed ectopic DNA synthesis and subsequently arrested with a 4n DNA content.[49] Interestingly, arrested cells showed elevated expression of $p21^{CIP1}$, which may suggest that they were arrested in G_2. These results were later confirmed in mice in which *Rb* could be inactivated specifically in the CNS and PNS. In contrast to the CNS, the PNS showed significant apoptotic cell death upon ablation of pRb.[50]

Another striking example of cell cycle arrest rather than apoptosis following *Rb* loss was observed in the retina. In adult chimeric mice, the retina was the only tissue that showed strongly reduced contribution of $Rb^{-/-}$ cells.[47] Exclusion of $Rb^{-/-}$ cells started already at day 16 of embryonic development and was manifested by extensive apoptosis (Dannenberg JH, te Riele H, unpublished data). This was recently confirmed in mice in which *Rb* was conditionally inactivated in the retina: also here the majority of *Rb*-deficient retinal precursors underwent apoptotic cell death.[51-53] However, a subset of cells, in particular amacrine cells, survived.[53] Although it is unclear whether these cells arrested in G_2, this again demonstrates that the consequences of *Rb* loss are cell-type dependent: while certain cell types are doomed to death in the absence of *Rb*, there are also naturally death-resistant cell types that survive *Rb* loss and find a way to arrest the cell cycle.

Tumorigenesis by loss of pocket proteins. Somewhat remarkably, the tumor spectrum in mice heterozygous for *Rb* is limited to the intermediate lobe of the pituitary and thyroid glands. These tumors invariably show loss of the wild-type *Rb* allele.[18] Analyses of the earliest consequences of *Rb* deficiency in the pituitary gland has indicated that the absence of *Rb* gives rise to increased cell turn-over manifested by ectopic DNA synthesis and elevated apoptosis.[54] It has therefore been suggested that the acquisition of an additional event that suppresses apoptotic cell death is an essential step for tumor progression in this model.

Concomitant ablation of *Rb* and one of its homologues, either *p107* or *p130*, strongly expanded the tumor spectrum. E.g., chimeric mice generated from $Rb^{+/-}p107^{-/-}$ ES cells developed besides pituitary and thyroid tumors, osteosarcomas, lymphosarcomas, leiomyosarcomas and tumors located to the adrenal gland and ovary and in the majority of cases the wild-type *Rb* allele was lost.[17] Chimeric mice generated form either $Rb^{-/-}p107^{-/-}$ or $Rb^{-/-}p130^{-/-}$ ES cells developed retinoblastoma, a finding that was confirmed in *p107* and *p130*-deficient mice in which *Rb* could be conditionally inactivated in the retina.[17,51-53,55] Also in these models for retinoblastoma, early lesions showed ectopic DNA synthesis and massive apoptosis. This again suggested that tumorigenic outgrowth of pocket-protein-defective retinoblasts would require events to counteract apoptosis. However, this view was challenged by the observation that in the chimeric and hereditary mouse models, retinoblastomas showed characteristics of amacrine cells indicating that tumors did not originate from cells that had evaded apoptosis, but from the small subset of cells that survived loss of pocket proteins. Thus, retinoblastoma development in *Rb/p107*- and *Rb/p130*-deficient retinas requires alleviation of cell cycle arrest rather than suppression of apoptosis. Although it is at present unclear whether or not *Rb/p107*- or *Rb/p130*-deficient amacrine precursor cells arrest in G_2, this observation indicates that at least in certain cell types, cell cycle arrest rather than apoptosis is the blockade that needs to be alleviated for unleashing the increased proliferative capacity of pocket-protein-deficient cells.

Acceleration of tumorigenesis by loss of Rb and other cell cycle inhibitors. The combination of constitutive or conditional knockout alleles for *Rb* and *p53* revealed a strong synergism between the loss of these two tumor suppressor genes in tumor development, e.g., somatic loss of *Rb* and *p53* by Cre-mediated recombination allowed the generation of murine models for pinealoblastoma,[56] medulloblastoma,[57] breast cancer[58] and SCLC.[59] $Rb^{+/-}p53^{-/-}$ mice developed tumor types that were not seen in the single knockouts, including islet cell tumors and pineal gland tumors.[60] Thus far, the synergism between loss of *Rb* and loss of *p53* has been interpreted to reflect a requirement for alleviation of apoptosis in *Rb*-deficient cells. While this may be true for certain tumor types, this appears not to be the case for pituitary and thyroid gland tumors, which in $Rb^{+/-}p53^{+/-}$ mice generally developed without loss of the wild-type *p53* allele.[60] Also in retinoblastoma, no evidence was found for loss of *p53*.[17,55] This may indicate that in these tissues progression of *Rb*-deficient lesions does not require suppression of apoptosis, but rather alleviation of cell cycle arrest that is effectuated by suppression of CDK activity. Although in mitogen-deprived pocket-protein-deficient MEFs, the G_2 arrest could be alleviated by down regulation of *p53*, this may not necessarily be sufficient in vivo, perhaps because of a dominant role of the CDK inhibitor $p27^{KIP1}$. In this respect it is of interest that the level of $p27^{KIP1}$ protein and expression of $p27^{KIP1}$ mRNA was strongly suppressed in pituitary gland tumors in $Rb^{+/-}$ mice.[61] Furthermore, development of both pituitary and thyroid gland tumors in *Rb* heterozygous animals were strongly accelerated by concomitant ablation of $p27^{KIP1}$. Also, tumors in $Rb^{+/-}p27^{KIP1-/-}$ mice were more aggressive than in $Rb^{+/-}$ littermates.[61] Although it is unclear whether *Rb*-deficient precursor lesions in the pituitary gland have been arrested in G_2, inhibition of Cyclin-Cdk activity by $p27^{KIP1}$ may provide a strong barrier to tumor progression.

CONCLUDING REMARKS

Ablation of the G_1/S checkpoint is an essential step in tumor development. However, it is clearly not sufficient as both in vitro and in vivo evidence has shown that both apoptosis and cell cycle arrest by accumulation of cyclin-dependent kinase inhibitors impose a strong barrier to unconstrained proliferation. Whether pocket-protein-defective cells undergo apoptotic cell death or cell cycle arrest seems to be highly cell-type dependent. There is at least one example, retinoblastoma in mice, where cell cycle arrest rather than apoptosis is the major barrier to malignant outgrowth of pocket

protein-defective retinoblasts. This mechanism may be more widespread. While the acquisition of an additional genetic event that alleviates cell cycle arrest may lead to tumor progression and development of cancer, arrested cells are expected to remain present in the body. As such cells may be refractory to therapeutic regimens that are targeting cycling cells and the G_2 arrest is reversible, they may become a source of recurrent cancer. Elucidation of the mechanistic details and significance in cancer of the G_2 arrest will be of critical importance to understand the behavior of occult premalignant cells and may lead to strategies to impede their progression to malignancy.

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