Mitogen requirement for cell cycle progression in the absence of pocket protein activity

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Summary

Primary mouse embryonic fibroblasts lacking expression of all three retinoblastoma protein family members (TKO MEFs) have lost the G_1 restriction point. However, in the absence of mitogens these cells become highly sensitive to apoptosis. Here, we show that TKO MEFs that survive serum depletion pass G_1 but completely arrest in G_2. $p21^{CIP1}$ and $p27^{KIP1}$ inhibit Cyclin A-Cdk2 activity and sequester Cyclin B1-Cdk1 in inactive complexes in the nucleus. This response is alleviated by mitogen restimulation or inactivation of p53. Thus, our results disclose a cell cycle arrest mechanism in G_2 that restricts the proliferative capacity of mitogen-deprived cells that have lost the G_1 restriction point. The involvement of p53 provides a rationale for the synergism between loss of *Rb* and *p53* in tumorigenesis.

Introduction

Proliferation of cells in culture is dependent on the presence of mitogenic stimuli. In the absence of mitogens, cells fail to progress through the so-called restriction point and arrest in G_0/G_1. The restriction point was originally defined by Pardee as the event in G_1 after which cells are committed to progress through the cell cycle independently of mitogens (Pardee, 1974). Most physiological signals that force cells to pass the G_1 restriction point activate Ras signaling, ultimately resulting in increased transcription of *Cyclin D1*. Additionally, Cyclin D1 protein levels are stabilized by activation of the PI3 kinase pathway (Malumbres and Barbacid, 2001; Schmidt et al., 2002). Cyclin D1 with its catalytic subunits, the cyclin-dependent kinases (Cdks) Cdk4 and Ckd6, mediates the extensive phosphorylation and partial inactivation of the retinoblastoma protein pRb during G_1 (Planas-Silva and Weinberg, 1997). pRb hyperphosphorylation triggers the activity of E2F transcription factors, leading to enhanced Cyclin E levels. Increased Cyclin E/Cdk2 activity allows further pRb hyperphosphorylation, G_1 progression, and initiation of S phase. Conversely, mitogen starvation results in reduced transcription of *Cyclin D1*, destabilization of Cyclin D1 protein, and induction of the Cdk2 inhibitor $p27^{KIP1}$, resulting in hypophosphorylated pRb, subsequent inhibition of E2F activity, and arrest in G_0/G_1 (Blagosklonny and Pardee, 2002).

Despite the critical role of pRb in controlling the G_1 restriction point, pRb knockout primary mouse embryonic fibroblasts (MEFs) do not proliferate in the absence of mitogens but still arrest (Herrera et al., 1996; Almasan et al., 1995). This could be explained by the redundant functions of the pRb family members p107 and p130 (Mulligan and Jacks, 1998), since these so-called pocket proteins collectively regulate the activity of E2F transcription factors. We and others have shown that complete disruption of the pocket proteins in MEFs (*triple* knockout MEFs or TKO MEFs) resulted in abrogation of the G_1 restriction point (Dannenberg et al., 2000; Sage et al., 2000). Also, viral oncoproteins like E1A, E7, or Large T, which are encoded by adenoviruses, human papillomaviruses, and polyomaviruses, respectively, can overcome the G_1 restriction point by direct interaction with the pRb family members (Helt and Galloway, 2003).

The function of pocket proteins is often compromised in human cancer through either expression of viral oncogenes, overexpression of *Cyclin D1*, or loss of the tumor suppressor gene $p16^{INK4A}$, suggesting that loss of the G_1 restriction point is a critical step in the development of cancer (Pardee, 1974; Malumbres and Barbacid, 2001). However, here we show that ablation of the pocket proteins in primary cells in vitro does not result in complete alleviation of cell cycle control. We provide evidence for a p53-dependent control mechanism that acts in the G_2

SIGNIFICANCE

Cancer is a multistep process resulting from abrogation of several barriers to uncontrolled proliferation. One of these barriers is formed by the retinoblastoma protein family that prevents cells from undesired replication of DNA. We have identified a cell cycle barrier that prevents unbalanced cell division of mitogen-starved cells that have passed the restriction point. We show that loss of these barriers, e.g., by combined inactivation of retinoblastoma proteins and p53, allows proliferation of cells in the absence of growth stimuli. This observation gives insight into the causes of oncogenic transformation, providing opportunities for therapeutic intervention.

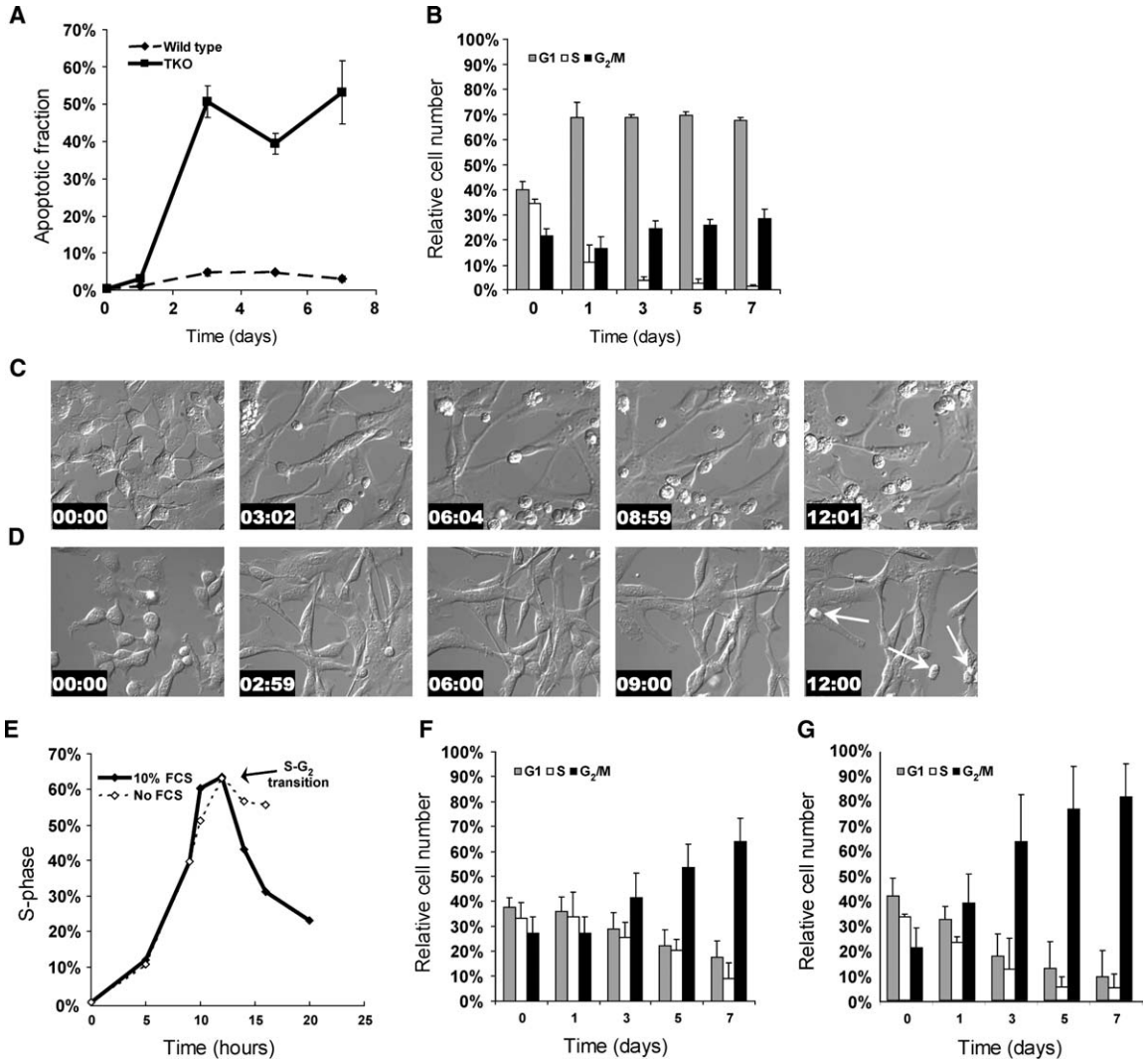

Figure 1. Serum starvation of TKO MEFs results in massive apoptosis from G_2 and a G_2 arrest in the surviving cells

A: Apoptosis induction in wild-type and TKO MEFs upon serum starvation. Graphs represent the average of three independent experiments; error bars represent standard deviations.

B: Cell cycle distribution of serum-starved wild-type MEFs. Graphs represent the average of three independent experiments; error bars represent standard deviations.

C and **D:** Induction of apoptosis in asynchronously (**C**) and synchronously (**D**) growing TKO MEFs by serum depletion. Time is given in hh:mm after serum starvation.

E: S phase progression of TKO MEFs in the presence and absence of serum after mitosis as determined by BrdU incorporation.

F and **G:** Cell cycle distribution of serum-starved TKO (**F**) and TKO-Bcl2 MEFs (**G**). Graphs represent the average of three independent experiments; error bars represent standard deviations.

phase of the cell cycle to arrest or eliminate serum-deprived cells lacking a functional G_1 restriction point.

Results

Mitogen starvation in the absence of pocket proteins results in massive apoptosis and arrest in G_2

Consistent with previous results (Sage et al., 2000; Dannenberg et al., 2000), serum deprivation in wild-type MEFs led to the accumulation of cells with a 2n and, to a lesser extent, 4n DNA content, but not to apoptosis (Figures 1A and 1B). In contrast, TKO MEFs underwent massive apoptosis upon serum starvation (Figure 1A). We used time-lapse microscopy to monitor the onset of apoptosis in serum-starved TKO MEF cultures. Several hours after serum withdrawal, the first apoptotic events could be observed (floating cells, Figure 1C). To examine if programmed cell death occurred at a specific point in the cell cycle, we synchronized TKO MEFs by mitotic shake-off and cultured them under serum-free or high-serum conditions. Time-lapse microscopy revealed that, in the absence of serum, the first apoptotic events (indicated by arrows in Figure 1D) occurred at approximately 12 hr after exit from mitosis. Simultaneously, we followed the progression of synchronized cells by measuring 5-bromodeoxyuridine (BrdU) incorporation. Figure 1E shows that the kinetics of progression through G_1 and entry into S phase of TKO cells were independent of serum, consistent with loss of the G_1 restriction point (Dannenberg et al., 2000; Sage et al., 2000). The appearance of apoptotic cells coincided with

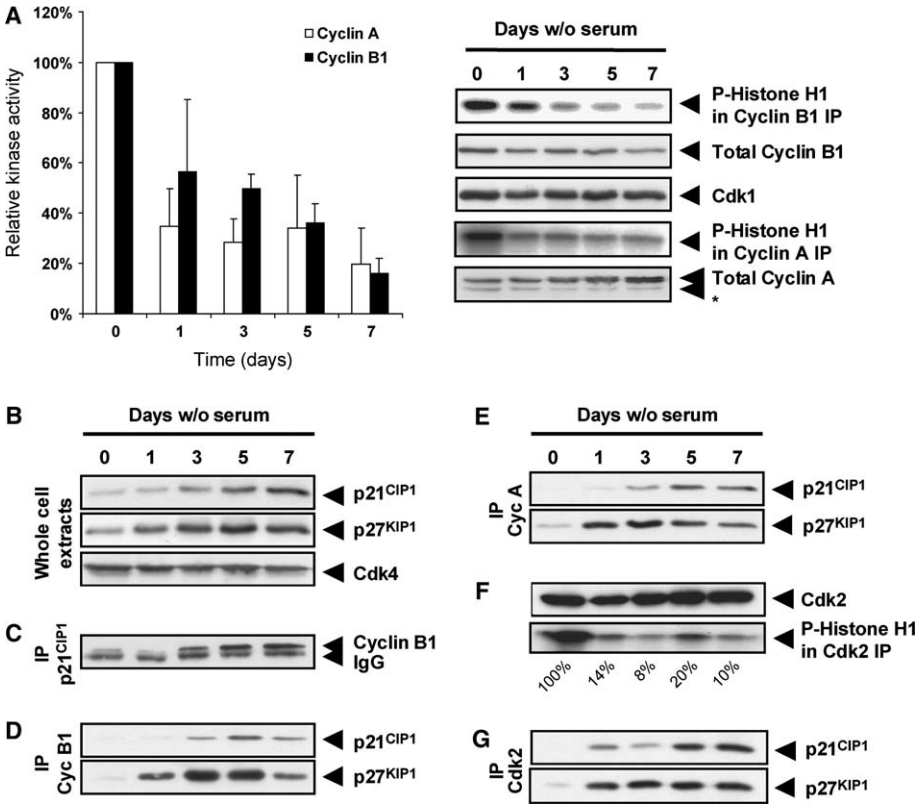

Figure 2. Serum starvation-induced G_2 arrest in apoptosis-resistant TKO MEFs is associated with $p21^{CIP1}$- and $p27^{KIP1}$-dependent inhibition of Cyclin A and B1 kinase activity

A: Cyclin A- and B1-associated kinase activities upon serum starvation and total levels of cyclins and Cdk1. Graphs represent the average values of three independent experiments, and error bars represent standard deviations; blots represent the results from a representative experiment.

B: Total levels of $p21^{CIP1}$ and $p27^{KIP1}$ in serum-starved apoptosis-resistant TKO MEFs. Cdk4 functions as a loading control.

C–E: Immunoprecipitations showing interactions of $p21^{CIP1}$ and $p27^{KIP1}$ with Cyclin B1 (**C** and **D**) and Cyclin A (**E**) from serum-starved TKO-Bcl2 MEFs.

F: Total Cdk2 levels and Cdk2 kinase activity in serum-starved TKO-Bcl2 MEFs. Percentages represent quantified values of labeled Histone H1.

G: Immunoprecipitation of Cdk2 from serum-starved TKO-Bcl2 MEFs.

exit from S phase and onset of G_2. Clearly, this apoptotic response could act as an effective safety strategy to prevent propagation of G_1 restriction point-defective cells in the absence of mitogens. Strikingly, however, we observed that the small fraction of TKO MEFs that survived serum starvation did not proliferate but arrested in the G_2 phase of the cell cycle (Figure 1F). In order to study this cell cycle arrest in detail, we generated apoptosis-resistant TKO MEFs by expressing the antiapoptotic protein Bcl2 using retroviral transduction. TKO-Bcl2 cells largely survived serum starvation but arrested in G_2 with the same kinetics as TKO MEFs (Figure 1G).

Inhibition of Cyclin A- and B1-associated kinase activity

Cdk1 kinase activity is essential for progression into mitosis and is dependent on association with Cyclin A or Cyclin B1 (Nigg, 2001). We therefore determined these Cyclin-associated kinase activities in apoptosis-resistant TKO MEFs after serum starvation. Indeed, Cyclin B1-Cdk1 kinase activity was downmodulated 5-fold compared to B1-associated kinase activity in asynchronously growing cultures, although total levels of Cyclin B1 and Cdk1 remained constant. Also, Cyclin A-associated kinase activity was reduced upon serum starvation, while the levels of Cyclin A slightly increased (Figure 2A). Cyclin-associated kinases can be inhibited by cyclin-dependent kinase inhibitors (CKIs) (Coqueret, 2003). We therefore determined the levels of the CKIs $p21^{CIP1}$ and $p27^{KIP1}$ in serum-starved apoptosis-resistant TKO MEFs. Figure 2B shows that both $p21^{CIP1}$ and $p27^{KIP1}$ levels were strongly increased upon serum starvation. Previous reports have suggested that $p21^{CIP1}$ can inhibit Cyclin B1-associated kinase activity by direct interaction (Smits et al., 2000b; Baus et al., 2003). Figure 2C shows that upon serum starvation the amount of Cyclin B1 coprecipitating with $p21^{CIP1}$ increased and reached a maximum level after 5 and 7 days of serum starvation. Similarly, $p21^{CIP1}$ increasingly coimmunoprecipitated with Cyclin B1-Cdk1. Thus, Cyclin B1-Cdk1 and $p21^{CIP1}$ were present in the same complex at 5 and 7 days after serum deprivation (Figure 2D). Interestingly, we observed that also $p27^{KIP1}$ was sequestered into Cyclin B1 complexes in these cells, although here the level of interaction reached a maximum after 3–5 days of serum starvation and then decreased (Figure 2D, lower panel). Since $p27^{KIP1}$ was absent in $p21^{CIP1}$ immunoprecipitates, we hypothesize that $p21^{CIP1}$ and $p27^{KIP1}$ compete for similar Cyclin B1-Cdk1 binding sites (data not shown). In parallel, we found that also Cyclin A increasingly associated with $p21^{CIP1}$ and $p27^{KIP1}$ upon serum withdrawal (Figure 2E). In agreement with this, increasing levels of $p21^{CIP1}$ and $p27^{KIP1}$ also inhibited the kinase activity of Cdk2 (Figure 2F) through direct association (Figure 2G). Thus, inhibition of Cyclin A-Cdk2 may contribute to the G_2 arrest, which is consistent with a role for Cyclin A-Cdk2 in progression from G_2 to mitosis (Furuno et al., 1999).

Individual roles of the CKIs in the G_2 arrest

To evaluate the individual roles of $p21^{CIP1}$ and $p27^{KIP1}$ in the G_2 arrest, we asked whether high levels of $p27^{KIP1}$ could block TKO-Bcl2 MEFs in G_2. We synchronized TKO-Bcl2 MEFs by mitotic shake-off and microinjected YFP-tagged $p27^{KIP1}$ 12 hr later (Connor et al., 2003), when the majority of the cells were in G_2. Time-lapse microscopy showed that control-injected G_2 cells readily progressed through mitosis, whereas none of the YFP-$p27^{KIP1}$-expressing G_2 cells entered mitosis, indicating that $p27^{KIP1}$ is capable of establishing a G_2 arrest (data not shown).

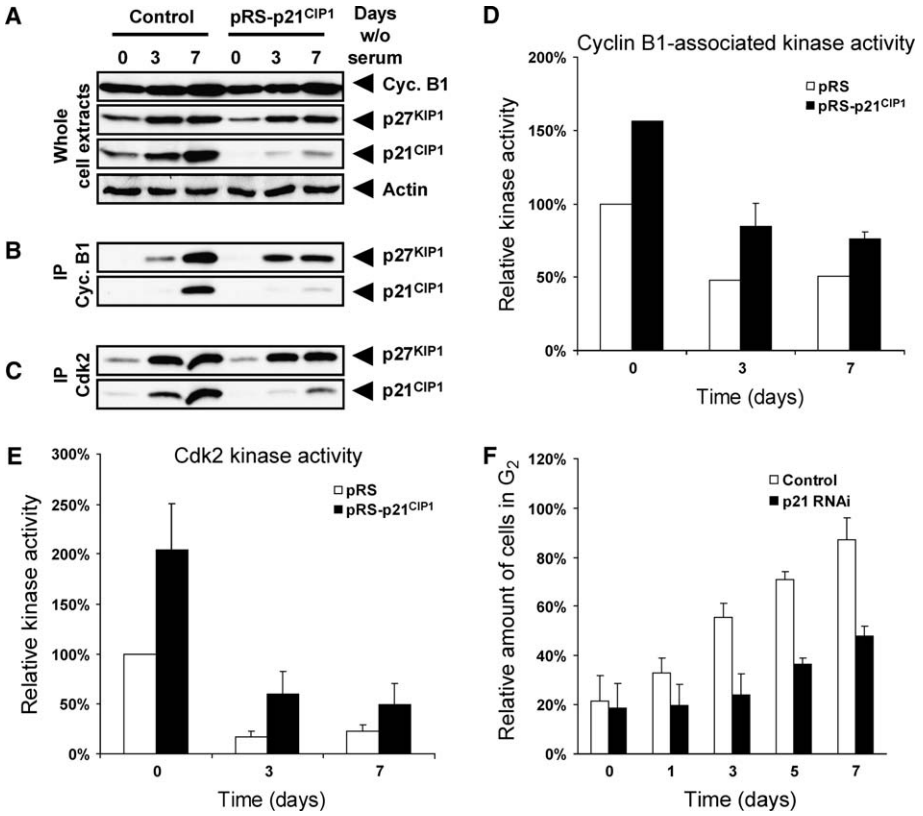

Figure 3. Inhibition of $p21^{CIP1}$ delays the G_2 arrest

A: Total levels of Cyclin B1, $p27^{KIP1}$, and $p21^{CIP1}$ in pRS control- and pRS-$p21^{CIP1}$-infected TKO-Bcl2 MEFs upon serum starvation. Actin functions as a loading control.

B and **C:** Immunoprecipitations of Cyclin B1 (**B**) and Cdk2 (**C**) from serum-starved control and pRS-$p21^{CIP1}$-infected TKO Bcl2 MEFs probed for the presence of $p21^{CIP1}$ and $p27^{KIP1}$.

D and **E:** Cyclin B1-associated kinase activity (**D**) and Cdk2 kinase activity (**E**) in serum-starved TKO-Bcl2 MEFs infected with either pRS control or pRS-$p21^{CIP1}$ viruses. Error bars represent standard deviations of two independent experiments.

F: G_2 accumulation of serum-starved pRS control- or pRS-$p21^{CIP1}$-infected TKO-Bc2 MEFs as determined by FACS analysis. Graphs represent the average of two independent experiments; error bars represent standard deviations.

In another approach, we have attempted to knock down the CKIs using vector-driven RNAi constructs. Although we were unsuccessful in knocking down $p27^{KIP1}$ in TKO-Bcl2 MEFs, we succeeded in knocking down $p21^{CIP1}$. Figure 3A shows that $p21^{CIP1}$ was hardly induced in TKO-Bcl2 MEFs expressing pRetroSuper $p21^{CIP1}$ upon serum starvation, whereas it readily accumulated in control-infected cells. pRetroSuper $p21^{CIP1}$ did not affect Cyclin B1 expression and allowed normal $p27^{KIP1}$ accumulation upon serum starvation (Figure 3A). Inhibition of $p21^{CIP1}$ expression resulted in less $p21^{CIP1}$ associated to Cyclin B1 (Figure 3B, lower panel) and Cdk2 (Figure 3C, lower panel). This resulted in a strong increase of Cyclin B1-associated and Cdk2 kinase activities (Figures 3D and 3E) in cells growing in high serum. During serum starvation, these activities were reduced, most likely due to the strong association of $p27^{KIP1}$ to Cyclin B1 and Cdk2 (Figures 3B and 3C), but not to the same level as in pRS control cells (Figures 3D and 3E). Consistently, FACS analysis revealed that the accumulation of cells in G_2 was reduced upon expression of $p21^{CIP1}$-RNAi (Figure 3F). As cell numbers only moderately increased (data not shown), these data suggest that inhibition of $p21^{CIP1}$ delayed the G_2 arrest but was not sufficient for unconstrained proliferation under mitogen-deprived conditions.

These results demonstrate that in serum-starved TKO MEFs association of both $p21^{CIP1}$ and $p27^{KIP1}$ to Cyclin B1-Cdk1 complexes and Cdk2 contribute to inhibition of mitotic cyclins, leading to a sustained G_2 arrest.

The mitogen starvation-induced G_2 arrest is reversible

If the G_2 arrest displays typical characteristics of a restriction point, serum-starved apoptosis-resistant TKO MEFs should be capable of reentering the cell cycle upon serum addition. Time-lapse microscopy revealed the appearance of mitotic cells 18–24 hr after serum readdition to G_2-arrested cells (data not shown). Using MPM2 as a marker for the appearance of mitotic cells, we confirmed by FACS that the cells entered mitosis at 18–24 hr after serum restimulation (Figure 4A). To verify the relevance of complexes of Cyclin B1 with $p21^{CIP1}$ and $p27^{KIP1}$, we determined whether they were affected by serum readdition. $p27^{KIP1}$ completely disappeared from the Cyclin B1 kinase complex within 15 hr after the addition of serum (Figure 4B). $p21^{CIP1}$-Cyclin B1 complexes slightly increased after 15 hr but gradually decreased when the cells entered mitosis, although they did not completely disappear. Consistently, $p27^{KIP1}$ levels dropped promptly, whereas $p21^{CIP1}$ levels only moderately decreased. Importantly, Cyclin A-associated kinase activity reappeared within 15 hr after serum addition, reaching a maximum at 21 hr, and Cyclin B1-Cdk1 was reactivated within 18 hr and peaked at 24 hr after serum stimulation (Figure 4C). These results show that mitogen stimulation results in release of CKIs, reactivation of Cyclin A- and Cyclin B1-associated kinase activities, G_2 progression, and mitotic entry, demonstrating that the G_2 arrest is reversible.

The mitogen starvation-induced G_2 arrest is different from a DNA damage response

During G_2 phase and during a DNA damage-induced G_2 arrest, Cyclin B1 localizes to the cytoplasmic compartment in an inactive state (Toyoshima et al., 1998). We wanted to compare the G_2 serum starvation arrest with a DNA damage-induced arrest. In TKO MEFs, DNA damage indeed induced cytoplasmic retention of Cyclin B1. In contrast, serum starvation resulted in

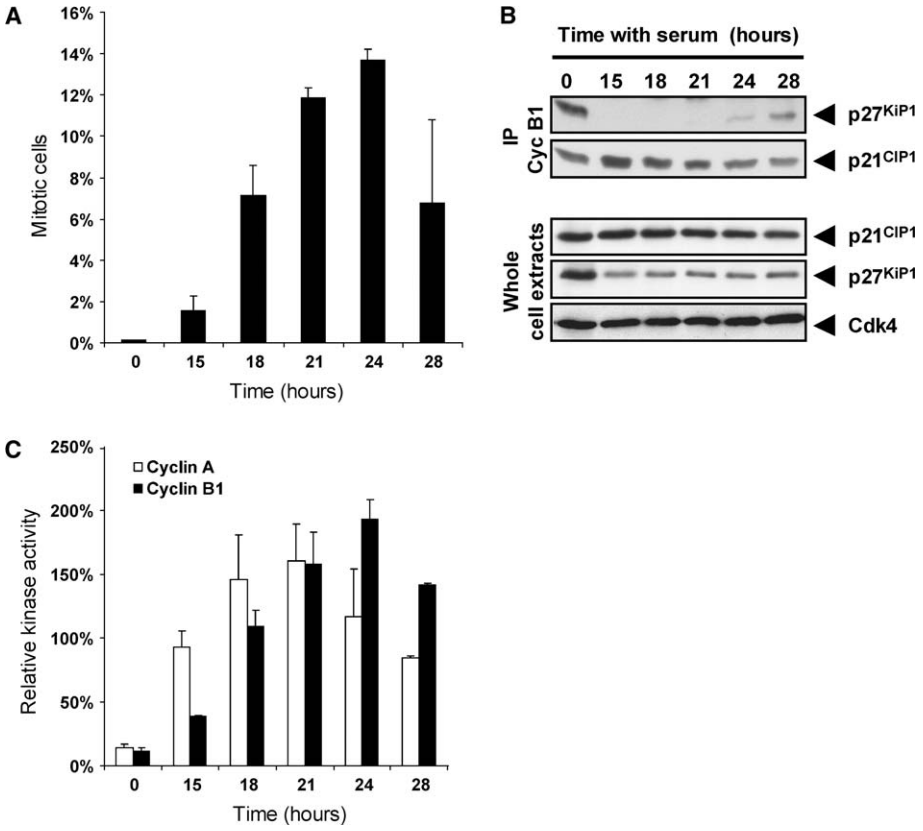

Figure 4. The serum starvation-induced G_2 arrest is reversible

A: Mitotic entry measured by MPM2 positivity of serum-starved TKO-Bcl2 MEFs upon serum readdition. Graph represents average values of two independent experiments; error bars show standard deviations.

B: Disruption of the CKI/Cyclin B1 kinase complexes in serum-stimulated TKO-Bcl2 MEFs. Lower panel shows whole-cell lysates. Cdk4 functions as a loading control.

C: Induction of Cyclin A- and B1-associated kinase activities upon serum stimulation of G_2-arrested/apoptosis-resistant TKO MEFs. Graph represents average values of two independent experiments; error bars show standard deviations.

accumulation of Cyclin B1 in the nucleus (Figure 5A). Additionally, inhibition of the DNA damage-induced kinases ATM/ATR by caffeine could not release the cells from the G_2 block, whereas serum readdition resulted in progression into mitosis (Figure 5B). Although inhibition of ATM/ATR kinase activity did not result in mitotic entry, we found that $p21^{CIP1}$ almost completely disappeared from the Cyclin B1-Cdk1 kinase complex (Figure 5C, upper panel) and that total $p21^{CIP1}$ levels moderately decreased (Figure 5C, lower panel). However, total levels of $p27^{KIP1}$ as well as the amount of $p27^{KIP1}$ associated to Cyclin B1 increased in caffeine-treated cells, in contrast to serum-stimulated cells, where $p27^{KIP1}$ levels and Cyclin B1-associated $p27^{KIP1}$ levels dramatically dropped (Figure 5C). Concordantly, we found that inhibition of ATM/ATR kinase activities was not sufficient to reactivate Cyclin B1- and Cyclin A-associated kinase activities under mitogen-deprived conditions (Figure 5D). This suggests that $p21^{CIP1}$ but not $p27^{KIP1}$ is induced by ATM/ATR-dependent signaling upon serum starvation in apoptosis-resistant TKO MEFs. Therefore, we hypothesize that the serum starvation-induced G_2 arrest might partially depend on the DNA damage signaling pathway for the induction of $p21^{CIP1}$. However, when the DNA damage pathway is inhibited during an already established G_2 arrest, $p27^{KIP1}$ is sufficient to prevent reactivation of Cyclin B1- and Cyclin A-associated kinases and alleviation of the G_2 arrest.

Increased inhibition of pocket protein activity correlates with increased G_2 arrest

To determine whether the G_2 arrest also occurs in MEFs lacking one or two pocket proteins, we compared the serum starvation responses of wild-type, $Rb^{-/-}$, $p107^{-/-}p130^{-/-}$, $Rb^{-/-}p130^{-/-}$, $Rb^{-/-}p107^{-/-}$, and apoptosis-resistant TKO MEFs by determining the G_2 over G_1 ratios. While $p107^{-/-}p130^{-/-}$ MEFs responded in the same way as wild-type MEFs, loss of *Rb* alone resulted in an increased G_2/G_1 ratio (0.69 versus 0.45 in wild-type MEFs) (Figure 6A, upper three histograms). $Rb^{-/-}p130^{-/-}$ MEFs behaved like $Rb^{-/-}$ MEFs, whereas combinational loss of *p107* and *Rb* resulted in an increased G_2/G_1 ratio when serum-starved (1.1 versus 0.69 in *Rb*-deficient MEFs; Figure 6A, lower three histograms). Serum-starved $Rb^{-/-}p107^{-/-}$ MEFs showed a substantial fraction of polyploid cells, whereas this feature was less prominent in apoptosis-resistant TKO MEFs. Therefore, a part of the 4n cells observed in the serum-starved $Rb^{-/-}p107^{-/-}$ MEFs may be tetraploid cells that have arrested in G_1, while the 8n cell population contributes to the G_2 fraction. We did not observe a substantial induction of apoptosis in either of the cell cultures (indicated by "A" in Figure 6A).

We next determined the complex formation between Cyclin B1 and $p27^{KIP1}$ and Cyclin B1 and $p21^{CIP1}$ in serum-starved cells. The $p27^{KIP1}$-Cyclin B1 interaction could be detected in all serum-starved knockout MEFs except for the $p107^{-/-}p130^{-/-}$ MEFs (Figure 6B) and correlated with the fraction of G_2 cells. Since no Cyclin B1 could be detected in serum-starved wild-type and $p107^{-/-}p130^{-/-}$ MEFs (Figure 6B), we envisage that the cells arrested with a 4n DNA content are tetraploid cells arrested in G_1, a described tendency for MEFs upon passaging (Borel et al., 2002).

These results suggest that also in MEFs in which the pocket protein family is only partially ablated, serum starvation induces a G_2 arrest that is mediated by a $p27^{KIP1}$-Cyclin B1 interaction. However, the serum starvation-induced association of $p21^{CIP1}$ to Cyclin B1 appears to be unique for TKO MEFs (Figure 6B).

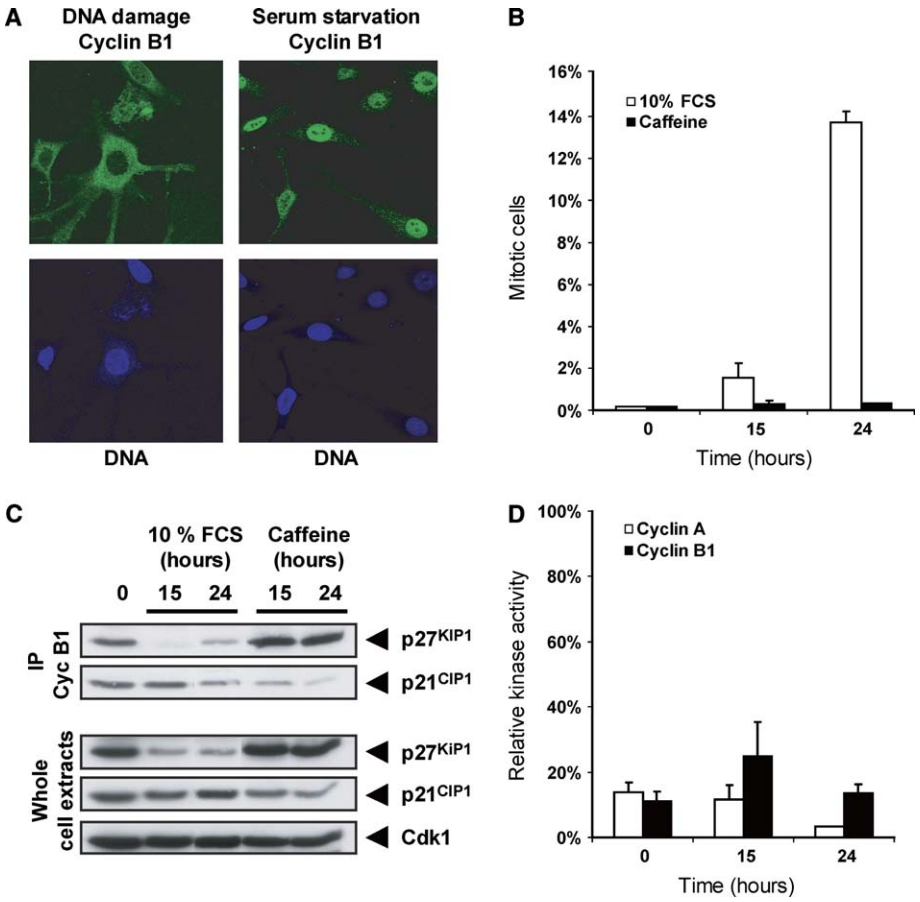

Figure 5. The G_2 arrest is partially different from a DNA damage response

A: Double immunofluorescence staining for Cyclin B1 (green) and DNA (blue) showing DNA damage-induced cytoplasmic localization and serum starvation-induced nuclear localization of Cyclin B1.

B: Mitotic entry determined by MPM2 positivity of serum-starved TKO-Bcl2 MEFs stimulated with either 10% serum (white bars) or 5 mM caffeine (black bars). Graph represents average values of two independent experiments; error bars show standard deviations.

C: $p21^{CIP1}$-Cyclin B1 and $p27^{KIP1}$-Cyclin B1 complex formation in serum-starved TKO-Bcl2 MEFs stimulated with either 10% serum or 5 mM caffeine. Cdk1 functions as a loading control.

D: Cyclin A- and B1-associated kinase activities of serum-starved TKO-Bcl2 MEFs treated with 5 mM caffeine. Values represent relative kinase activities as compared to asynchronously proliferating TKO-Bcl2 MEFs. Error bars show standard deviations of two experiments.

In human cancer, the G_1 restriction point is often affected by mechanisms other than genetic ablation of pocket proteins, such as expression of viral oncogenes, loss of *p16*^{INK4A}, or mutation or amplification of *Cyclin D1*. Therefore, we investigated whether the responses to serum starvation of *p16*$^{INK4A-/-}$ MEFs, wild-type MEFs expressing a nondegradable form of Cyclin D1 (Agami and Bernards, 2000), and wild-type MEFs stably expressing E1A were similar to that of pocket protein knockout MEFs. Since E1A-expressing MEFs underwent massive apoptosis as previously described (data not shown) (Mymryk et al., 1994), we introduced Bcl2 in these cells. $p16^{INK4A}$ null MEFs responded like wild-type MEFs upon serum starvation: they arrested in G_1 phase of the cell cycle and had completely lost Cyclin B1 expression (data not shown). In contrast, MEFs expressing E1A and Bcl2 accumulated in G_2 almost to the same extent as apoptosis-resistant TKO MEFs (Figure 6C). This arrest correlated with inhibition of Cyclin B1-associated kinase activity (Figure 6E) and binding of $p27^{KIP1}$ and $p21^{CIP1}$ to Cyclin B1 (Figure 6F). Although in E1A-expressing cells a clear interaction of $p21^{CIP1}$ and Cyclin B1 was already present in proliferating cells and the levels of Cyclin B1 decreased during serum starvation, the fraction of Cyclin B1 associated to $p21^{CIP1}$ increased upon mitogen-starved conditions (Figure 6F).

MEFs expressing a nondegradable form of Cyclin D1 accumulated in G_2 to the same extent as *Rb*$^{-/-}$ MEFs, and approximately 10% of the cells underwent apoptosis when serum starved (Figure 6D). G_2 arrest correlated with loss of Cyclin B1 kinase activity (Figure 6E) through binding of the CKIs (Figure 6F).

Together, these results show that interference with the G_1 restriction point by events frequently seen in tumor development is not sufficient for unhindered proliferation under mitogen-deprived conditions. Instead, such cells arrest in G_2 due to inhibition of Cyclin B1 by $p21^{CIP1}$ and/or $p27^{KIP1}$.

p53 is essential for the G_2 arrest

p53 is an important mediator of apoptosis and growth arrest under many stress conditions (Hanahan and Weinberg, 2000). To investigate the role of p53 in apoptosis and growth arrest of serum-deprived TKO MEFs, we inactivated p53 function in TKO MEFs by RNA interference using pRetroSuper-p53. Figure 7A shows that, in TKO-p53RNAi MEFs, the levels of p53 and one of its target proteins, $p21^{CIP1}$, were strongly reduced compared to the levels in control-infected TKO and apoptosis-resistant TKO-Bcl2 MEFs. Knocking down *p53* expression almost completely prevented apoptosis in serum-starved TKO MEFs and was even more effective as the expression of Bcl2 (Figure 7B). Furthermore, unlike TKO-Bcl2 MEFs, serum-starved TKO-p53RNAi MEFs continued to incorporate BrdU while the accumulation of cells in G_2 was strongly diminished (Figure 7C). Importantly, simultaneous ablation of pocket proteins and p53 resulted in prolonged proliferation in the absence of mitogens (Figure 7D; Figure S1 in the Supplemental Data available with this article online): TKO-p53RNAi cells grew

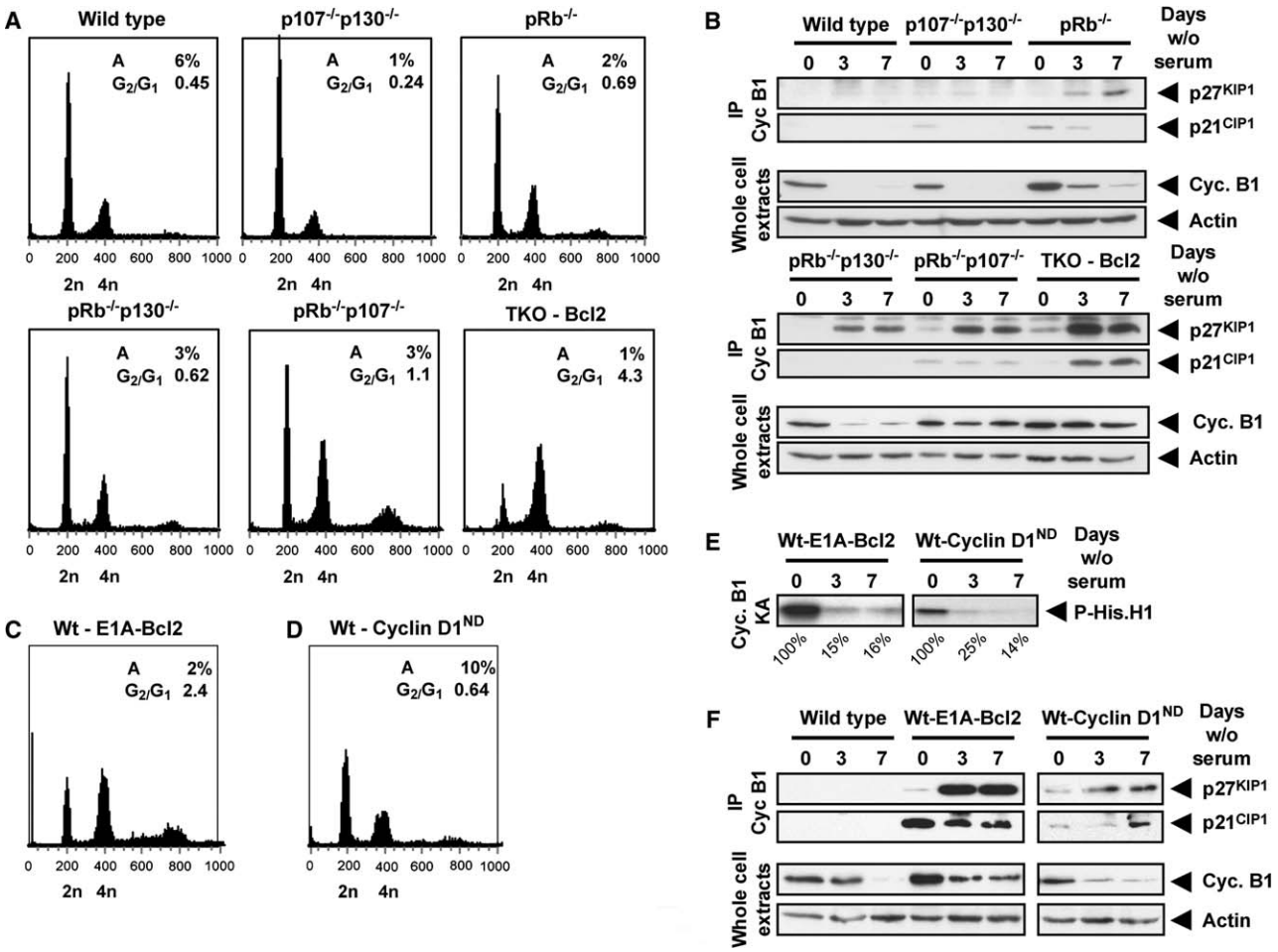

Figure 6. Increasing pocket protein loss results in increased G_2 arrest and increased $p27^{KIP1}$-Cyclin B1 complex formation under serum-starved conditions

A: Histograms showing cell cycle distribution of pocket protein knockout MEFs at 3 days of serum starvation (''A'' refers to the apoptotic population [<2n]).

B: Immunoprecipitation of Cyclin B1 in serum-starved wild-type and pocket protein knockout MEFs. Actin in whole-cell lysates functions as a loading control.

C and **D:** Histograms showing cell cycle distributions of wild-type MEFs expressing either E1A and Bcl2 (**C**) or a nondegradable form of Cyclin D1 (Cyclin $D1^{ND}$) (**D**).

E: Cyclin B1-associated kinase activity of serum-starved E1A-Bcl2 MEFs and Cyclin $D1^{ND}$ MEFs. Percentages represent a quantification of the intensity of the kinase activity.

F: Immunoprecipitations (upper panels) and whole-cell lysates (lower panels) from Cyclin B1 of serum-starved wild-type, E1A-Bcl2, and Cyclin $D1^{ND}$ MEFs. Actin functions as a loading control.

exponentially for at least three population doublings in the absence of serum, although the generation time had increased from approximately 1 day under high-serum conditions to 2 days. However, when reaching confluency, many TKO-p53 RNAi cells died. Unfortunately, we could not assess the growth potential of TKO-p53RNAi cells for a longer time, as we could not passage these cells under serum-free conditions. Time-lapse microscopy revealed that TKO-p53RNAi MEFs being passaged under serum-free conditions did not reattach and subsequently died, suggesting that serum contains factors essential for cell attachment (data not shown). Alternatively, cell death could be a consequence of residual trypsin activity, which is normally inhibited by serum.

As neither Cyclin A- and B1-associated kinase activities (Figure 7E) nor Cdk2 kinase activity (Figure 7F) in these cells was inhibited until day 7, we compared the presence of $p21^{CIP1}$ and $p27^{KIP1}$ in the Cyclin B1-Cdk1 complexes in TKO-Bcl2 and TKO-p53RNAi cells. Figure 7G shows that $p21^{CIP1}$ and $p27^{KIP1}$ were virtually absent from Cyclin B1-Cdk1 kinase complexes isolated from TKO-p53 RNAi MEFs. Similarly, Cdk2 immunoprecipitates showed no association to $p21^{CIP1}$ and a weak interaction to $p27^{KIP1}$, which was not induced by serum starvation (Figure 7H). As is shown in Figure 7I (middle panel), $p21^{CIP1}$ expression was almost completely abolished in mitogen-starved TKO-p53RNAi MEFs, while $p27^{KIP1}$ expression was markedly reduced (Figure 7I, upper panel). This suggests that in the absence of p53 the accumulation of both $p21^{CIP1}$ and $p27^{KIP1}$ was largely suppressed, allowing mitogen-independent proliferation of pocket protein-deficient cells.

In another approach, we expressed E1A in $p53^{-/-}$ MEFs. Whereas in control-infected cells proliferation was inhibited as assayed by BrdU incorporation, E1A-expressing $p53^{-/-}$ MEFs continued to incorporate BrdU up to 7 days of mitogen starvation, although the percentage of BrdU-positive cells gradually decreased (Figure 7J).

In conclusion, these results indicate that the combined abrogation of p53 and pocket protein functions strongly potentiates the ability of cells to proliferate independently of mitogens.

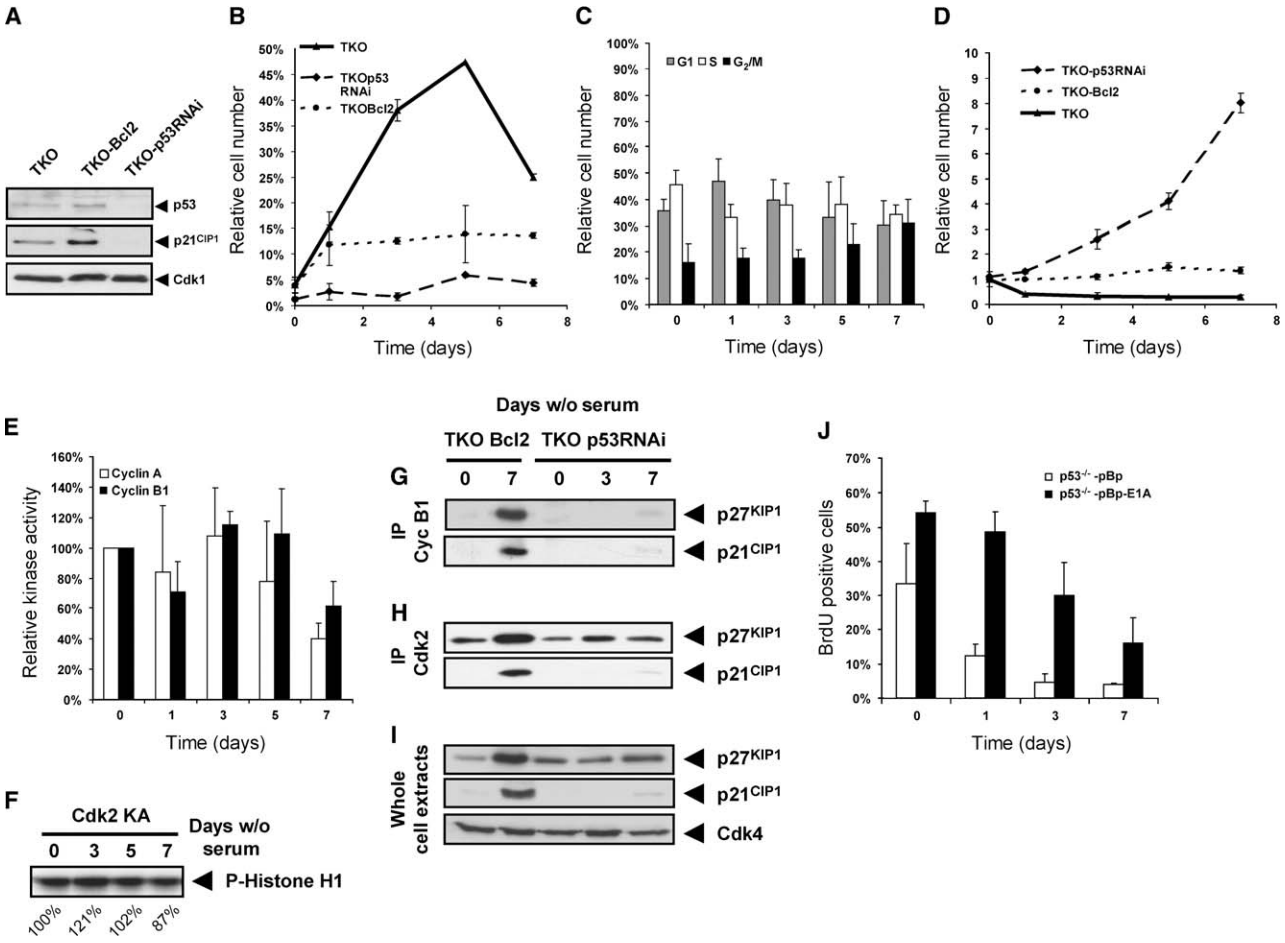

Figure 7. Serum starvation-induced apoptosis and G_2 arrest in TKO MEFs are dependent on p53

A: Levels of p53 and $p21^{CIP1}$ in TKO, TKO-Bcl2, and TKO-p53RNAi MEFs. Cdk1 functions as a loading control.

B: Induction of apoptosis by serum starvation in TKO, TKO-p53RNAi, and TKO-Bcl2 MEFs. Values represent the average of two experiments; error bars represent standard deviations.

C: Cell cycle distribution of serum-starved TKO-p53RNAi MEFs. Values represent the average of three independent experiments; error bars show standard deviations.

D: Growth curves showing serum-independent proliferation of p53-deficient TKO MEFs. Experiment was performed in triplo; error bars show standard deviations.

E: Cyclin A- and B1-associated kinase activities upon serum starvation of TKO-p53 RNAi MEFs. Graph represents average values of four independent experiments; error bars show standard deviations.

F: Cdk2 kinase activity of serum-starved TKO-p53-RNAi MEFs. Percentages represent quantification of labeled Histone H1.

G and **H:** Immunoprecipitations of Cyclin B1 (**G**) and Cdk2 (**H**) from serum-starved TKO-Bcl2 MEFs and TKO-p53RNAi MEFs.

I: Whole-cell lysates of serum-starved TKO-Bcl2 and TKO-p53RNAi MEFs. Cdk4 functions as a loading control.

J: Proliferating cells as determined by BrdU incorporation in control- and E1A-infected $p53^{-/-}$ MEF cultures. Error bars represent standard deviations of two experiments.

Discussion

Loss of pocket protein function results in deregulated E2F activity and consequently elevated E2F target gene expression. Amongst E2F targets are many proapoptotic genes like Apaf-1, caspases, Bad, and Bak1 (Stanelle et al., 2002; Muller et al., 2001). Serum starvation activates the FOXO family of transcription factors and thereby expression of proapoptotic genes like FasL and Bim (Burgering and Kops, 2002). It is likely that the combinatorial effects of elevated expression of proapoptotic E2F and FOXO target genes cause the massive apoptosis that we have observed in serum-starved TKO MEFs. We have also shown that loss of p53 suppresses serum starvation-induced apoptosis. A simultaneous requirement for p53 and free E2F as was shown for Apaf-1 induction could explain this observation (Moroni et al., 2001). Our results are consistent with previous reports showing that mitogen-starved E1A-expressing murine cells are highly sensitive to apoptosis (Mymryk et al., 1994), which could be suppressed by inactivation of p53 by adenoviral E1B (Rao et al., 1992).

Protection from apoptosis is a hallmark of cancer. The antiapoptotic protein Bcl2 can suppress apoptosis induced in mitogen-starved fibroblasts that express high levels of the oncogenes c-Myc (Hanahan and Weinberg, 2000) or E1A (Rao et al., 1992). We therefore overexpressed Bcl2 in TKO MEFs and studied the cell cycle progression of these cells upon

mitogen starvation. Indeed, apoptosis was suppressed, but interestingly the majority of surviving cells arrested with a G_2 DNA content. Additionally, we have shown that partial abrogation of the G_1 restriction point by loss of Rb alone, combined loss of Rb and p107, expression of the adenoviral oncogene E1A, or expression of a nondegradable form of Cyclin D1 also resulted in an accumulation of cells in G_2 under mitogen-deprived conditions. On the other hand, loss of the Cyclin D1 inhibitor *p16^{INK4A}* did not result in a partially defective G_1 restriction point, which is not surprising, since Cyclin D1 completely disappears under mitogen-starved conditions (Blagosklonny and Pardee, 2002).

Our results show that alleviation of both the G_1 restriction point *and* the apoptotic response is not sufficient for mitogen-independent proliferation. Instead, cells lacking a functional G_1 restriction point can activate a pocket protein-independent arrest in G_2 that serves as an emergency brake to prevent unconstrained proliferation under mitogen-deprived conditions. We are currently analyzing Rb-deficient premalignant lesions to investigate whether this mechanism operates in vivo.

Serum starvation also induces FOXO-dependent transcription of p27^{KIP1} (Medema et al., 2000). In wild-type cells, p27^{KIP1} induction inhibits Cyclin E-Cdk2 activity, which contributes to hypophosphorylation of the pocket proteins and G_1 arrest. Here, we show that in pocket protein-compromised MEFs, induction of p27^{KIP1} and p21^{CIP1} upon serum starvation is responsible for the G_2 arrest through inhibitory interactions with Cyclin B1 and Cyclin A. As Cdk2 is not essential for cell cycle progression (Ortega et al., 2003), one may argue that inhibition of Cdk1, rather than Cdk2 is critical for serum starvation-induced G_2 arrest. However, others have shown that Cyclin A-Cdk2 and Cyclin B1-Cdk1 kinase activities are essential for mitotic entry (Furuno et al., 1999; Nurse, 1990; Nigg, 2001), Therefore, we think that inhibition of both activities is relevant to the serum starvation-induced G_2 arrest. This is consistent with our finding that readdition of serum caused reappearance of Cyclin B1- and Cyclin A-associated kinase activities and propagated entry of mitogen-starved cells into mitosis. We also consider the activity of both p27^{KIP1} and p21^{CIP1} to be critical to the G_2 arrest. We could confirm that overexpression of p27^{KIP1} alone under high-serum conditions was able to block TKO-Bcl2 MEFs in G_2. This is consistent with recent work showing an inhibitory interaction of p27^{KIP1} and Cyclin B1 (Nakayama et al., 2004; Martin et al., 2005; Aleem et al., 2005). Furthermore, inhibition of p21^{CIP1} by RNA interference markedly delayed the G_2 arrest, although it was not sufficient for unrestrained proliferation under mitogen-starved conditions. Others have previously suggested a role for p21^{CIP1} in a G_2 arrest upon DNA damage by inhibition of Cyclin B1 kinase activity in the nucleus (Smits et al., 2000b; Baus et al., 2003). It is likely that p21^{CIP1} RNAi-treated cells ultimately arrest in G_2 through inhibition of Cyclin A- and B1-associated kinase activities by p27^{KIP1}.

Interestingly, we found both p21^{CIP1} and p27^{KIP1} associated to Cyclin B1 in cells expressing E1A and Bcl2 or cells expressing nondegradable Cyclin D1, indicating that the G_2 emergency brake also operates in cells with partially inhibited pocket protein activity. Indeed, the interaction between p27^{KIP1} and Cyclin B1 was also seen in serum-starved *Rb$^{-/-}$* MEFs and became increasingly evident when additional pocket proteins were ablated, providing a possible explanation for the observed synergy of *Rb* and *p27^{KIP1}* loss in murine tumorigenesis (Park et al., 1999). Our results are summarized in Figure 8, showing the CKIs as central mediators of restriction points operating in G_1 or G_2, depending on the presence of functional pocket proteins.

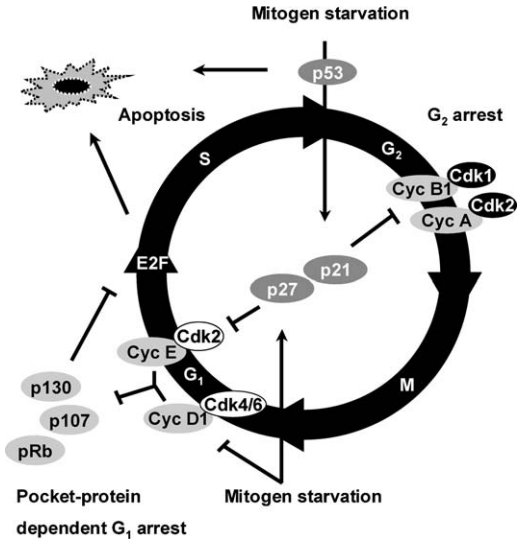

Figure 8. p21^{CIP1} and p27^{KIP1} as central players in a mitogen starvation-induced G_1 and G_2 cell cycle arrest

The interaction of Cyclin B1 and p21^{CIP1} in arrested cells could be disrupted by blocking the ATM/ATR kinases, suggesting that part of the G_2 arrest is elicited by a DNA damage response. However, the Cyclin B1-p27^{KIP1} interaction appears to be independent from a DNA damage response, since blocking of ATM/ATR kinase activity did not decrease the stability of this complex. Apparently, the p27^{KIP1}-mediated inhibition of Cyclin B1 (and possibly Cyclin A) is sufficient to maintain the G_2 arrest.

Loss of p53 suppressed apoptosis and abrogated the G_2 arrest in serum-starved TKO MEFs. Thus, besides its role in protecting cells from entering mitosis after DNA damage (Taylor and Stark, 2001), p53 activity is also involved in mitogenic signaling during G_2. p53 might act as a sensor of mitogenic signaling; however, it might also merely act as a factor that allows cells to accumulate sufficient levels of p21^{CIP1} during serum starvation. In p53 knockdown TKO MEFs, p21^{CIP1} does not accumulate because it is a direct transcriptional target of p53. The reduced inhibition of Cdk2 activity in these cells might result in phosphorylation and hence degradation of p27^{KIP1} (Morisaki et al., 1997). However, why then was inhibition of p21^{CIP1} by RNAi not sufficient for unhindered proliferation under mitogen-deprived conditions, whereas p53 inhibition was? A trivial explanation is that p21^{CIP1} was more effectively reduced by p53 RNAi than by p21^{CIP1} RNAi (compare Figures 3B and 3C and Figures 7G and 7H). Other possibilities we are currently addressing are that p53 targets other than p21^{CIP1} contribute to the reduction of Cdk2 activity upon serum starvation and that p27^{KIP1} may be a direct p53 target as was suggested before (Fontemaggi et al., 2002).

Another question is whether TKO-p53RNAi MEFs have become fully serum independent. On the one hand, this seemed to be the case as cells reached confluency by exponential growth in the absence of serum. On the other hand, the generation time had doubled, suggesting that TKO-p53RNAi cells

are still dependent on serum for optimal growth. A procedure allowing passaging of cells under serum-deprived conditions should reveal whether these growth characteristics persist beyond three population doublings.

Loss of the G_1 restriction point is believed to be an essential step in the development of cancer (Hanahan and Weinberg, 2000). Dependent on the cell type or type of cancer, this defect can be the result of overexpression of *Cyclin D1*, loss of one or more of the pocket proteins, or expression of a viral oncogene or combination of these. However, our results suggest that, besides loss of the G_1 restriction point, unconstrained proliferation also requires suppression of apoptosis and alleviation of a G_2 cell cycle arrest. Recent studies on retinoblastoma in mice may provide an example of this scenario. Inactivation of *Rb* in the murine retina resulted in increased proliferation of retinal progenitors, which was enhanced by additional loss of *p107*. While the majority of the retinal cells (rods, cones, and ganglion and bipolar cells) subsequently underwent apoptosis, amacrine precursors arrested. The latter compartment gave rise to retinoblastomas in *Rbp130*- and *Rbp107*-deficient retinas, suggesting that alleviation of cell cycle arrest rather than suppression of apoptosis was required for oncogenic transformation of pocket protein-deficient cells (Dannenberg et al., 2004; Robanus-Maandag et al., 1998; MacPherson et al., 2004). Although no loss of p53 was observed in murine retinoblastoma, we speculate that other events impinging on the G_2 arrest may have occurred to allow oncogenic transformation (Robanus-Maandag et al., 1998; Dannenberg et al., 2004). On the other hand, loss of *Rb* and loss of *p53* do strongly synergize in murine tumorigenesis. For example, combinational loss of *Rb* and *p53* in the CNS of mice resulted in medulloblastoma at 3–4 months of age, whereas individual loss of either *Rb* or *p53* did not cause this malignancy (Marino et al., 2000). Our findings provide a novel explanation for this strong synergy.

Experimental procedures

Isolation and culturing of MEFs

MEFs were isolated from chimeric embryos as described before (Dannenberg et al., 2000) and cultured in GMEM (Invitrogen-GIBCO) supplemented with 10% fetal calf serum, 1 mM nonessential amino acids (Invitrogen-GIBCO), 1 mM sodium pyruvate (Invitrogen-GIBCO), 100 μ/ml penicillin, 100 μg/ml streptomycin (Invitrogen-GIBCO), and 0.1 mM β-mercaptoethanol (Merck). For serum starvation experiments, cells were trypsinized and allowed to attach under high-serum conditions for 1 hr. Subsequently, cells were washed with PBS and supplemented with serum-free medium. For serum stimulation experiments, serum-free medium was replaced by complete medium for the indicated times. In the case of caffeine treatment, cells were fed with serum-free medium containing 5 mM caffeine (Sigma). Growth curves were performed as described previously (Dannenberg et al., 2000). For synchronization in mitosis, MEFs were cultured in the presence of 250 ng/ml nocodazole for 8 hr. Mitotic cells were isolated by shake-off and replated at indicated conditions.

Constructs, transfection, and retroviral infections

The pRetroSuper system and pRetroSuper-p53 were described previously (Dirac and Bernards, 2003). A retroviral vector encoding a nondegradable form of Cyclin D1 was obtained from R. Agami (Agami and Bernards, 2000). The 19-mer $p21^{CIP1}$ targeting sequence in pRetroSuper-$p21^{CIP1}$ is GCCCTCACTCTGTGTGTCT. The LZRS-Bcl2-Zeo plasmid was obtained from A. Werner (Werner et al., 2002). Ecotropic retroviral supernatants were produced by transfecting Phoenix cells by calcium phosphate coprecipitation. Thirty-six hours posttransfection, retroviral supernatants were filtered through a 0.45 μm filter and used to infect MEFs. MEFs were sequentially infected three times, each time for at least 6 hr in the presence of 4 μg/ml polybrene. In the case of the pRetroSuper constructs, cells were allowed to recover from the retroviral infection for 7 days and subsequently used for BrdU incorporation assays. Bcl2-expressing MEFs were selected in 25 μg/ml bleomycin for 7 days.

FACS analysis

To measure BrdU incorporation, cells were incubated with 10 mM BrdU for 1 hr. Cells were fixed in 70% ethanol at 4ºC. Cells were stained with mouse anti-BrdU (Becton Dickinson) as described in Brugarolas et al. (1998) or stained for MPM2 positivity (Smits et al., 2000a) and subsequently analyzed on FACS using "Cell Quest" software and "FACS Express" software (De Novo Software).

Time-lapse microscopy and immunofluorescence

For time-lapse microscopy experiments, cells were plated on 35 mm culture dishes (Wilco dishes) (van Vugt et al., 2004). Microinjection was performed as described previously (van Vugt et al., 2004). For immunofluorescence microscopy, cells were grown on coverslips and grown in the absence of serum or irradiated with 10 Gy of γ irradiation. Cells were fixed in 4% formaldehyde and permeabilized using 0.2% Triton X (Sigma) in PBS. Aspecific staining was blocked by incubation in PBS containing 5% BSA for 30 min. Slides were incubated with indicated antibodies in PBS containing 5% of BSA for 2 hr at room temperature. DNA was stained using To-Pro3 Dye (Molecular Probes), and slides were mounted in the presence of Vectashield to prevent bleaching (Vector Laboratories). Slides were analyzed by confocal microscopy as described previously (van Vugt et al., 2004).

Immunoprecipitations, immunoblots, and antibodies

Cells were harvested and subsequently lysed for 30 min in ELB (150 mM NaCl, 50 mM HEPES [pH 7.5], 5 mM EDTA, 0.1% NP-40) containing protease inhibitors (Complete; Roche) and phosphatase inhibitors (5 mM NaF, 0.5 mM sodium vanadate, and 20 mM β-glycerolphosphate). Protein concentrations were determined using the Bradford assay (Bio-Rad). For immunoprecipitations, 60 mg of protein was incubated with 0.4 μg of immobilized antibody overnight at 4ºC while being rotated. In the case of immunoprecipitations for kinase assays, 20 μg (Cyclin B1) or 50 μg (Cyclin A) of protein was used. For kinase assays, immunoprecipitation reactions were washed four times in ELB and once in kinase buffer (50 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM DTT). Each reaction was performed in 30 μl of kinase buffer with 5 mg of Histone H1 (Roche) and [γ-^{32}P] dATP for 30 min at 37ºC. Subsequently, the reaction mixture was separated by SDS-PAGE, and after gel drying, the signal was quantified using phospho-imaging screens and TINA software. The antibodies used were rabbit anti-Cyclin A (C19), rabbit anti-Cyclin B1 (H433), mouse anti-Cyclin B1 (GNS1), rabbit anti-p21 (C19), mouse anti-p21 (F5), mouse anti-Cdk1 (17), rabbit anti-Cdk2 (M2), rabbit anti-Cdk4 (C22), goat anti-Actin (I19) (Santa Cruz), mouse anti-Cyclin A (Ab2) (Neomarkers), mouse anti-p27 (BD Transduction laboratories), MPM2 (Upstate), Alexa Fluor 488 anti-mouse and 568 anti-rabbit secondary antibodies (Molecular Probes), or HRP-conjugated goat anti-mouse, HRP-conjugated goat anti-rabbit, and HRP-conjugated rabbit anti-goat (Dako) secondary antibodies.

Supplemental data

The Supplemental Data include one supplemental figure and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/6/455/DC1/.

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