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Discrete signaling pathways participate in RB-dependent responses to chemotherapeutic agents

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The retinoblastoma (RB) tumor suppressor has been proposed to function as a key mediator of cell cycle checkpoints induced by chemotherapeutic agents. However, these prior studies have relied on embryonic fibroblasts harboring chronic loss of RB, a condition under which compensation of RB functions is known to occur. Here we utilized primary adult fibroblasts derived from mice harboring loxP sites flanking exon 3 of the *Rb* gene to delineate the action of RB in the chemotherapeutic response. In this system we find that targeted disruption of *Rb* leads to little overt change in cell cycle distribution. However, these cells exhibited deregulation of RB/E2F target genes and became aneuploid following culture in the absence of RB. When challenged with both DNA damaging and antimetabolite chemotherapeutics, RB was required for primary adult cells to undergo DNA damage checkpoint responses and loss of RB resulted in enhanced aneuploidy following challenge. In contrast, following spontaneous immortalization and the loss of functional p53 signaling, the antimetabolite 5-fluorouracil (5-FU) failed to induce arrest despite the presence of RB. In these immortal cultures RB/E2F targets were deregulated in a complex, gene-specific manner and RB was required for the checkpoint response to camptothecin (CPT). Mechanistic analyses of the checkpoint responses in primary cells indicated that loss of RB leads to increased p53 signaling and decreased viability following both CPT and 5-FU treatment. However, the mechanism through which these agents act to facilitate cell cycle inhibition through RB were distinct. These studies underscore the critical role of RB in DNA-damage checkpoint signaling and demonstrate that RB mediates chemotherapeutic-induced cell cycle inhibition in adult fibroblasts by distinct mechanisms.

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Introduction

Cell cycle checkpoints maintain genome integrity by coordinating the order of cell cycle events, thereby ensuring faithful replication and partitioning of the genome (Hartwell and Weinert, 1989; Nyberg *et al*., 2002). In response to genotoxic stress, cell cycle checkpoints function to activate DNA repair mechanisms and elicit cell cycle arrest (Bartek and Lukas, 2001). Delays in cell cycle progression are believed to allow time for damaged DNA to be repaired, thus preventing the replication and propagation of potentially deleterious mutations. When DNA damage is not repairable, checkpoints eliminate potentially hazardous cells by inducing permanent cell cycle arrest or apoptosis (Bartek and Lukas, 2001). As such, inactivation of key checkpoint genes predisposes cells to development of genomic instability and cancer (Bartkova *et al*., 1997; Flatt and Pietenpol, 2000). Importantly, most chemotherapeutic agents elicit checkpoint responses. Therefore, understanding the relationship between genetic lesions commonly observed in cancers and checkpoint responses is critical for efficacious therapy.

The retinoblastoma (RB) tumor suppressor plays a central role in DNA damage-induced cell cycle checkpoints and is required for induction of G1 and S-phase arrest following DNA damaging events (Harrington *et al*., 1998; Brugarolas *et al*., 1999; Knudsen *et al*., 2000). Active, hypophosphorylated RB is a potent negative regulator of cell cycle progression from G1 into and through S phase (Weinberg, 1995; Knudsen *et al*., 1998; Harbour and Dean, 2000). RB elicits cell cycle arrest by binding members of the E2F family of transcription factors, inhibiting the ability of E2Fs to stimulate transcription (Lam and La Thangue, 1994; Dyson, 1998). Moreover, the RB-E2F complexes that form on the promoters of target genes actively repress transcription by recruiting chromatin modifying complexes (Zhang and Dean, 2001). In response to mitogenic signaling, RB is inactivated by sequential hyperphosphorylation catalysed by cyclin D-cdk4/6 and cyclin E-cdk2 complexes; these modifications are sufficient to disrupt interactions with E2F and to allow progression through the cell cycle (Lundberg and Weinberg, 1998; Mittnacht, 1998; Harbour *et al*., 1999). An important role for the RB family of pocket

proteins in the DNA damage response was initially suggested by studies with human papilloma virus-16 protein E7, which antagonizes the ability of pocket proteins to interact with E2F (Helt and Galloway, 2003). These experiments demonstrated that expression of E7 abrogated cell cycle arrest following exposure to γ-irradiation or actinomycin D (Demers *et al*., 1994; Slebos *et al*., 1994; Song *et al*., 1998). Subsequently, the direct requirement of RB, but not p107 or p130, for the DNA-damage checkpoint response was revealed by the observation that exposure to γ-irradiation, UV-irradiation or a variety of chemotherapeutic drugs inhibited G1–S-phase progression in $Rb^{+/+}$ mouse embryo fibroblasts (MEFs) but not in $Rb^{-/-}$ MEFs (Harrington *et al*., 1998). Additionally, an RB-dependent intra-S-phase response to DNA damage was identified by the observation that treatment with cisplatin (cis-diamminedichloroplatinum II (CDDP)), etoposide or mitomycin C (MMC) inhibited S-phase progression in $Rb^{+/+}$ MEFs, but not in $Rb^{-/-}$ MEFs (Knudsen *et al*., 2000).

Cell cycle checkpoints elicited by a number of clinically relevant agents signal for growth arrest through RB via the key upstream checkpoint genes p53 (Kastan *et al*., 1992; Slebos *et al*., 1994; Smith *et al*., 1994) and $p21^{cip1}$ (Brugarolas *et al*., 1995; Deng *et al*., 1995). Following damage, p53 is stabilized and activated by post-translational modifications (Appella and Anderson, 2001). Activated p53 signals to critical downstream targets, including $p21^{Cip1}$, an inhibitor of cyclin-dependent kinase (cdk) activity (el Deiry *et al*., 1993). Increased $p21^{Cip1}$ levels blocks cdk2 activity resulting in RB hypophosphorylation and cell cycle arrest (Brugarolas *et al*., 1999). Additional p53-independent mechanisms that regulate cdk2 activity could also signal to inhibit RB phosphorylation and arrest cell cycle progression following DNA damage. Specifically, Cdc25A and cyclin D1 levels are attenuated by degradation in response to DNA damage signals (Agami and Bernards, 2000; Mailand *et al*., 2000; Bartek and Lukas, 2001; Lan *et al*., 2002). Decreased levels and activity of Cdc25A phosphatase results in maintenance of cdk2 phosphorylation and inhibition of cyclin E/cdk2 activity (Mailand *et al*., 2000). Likewise, ubiquitin-mediated proteolysis of cyclin D1 following DNA damage releases $p21^{cip1}$ from cyclin D1/cdk4 complexes resulting in inhibition of cyclin E/cdk2 activity (Agami and Bernards, 2000). Thus, many effectors of the DNA damage response can ultimately impinge on RB action.

Although upstream signaling to RB has been well characterized, little is known about the molecular events downstream of RB signaling that mediate cell cycle arrest in response to DNA damage. Additionally, the role of RB in DNA damage checkpoint signaling in primary and immortalized adult somatic cells has not been characterized. Therefore, we investigated the consequences of acute RB loss on target gene regulation and chemotherapeutic response in adult somatic cells utilizing the Cre/LoxP system to conditionally eliminate RB function. Using mice harboring loxP sites flanking exon 3 of the *Rb* gene (Sage *et al*., 2003), we established controlled acute RB elimination mediated by adenoviral expression of Cre recombinase in both primary and immortal stage adult fibroblasts. We demonstrate that RB loss in primary adult fibroblasts deregulated the expression of RB/E2F targets, induced aneuploidy and compromised cell cycle arrest following exposure to DNA damaging and antimetabolite agents. RB also played a requisite role in eliciting cell cycle arrest in an immortalized fibroblastic line (with mutated p53) exposed to the DNA damaging agent camptothecin (CPT). In contrast, these immortal cells failed to arrest following exposure to the antimetabolite 5-fluorouracil (5-FU), despite the presence of RB. Analysis of the checkpoint responses in primary cells revealed that RB was required for G1/S and intra-S-phase cell cycle arrest following exposure to both CPT and 5-FU. Furthermore, RB loss led to enhanced aneuploidy and increased p53 signaling following treatment with CPT and 5-FU. Finally, we present evidence that CPT and 5-FU signal through disparate RB-dependent mechanisms to elicit cell cycle arrest.

Results

Efficient deletion of RB from adult cells

To assess the consequences of acute RB loss in adult cells, we employed fibroblasts isolated from adult mice (murine adult fibroblasts: MAFs). These mice harbor conditional *Rb* alleles ($Rb^{loxP/loxP}$) in which loxP sites flank exon 3 of the *Rb* gene. Details of the generation and characterization of these mice are described elsewhere (MacPherson *et al*., 2003; Sage *et al*., 2003). Initially, we verified Cre-mediated recombination at the *Rb* locus. To introduce Cre recombinase, MAFs were infected with recombinant adenoviruses expressing both GFP and Cre (Ad-GFP-Cre) or GFP alone (Ad-GFP) as control. Under the conditions utilized, greater than 90% Ad-GFP- and Ad-GFP-Cre-infected cells were positive for GFP fluorescence 16–24 h postinfection (data not shown). To demonstrate recombination, primers were designed to anneal to regions flanking exon 3 of the *Rb* gene. Efficient recombination deletes exon 3, resulting in a shortened transcript, which can be detected by RT–PCR (Figure 1a). RNA was prepared from uninfected MAFs (0 h) and at 12, 24 and 48 h post-Ad-GFP-Cre infection. RT–PCR analysis of the *Rb* transcript revealed the accumulation of the Δexon3-PCR product relative to uninfected cells (compare lane 2 with lanes 3–5), indicating effective recombination at this locus (Figure 1b). To establish that recombination resulted in loss of RB protein, polyclonal anti-RB antisera was utilized to immunoprecipitate lysates prepared from Ad-GFP- or Ad-GFP-Cre-infected MAFs. Western blotting with anti-RB monoclonal antibody demonstrated that Ad-GFP-Cre-infected MAFs were devoid of RB protein when compared to MAFs infected with control adenovirus (Figure 1c). These results confirm effective use of Cre recombinase to

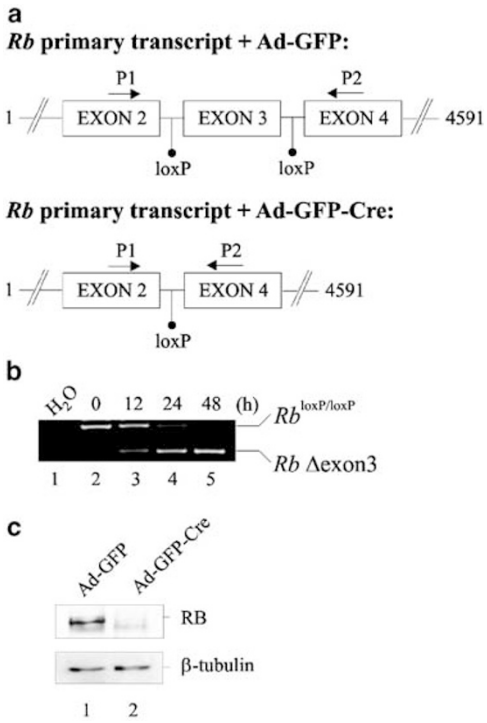


Figure 1 Infection of primary $Rb^{loxP/loxP}$ MAFs with adenoviral Cre mediates efficient recombination and loss of RB protein. **(a)** Schematic representation of PCR strategy to demonstrate Cre-mediated recombination in $Rb^{loxP/loxP}$ MAFs. Primers (P1 and P2) were designed to anneal upstream and downstream of lox P sites flanking exon 3 of mouse *Rb* gene. Recombination removes exon 3, resulting in a shorter primary transcript. Consequently, P1 and P2 primers are in closer proximity resulting in shorter RT–PCR product. **(b)** Asynchronously proliferating primary $Rb^{loxP/loxP}$ MAFs were infected with Ad-GFP or Ad-GFP-Cre. At 0, 12, 24 and 48 h postinfection, RNA was isolated and RT–PCR using primers P1 and P2 was performed to evaluate recombination at the floxed *RB* locus. **(c)** Asynchronously proliferating primary MAFs infected with either Ad-GFP or Ad-GFP-Cre were harvested and equal amounts of protein were subject to immunoprecipitation using RB polyclonal anti-sera. Immunoprecipitated proteins were separated by SDS–PAGE and RB was detected by immunoblotting with monoclonal RB antibody. Lysates were immunoblotted for β-tubulin to demonstrate equal input into immunoprecipitations

acutely ablate RB protein from adult fibroblasts containing *RB* flanked by loxP sites.

Acute loss of RB deregulates targets of RB-mediated repression and promotes spontaneous aneuploidy

To evaluate whether acute loss of RB influenced cell cycle distribution, asynchronously dividing uninfected, Ad-GFP- and Ad-GFP-Cre-infected MAFs (6 days postinfection) were harvested, fixed and stained with propidium iodide. Flow cytometric analysis revealed no significant differences in cell cycle distribution between primary cells with acute RB loss and control cells (Figure 2a). Such results are consistent with the observed lack of gross cell cycle deregulation in unchallenged $Rb^{-/-}$ MEFs (Herrera *et al*., 1996).

To determine the biochemical influence of RB loss, the expression of downstream targets of the RB/E2F signaling axis was analysed. Ad-GFP- and Ad-GFP-Cre-infected primary MAFs were harvested and the levels of proteins involved in cell cycle control (cyclins E, A and B1, cdc2, cdk4) or DNA synthesis (PCNA, MCM7) were determined. Relative to control MAFs, cells lacking RB showed deregulation of previously characterized RB targets (Figure 2b, compare lanes 1 and 2). In contrast, levels of cdk4, which functions upstream of RB, were unchanged by acute RB knock out (Figure 2b). In cells that do not carry $Rb^{loxP/loxP}$ alleles, infection with Ad-GFP-Cre had no effect on RB targets (data not shown). These data demonstrate that acute loss of RB in adult primary cells results in the deregulated expression of critical downstream RB targets.

Due to the significant deregulation of these target genes, we investigated whether the acute loss of RB could translate into mitogen-independent proliferation. Ad-GFP- and Ad-GFP-Cre-infected MAFs were cultured in 0.1% FBS for 72 h and labeled with bromodeoxyuridine (Brd-U) to detect the fraction competent for passage through S phase. As expected, the Ad-GFP-infected MAFs were dependent on serum for cell cycle progression and Brd-U incorporation was significantly attenuated by serum withdrawal (Figure 2c). Similarly, Ad-GFP-Cre-infected MAFs were also dependent on mitogen for cell cycle progression (Figure 2c). Therefore, although loss of RB results in substantive target gene deregulation, it is not sufficient to overcome the mitogen dependence of primary adult fibroblasts.

Given the potential connection between RB loss and genome instability (Zheng and Lee, 2002), and the role of RB/E2F signaling in the regulation of many proteins involved in licensing and replicating DNA (Muller *et al*., 2001; Markey *et al*., 2002; Polager *et al*., 2002; Ren *et al*., 2002) we investigated whether unchallenged RB-deficient cells become aneuploid in culture. Asynchronously dividing MAFs were infected with Ad-GFP and Ad-GFP-Cre and at 3, 8 and 12 days postinfection, cells were harvested and FACS analysis was utilized to determine DNA content. RB-deficient MAFs (Ad-GFP-Cre) accumulated an increasing population of cells with 8N DNA content by day 12 (Figure 2d). In contrast, Ad-GFP-infected MAFs exhibited only 2N and 4N DNA content, demonstrating a role for RB in maintaining ploidy under standard culturing conditions.

Acute RB knock out in immortalized MAFs lacking functional p53

Although inactivation of the RB pathway may be a defining event in the majority of tumors, it is critical to understand how RB functions in modifying chemotherapeutic responses in the context of additional mutations associated with tumorigenesis. Therefore, to study how the accumulation of growth enhancing mutations influences RB action in adult cells, we utilized two independently immortalized $Rb^{loxP/loxP}$ MAF cultures. These cultures were immortalized using a standard 3T3

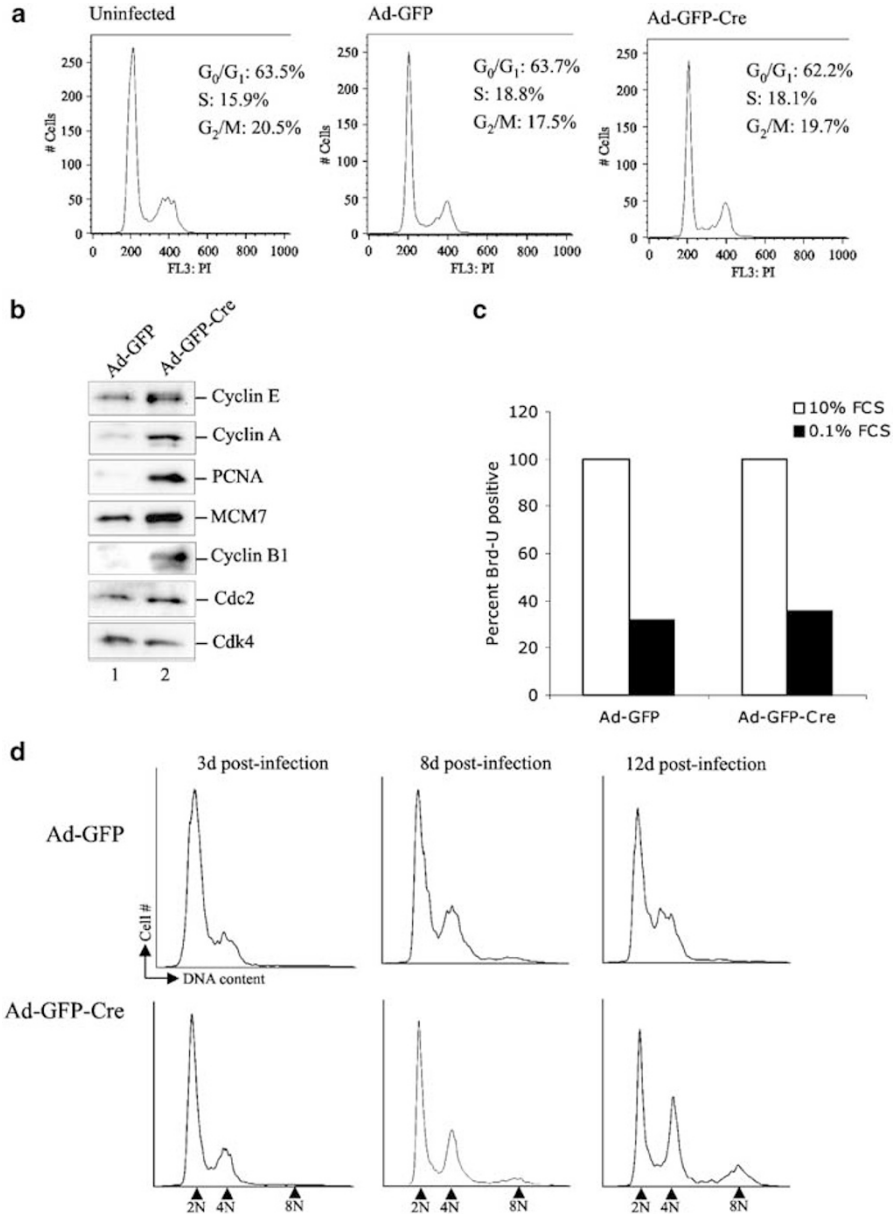
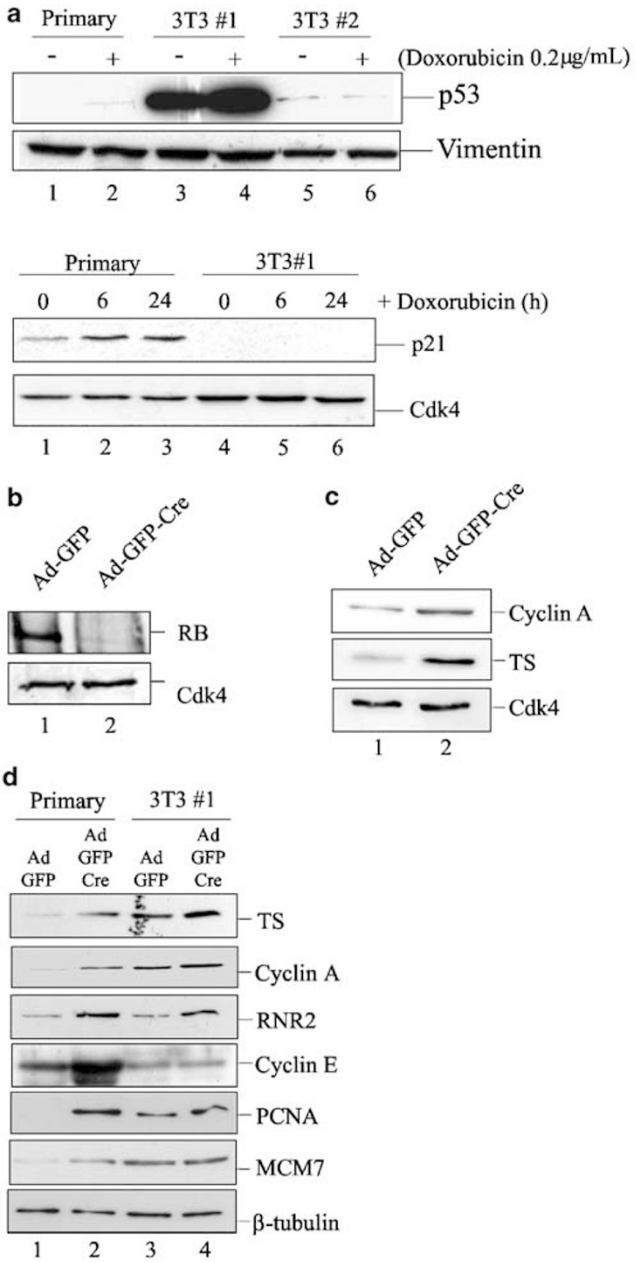


Figure 2 Acute RB loss in primary MAFs deregulates RB/E2F targets and promotes spontaneous aneuploidy. **(a)** Cell cycle distribution of uninfected, Ad-GFP- and Ad-GFP-Cre-infected primary *Rb*$^{loxP/loxP}$ MAFs. Asynchronously proliferating cells were harvested and processed for flow cytometry by propidium iodide staining. G_o/G_1, S and G_2/M population, determined by ModFit software, are indicated as percentages of the total population. **(b)** Equal amounts of total protein lysate from asynchronously proliferating Ad-GFP- or Ad-GFP-Cre-infected primary MAFs were resolved by SDS–PAGE. Effect of acute RB loss on levels of indicated proteins was determined by immunoblotting. **(c)** Ad-GFP- or Ad-GFP-Cre-infected primary MAFs were cultured for 72 h in media containing 0.1% FCS, and pulse labeled with Brd-U for 8 h. The effects of serum withdrawal on cell cycle progression were determined by evaluating Brd-U incorporation. Data is represented as the percent of Brd-U positive cells cultured in 0.1% serum relative to controls cultured in 10% serum. **(d)** Asynchronously growing Rb$^{loxP/loxP}$ MAFs were infected with either Ad-GFP or Ad-GFP-Cre. Following 3, 8 and 12 days of standard culture cells were harvested and fixed with 70% ethanol. Identification of 2N, 4N and 8N DNA content was determined by FACS analysis following propidium iodide staining

protocol by counting and replating 3×10^5 uninfected *Rb*$^{loxP/loxP}$ MAFs per 6 cm dish every 3 days (Todaro and Green, 1963). In rodent models it is well established that the spontaneous immortalization of fibroblasts is mediated largely through the loss of functional p53 (Harvey and Levine, 1991; Rittling and Denhardt, 1992). Therefore, we initially analysed the p53 status of the two immortalized MAF cultures. Uninfected primary and immortalized MAFs (designated 3T3#1 and 3T3#2) were incubated with doxorubicin (DOX) and protein lysates prepared. Immunoblotting revealed increased p53 levels in primary cells (Figure 3a; top panel, compare lanes 1 and 2). Compared to primary cells, levels of p53 were significantly elevated in the

unstressed immortal cultures (3T3#1 >3T3#2), indicating that p53 in these cells has acquired point mutations increasing protein stability (Figure 3a; top panel, compare lanes 1, 3 and 5). To confirm the presence of mutant p53 in these cells, lysates from untreated or DOX-treated cells were immunoblotted for the p53 target $p21^{Cip1}$, which is upregulated by p53 in response to DNA damage (el Deiry *et al*., 1993). Similar to primary cells, exposure of 3T3#2 to DOX-induced $p21^{Cip1}$ expression (data not shown). However, in contrast to primary cells, $p21^{Cip1}$ levels failed to increase in 3T3#1 MAFs following exposure to DOX (Figure 3a; bottom panel, compare lanes 1–3 and 4–6). Indeed, $p21^{Cip1}$ was undetectable in 3T3#1 MAFs, indicating that the p53 pathway is completely compromised in 3T3#1 cells. Therefore, 3T3#1 was chosen for further study.



To investigate the effect of acute RB loss in immortal cells already harboring additional genetic lesions, 3T3#1 MAFs were infected with either Ad-GFP or Ad-GFP-Cre. To document RB loss following Ad-GFP-Cre infection of immortal MAFs, the recombined *Rb* mRNA transcript (RbΔexon3) was detected by RT–PCR (data not shown). In addition, lysates from Ad-GFP- and Ad-GFP-Cre-infected immortal MAFs were immunoprecipitated with RB antisera and the absence of RB protein in Ad-GFP-Cre-infected cells was confirmed by immunoblotting (Figure 3b). Furthermore, immunoblotting for cyclin A and thymidylate synthase (TS) revealed that acute RB loss significantly deregulated expression of these genes (Figure 3c). Importantly, these data demonstrate that these RB/E2F targets are still subordinate to RB signaling in these cells and that RB function was not lost during immortalization. However, when levels of RB targets in primary and immortal cells were directly compared we observed that immortalized MAFs contained higher basal levels of TS, cyclin A, PCNA and MCM7 than primary MAFs (Figure 3d, compare lanes 1 and 3), indicating that spontaneous immortalization resulted in deregulated expression of these genes, even in the presence of functional RB. In contrast, the basal expression of cyclin E and RNR2 was not increased in immortal MAFs, suggesting that increased expression of RB/E2F targets was not due to increased cell cycle rate in immortal *versus* primary cultures. This notion is supported by the observation that the population doubling times of uninfected early passage primary and immortal cells were not significantly different (2.5 ± 0.11 and 2.07 ± 0.46 days, respectively). Analysis of the effects of acute RB loss in immortal cells revealed divergent effects on the expression of RB/E2F target genes. For example, levels of TS, RNR2 and cyclin A were increased upon acute RB ablation (Figure 3d, compare lanes 3 and 4). However, levels of cyclin E, PCNA and MCM7 were not appreciably influenced by RB loss (Figure 3d, compare lanes 3 and 4). These data indicate that despite retention of functional RB,

Figure 3 Disparate consequences of acute RB loss on target gene expression in immortalized MAFs lacking functional p53. **(a)** Two primary $Rb^{loxP/loxP}$ MAF cultures were independently immortalized by following a standard 3T3 protocol. Uninfected primary and immortal MAFs cultures (3T3#1 and 3T3#2) were treated with 0.2 μg/ml DOX for 24 h. Equal total protein was separated by SDS–PAGE and levels of p53 were analysed by immunoblotting (upper panel). Primary and 3T3#1 MAFs were exposed to 0.2 μg/ml DOX for 24 h and induction of $p21^{Cip1}$ was evaluated by immunoblot (lower panel). **(b)** Asynchronously proliferating immortalized 3T3#1 MAFs infected with Ad-GFP or Ad-GFP-Cre were harvested and equal amounts of protein were immunoprecipitated using RB polyclonal anti-sera. Immunoprecipitated RB was detected by immunoblotting with monoclonal RB. Lysates were immunoblotted for cdk4 to demonstrate equal input into immunoprecipitations. **(c)** Equal amounts of total protein lysate from immortalized Ad-GFP- or Ad-GFP-Cre-infected 3T3#1 MAFs were resolved by SDS–PAGE. Levels of TS, cyclin A and Cdk4 proteins were determined by immunoblotting. **(d)** Asynchronously proliferating primary (p4) and immortal 3T3#1 (p27) MAFs were harvested 6 days postinfection with either Ad-GFP or Ad-GFP-Cre. Equal amounts of protein were separated by SDS–PAGE and levels of indicated proteins determined by immunoblotting. β-tubulin served as loading control

spontaneous immortalization of adult fibroblasts can compromise regulation of specific RB/E2F target genes and confer divergent effects of acute RB loss.

Primary and immortal adult fibroblasts lacking RB are defective in cell cycle response to DNA damaging agents

We next challenged the role of RB in the response of primary and immortalized cells to chemotherapeutic drugs. Asynchronously proliferating primary MAFs (infected with Ad-GFP or Ad-GFP-Cre) were exposed to ionizing radiation or DNA damaging compounds. Agents were chosen to reflect a diversity of DNA damaging mechanisms including: DNA alkylation (CDDP and MMC), DNA intercalation (DOX) and topoisomerase I inhibition (CPT). Following each treatment the proliferative fraction of treated cells was determined by analysing Brd-U incorporation (8 h pulse beginning after drug removal). We found that each DNA damaging agent induced a dose-dependent cell cycle inhibition in control MAFs (Ad-GFP) containing functional RB (Figure 4a). In contrast, acute RB loss (Ad-GFP-Cre) abrogated the ability of primary MAFs to arrest (Figure 4a). For example, compared to untreated controls, less than 20% of Ad-GFP-infected MAFs incorporated Brd-U following exposure to CPT (5 μM). In contrast, following exposure to the same dose of CPT, Ad-GFP-Cre-infected cells incorporated Brd-U at the same rate as untreated cells (Figure 4a). These results demonstrate that RB maintains a critical role in the DNA damage response in primary somatic adult cells.

Given the importance of understanding how RB influences chemotherapeutic responses in cells containing growth enhancing mutations associated with tumorigenesis, we next evaluated the role of RB in influencing the response of immortal stage MAFs to DNA damaging agents. Asynchronously proliferating 3T3#1 MAFs, infected with Ad-GFP or Ad-GFP-Cre, were incubated with either CPT or DOX and labeled with Brd-U as above. Exposure of Ad-GFP-infected MAFs to CPT resulted in significant inhibition of Brd-U incorporation relative to untreated controls, although these cells were slightly more resistant to CPT than primary cells (Figure 4b). However, similar to primary cells, acute loss of RB in these cultures significantly compromised induction of cell cycle arrest (Figure 4b). Therefore, these data demonstrate that RB plays a critical role in the checkpoint response of both primary and immortal stage fibroblasts to specific DNA damaging agents.

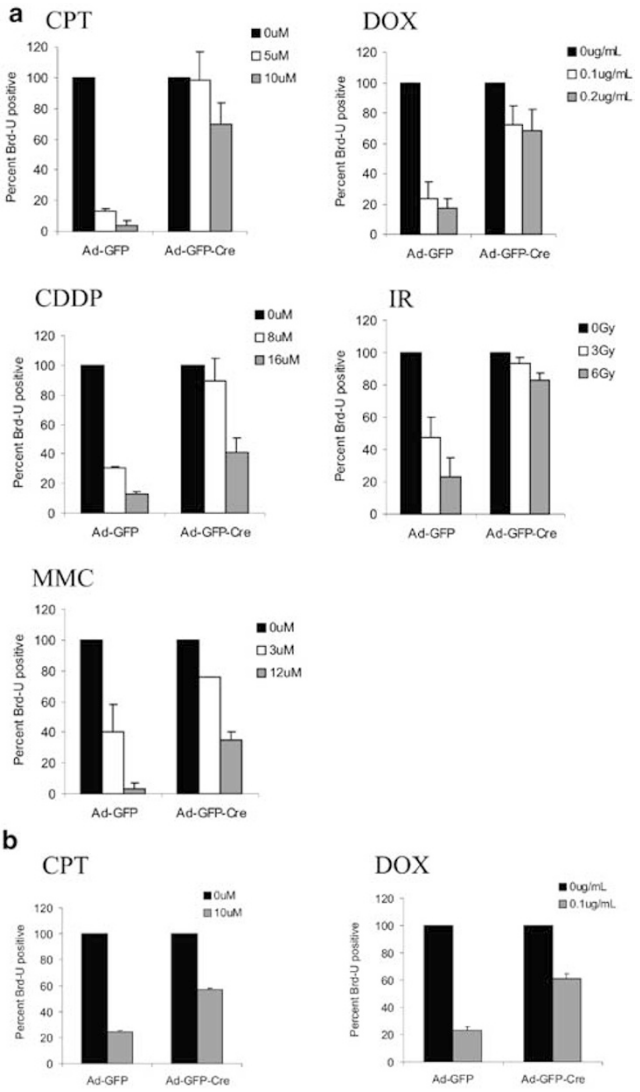


Figure 4 RB is required for G1–S checkpoint in primary and immortal adult fibroblasts exposed to DNA damaging agents. **(a)** Asynchronously proliferating, primary $Rb^{loxP/loxP}$ MAFs, infected with Ad-GFP or Ad-GFP-Cre, were treated with DNA damaging agents: ionizing radiation (IR), CDDP, MMC, DOX or CPT at the indicated doses. **(b)** Asynchronously growing 3T3#1 immortal MAFs, infected with Ad-GFP or Ad-GFP-Cre, were treated with CPT or DOX at the indicated doses. Primary and immortal cells were exposed to drugs for 16 h, washed and cultured in drug-free media containing Brd-U for an additional 8 h. Induction of cell cycle arrest was assessed by Brd-U incorporation. Data is shown as the percent of cells staining positively for Brd-U incorporation (mean $\pm$ standard deviation), with untreated controls set arbitrarily to 100%. Results are from at least two independent experiments performed in duplicate with a minimum of 150 cells counted per condition

Disparate RB-dependent checkpoint responses of primary and immortal MAFs to antimetabolites

The growth-suppressive effects of various antimetabolites, including 5-FU, methotrexate (MTX) and hydroxyurea (HU) have been demonstrated to be compromised in RB-deficient cells. This is attributed to elevated levels of the metabolic enzymes targeted by these compounds (TS, dihydrofolate reductase and ribonucleotide reductase (RNR)) (Almasan *et al*., 1995; Li *et al*., 1995; Angus *et al*., 2002). Therefore, we next probed the consequences of acute RB loss on the response of primary and immortal cultures to antimetabolites. Ad-GFP-infected primary MAFs exhibited a dose-dependent reduction in Brd-U incorporation upon exposure to 5-FU and HU, indicating inhibition of cell cycle progression after drug treatment (Figure 5a).

In contrast, Ad-GFP-Cre-infected MAFs incorporated Brd-U at significantly higher levels than controls (Ad-GFP), indicating that RB loss abrogated the ability to arrest after antimetabolite treatment (Figure 5a). We next tested the consequences of acute RB loss on the response of immortalized MAFs to antimetabolites. In contrast to primary cells, treatment of immortal 3T3#1 MAFs with 5-FU did not result in cell cycle arrest, regardless of RB status (Figure 5b), indicating that immortalization abrogated the ability of RB to signal for arrest following 5-FU exposure. Together, these data demonstrate the requirement of RB for DNA damage and antimetabolite-induced cell cycle arrest in primary adult fibroblasts, underscoring the critical role of RB in the G1–S cell cycle checkpoint. However, although immortalized MAFs (containing mutant p53) exhibited an RB-dependent checkpoint to DNA damaging agents, these cells became resistant to the antimetabolite 5-FU despite retention of functional RB.

Acute RB loss in primary cells abrogates G1- and S-phase checkpoint and promotes aneuploidy following CPT and 5-FU exposure

To determine the role of RB in cell cycle transitions following exposure to chemotherapeutic agents we employed bivariate flow cytometry (Figure 6). Using this approach incorporation of Brd-U (to indicate DNA synthesis) and DNA content (an indicator of cell cycle phase) were measured concurrently. CPT and 5-FU were employed as representative DNA damaging and antimetabolite agents that induced RB-dependent checkpoint responses (Figures 5 and 6). Primary MAFs infected with Ad-GFP or Ad-GFP-Cre were incubated for 16 h with CPT (10 μM) or 5-FU (20 μM). Cells were then washed, incubated in drug-free media and pulse labeled with Brd-U for 2 h at 24, 48 and 72 h after drug addition. During pulse labeling, only cells in S phase will incorporate Brd-U. Untreated RB-proficient and RB-deficient MAFs readily incorporated Brd-U in S phase. However, consistent with our earlier findings (Figure 2d), unchallenged RB-deficient MAFs exhibited a population of cells with a DNA content >4N. Treatment of Ad-GFP-infected MAFs with CPT initially resulted in arrest in all phases of the cell cycle (Figure 6a, top two panels). Subsequently a fraction of cells underwent DNA synthesis and accumulated in G2/M (Figure 6a). In contrast, RB-deficient MAFs continued to incorporate Brd-U following CPT treatment and were able to complete S phase, indicated by accumulation of cells in G2/M (Figure 6a, bottom two panels). Interestingly, CPT-treated, RB-deficient cells apparently continued to incorporate Brd-U in the absence of cytokinesis, as indicated by the significant accumulation of cells with a DNA content greater than 4N.

RB-proficient cells exposed to 5-FU failed to accumulate in any phase of the cell cycle, indicating that 5-FU initiated arrest in all phases of the cell cycle (Figure 6b, bottom two panels). However, Ad-GFP-Cre-infected MAFs continued to synthesize DNA and accumulated 4n DNA content following 5-FU treatment, indicating that RB loss subverts G1 and S-phase checkpoints and 5-FU induces a G2/M arrest in RB-deficient cells. RB-deficient cells exposed to 5-FU also accumulated a population of cells with >4n DNA content, although this effect was less pronounced than that associated with CPT.

Since it is known that RB-deficient cells are sensitized to cell death following treatment with chemotherapeutic agents (Almasan *et al*., 1995), we next assessed the long-term consequence of exposure of RB-proficient and RB-deficient cells to CPT and 5-FU. For this purpose we utilized the MTT assay, which is based on the cleavage of the MTT reagent by mitochondrial dehydrogenases of viable cells. Asynchronously growing Ad-GFP- and Ad-GFP-Cre-infected cells were treated with either 10 μM CPT or 20 μM 5-FU for 16 h after which cells were washed and incubated in drug-free media. At 72 h after drug addition MTT reagent was added to cells for 2 h to indicate the relative numbers of viable cells. Compared to untreated controls (normalized to 100%), there were significantly fewer viable Ad-GFP-Cre-infected cells than Ad-GFP-infected cells following 5-FU and CPT treatment (Figure 6c), indicating that RB loss enhances the cytotoxicity of these agents.

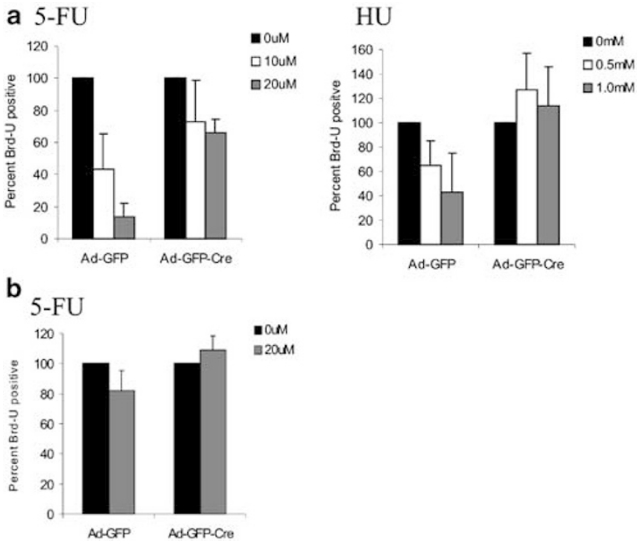
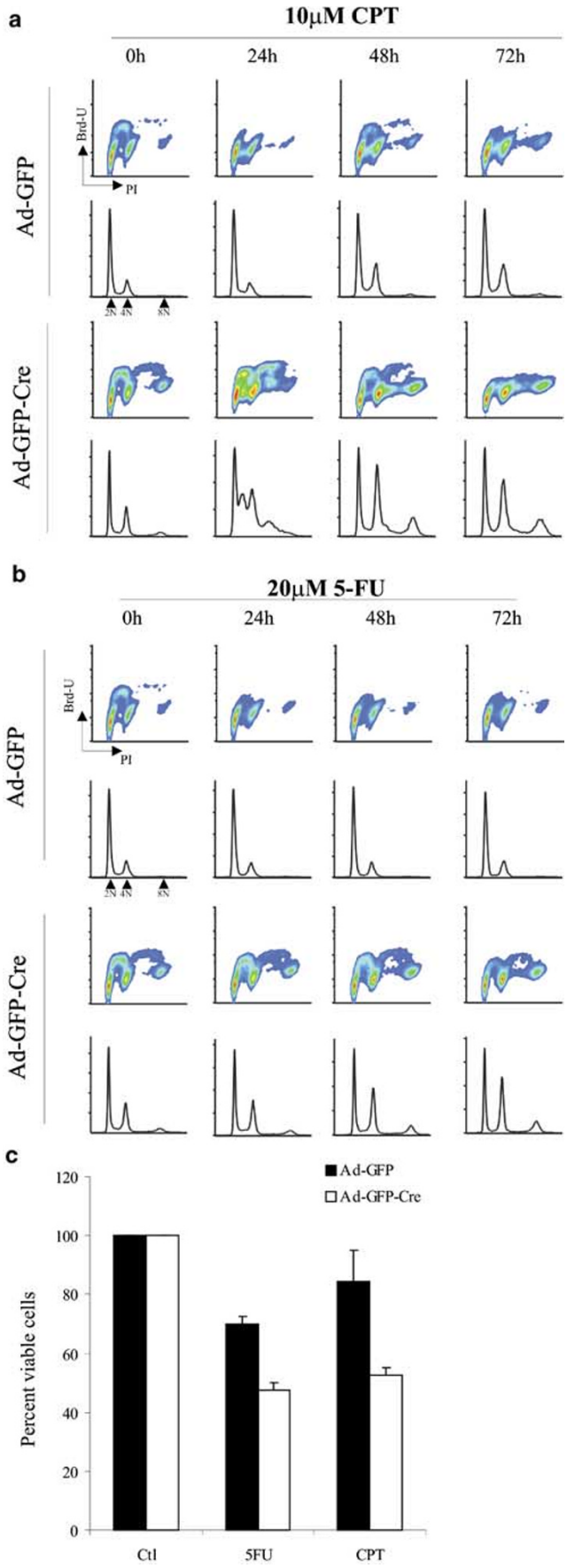


Figure 5 Distinct RB-dependent checkpoint responses of primary and immortal MAFs to 5-FU. **(a)** Asynchronously proliferating, primary $Rb^{loxP/loxP}$ MAFs, infected with Ad-GFP or Ad-GFP-Cre, were treated with the antimetabolites 5-FU or HU. **(b)** Asynchronously proliferating, immortal 3T3#1 MAFs, infected with Ad-GFP or Ad-GFP-Cre, were treated with (5-FU). Primary and immortal cells were exposed to drugs for 16 h, washed and cultured in drug-free media containing Brd-U for an additional 8 h. Drug effects on cell cycle progression were assessed by Brd-U incorporation. Data is shown as the percent of cells staining positively for Brd-U incorporation (mean ± standard deviation), with untreated controls set arbitrarily to 100%. Results are from at least two independent experiments performed in duplicate with a minimum of 150 cells counted per condition



Hyperactivation of p53 signaling by 5-FU in RB-deficient MAFs

The mechanism of 5-FU cytotoxicity is ascribed to the incorporation of 5-FU metabolites into RNA and DNA, as well as its ability to inhibit TS (Longley *et al*., 2003). However, TS inhibition is believed to be the principle mechanism of action of 5-FU (Peters *et al*., 2002). TS is a well established target of the RB/E2F signaling axis (Almasan *et al*., 1995; DeGregori *et al*., 1995; Li *et al*., 1995), and RB-deficient cell lines display increased TS expression and activity that is associated with resistance to agents that target TS (Li *et al*., 1995). Therefore, we next sought to determine the effects of acute RB loss on TS expression in primary MAFs and how this contributes to the observed lack of cell cycle checkpoint response to 5-FU in RB-deficient MAFs. We initially investigated the effect of acute RB loss on the activity of the TS promoter. Ad-GFP- or Ad-GFP-Cre-infected primary MAFs were transiently transfected with a TS-luciferase reporter plasmid under control of the rat TS promoter (Siddiqui *et al*., 2003). As shown in Figure 7a, luciferase activity was approximately three-fold higher in Ad-GFP-Cre- than in Ad-GFP-infected MAFs, demonstrating that acute RB loss deregulates TS promoter activity. Furthermore, TS RNA (Figure 7b, compare lanes 1 and 2) and protein levels (Figure 7c, compare lanes 1 and 2) were increased following acute RB loss. As resistance to 5-FU is mostly attributed to high levels of TS in tumors (Banerjee *et al*., 2002), we reasoned that the lack of a cell cycle checkpoint to 5-FU in RB-deficient (Ad-GFP-Cre) cells might be due to the intrinsically higher TS levels in these cells, resulting in inability of 5-FU to sufficiently inactivate TS and failure to activate checkpoint signaling. Therefore, we investigated the ability of 5-FU to inactivate TS in RB-proficient and RB-deficient cells. 5-FU must be converted to the active metabolite fluorodeoxyuridine monophosphate (FdUMP) in order to exert its effects on TS (Longley *et al*., 2003). FdUMP binds to the nucleotide binding site of TS, blocking binding of the endogenous TS substrate (dUMP), resulting in inhibition of dTMP synthesis (Santi *et al*., 1974; Sommer and Santi, 1974). When bound to TS, FdUMP forms a stable ternary complex with TS and 5,10-methyl-tetrahydrofolate, resulting in an inactive higher molecular weight complex that can be detected as a shift by TS

Figure 6 Loss of RB abrogates G1/S and intra-S arrest, promotes aneuploidy and enhances cytotoxicity following CPT and 5-FU exposure. Asynchronously proliferating primary MAFs infected with either Ad-GFP or Ad-GFP-Cre were treated with **(a)** CPT (10 μM) or **(b)** 5-FU (20 μM) for 16 h, washed and cultured in drug-free media. At 24, 48 and 72 h postdrug addition, cells were pulse labeled for 2 h with Brd-U. Cells were then harvested, fixed, stained for Brd-U incorporation (*y*-axis) and DNA content (*x*-axis) and analysed by FACS analysis. **(c)** Asynchronously growing Ad-GFP- and Ad-GFP-Cre-infected MAFs were treated for 16 h with CPT (10 μM) or 5-FU (20 μM). Cells were then washed and incubated in drug-free media. 72 h after drug addition, cytotoxicity was determined by MTT assay as described in Materials and methods. Each point represents the mean $\pm$ standard deviation from two independent experiments performed in triplicate

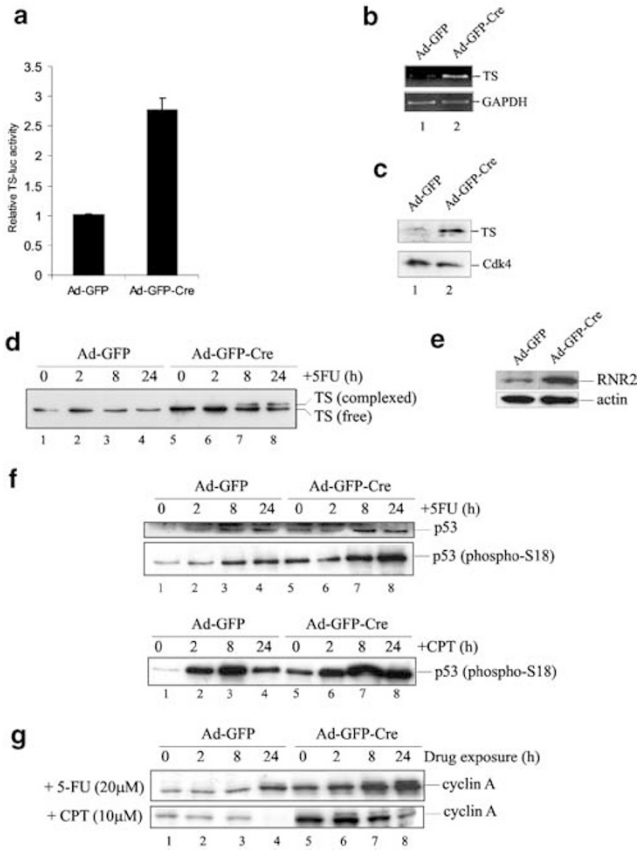


Figure 7 Acute RB loss in primary MAFs results in increased TS expression but does not abrogate 5-FU-induced checkpoint signaling. **(a)** Ad-GFP- and Ad-GFP-Cre-infected primary $Rb^{loxP/loxP}$ MAFs were transiently cotransfected with reporter constructs encoding luciferase regulated by the TS promoter and β-galactosidase. At 48 h post-transfection, lysates analysed for luciferase activity. Levels of β-galactosidase were used to normalize for transfection efficiency. **(b)** Asynchronously proliferating, Ad-GFP- and Ad-GFP-Cre-infected primary $Rb^{loxP/loxP}$ MAFs were harvested and total RNA extracted. The effect of acute RB loss on relative levels of TS mRNA was established by RT–PCR. Analysis of GAPDH levels was performed as loading control. **(c)** Equal amounts of total protein lysate from asynchronously proliferating Ad-GFP- or Ad-GFP-Cre-infected primary MAFs were resolved by SDS–PAGE. Effect of acute RB loss on TS levels was determined by immunoblotting. **(d)** Ad-GFP- and Ad-GFP-Cre-infected MAFs were treated with 5-FU (20 μM). Following 2, 8 and 24 h of treatment, cells were harvested and the effects of 5-FU on TS mobility were determined by immunoblot. **(e)** Asynchronously proliferating, Ad-GFP- and Ad-GFP-Cre-infected primary $Rb^{loxP/loxP}$ MAFs were harvested and equal total protein resolved by SDS–PAGE. RNR2 and β-actin levels were determined by immunoblot. **(f)** $Rb^{loxP/loxP}$ MAFs, infected with Ad-GFP (lanes 1–4) or Ad-GFP-Cre (lanes 5–8) were treated with 20 μM 5-FU or 10 μM CPT and proteins harvested following 2, 8 and 24 h of treatment. Equal total protein was resolved by SDS–PAGE and the influence of drug treatment on levels of total p53 and phospho-p53 (serine 18) were determined by immunoblot. **(g)** Ad-GFP- and Ad-GFP-Cre-infected MAFs were treated with 5-FU (20 μM) or CPT (10 μM) as described in (**f**). Effects of drug treatment on cyclin A protein levels in RB-proficient and RB-deficient cells was determined by immunoblot

immunoblot. Therefore, to investigate TS inactivation, Ad-GFP- and Ad-GFP-Cre-infected primary cells were exposed to 20 μM 5-FU and lysates were immunoblotted for TS. Following 5-FU exposure, we surprisingly found that despite induction of cell cycle arrest (Figure 5a), there was minimal change in the level of free TS in RB-proficient (Ad-GFP) MAFs (Figure 7d, lanes 1–4), indicating that complete inactivation of TS is not required to elicit downstream checkpoint signaling. Interestingly, we found that significantly more TS in RB-deficient MAFs was in the ternary complex than the equivalent RB-proficient MAFs (Figure 7d, compare lanes 1–4 and 5–8). This suggested that 5-FU might be more efficiently activated to FdUMP in RB-deficient cells than in RB-proficient cells. Consistent with this concept, the promoters of two genes encoding enzymes involved in 5-FU metabolism to FdUMP, thymidine kinase and RNR (Longley *et al*., 2003), are regulated by the RB/E2F axis (Mudrak *et al*., 1994; Angus *et al*., 2002). Indeed, expression of the RNR small subunit (RNR2) was significantly elevated following acute RB loss in primary MAFs (Figure 7e). Thus, these findings suggest that increased expression of RB targets following RB loss may provide conditions that enhance the conversion of 5-FU to its active metabolites. To address whether checkpoint signaling upstream of RB was activated by 5-FU and CPT exposure we investigated the ability of these compounds to activate p53 in RB-proficient and RB-deficient cells. In response to various stress conditions, the human p53 protein is stabilized by phosphorylation on serine 15 (homologous to serine 18 in mouse p53), resulting in transcriptional upregulation of p53 target genes including $p21^{Cip1}$ (Stewart and Pietenpol, 2001). Exposure of both Ad-GFP- and Ad-GFP-Cre-infected cells to 5-FU resulted in increased p53 stabilization and phosphorylation on serine 18 that was of greater magnitude in RB-deficient cells (Figure 7f upper panel, compare lanes 1–4 and 5–8). However, 5-FU treatment resulted in similar rates of accumulation of p53 and phospho-p53 (ser18), indicating that checkpoint signaling was activated with similar kinetics in both RB-proficient and RB-deficient cells (Figure 7f upper panel, compare lanes 1–4 and 5–8). Similarly, exposure to CPT resulted in dramatic phosphorylation of p53 that was enhanced in RB-deficient cells (Figure 7f lower panel, compare lanes 1–4 and 5–8). Interestingly, basal levels of p53 phosphorylated on serine 18 were higher in RB-deficient (Ad-GFP-Cre) cells than RB-proficient (Ad-GFP) cells (Figure 7d, compare lanes 1 and 5).

Cyclin A is not a universal target of RB-dependent checkpoint signaling

Having shown that checkpoint signaling upstream of RB is intact in RB-deficient cells exposed to 5-FU and CPT, we next investigated the effects of these drugs on expression of the critical downstream RB target cyclin A. We have previously shown that cyclin A is down-regulated in an RB-dependent manner following exposure to the DNA damaging agent CDDP (Knudsen *et al*., 2000; Lan *et al*., 2002). To address whether cyclin A is also an important target of 5-FU and CPT-induced checkpoint signaling, we incubated Ad-GFP- and

Ad-GFP-Cre-infected primary MAFs with 20 μM 5-FU or 10 μM CPT for 24 h. At 2, 8 and 24 h after drug addition, cells were harvested and cyclin A levels determined by immunoblot. Interestingly, we found that 5-FU failed to reduce cyclin A levels in both RB-proficient and RB-deficient cells (Figure 7g). In contrast, analysis of the effects of CPT revealed dramatic reductions in cyclin A levels in Ad-GFP-infected cells (Figure 7g). This reduction was not completely RB dependent because there was also a reduction in cyclin A levels in Ad-GFP-Cre-infected cells. However, due to the intrinsically higher basal cyclin A levels in these cells, cyclin A levels in CPT-treated RB-deficient MAFs were still significantly higher than in RB-proficient MAFs (Figure 7e, compare lanes 4 and 8). Collectively, these data argue against a model whereby 5-FU fails to induce cell cycle arrest in RB-deficient cells because the high TS levels in these cells cannot be sufficiently inhibited to elicit checkpoint signaling. In contrast, these data demonstrate that checkpoint signaling upstream of RB is induced by exposure of RB-deficient cells to 5-FU, and that the checkpoint signaling defect is downstream of RB and independent of repression of cyclin A. Therefore, although 5-FU induces an RB-dependent cell cycle arrest, the mechanism through which RB signals is mechanistically distinct from that following exposure to DNA damaging agents CDDP and CPT.

Discussion

Mutation or functional inactivation of RB is a common event in human cancer (Lee *et al*, 1988; Harbour *et al*., 1988; Horowitz *et al*., 1990; Hall and Peters, 1996). Therefore, understanding how RB influences the response to chemotherapeutic agents is important for the design of optimal treatment. RB has been proposed to function as a critical component of checkpoint signaling by eliciting cell cycle arrest following exposure to chemotherapeutic agents (Almasan *et al*., 1995; Harrington *et al*., 1998; Knudsen *et al*., 2000). The most widely utilized system for delineating the role of endogenous RB in chemotherapeutic response are murine embryonic fibroblasts (MEFs) derived from the mating of $Rb^{+/-}$ animals. The use of MEFs is a requirement due to the embryonic lethality following germline loss of both *Rb* alleles (Clarke *et al*., 1992; Jacks *et al*., 1992; Lee *et al*., 1992). Here, we have used the Cre/LoxP system to achieve conditional RB knock out in adult fibroblasts. There are several readily defineable advantages of the conditional knock out of RB over the utilization of $Rb^{-/-}$ MEFs. In the case of $Rb^{-/-}$ MEFs the *Rb* gene has been lost through the development of the embryo. It is quite clear that under these conditions related proteins (i.e. p107) can compensate for the loss of RB (Sage *et al*., 2003). Additionally, using conditional RB inactivation cells can first be modified (e.g. *in vitro* immortalization or senescence) before inactivation of RB. Thus, it is possible to evaluate the specific consequence of RB loss upon a pre-established condition. Lastly, one can monitor the influence of RB loss in a kinetically tractable fashion. In our study we exploit this aspect of the model and demonstrate the kinetic accumulation of cells with abnormal (8N) DNA content following acute RB loss. Together, these attributes make this model attractive for a variety of studies pertaining to the action of RB.

Using this model we demonstrate that the targeted disruption of RB has specific influences on adult fibroblasts. The loss of RB is accompanied by deregulated expression of a number of known RB/E2F target genes. These targets are similar to those observed to be deregulated in $Rb^{-/-}$ MEFs (Herrera *et al*., 1996; Hurford *et al*., 1997; Philips *et al*., 1998; Angus *et al*., 2002). Analysis of serum dependence in the presence of these targets revealed that the RB-deficient adult fibroblastic cultures are still serum dependent. This result contrasts with observations made with MEF cultures following acute loss of RB (Sage *et al*., 2003). However, the principal cell cycle aberration we observe with the loss of RB is the development of aneuploidy arising with passage following the loss of RB. This finding extends previous observations that showed a role for RB in preventing DNA rereplication following challenge with microtubule destabilizing drugs (Di Leonardo *et al*., 1997; Lentini *et al*., 2002) and CDDP (Lan *et al*., 2002) and suggests a role for RB in maintaining ploidy in unstressed cells. There are a number of possible mechanisms for the development of spontaneous aneuploidy given the role of RB in replication control (Knudsen *et al*., 1998; Avni *et al*., 2003) or centrosome duplication (Meraldi *et al*., 1999; Balsitis *et al*., 2003). We are presently investigating the mechanism through which aneuploidy arises in these cultures. That chemotherapeutic challenge further promotes aneuploidy argues that there is a general loosening of the coupling between S phase and mitosis following the loss of RB that is further exacerbated following replicative stresses. Clearly, the development of aneuploidy suggests one means through which loss of RB could directly promote tumor progression through the development of additional mutations.

While it is known that RB plays a pivotal role in the checkpoint response to chemotherapeutic agents, the consequence of acute RB loss in cells with simple (i.e. primary) or more complex (i.e. p53 deficient) genetic backgrounds has not been elucidated. We evaluated a number of DNA damaging (ionizing radiation, CDDP, DOX, CPT and MMC) and antimetabolite (5-FU and HU) chemotherapeutic agents following acute loss of RB in adult fibroblasts. In primary cells we found that RB was required for the checkpoint response to all of these agents. These results reinforce the critical action of RB for the induction of G1/S-phase arrest to a wide-range of clinically relevant agents with different primary mechanisms of action. In the case of 5-FU and CPT as representative members of each class of agent, we investigated the action of RB in a spontaneously immortalized cell line containing functionally inactivate p53. These immortal cells were 5-FU resistant even in the presence of RB. This result could be due to the

known influence of p53 on sensitivity to 5-FU (Bunz *et al*., 1999). In contrast, the p53-deficient immortal cells were still sensitive to CPT-mediated cell cycle inhibition and this response was RB dependent. Interestingly, similar results were observed with DOX, suggesting that DNA damaging chemotherapeutics have a strong dependence on RB even in the absence of functional p53. Whether these findings in the immortalized cell line are broadly applicable to immortalized cultures is under investigation.

In this study we specifically evaluated the activity of p53 in the presence or absence of RB. Interestingly, basal levels of activated p53 were higher in RB-deficient cells. Moreover, exposure to 5-FU and CPT enhanced activation of p53 and resulted in reduced viability of RB-deficient cells. This finding supports previous data that suggests that RB-deficient cells that retain p53 signaling are more sensitive to induction of apoptosis following chemotherapeutic challenge (Almasan *et al*., 1995). A possible explanation for the increased basal p53 activity in RB-deficient cells is unrestrained E2F1 activity. Human p53 is directly phosphorylated on serine 15 (serine 18 in mice) by the protein kinase ATM following DNA damage (Banin *et al*., 1998; Canman *et al*., 1998). E2F1 has recently been shown to positively regulate the ATM promoter, resulting in increased phosphorylation of p53 on serine 15 (Berkovich and Ginsberg, 2003). Furthermore, E2F1 has been shown to enhance the stabilization, activation and accumulation of p53 protein via the ARF pathway (Pomerantz *et al*., 1998). A likely possibility is that the enhanced activation of p53 in RB-deficient cells provides the enhanced sensitivity of these cells to death following 5-FU or CPT challenge.

While it is well established that p53 and additional DNA damage signaling pathways function upstream of RB, the critical downstream targets of RB action in checkpoint pathways have remained largely undetermined. In the case of 5-FU, it has been postulated that the high levels of endogenous TS in RB-deficient cells contribute to the resistance to this agent. Therefore, because acute RB loss resulted in high levels of TS, we initially speculated that virtually all of the TS would be converted to the inactive ternary complex by 5-FU in RB-proficient cells, while in RB-deficient cells sufficient free TS would remain for the observed cell cycle progression following treatment. However, analysis of the levels of free : bound TS revealed that TS remains largely in its free form in RB-proficient cells following 5-FU treatment, despite induction of cell cycle arrest. This finding suggests that bulk differences in the ratio of bound : free TS in 5-FU-treated RB-proficient and RB-deficient cells cannot explain the lack of cell cycle arrest in RB-deficient cells. Interestingly, we detected increased formation of the inactive ternary complex in RB-deficient cells exposed to 5-FU. We speculate that this was due to the RB/E2F-targets RNR and thymidylate kinase (TK) that are elevated in RB-deficient cells (Ogris *et al*., 1993; Mudrak *et al*., 1994; DeGregori *et al*., 1995; Angus *et al*., 2002). Analysis of upstream DNA damage checkpoint signaling revealed similar kinetics of activation of p53 by 5-FU in both RB-proficient and RB-deficient cells. This finding confirms that inability of 5-FU to adequately inhibit TS activity due to high TS levels, with concomitant failure to activate checkpoint signaling, cannot explain the lack of cell cycle arrest in RB-deficient cells exposed to 5-FU. This conclusion is supported by the work of Longley *et al*. (2002) who demonstrated that exogenous TS expression did not abrogate the stabilization of p53 by 5-FU in MCF7 breast cancer cells. Indeed, uridine (which competes with 5-FU metabolites for incorporation into RNA) inhibits p53 stabilization following 5-FU exposure, indicating that interference with RNA metabolism may be the critical mechanism of 5-FU cytotoxicity (Longley *et al*., 2002). Taken together, these data support the notion that high levels of TS are not responsible for the 5-FU checkpoint defect in RB-deficient MAFs and indicate that checkpoint signaling downstream of RB is abrogated. Furthermore, these data suggest that the activation of 5-FU is enhanced in RB-deficient cells, possibly due to increased levels of enzymes involved in the metabolic activation of 5-FU.

A number of studies have suggested that a critical target of DNA damage checkpoint signaling in G1/S are cdks, the activity of which is inhibited by a variety of mechanisms following DNA damage (Agami and Bernards, 2000; Mailand *et al*., 2000). Therefore, it is believed that the cell cycle checkpoint defect in RB-deficient cells may be due to high cdk2 activity because cyclins A and E levels are elevated in these cells. Previous work from our laboratory has demonstrated that transcriptional downregulation of cyclin A and resultant cdk inhibition by RB is required for cell cycle arrest following exposure to the DNA damaging agent CDDP (Knudsen *et al*., 2000; Lan *et al*., 2002). Analysis of the effects of CPT and 5-FU exposure revealed differences in signaling downstream of RB. Although both agents elicited RB-dependent cell cycle arrest, CPT induced significant downregulation of cyclin A in RB-proficient cells. This reduction was not completely RB dependent because there was also a reduction in cyclin A levels in Ad-GFP-Cre-infected cells, possibly due to damage-induced post-transcription regulation (Guo *et al*., 2000). However, cyclin A levels remained high in RB-deficient cells, suggesting that CPT functions in a similar manner to CDDP. In contrast, 5-FU failed to elicit cyclin A downregulation. Indeed, cyclin A levels were significantly increased by 5-FU in both RB-proficient and RB-deficient cells. These results demonstrate that cyclin A is not a universal target of RB checkpoint signaling. We are currently investigating critical targets of RB following 5-FU treatment.

Together, these studies demonstrate that the acute loss of RB leads to specific phenotypic consequences in adult cells. Conditional loss of RB leads to target gene deregulation, facilitates the spontaneous development of aneuploidy and compromises the response to chemotherapeutic agents. These activities of RB can be only partially circumvented upon the loss of p53 activity during immortalization. Analyses of the mechanisms underlying sensitivity to 5-FU and CPT illustrate that

RB loss primes cells for p53 activation and subsequent cell death. Interestingly, 5-FU and CPT act in discrete fashions downstream from RB to elicit cell cycle inhibition and indicate that loss of RB bypasses chemotherapy-induced cell cycle inhibition through multiple pathways.

Materials and methods

Isolation of primary Rb$^{loxP/loxP}$ *MAFs*

Floxed *Rb* mice (*Rb*$^{loxP/loxP}$) of mixed 129/FVBN background (MacPherson *et al*., 2003; Sage *et al*., 2003), at least 5 weeks of age, were killed by CO_2 anaesthetization followed by cervical dislocation. Fibroblasts were isolated from the peritoneal fascia as follows. The peritoneum was excised, minced into small pieces and dissociated by constant agitation for 40 min at 37°C in 0.2 mg/ml collagenase (Type I, Sigma) supplemented with 100 U DNase I (Roche). After washing in PBS, dissociated tissue was incubated for 20 min at 37°C in 0.25% trypsin (Gibco) with constant agitation. Isolated cells were then washed twice and plated in tissue culture dishes. These cells were designated as passage one.

Rb$^{loxp/loxp}$ *MAF culture, recombinant adenoviral infections and immortalization*

Primary *Rb*$^{loxP/loxP}$ MAFs were propagated by routine subculturing in DMEM containing 10% FCS supplemented with 100 U/ml penicillin/streptomycin and 2 mM L-glutamine at 37°C in air containing 5% CO_2. Primary cells used in this study were between passages 2 and 6. For generation of immortalized MAFs a standard 3T3 protocol was conducted (Todaro and Green, 1963). Briefly, 3×10^5 MAFs were plated in 6 cm culture dishes. After 3 days in culture, cells were counted and replated at 3×10^5 cells per 6 cm dish. Immortalized MAFs were used in experiments between passages 25 and 40. Replication-defective recombinant adenovirus expressing green fluorescent protein alone (Ad-GFP) or GFP and Cre recombinase (Ad-GFP-Cre) were obtained from G Leone (Department of Molecular Genetics, Ohio State University). For conditional RB knock out in primary and immortal *Rb*$^{loxP/loxP}$ MAFs, cells were infected with adenovirus at an MOI of approximately 20 (actual infection efficiency was 90–95%, as determined by GFP immunofluorescence). Cells were cultured for at least 3 days postadenoviral infection before use. All experiments were controlled by using Ad-GFP-Cre-infected MAFs matched with Ad-GFP-infected MAFs of the same passage number infected for the same length of time. To determine dependence of primary cells on serum for growth, asynchronously growing Ad-GFP- and Ad-GFP-Cre-infected MAFs were cultured in 0.1% FCS for 72 h. For cell cycle analysis, cells were washed with PBS, trypsinized and fixed with 70% ethanol. Cells were then stained with propidium iodide and processed for flow cytometric analysis as previously described (Knudsen *et al*., 1998).

RT–PCR analysis and reporter assays

Total RNA was extracted with Trizol (Gibco) and first-strand cDNA was synthesized from 1 μg of RNA using the SuperScript RT–PCR system (Gibco) following the manufacturers protocols. For recombination analysis, cDNAs were subjected to PCR amplification using the following primers: 5′-CTGGCCAGGCTTGAGTTTGAAG-3′ (sense) and 5′-CAG TAGATAACGCACTGCTG-3′ (antisense) that anneal to nucleotides 210–231 and 611–630 in mouse *Rb* cDNA, respectively. PCR conditions consisted of initial denaturation for 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 51°C and 1 min at 72°C, followed by a final extension for 5 min at 72°C. For TS mRNA analysis cDNAs were subjected to PCR using the following primers: 5′-GGCAGGTGGAACA CATTTTGC-3′ (sense) and 5′-TCCCAGATTCTCACTCCCT TGG-3′ (antisense). Cycling conditions consisted of 32 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 30 s. Loading was controlled by evaluation of GAPDH mRNA levels with the following primers: 5′-ACCCAGAAGACTGTGGATGG-3′ (sense) and 5′-AGGAGACAACCTGGTCCTCA-3′ (antisense). PCR conditions were 20 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 30 s. In all, 10 µm of each PCR product were separated by agarose gel electrophoresis and products visualized by ethidium bromide staining. Analysis of TS promoter activity was performed by transient transfection (FuGENE 6, Roche) of pGL2-Basic firefly luciferase expression vector under the control of the rat TS promoter (Siddiqui *et al*., 2003). For all transfections pCMV-β galactosidase was cotransfected to normalize for transfection efficiency. Analysis of luciferase activity was performed 48 h post-transfection as previously described (Knudsen *et al*., 1999).

Immunoblotting and immunoprecipitation

For immunoblotting, cells infected with Ad-GFP or Ad-GFP-Cre were harvested by scraping in ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 1.0% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM PMSF, 50 mM NaF, 13 mg/ml β-glycerophosphate and 120 μg/ml sodium vanadate). Insoluble material was removed by centrifugation (13 000 r.p.m., 10 min at 4°C) and soluble protein concentrations determined by DC Protein assay (BioRad). Equal amounts of protein from each lysate were boiled in SDS–PAGE sample buffer and resolved by SDS–PAGE. Specific proteins were detected by standard immunoblotting procedures using the following primary antibodies (Santa Cruz, 1 : 500 dilution): Cyclins B1(GNS1), A (C19) and E (HE12), p21 (C19), Cdc2 (17), PCNA (pc10), β-tubulin (D10), MCM7 (141.2), Cdk4 (H22) and RNR2 subunit (I15). Detection of total p53 was with monoclonal anti-p53 (Ab-1, Oncogene). p53 phosphorylated on serine 18 was detected with polyclonal anti-phosphor p53 (ser15) antibody (Cell Signaling Technology). Polyclonal TS antibody was a kind gift from Dr M Fukushima (Taiho Pharmaceutical). After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies, immune complexes were detected by enhanced chemiluminescence (NEN).

For immunoprecipitation, extracts of cells infected with either Ad-GFP or Ad-GFP-Cre were prepared in RIPA buffer. After clarification by centrifugation (13 000 r.p.m./15 min) approximately 2 mg total protein was immunoprecipitated by incubation with RB 851 polyclonal antisera (Knudsen *et al*., 2000) and protein A-agarose beads for 16 h at 4°C with rotation. After extensive washing in RIPA buffer, immune complexes were recovered by boiling in SDS–PAGE sample buffer and resolved by 7% SDS–PAGE. RB protein was detected by immunoblotting with a monoclonal anti-RB antibody (G3-245, Becton Dickson, 1 : 100 dilution).

DNA damage, Brd-U labeling and bivariate flow cytometry

Primary or immortalized MAFs infected with Ad-GFP or Ad-GFP-Cre were seeded on coverslips in six- or 24-well plates and allowed to attach overnight. Cells were then irradiated with a Cs137 source (3 and 6 Gy) or incubated with

chemotherapeutic drugs at the indicated concentrations for 16 h. Drugs were then removed by washing cells (3×5 min) with tissue culture media and cells labeled with Brd-U (Amersham Pharmacia Biotech) to detect DNA synthesis. Following 8 h of labeling, cells were washed and fixed in 3.7% formaldehyde and processed to detect Brd-U incorporation by indirect immunofluorescence as previously described (Knudsen *et al*., 1998). CPT, MMC and DOX were purchased from Sigma, 5-FU from Calbiochem and clinical grade CDDP from Bristol Oncology. For flow cytometric analysis of cell cycle distribution, Ad-GFP- and Ad-GFP-Cre-infected MAFs were fixed with 70% ethanol and processed for propidium iodide staining as previously described (Knudsen and Wang, 1997). Bivariate flow cytometric analysis of DNA synthesis (Brd-U incorporation) and DNA content (PI) was performed as previously described (Knudsen *et al*., 2000).

MTT cytotoxicity assay

To establish cytotoxicity of chemotherapeutic drugs 4.5×10^3. Ad-GFP- and Ad-GFP-Cre-infected MAFs were plated per well in a 96-well microplate and either left untreated or treated with drugs at the indicated concentrations for 16 h. Cells were then washed and cultured in drug-free media. At 72 h after drug addition, cells were incubated for 2 h in the presence of 0.5 mg/ml MTT reagent (Sigma). The formazan product formed by viable cells was then solubilized overnight by addition of an equal volume of solubilization buffer (10% SDS in 0.01 N HCl). The absorbance of each well at 695 nm was then determined using a microtitre plate reader. Effect of drug treatment on cell viability was calculated by comparing the absorbance in wells containing drug-treated cells to the absorbance in wells containing untreated cells.

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