p130/p107/p105Rb-dependent transcriptional repression during DNA-damage-induced cell-cycle exit at G2

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Summary

The progression of normal cells from G2 into mitosis is stably blocked when their DNA is damaged. Tumor cells lacking p53 arrest only transiently in G2, but eventually enter mitosis. We show that an important component of the stable G2 arrest in normal cells is the transcriptional repression of more than 20 genes encoding proteins needed to enter into and progress through mitosis. Studies from a number of labs including our own have shown that, by inducing p53 and p21/WAF1, DNA damage can trigger RB-family-dependent transcriptional repression. Our studies reported here show that p130 and p107 play a key role in transcriptional repression of genes required for G2 and M in response to DNA damage. For *plk1*, repression is partially abrogated by loss of p130 and p107, and is completely abrogated by loss of all three RB-family proteins. Mouse cells lacking RB-family proteins do not accumulate with a 4N content of DNA when exposed to adriamycin, suggesting that all three RB-family proteins contribute to G2 arrest in response to DNA damage. Stable arrest in the presence of functional p53-to-RB signaling is probably due to the ability of cells to exit the cell cycle from G2, a conclusion supported by our observation that KI67, a marker of cell-cycle entry, is downregulated in both G1 and G2 in a p53-dependent manner.

Key words: E2F, Mitosis, Checkpoint

Introduction

Progression through the cell cycle is controlled by checkpoints that ensure that previous events have been completed and that detect whether cellular components such as DNA have been damaged (Elledge, 1996). Transient cell-cycle arrest provides time for repair. Permanent arrest has also been observed and blocks the proliferation of cells containing the damage (Linke et al., 1996). Alternatively, cells can undergo apoptosis in response to stress (Lowe et al., 1993). Like apoptosis, cell-cycle arrest is essential to suppress the propagation of damaged DNA, and to inhibit the formation and progression of tumors. Multiple pathways affect several different cell-cycle checkpoints when DNA becomes damaged. G1 arrest is controlled primarily by the tumor suppressor p53, which is activated in response to various forms of stress, binds to the promoters of its target genes and induces their transcription (Kastan et al., 1991; Vogelstein et al., 2000). Induction of the cyclin-dependent kinase (CDK) inhibitor p21/WAF1 by p53 blocks the entry of cells into S phase to cause G1 arrest (Dulic et al., 1994). p53 also mediates a protective arrest within S phase when nucleotide pools are imbalanced (Agarwal et al., 1998). The mutation of p53 that occurs in more than half of all human cancers eliminates important cell-cycle arrest and apoptosis responses to damaged DNA, leading to genomic instability that drives cancer progression (Vogelstein et al., 2000).

Cells from diverse organisms are blocked in G2 when DNA is damaged. Although p53-independent pathways initiate the arrest, p53 is essential for the long-term maintenance of G2 arrest (reviewed by Taylor and Stark, 2001). The initiation pathway involves the inhibition of CDK1 (also called CDC2), which is the CDK that drives cells into mitosis. Inhibition of CDK1 results from inhibition of its activator, CDC25C, by the kinases CHK1 and CHK2, which themselves are phosphorylated and activated by the DNA-damage-inducible kinases ATM and ATR (Smits and Medema, 2001). This cascade rapidly inhibits CDK1 in cells with or without p53. However, cells lacking p53 are unable to maintain the G2 arrest and eventually enter mitosis (Bunz et al., 1998). Also, p53 can arrest cells in a G2-like state when it is overexpressed, even in the absence of stress-induced activation (Agarwal et al., 1995).

It was recently shown that p21/WAF1-mediated inhibition of CDK1/Cyclin B and CDK2/Cyclin A, both of which are needed to enter mitosis, helped to maintain the G2 arrest that occurs in response to DNA damage (Baus et al., 2003). Cells in which p53 was inactivated by the E6 protein from human papilloma virus could not stably arrest when DNA was damaged with bleomycin. Under these conditions, the cells initially arrested in G2, some cells escaped into mitosis and many cells died, whereas cells that did not express E6 were stably arrested. RB dephosphorylation, which normally

occurs when cells exit the cell cycle from G1, was also observed when cells with an intact p53 pathway were blocked in G2 by DNA damage (Baus et al., 2003). This suggested that the cells had exited the cell cycle from G2 in a p53-dependent manner.

Previous work has shown that, when DNA is damaged and cells arrest in G2, many genes required for G2 and M are downregulated (Chang et al., 2002; Crawford and Piwnica-Worms, 2001; Ren et al., 2002). Here we show that both the G2 arrest and the downregulation of genes required for G2 and M are critically dependent on the Rb family, especially p130 and p107. Many of the genes repressed during the DNA damage response are known to be E2F targets that are downregulated when cells exit the cell cycle from G1. We also show that the well-known proliferation marker KI67 is downregulated in cells with a 4N complement of DNA when DNA is damaged and that this response requires p53. These results suggest that p53 drives G2 cells out of the cell cycle. By blocking the segregation of damaged genomes, this stable block to mitotic entry provides an additional mechanism to maintain genomic stability and suppress tumor formation.

Materials and Methods

Cell lines and culture conditions

Cell lines were grown in a humidified atmosphere containing 10% CO_2 in Dulbecco's minimal essential medium (Gibco), supplemented with antibiotics and 10% fetal bovine serum (Gibco). GSE and WT cells were described previously (Clifford et al., 2003) Briefly, the HT1080 human fibrosarcoma-derived cell line, containing wild-type p53 (our unpublished observations), was infected with a retrovirus expressing GSE56, a dominant-negative carboxyl-terminal fragment of p53 (Ossovskaya et al., 1996). Uninfected cells were removed by G418 selection to produce the pool of infected 'GSE' cells. HT1080 cells infected with the empty LXSN virus were used to produce the pool of 'WT' cells. Cell-cycle content was determined by scanning propidium iodide-stained cells with a FACScan (Becton Dickinson) and FACS data were analyzed using Modfit 3.0 software. Etoposide was from Bristol-Myers Squibb and adriamycin from Sigma. Primary MEFs were used within 5 to 6 passages. TR9-7 and NARF2 cells have been described previously (Agarwal et al., 1995; Stott et al., 1998). To create NARF2 derivatives, we first prepared a pool of NARF2 cells expressing the ecotropic receptor by infecting parental NARF2 cells with an amphotropic retrovirus containing a pBabe-Bleo-Eco vector, which contains an expression cassette for the ecotropic receptor (uninfected cells were removed by selection with 200 μg/ml Zeocin). Next, the pool was individually infected with ecotropic retroviruses produced in Bosc cells using the following vectors: pLXSN, pLXSN-GSE56, pLXSN-Cyclin E, pLXSN-E7, pBabe-Puro, pBabe-puro-Cyclin D1/Cdk fusion (Chytil et al., 2004) or lentiviral vectors encoding the Hdm2 cDNA (LV-Hdm2) or short-hairpin-RNA-targeting p53 (shp53) (Brummelkamp et al., 2002) produced by transfection of 293T cells with a second-generation packaging construct (pCMV-dR8.74) and pMD2G (both provided by D. Trono, University of Geneva).

Northern analysis

Total RNA was extracted with Trizol reagent (Gibco) according to the manufacturer's instructions, separated by electrophoresis in a denaturing agarose gel, transferred to Hybond-N+ nylon membranes (Amersham) and probed with ^{32}P-labeled DNA probes. Probes were obtained from the I.M.A.G.E. consortium and confirmed by sequencing.

Western analysis

Whole cell extracts were prepared by incubating cell pellets in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1.0% NP-40, 10 μg/ml aprotinin, 100 μg/ml phenylmethane sulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin and 1 mM sodium metavanadate. Extracts containing equal quantities of proteins, determined by the Bradford method, were separated by SDS-PAGE (12.5% acrylamide) and transferred to polyvinylidene difluoride membranes (Millipore). Antibodies to p53 and p21/WAF1 were from Santa Cruz, to p14ARF and β-actin were from Neomarkers, and to PLK1 were from Oncogene Research Products. Bound antibodies were detected with goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (Hoffman-La Roche), using enhanced chemiluminescence (Dupont).

FACS analysis of KI67

Cells harvested with trypsin (including floating cells) were washed once with phosphate-buffered saline (PBS) and fixed by dropping them into 70% ethanol at –20°C. The cells were incubated in PBS + 0.25% Triton X-100 on ice for 15 minutes, and then in PBS containing 0.1% bovine serum albumin (w/v) and 0.02% NaN_3 (w/v) at room temperature for 30 minutes. Cells were then incubated for 2 hours at room temperature with monoclonal antibodies specific for KI67 (Oncogene Research Products), diluted in 0.1% bovine serum albumin (w/v), 0.02% NaN_3 (w/v) in PBS. KI67 antibodies were detected with a goat anti-mouse antibody labeled with fluorescein isothiocyanate (FITC; Sigma). The cells were washed, counterstained with a solution containing propidium iodide (50 μg/ml) and RNAse (2 units/ml) and analyzed by two-parameter FACScanning. For each condition, 20,000 cells were analyzed.

Since different treatments altered the background fluorescence slightly, each sample was split, with half receiving antibody to KI67 and the other half not. Gates were created to analyze cells separately with either 2N or 4N content of DNA and histograms of KI67 staining were generated separately for these sets of data. Next, regions ('M') were generated for each histogram to detect positive staining. In order to position the region, it was first placed on the negative control for each sample, in such a way that between 0.5 and 1% of the cells were included in the region. The same region was then transposed to the corresponding stained sample to detect positive cells. Thus, each experimental condition was analyzed by measuring the number of cells in a region determined based on background fluorescence.

Analysis of Affymetrix data

U74A microarrays (Affymetrix), which contain 12,000 known genes and expressed sequence tags (ESTs), were used. Approximately 50% of the genes on the array were expressed in both the untreated wild-type and p130/p107-null cells. Genes repressed in a p130/p107-dependent manner were identified as follows: first, we included only those genes repressed by at least 2-fold in the wild-type cells. To find genes regulated similarly to *cdk1*, we excluded all genes that were induced more than 1.5-fold in the p130/p107-null cells. Next we normalized each gene to correct for differences in basal expression in the two cell types. Finally, we retained in the data set only those genes that showed a 2-fold difference in the normalized, treated expression levels between the cell types. The genes that met these criteria for both the 12- and 24-hour time points are presented in Table 1.

Results

p130/p107-dependent repression of cell-cycle regulatory genes

Treatment of mouse embryo fibroblasts (MEFs) lacking p130

and p107 with adriamycin does not cause the downregulation of CDK1, unlike the response of wild-type MEFs, in which CDK1 protein is downregulated 24 hours after adding adriamycin (Taylor et al., 2001). Affymetrix microarrays were used to search for additional genes that required p130 and p107 to be repressed by DNA damage. We compared MEFs from normal mice with those from mice in which both p130 and p107 had been eliminated by homologous recombination (Hurford et al., 1997). Cells were treated with adriamycin for 12 or 24 hours and mRNA was analyzed, using arrays containing 12,000 genes and ESTs.

We identified a group of genes that, similarly to *cdk1*, were repressed in wild-type cells but not in p130/p107-null cells (Table 1). The products of many such genes are required either for entry into mitosis (e.g. CDK1, Cyclin B1, PLK1) (Pines, 1995; Toyoshima-Morimoto et al., 2001) or for progress through mitosis (e.g. SMC4L1 for chromatin condensation, STATHMIN for spindle formation, and PRC1 and RB6K for cytokinesis) (Fontijn et al., 2001; Hirano, 2000; Mistry and Atweh, 2002; Mollinari et al., 2002) (Table 1). We also found that many genes whose products are involved in DNA synthesis were repressed better in the presence of p130 and p107, including components required at origins of replication (MCM3, 4 and 5) (Tye, 1999) and enzymes that provide substrates for DNA synthesis (DCK and RR-R2; Momparler and Fischer, 1968; Reichard, 2002) (Table 1). p130 and p107 are also required to repress several genes whose products are required for the spindle checkpoint. Fourteen genes that encode proteins with various known functions (e.g. signal transduction, chromatin architecture, RNA splicing, DNA damage signaling) and 10 with poorly defined functions were also repressed more efficiently in cells containing p130 and p107 (data not shown).

Repression is not secondary to alterations in cell-cycle distribution

RB-family proteins are needed for G1 arrest in response to DNA damage (Harrington et al., 1998). Also, some of the genes we identified are poorly expressed during G1, with increased expression in late S phase and G2 (Zwicker et al., 1995). Thus, the changes in gene expression that we have observed might have been caused by differences in cell-cycle distribution between wild-type and p130/p107-null cells. The cell-cycle distributions of untreated wild-type and p130/p107-null MEFs were very similar, suggesting that there were no gross differences in proliferative ability (Fig. 1). Twelve hours after adding adriamycin, S-phase cells were reduced in wild-type, but not p130/p107-null cells (Fig. 1). By 24 hours after adding adriamycin, both wild-type and p130/p107-null cells showed reduced numbers in S phase. Importantly, the numbers of wild-type and p130/p107-null cells in G2/M were very similar, suggesting that the changes in gene expression that we have observed are not secondary to alterations in cell-cycle distribution in the p130/p107-null cells (Fig. 1). Although there were approximately twice as many p130/p107-null cells in S phase after treatment, the total number of cells in S phase was low (~10%) and thus could not have contributed significantly to the observed levels of gene expression.

We used northern analysis to assess the levels of some of the mRNAs identified in the array experiment. Wild-type MEFs treated with adriamycin showed repression of *kif4a*, *survivin*, *ect2* and *aurora-a*, whereas these genes were not efficiently repressed in MEFs lacking p130 and p107 (Fig. 2A). Analysis of northern transfers using a phosphorImager indicated that several additional genes were repressed better in the wild-type cells (*aurora-a*, 8.8-fold; *bub1*, 2.1-fold; *tacc3*, 2.6-fold; and *ect2*, 1.5-fold) than in the p130/p107-null MEFs (*aurora-a*, 1.5-fold; *bub1*, 0.6-fold; *tacc3*, 0.7-fold; and *ect2*, 0.8-fold). Thus, when DNA is damaged, p130, p107, or both mediate downregulation of a number of genes involved in mitosis.

Table 1. Genes repressed in a p130/p107-dependent manner

	Accession number	Fold repression*			
		12 hours ADR		24 hours ADR	
		KO†	WT†	KO	WT
Mitosis					
rb6k	AV059766	–2.1	***–37***‡	–3.1	***–38***
prc1	AA856349	–2	***–31***	–2.8	***–33***
topIIa	U01915	–1.7	***–22***	–4.1	***–24***
plk1	U01063	–2.8	***–18***	–4.1	***–23***
ki67	X82786	–2.2	–8.7	–4.9	***–20***
cdk1	M38724	–1.6	–6.9	–2.8	–18
knsl5	AI591702	–1.6	–9.6	–8.6	***–17***
cycB1	X64713	–1.3	***–19***	–2.1	–14
aurora-a	U80932	–1.6	–7.5	–2.5	***–13***
ect2	L11316	–1.2	–5.9	–1.3	–10
smc2l1	U42385	–1.1	–5.7	–1.2	–9.3
anilin	AW123269	1.1	–5.1	1.2	–8.3
survivin	AB013819	–1.2	–4.5	–2.8	–9.4
knsl1	AJ223293	–1.5	***–11***	–2.7	–8.9
cdc25c	L16926	1	–4.2	–1.5	***–8.8***
tacc3	AW209238	1.2	–3.6	–1.1	***–7.2***
stathmin	AI838080	–1.4	–3.7	–2	–5.9
kif4a	D12646	–1.3	–5.7	–1.7	–5.1
mcak	AA007891	–1.5	–4.7	–2	–4.9
cks2	AA681998	–1.1	–4.2	–1	–4.8
cycB2	X66032	–1.3	–3.5	–2.3	–4.8
sak-b	L29480	–1	–4.6	1.1	–4
cks1	AB025409	–1.1	–3.1	–1.1	–3.8
smc4l1	AA032310	–1.3	–2.9	–1.3	–3
Spindle checkpoint					
bub1	AF002823	–1.2	***–9.9***	–2.1	***–10***
p55cdc	AW061324	–1.3	–4	–1.9	–9.1
mad2	U83902	–1.1	–4.9	–1.4	–7.2
DNA synthesis					
mcm5	D26090	–2.6	–6.7	–2.9	***–34***
mcm3	X62154	–1.6	–4.3	–2	***–12***
topbp1	AI843655	–1.2	–3.3	–1.2	–6.4
rr-r2	M14223	1.3	–2.1	–2.3	–5.2
p58 primase	D13545	–1.2	***–3.6***	–2	***–5.1***
mcm4	D26089	–1.4	–3.2	–1.4	–4.8
fen1	L26320	–1.4	–4.1	–1.4	–4.2
rpa3/rf-a	AI848299	–1.1	***–6.8***	–1.2	–3.9
dck	X77731	1.3	–2.3	–1.3	–3.6
rpa1	AI843650	–1.3	–3.3	–1.6	–3.1
Novel	10 genes				
Misc.	14 genes				

*Fold repression at 12 and 24 hours after adding adriamycin (ADR; 345 nM) is shown. Genes on the list showed at least a twofold reduction in response to adriamycin, and at least a twofold change relative to the p130/p107-null cells at both times.

†KO, knockout; WT, wild type.

‡Numbers in bold italics are estimates (owing to very low expression in treated sample).

Repression of p130/p107 target genes by overexpressed p53

The p130/p107-dependent repression observed in our experiments was probably triggered by p53, which upregulated p21/WAF1, thereby inhibiting CDK activity and causing the formation of repressor complexes containing p130/p107 and E2F. Experiments with a mouse cell line containing a temperature-sensitive mutant of p53 were consistent with this idea. The mutant p53 protein used has wild-type transcriptional activation functions at 32°C but is defective at 37°C (Martinez et al., 1991). We analyzed several genes that were repressed in a p130/p107-dependent manner in response to DNA damage. A temperature shift to 32°C caused the downregulation of all genes tested (Fig. 2B). These results show that overexpression of functional p53 without damage-induced modification is sufficient to downregulate a number of genes that are repressed by p130 and p107. Our experiments with several human cell lines are also consistent with this idea (see below). To gain additional insight into the mechanism of repression, we carried out a more detailed analysis of one of the repressed genes. PLK1 is of particular interest because it is upregulated in many different types of tumors and controls multiple mitotic processes (Dai et al., 2003; Wolf et al., 2000). Our experiments with the temperature-sensitive p53 provided direct evidence that *plk1* is repressed in response to p53 overexpression (Fig. 2B). In addition, previous work has shown that *plk1* is downregulated in cells expressing high levels of p21/WAF1 (Chang et al., 2002; Zhu et al., 2002) and that the CDE/CHR elements were particularly important for p21-mediated repression. When *plk1* repression was examined in the p130/p107-null MEFs, it was noted that, although delayed, *plk1* was efficiently repressed (Fig. 3A). To determine whether loss of all RB-family members could abrogate *plk1* repression, we examined p130/p107/p105RB triple-knockout (TKO) MEFs (Sage et al., 2000). Treatment of the TKO cells with adriamycin resulted in the loss of nearly all *plk1* repression when normalized for loading (Fig. 3B). There is a 10% reduction in *plk1* levels at 18 hours, which recovers at later time points. These fluctuations might be due to slight

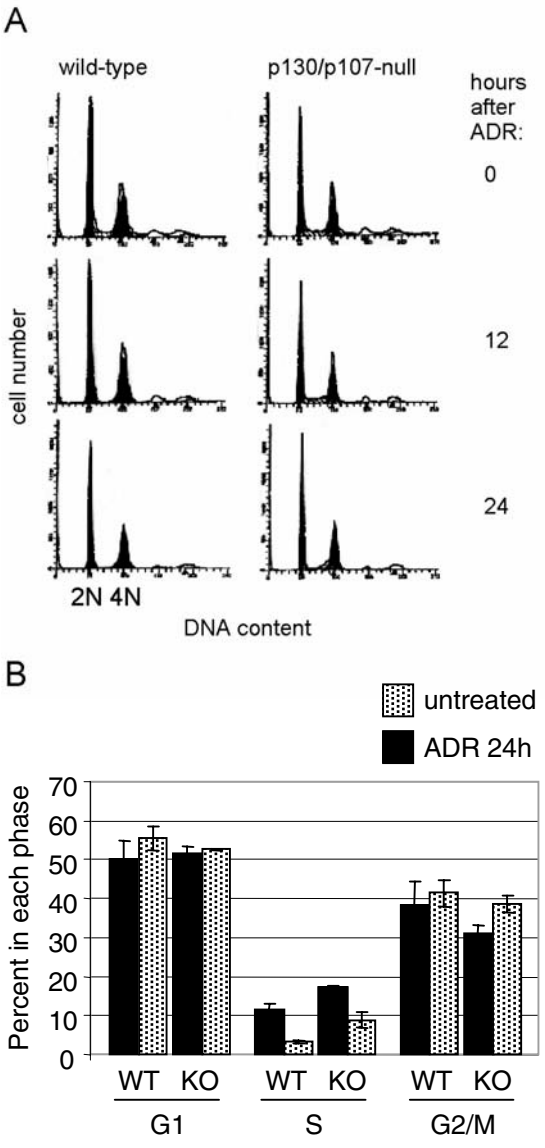

Fig. 1. Cell-cycle distribution of MEFs. Cells were treated for 12 or 24 hours with adriamycin (345 nM) and fixed. The DNA content of propidium iodide-stained cells was determined by FACS. (A) Flow cytometry profiles of MEFs treated with adriamycin. (B) Percentage of MEFs in different phases of the cell cycle. Standard errors are shown.

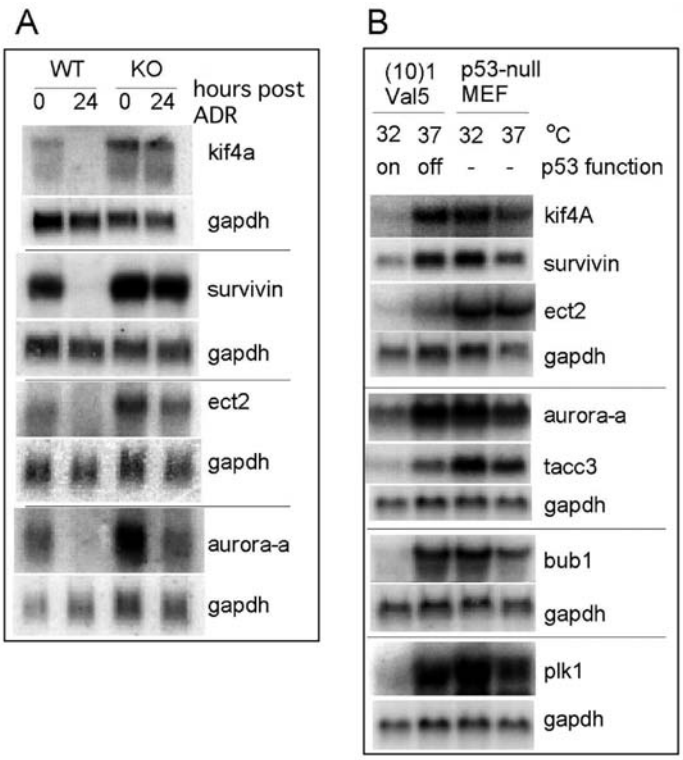

Fig. 2. Analysis of gene expression. Total RNA was analyzed by northern blot. Transfers were stripped and probed for the expression of *gapdh* to assess loading. Results from two representative experiments are shown. (A) Gene expression in wild-type and p130/p107-null MEFs. MEFs were treated with adriamycin (345 nM) and northern blots were analyzed with the probes indicated. (B) Repression by p53. The effect of p53 was determined using (10)1 Val5 cells with a temperature-sensitive p53. p53-null MEFs were used as a control.

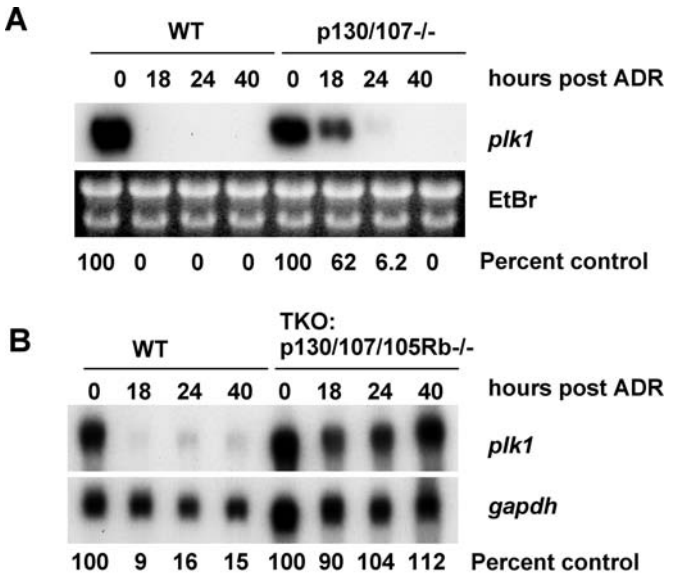

Fig. 3. The role of the RB family in the repression of *plk1*. MEFs were exposed to adriamycin (ADR, 345 nM) and total RNA was analyzed by northern blot. (A) *plk1* mRNA expression in wild-type and p130/p107-null MEFs. To indicate loading, the gel is shown stained with ethidium bromide before transfer. (B) *plk1* mRNA expression in cells lacking all three RB-family proteins (triple-knockout, TKO cells). The transfer was stripped and probed for the expression of *gapdh* to assess loading.

differences in cell-cycle distribution as the TKO cells progress through S phase and into G2 in the presence of the drug (see below).

RB-family proteins are required for damage-induced G2 arrest

Since we predicted that the repression of genes required for G2 and M is an important part of the mechanism that blocks cells from entering mitosis in response to stress, we analyzed the cell-cycle responses to DNA damage in the TKO MEFs. Interestingly, in TKO cells, the response to DNA damage caused either by adriamycin or etoposide was altered dramatically. Wild-type cells were gradually blocked with either a 2N or 4N content of DNA when exposed to etoposide (Fig. 4A). Visual examination indicated that there were very few mitotic cells, suggesting that the cells with 4N DNA were in G2 (data not shown). By contrast, TKO cells appeared to progress slowly through S phase and failed to accumulate with a 4N content of DNA (Fig. 4A,B). The accumulation of wild-type but not RB-family-null cells with a 4N content of DNA was also observed in response to adriamycin (Fig. 4C). Thus, the RB family appears to play a crucial role in the block to mitotic entry that occurs in response to DNA damage. Interestingly, many more TKO cells than wild-type cells acquired a sub-G1 content of DNA when exposed to etoposide or adriamycin, suggesting that the loss of RB proteins sensitizes cells to killing by this drug (Fig. 4A and data not shown).

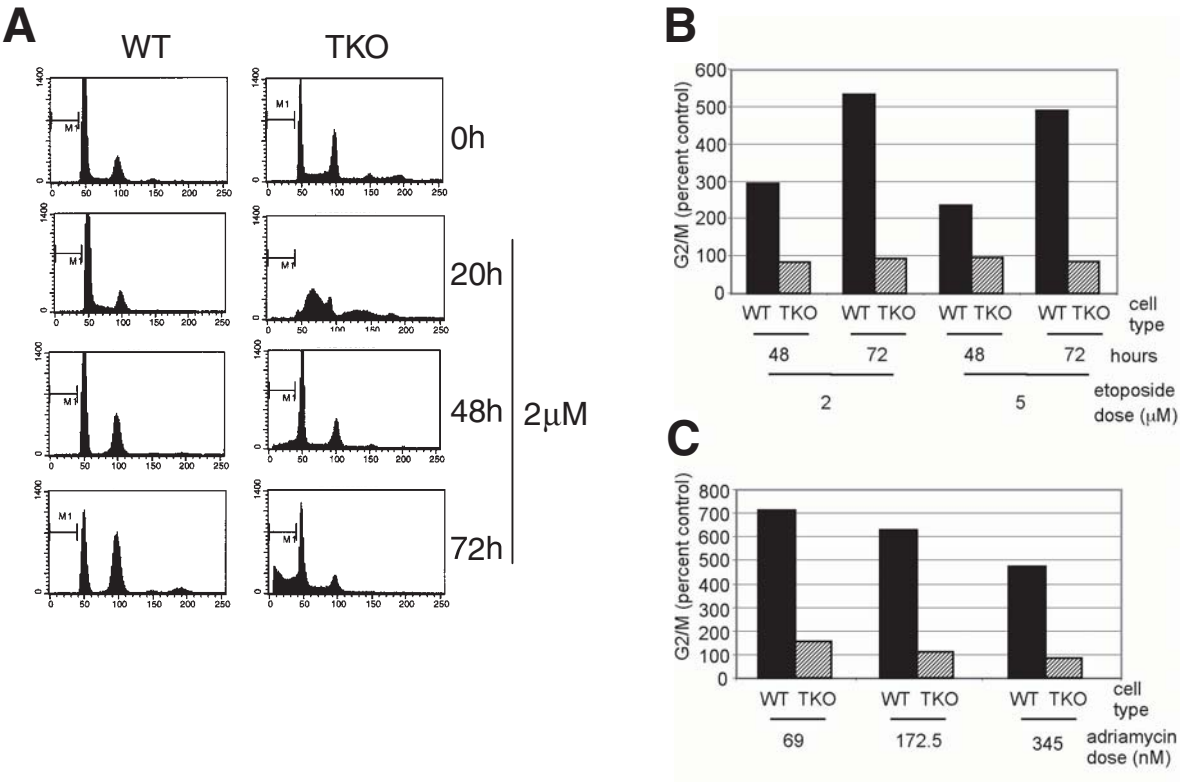

Fig. 4. Cell-cycle distribution of cells lacking RB proteins in response to DNA damage. Primary MEFs were treated with etoposide or adriamycin, and cell-cycle distributions were determined using FACS. (A) Effect of etoposide on wild-type (WT) and p130/p107/p105RB-null (triple-knockout, TKO) MEFs. Histograms of DNA content are shown. (B and C) Effect of etoposide and adriamycin on G2 arrest. The percentage of cells with a 4N content of DNA in treated samples is shown relative to the percentage in untreated wild-type or TKO MEFs. In (C), the cells were exposed to adriamycin for 72 hours. Results from two independent experiments are shown.

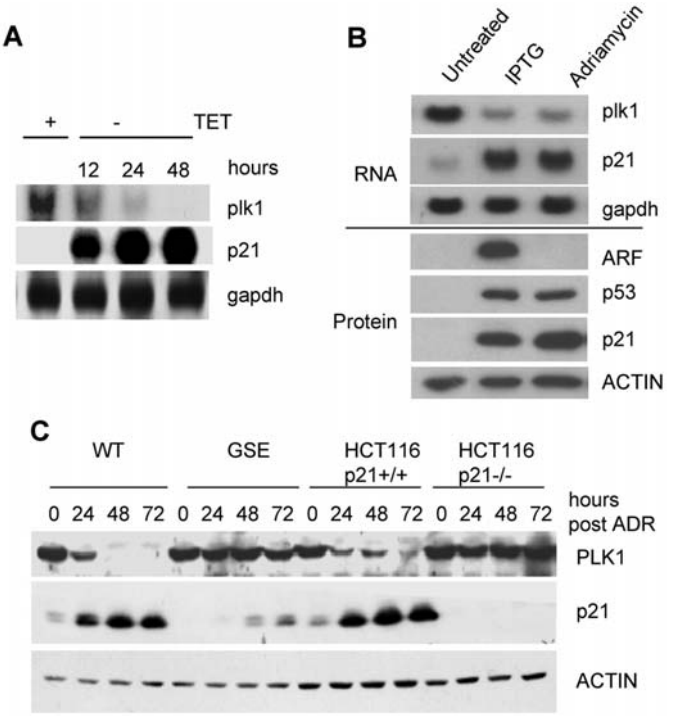

Fig. 5. Analysis of PLK1 expression in response to p53 signaling. The levels of PLK1 protein and mRNA were measured using western and northern blots, respectively. To assess loading, northern and western blots were stripped and reprobed for the expression of *gapdh* (northern) or actin (western). (A) Expression of *plk1* and *p21/waf1* mRNAs in response to the overexpression of p53. TR9-7 cells containing a tetracycline-regulated p53 were incubated in the presence (p53 off) or absence (p53 on) of tetracycline. (B) The effects of p53 induced by DNA damage or high levels of p14ARF. NARF2 cells containing IPTG-inducible p14ARF were incubated in the presence of IPTG for 24 hours or in the presence of adriamycin (345 nM) for 24 hours without adding IPTG. (C) The role of p53 and p21/WAF1 in the downregulation of the PLK1 protein in response to DNA damage. HT1080 cells with (GSE) and without (WT) the dominant-negative p53 fragment GSE56 were analyzed, as well as HCT116 cells in which *p21/waf1* was deleted and the corresponding parental cells. The cells were exposed to adriamycin (345 nM), and the total protein was extracted and analyzed by western blot.

The role of p53, p21/WAF1 and the RB family in the repression of *plk1*

Our examination of *plk1* repression in a number of human cell systems consistently implicated the signaling cascade from p53 and p21/WAF1 to CDK inhibition and ultimately RB-family-mediated repression. Using human cell lines expressing inducible p53 (TR9-7) or p14ARF (NARF2), we observed efficient repression of *plk1* upon p53 overexpression or adriamycin-induced p53 stabilization (Fig. 5A,B). We have previously characterized the TR9-7 cell line, which shows high levels of p53 expression after removal of tetracycline (Agarwal et al., 1995). The induction of p53 activity was confirmed by the induction of p21/WAF1 (Fig. 5A). NARF2 cells have been previously described (Stott et al., 1998). The use of the NARF2 cells parallels the effects of oncogene activation and demonstrates an additional signaling cascade that results in the repression of *plk1*. This observation rules out the need for phosphorylation on many of the N-terminal serine residues as p53 induced in TR97 and NARF2 cells under non-damage conditions is undetectably phosphorylated at serines 6, 9, 15, 20 and 37 in contrast to p53 stabilized by adriamycin treatment (Jackson et al., 2004).

Next, HT1080 cells, which contain wild-type p53 (WT cells), and a derivative expressing a dominant-negative p53 protein (GSE cells) (Clifford et al., 2003), were examined. Exposure of GSE cells to adriamycin did not cause the downregulation of PLK1, unlike WT cells, in which PLK1 was downregulated (Fig. 5C). Finally, PLK1 protein levels were examined in HCT116 cells lacking p21/WAF1. Unlike in the parental cells, in which PLK1 was downregulated, p21/WAF1-null cells did not downregulate the levels of PLK1 protein (Fig. 5C).

The results presented thus far indicate that the loss of PLK1 expression in response to DNA damage requires p53, p21/WAF1 and the RB family. To examine further the requirements of *plk1* gene repression, we created NARF2 cell lines in which p53 was inactivated by short-hairpin RNA directed at p53 (shp53), by a dominant-negative form of p53 (GSE56) or by overexpression of Hdm2. In all three instances, inactivation of p53 resulted in the inability of p14ARF to downregulate PLK1 protein levels (Fig. 6). We next examined effectors downstream of p21/WAF1, including Cyclin E and Cyclin D1/CDK2. Upon retroviral delivery of either Cyclin E or a fusion between CDK2 and Cyclin D1, p14ARF-mediated PLK1 repression was again inhibited (Fig. 6). The CDK2/Cyclin D1 fusion mimics a complex between the two proteins that has been observed upon overexpression of Cyclin D1 in certain cancers (Chytil et al., 2004). The fusion is capable of phosphorylating RB, suggesting that the downregulation of PLK1 we have observed is mediated by the RB/E2F system (Chytil et al., 2004). Finally, expression of HPV-E7, which is known to inactivate the RB-family members, also prevented PLK1 downregulation by the p14ARF/p53 pathway (Fig. 6).

Features present in the *plk1* promoter suggest that the p53/RB pathway causes repression at the transcriptional level.

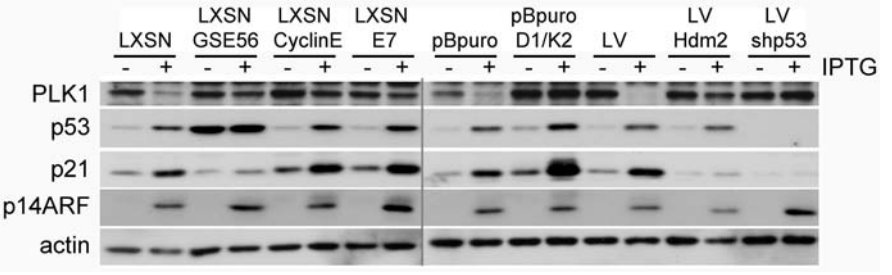

Fig. 6. Requirements for PLK1 downregulation in NARF2 cells. The expression levels of the indicated proteins were determined using western blot with extracts of NARF2 cells infected with viruses encoding the indicated genes. LXSN, pBabe-Puro and LV serve as controls. The NARF2 populations were exposed to IPTG for 48 hours and analyzed using antibodies to PLK1, p53, p21/WAF1, p14ARF and actin.

Table 2. Genes with CDE/CHR elements

Gene		CDE	Spacer	CHR	Repression‡	Position§
		G G C G G / C T C		T T T G A A		
cdk1	TTTAG	C G C G G	TGAG	T T T G A A*	↓	–8
cdc25c	GGGCT	G G C G G	AAGG	T T T G A A*	↓	–2
cyclin b2	TCAGC	G G C G C	GGTA	T T T G A A*	↓	+2
p130	TTCTT	C G C C G		T T T G A A*		+13
cenp-a	AAGTG	G G C G G	AGCGAGCGA	T T T G A A*	↓	–14
b-myb	CGCTT	G G C G G	GAGA	T **a g** G A A*		–32
rb6k	ACGCA	G C G C G	TAA	T T T **a** A A*	↓	–31
aurora-a	GTGTG	C G C C C		T T **a** A A *	↓	–34
cyclin a2	ATAGT	C G C G G	GATA	**c** T T G A A*	↓	–21
topo IIα	CTTCG	G G C G G	GCTA	**a a g** G A A*	↓	–37
survivin	CATTA	**a c** C G C	CAGA	T T T G A A*	↓	+24
plk1	TTCCC	**a** G C G C	CGCG	T T T G A A*	↓	+11
tk	CAGCA	C G C **c** G	TGG	T T T **a** A A*	↓	–16
cyclin b1	GGAGC	**a** G T G C	GGGG	T T T **a** A A*	↓	–17
p55cdc		**a** G C G G	AGAG	T T T **a** A **g***	↓	–12
mcak	GCTTG	C G C G C	GGGA	T T T **a** A A†	↓	–40
sak-b	CAAGC	G G C G G	GAGAT	T T T **c** A A†	↓	–11
kif4a	AACTT	G G C G G		T T **a** A A†	↓	–23
rr-r2	GCTCC	C G C G C	TGCG	**c** T T G A A†	↓	–38
rr-r1	GCGGG	C G C G G	GAAGGGGA	T T T G **g** A†		+61
prc1	CGCCC	C G T G G	CGCGG	T T T G A A†	↓	–55
knsl5	GGACG	G G C G C	**11 nt**	T T T G A A†	↓	–50

*Badie et al., 2000; Fajas et al., 2000; Fontijn et al., 2001; Lange-zu Dohna et al., 2000; Li and Altieri, 1999; Polager and Ginsberg, 2003; Tanaka et al., 2002; Uchiumi et al., 1997; Zwicker et al., 1995.

†Potential elements identified in this study.

‡Repressed by p130/p107 (see also Table 1).

§Position relative to the start of transcription.

Deviation from the consensus is underlined and bold.

For example, the *plk1* promoter contains a CDE/CHR element that is important for its repression by p21/WAF1 (Zhu et al., 2002) (Table 2). We used TR9-7 cells and isolated pools of cells transfected stably with *plk1* reporter constructs to test the importance of the CDE/CHR in regulating the promoter. Induction of p53 by removing tetracycline for 72 hours resulted in a 5.5-fold repression of the wild-type *plk1* promoter. Promoter constructs with mutations in either the CDE or the CHR were repressed less efficiently and this effect was statistically significant (mutant CDE: 3.3-fold repression, P=0.004; mutant CHR: 2.8-fold repression, P<0.001; data not shown). The wild-type *plk1* promoter was also repressed 7.1-fold when the TR9-7 pools were incubated in the presence of tetracycline to downregulate the exogenous p53 and then infected with an adenovirus to overexpress p21/WAF1. In this type of experiment, mutant promoters were less efficiently repressed (mutant CDE: 2.9-fold repression, P=0.002; mutant CHR: 1.8-fold repression, P<0.001; data not shown). Finally, the wild-type *plk1* promoter was induced 2.7-fold when the TR9-7 pools were infected with an adenovirus that overexpresses Cyclin E. Mutant promoters were less efficiently induced (mutant CDE: 1.8-fold repression, P=0.009; mutant CHR: 1.8-fold repression, P=0.006; data not shown). Although mutation of the CDE/CHR elements reduced the effects of p53, p21/WAF1 and Cyclin E, the effects were not eliminated. It is possible that other factors are important for the regulation of the *plk1* promoter.

In addition to the *plk1* promoter, we found that a number of genes containing CDE/CHR elements were repressed more efficiently by adriamycin in the presence of p130 and p107 (Table 2). A similar correlation between promoters with CDE/CHR elements and repression by p53 has been observed (Badie et al., 2000). One possibility is that the CDE/CHR mediates the DNA-damage-induced repression of multiple genes by p130 and p107. However, there are probably additional mechanisms at play in these various promoters. Together, our results are most consistent with the operation of a pathway that initiates upstream of p53 (with DNA damage or p14ARF), passes through p21/WAF1, CDKs and the RB family of proteins, and ultimately leads to repression of *plk1* transcription. Subsequently, loss of the function of this pathway in cells lacking RB-family proteins results in the diminished ability to generate and maintain a G2/M arrest.

p53 drives G2 cells into a non-proliferative state

p53 and the RB-family proteins are needed to block cells stably from entering mitosis when their DNA is damaged (Bunz et al., 1998; Clifford et al., 2003; Polager and Ginsberg, 2003) (Fig. 4). Interestingly, p53, p130 and p107 are involved in repressing many genes that are normally needed to progress through mitosis. Some of these repressed genes were previously shown to be induced by E2F (Ishida et al., 2001). The global reprogramming of gene expression that occurs in response to DNA damage parallels the repression of E2F targets that occurs when cells exit the cell cycle during terminal differentiation, senescence, or entry into G0 when deprived of growth factors (Furukawa et al., 1990; Ishida et al., 2001; Narita et al., 2003; Reichel et al., 1987). These similarities suggested that, instead of causing cells to simply arrest in G2, p53 and RB cause cells to exit the cell cycle from G2, thus

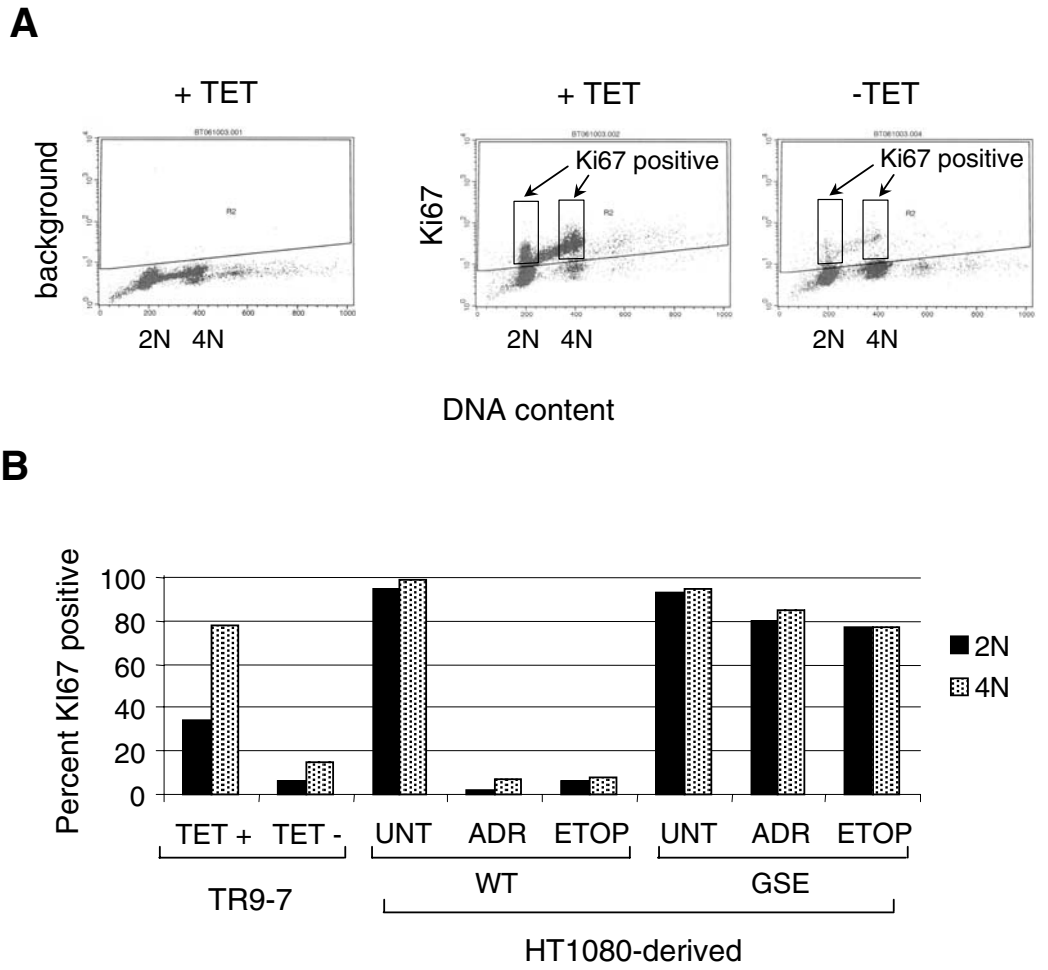

Fig. 7. FACS analysis of KI67 and DNA content in HT1080-derived cells. Flow cytometry was used to measure KI67 expression and DNA content simultaneously. (A) Representative FACScans of TR9-7 cells. TR9-7 cells were incubated in the presence or absence of tetracycline for 72 hours to induce p53. Cells were fixed and incubated with antibodies to KI67, followed by a secondary antibody labeled with FITC. Cells were also stained with propidium iodide for analysis of DNA content. Cells with 2N or 4N DNA content showing positive staining for KI67 are shown. Controls for each treatment were treated identically, except that the antibody to KI67 was not added. (B) Quantitation of the percentage of cells that are positive for KI67. TR9-7 cells were treated as described in (A). WT and GSE cells were left untreated or exposed to etoposide (10 μM) or adriamycin (345 nM) for 72 hours. KI67 and DNA content were measured as described in (A). The percentage of cells with 2N or 4N DNA content showing positive staining for KI67 is shown. The results represent two independent experiments.

ensuring a stable block to mitotic entry (Baus et al., 2003). To investigate this idea, we analyzed a well-known proliferation marker, KI67, since withdrawal from the cell cycle is strongly correlated with the loss of KI67 expression (Endl and Gerdes, 2000).

We used flow cytometry to analyze cells stained for both KI67 and DNA content. First, we analyzed TR9-7 cells with tetracycline-regulated p53. TR9-7 cells with a 2N content of DNA that were growing in the presence of tetracycline showed two populations, one with high levels of KI67 and another with background levels of KI67 staining (Figs 7A,B). Most cells (79%) with a 4N content of DNA expressed a high level of KI67, although a subpopulation (21%) with low levels was also observed. When p53 was induced by removing tetracycline, only 6.1% of cells with 2N DNA and 15% of the cells with 4N DNA showed KI67 staining above background levels. These results indicate that p53 on its own can suppress the expression of KI67 in cells with either a 2N or a 4N content of DNA. Next, the HT1080-derived GSE and WT cells were incubated in the presence or absence of etoposide or adriamycin to induce p53. More than 95% of untreated WT and GSE cells with either a 2N or a 4N content of DNA were positive for KI67 using this method (Fig. 7B). We observed that a very small percentage (8%) of WT cells with a 4N content of DNA were positive for KI67 after exposure to etoposide or adriamycin (Fig. 7B). By contrast, KI67 was not efficiently downregulated in GSE cells exposed to etoposide or adriamycin (Fig. 7B). Thus, the loss of KI67 in cells with a 2N or 4N content of DNA is a result of p53-dependent signaling. One interpretation of these results is that, in response to p53, cells exit the cell cycle from G2. The arrested cells have a 4N content of DNA but are not in mitosis (based on several criteria: absence of chromosome condensation; extensive attachment to the substratum; and undetectable levels of histone H3 phosphorylated on serine 10, which is a marker for mitosis) (Clifford et al., 2003, and data not shown). Unlike cells progressing through G2 during rapid proliferation, cells arrested in G2 by p53-dependent signaling lack many of the proteins required for mitosis (Table 1). Cells arrested by p53 signaling are viable, as at least 90% of them exclude Trypan Blue after arrest for 7 days (data not shown) and they can re-express cell-cycle proteins when p53 levels are lowered (our unpublished work).

Discussion

G2 arrest in response to spontaneous DNA damage blocks the segregation of damaged DNA and helps to avoid mutations that favor the development of neoplasia. G2 arrest also occurs in response to high levels of DNA damage induced by chemotherapeutic agents, and the ability to maintain G2 arrest is an important factor in the toxicity of these agents to tumor cells (Fan et al., 1995). Multiple p53-dependent and

-independent pathways ensure long-term G2 arrest when DNA is damaged (Taylor and Stark, 2001). One pathway that initiates G2 arrest involves ATM, ATR, CHK1 and CHK2, which rapidly block the ability of CDC25C to dephosphorylate and activate CDK1 (Fig. 8). Human colon tumor cells lacking either p53 or p21/WAF1 did not arrest stably in G2 in response to DNA damage (Bunz et al., 1998). Similarly, TKO cells lacking all three RB proteins did not accumulate with a 4N content of DNA in response to adriamycin or etoposide, suggesting that there were major defects in G2 arrest (Fig. 4). Since adriamycin and etoposide are frequently used in the treatment of cancer, and many cancer cells have a defective RB pathway, this might indicate that defective G2 arrest may be important in the clinical response to these agents. Adriamycin and etoposide have multiple cellular effects in addition to inducing DNA damage, which include inhibiting topoisomerase II (Larsen et al., 2003). Inhibiting topoisomerase II has been shown to activate a decatenation checkpoint that blocks progression through G2 and M when chromosomes are not decatenated (Downes et al., 1994). Thus, several checkpoints are probably activated by these agents, including the DNA damage G2 checkpoint and the decatenation checkpoint. At this point, we cannot determine if the RB family plays a specific role in these checkpoints, although G2 arrest is clearly abrogated in the null cells.

Our results with the TKO cells suggest that p53 and p21/WAF1 maintain G2 arrest in part by favoring the dephosphorylation of RB-family proteins, which then repress the genes needed for mitosis. RB binds to proteins other than E2F, including MDM2 and ABL (Welch and Wang, 1993; Xiao et al., 1995). Thus, it is possible that mis-regulation of E2F targets is not responsible for the failure of TKO cells to accumulate in G2 in response to DNA damage. However, overexpression of a dominant-negative version of E2F1 has been shown to abrogate the repression of *stathmin* and *aim1/aurora B*, and to overcome G2 arrest in response to DNA damage (Polager and Ginsberg, 2003). Therefore, repression by RB-family proteins appears to be an important factor in G2 arrest. Our results are consistent with an early paper in which overexpression of Rb was found to cause cells to accumulate in G2/M (Karantza et al., 1993). Also, G2 arrest in response to adriamycin was found to be defective upon overexpression of the E7 protein from HPV (Flatt et al., 2000). E7 can bind to several cellular proteins in addition to the Rb-family proteins, and it is possible that these other proteins might be important in the effects of E7 on G2 arrest. Our results using the TKO cells are, to our knowledge, the first demonstration that removing just Rb, p107 and p130 is sufficient to interfere with G2 arrest. Our results also suggest that the important factor in the effect of E7 is indeed its ability to inactivate the Rb family. The results of our cell-cycle studies with TKO cells are somewhat different to those previously published (Sage et al., 2000). In the earlier paper, etoposide was not investigated, and adriamycin was shown to cause a large accumulation of cells in G2. However, these measurements were carried out at 24 hours after adding adriamycin, whereas our studies focused on a longer time point (72 hours). This suggests that the G2 arrest cannot be stably maintained. Loss of all three RB proteins also sensitized cells to killing by etoposide and adriamycin. E2F1, 2 and 3 have been shown to induce apoptosis (Chen et al., 2000; Ziebold et al., 2001) and gel shift analysis has indicated

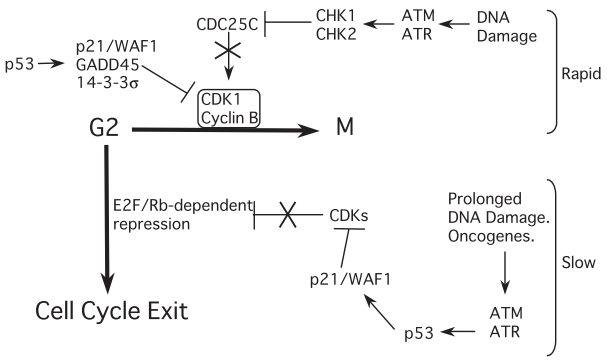

Fig. 8. Model of transcriptional repression and G2 arrest. A pathway involving ATM, ATR, CHK1 and CHK2 inactivates CDC25C in response to DNA damage, leaving the CDK1/Cyclin B1 complex inactive. p53 also helps to inactivate CDK1 by inducing p21/WAF1, GADD45 and 14-3-3σ. Cells blocked by these pathways are in G2, with high levels of all the proteins needed for mitosis. The block can be overcome by simple re-activation of CDK1. p53 also utilizes p21/WAF1 to block the phosphorylation of RB-family proteins by CDKs other than CDK1. Hypophosphorylated RB bound to E2F then downregulates the levels of proteins needed for mitosis. The similarity of this effect to the changes in gene expression that occur during G0 suggests that the p53/RB-dependent pathway drives cells out of the cell cycle from G2.

that TKO cells contain more free E2F than wild-type cells (Sage et al., 2000). In their unbound state in the absence of RB proteins, E2F may be responsible for the sensitization we have observed.

Several of the genes that we found to be downregulated by p53 and the RB-family proteins are known to be induced by E2F (Ishida et al., 2001). Interestingly, a reprogramming of gene expression similar to the one that occurs in response to p53 occurs when cells exit the cell cycle, for example, during starvation for growth factors (Furukawa et al., 1990; Ishida et al., 2001). The p53-dependent downregulation of KI67 in cells with a 4N content of DNA also suggests that p53 causes G2 cells to exit the cell cycle and a similar conclusion has been reached by Baus et al. (Baus et al., 2003). Once the repression program is set in motion, it may be difficult to re-express all the genes necessary with proper kinetics to re-enter the cell cycle (Chang et al., 2000). Ectopic expression of p21/WAF1 downregulates some of the same genes required for G2 and M that we have found to be downregulated by p53, p130 and p107 (Chang et al., 2000). It is likely that p21/WAF1 represses these genes by causing the dephosphorylation of RB-family proteins. When the expression of p21/WAF1 was turned off, some but not all of the repressed genes were re-expressed, resulting in cells that attempted mitosis but could not complete it (Chang et al., 2000). The particular genes that we identified as being repressed in a p130/p107-dependent manner overlap with genes identified in several recent microarray studies as genes that are modulated during the cell cycle. For example, many genes required for G2 and M are downregulated in response to ionizing radiation in Hela cervical carcinoma cells (Crawford and Piwnica-Worms, 2001). These effects may be independent of p53 as the p53 in Hela cells is targeted by the HPV E6 protein. Interestingly, irradiated samples had higher levels of p21/WAF1 than unirradiated controls, suggesting that the

changes in gene expression observed could have been influenced by p21/WAF1. The regulation of genes involved in G2 and M by the Rb/E2F system has been the subject of several studies. For example, many genes required for G2 and M are induced when E2F1 or E2F2 is overexpressed (Ishida et al., 2001). Also, gene expression in response to overexpression of Rb has been investigated, and overlap with the genes regulated in our study is evident (Markey et al., 2002). Also, some overlap with our gene list is evident in a recent study in which microarrays were used to analyze changes in basal gene expression levels upon knockout of Rb alone or p130 and p107 together (Black et al., 2003). To our knowledge, ours is the first analysis of how the loss of p130 and p107 affects the changes in gene expression that occur in response to DNA damage.

We have used NARF2 cells to investigate the reversibility of the arrest caused by p53, which was upregulated due to signaling from the inducible p14ARF locus. Although the repression of several genes including *plk1*, *cdk1*, *cyclin b1* and *ki67* is reversed upon removal of isopropyl-β-D-thiogalactopyranoside (IPTG), the cells were not able to divide (our unpublished work). Future work will address more precisely where in the cell cycle they are blocked. In agreement with our findings, Baus et al. have recently shown that RB-family proteins are hypophosphorylated in G2 cells exposed to bleomycin to damage DNA. In this state, they would be able to repress the genes that we have identified, thereby driving the cells out of the cell cycle. In their study, cell-cycle arrest induced by DNA damage was permanent (Baus et al., 2003). Our work implicates RB-family-dependent downregulation of a large number of proteins needed for the G2/M transition as an important mechanism that controls the exit of G2 cells from the cell cycle.

Our studies with an inducible p14ARF protein suggest that G2 arrest can occur in response to stimuli distinct from DNA damage. We have observed that inducing p14ARF by adding IPTG to NARF2 cells synchronously progressing through S phase caused approximately 70% of the cells to arrest with a 4N content of DNA (our unpublished work). Also, p53 induced by p14ARF in IPTG-treated NARF2 cells downregulated *plk1* as efficiently as p53 induced by DNA damage. p14ARF induced by activated oncogenes has been postulated to be a crucial mediator of a p53-dependent tumor suppressor function that eliminates cells when cell growth pathways are activated inappropriately (Sherr and Weber, 2000). Interestingly, activated RAS and high levels of MYC can cause cells to arrest with a 4N content of DNA (Felsher et al., 2000; Hirakawa and Ruley, 1988). Our results suggest that p53, induced by oncogenes or DNA damage, can drive G2 cells out of the cycle.

PLKs regulate several processes that are important for mitosis, and PLK1 is overexpressed in several types of human tumors (Dai et al., 2003; Wolf et al., 2000). We found that DNA damage causes the downregulation of PLK1 in a manner that depends on p53, p21/WAF1 and the RB family. Using an in vitro binding assay, we found that RB/E2F complexes can bind to the CDE/CHR of the *cdk1* promoter (Taylor et al., 2001). Also, both the CDE and CHR were required for optimal repression of *cdk1* in response to high levels of p53 (Taylor et al., 2001). In studies reported here, we found that full repression of the *plk1* promoter in response to p53 or p21/WAF1, or the full induction of the promoter by Cyclin E, depends on an intact CDE/CHR. In addition, downregulation of *plk1* mRNA and protein levels depends on the RB family. Interestingly, E2F4 was found to be associated with the *plk1* gene in experiments in which chromatin fragments bound to E2F4 were identified using chromatin immunoprecipitation followed by microarray analysis (Ren et al., 2002). These results are consistent with a model in which hypophosphorylated RB proteins associated with E2F factors bind to CDE/CHR elements to cause repression, although it is possible that other regions of the endogenous promoter may recruit E2F4. Mutation of the CDE/CHR elements did not completely abrogate the effects of p53, p21/WAF1 or Cyclin E, suggesting that other pathways might also regulate this promoter. Many of the genes that are repressed in a p130/p107-dependent manner in response to DNA damage also contain potential CDE/CHR elements. Whether all of the potential CDE/CHR elements mediate repression awaits more detailed analyses.

The G2/M transition is controlled by several pathways that might function at different times during the response to stress. The p53-dependent downregulation of proteins needed for mitosis requires several steps including the accumulation of p21/WAF1 protein, inhibition of CDK activity, accumulation of hypophosphorylated RB proteins, repression of E2F/RB target promoters and decay of the steady-state levels of the encoded proteins (Fig. 8). In this way, p53 drives G2 cells out of the cell cycle only after a significant delay, which might ensure that cells containing repairable damage do not permanently exit the cell cycle. Only cells that are delayed in G2 for longer times, perhaps owing to irreparable DNA damage, are driven out of the cell cycle, ensuring that they do not attempt to segregate damaged DNA.

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