protein interactions[10]. Thus, the role of TRIP-1 may involve a conformational change allowing recruitment of downstream proteins or its phosphorylation by RI in the RI–RII complex. The function of TRIP-1 may be analogous to GRB-2, which recruits signalling components to receptor tyrosine kinases.[19]

Considering the similarity in activities of activin and TGF-β and structures of their type II receptors, the lack of TRIP-1 association with activin RII suggests the existence of TRIP-1-related proteins for the related type II receptors. Thus, TRIP-1 may define a class of WD proteins involved in signalling by TGF-β superfamily members. Homology searches and polymerase chain reaction (PCR)-based cDNA cloning revealed a TRIP-1-related gene in *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. All three sequences are of similar length and have five WD repeats at corresponding positions and 39–46% amino-acid identity, including flanking regions between WD domains (Fig. 4*c*). These features suggest that these proteins are homologues. In *S. cerevisiae*, the induction of G1 arrest by α-factor[20] has similarities with the growth arrest by TGF-β. Studies of yeast TRIP-1 may shed light on the mechanism of TGF-β signal transduction. □

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Radiation-induced cell cycle arrest compromised by p21 deficiency

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THE protein p21 is a dual inhibitor of cyclin-dependent kinases[1–3] and proliferating-cell nuclear antigen (PCNA)[4], both of which are required for passage through the cell cycle. The *p21* gene is under the transcriptional control of p53 (ref. 5), suggesting that p21 might promote p53-dependent cell cycle arrest or apoptosis. p21 has also been implicated in cell senescence[6] and in cell-cycle withdrawal upon terminal differentiation[7–9]. Here we investigate the role of p21 in these processes using chimaeric mice composed partly of $p21^{-/-}$ and partly of $p21^{+/+}$ cells. Immunohistochemical studies of the $p21^{+/+}$ and $p21^{-/-}$ components of adult small intestine indicated that deletion of *p21* had no detectable effect on the migration-associated differentiation of the four principal intestinal epithelial cell lineages or on p53-dependent apoptosis following irradiation. However, $p21^{-/-}$ mouse embryo fibroblasts are impaired in their ability to undergo G1 arrest following DNA damage.

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To investigate the many proposed functions of p21, a null allele was generated in 129/Sv embryonic stem (ES) cells by replacing the *p21* coding sequence with a neomycin-resistance cassette (neo^r; Fig. 1*a–c*). Homozygous $p21^{-/-}$ ES cells were produced by subjecting two heterozygous $p21^{+/-}$ cell lines to selection in an increased concentration of G418. One $p21^{-/-}$ ES cell clone was recovered from each parental $p21^{+/-}$ line. p21 deficiency was verified by Southern blot analysis (Fig. 1*b*, *c*) and by immunoprecipitation of ES cell lysates with a p21 polyclonal antiserum (data not shown).

The small intestine was chosen as a model for assessing the role of p21 in proliferation, differentiation and cell death[10]. Studies of adult aggregation chimaeras indicate that each intestinal crypt is monoclonal whereas each villus receives cells from 2–3 surrounding crypts. Introduction of 129/Sv ES cells, such as those lacking p21, into normal B6 blastocysts results in B6↔129/Sv chimaeras that contain patches of B6 crypt-villus units and patches of 129/Sv crypt-villus units. These can be readily distinguished on the basis of differences in their ability to bind the lectin UEA-1 (ref. 11). In B6↔129/Sv chimaeras, proliferation was restricted to undifferentiated epithelial cells in both $p21^{+/+}$ and $p21^{-/-}$ crypts (Fig. 2*a–c*). Furthermore, multilabel immunohistochemical surveys using a well characterized panel of antibodies and lectins[11] revealed that loss of p21 had no detectable effects on the ability of the intestine to complete normal morphogenesis or to maintain the differentiation programs of four principal epithelial cell lineages (for example, see Fig. 2*f*). Low to moderate doses of γ-irradiation cause cell cycle arrest and p53-dependent apoptosis in crypts[12–14]. Irradiation caused the same 100–150-fold increase in the frequency of cell death in crypts composed either of $p21^{+/+}$ or of $p21^{-/-}$ cells (Fig. 2*g*, *h*), indicating that p21 is not required for p53-dependent cell death, at least in this system. $p21^{-/-}$ and $p21^{+/+}$ crypts also showed a similar reduction in 5-bromodeoxyuridine (BrdU)-positive cells following irradiation. Thus, radiation-induced cell cycle arrest occurred in undifferentiated intestinal epithelial cells regardless of p21 status (Fig. 2*d*, *e*). However, as cell cycle arrest in these cells has not been shown to require p53,

we examined the role of p21 in DNA-damage-induced G1 arrest in fibroblasts, which is p53 dependent.

Cultures of primary mouse embryo fibroblasts (MEFs) were prepared from B6↔129/Sv $p21^{-/-}$ chimaeras produced from two different $p21^{-/-}$ ES cell lines (p21 A and p21 B) and from B6↔129/Sv $p53^{-/-}$ chimaeras. MEFs were also prepared from wild-type embryos and from chimaeras generated from an ES cell line carrying a random integration of the *p21*-targeting vector. Initial MEF cultures were 30–70% 129/Sv (Fig. 3*a*). Highly pure MEF populations (>95% 129/Sv) were obtained following G418 selection (Fig. 3*a*). It should be noted that this enrichment required no more than two population doublings. The absence of p21 protein in the $p21^{-/-}$ MEFs was confirmed by immunoprecipitation (Fig. 3*b*, *c*), and the purity of p21-deficient cell populations was verified by immunofluorescence with a p21-specific antiserum (data not shown).

Immunoprecipitation with anti-CDC2 -CDK2 or -CDK6 antibodies showed that the pattern of cyclin–CDK complexes was indistinguishable in the $p21^{-/-}$ and $p53^{-/-}$ MEFs, although the $p21^{-/-}$ MEFs still contain wild-type p53, as judged by immunoprecipitation with conformation-specific anti-p53 antibodies (Fig. 3*d*). In these cells, p21 was absent from CDK2 and from CDC2 complexes, and CDK6 was primarily associated with p16 (Fig. 3*c*). In contrast to $p53^{-/-}$ MEFs, $p21^{-/-}$ cell populations did not show obvious differences in proliferation rates compared with normal controls (not shown).

The two $p21^{-/-}$ MEF populations, together with $p53^{-/-}$, random integration and wild-type controls, were treated with γ-radiation and subjected to cell-cycle analysis. The G1-phase radiation response was assessed by comparing the S-phase fraction in irradiated cells (at 18 hours after a dose of 5.5 Gy) to that in untreated cell populations. Wild-type cells and cells

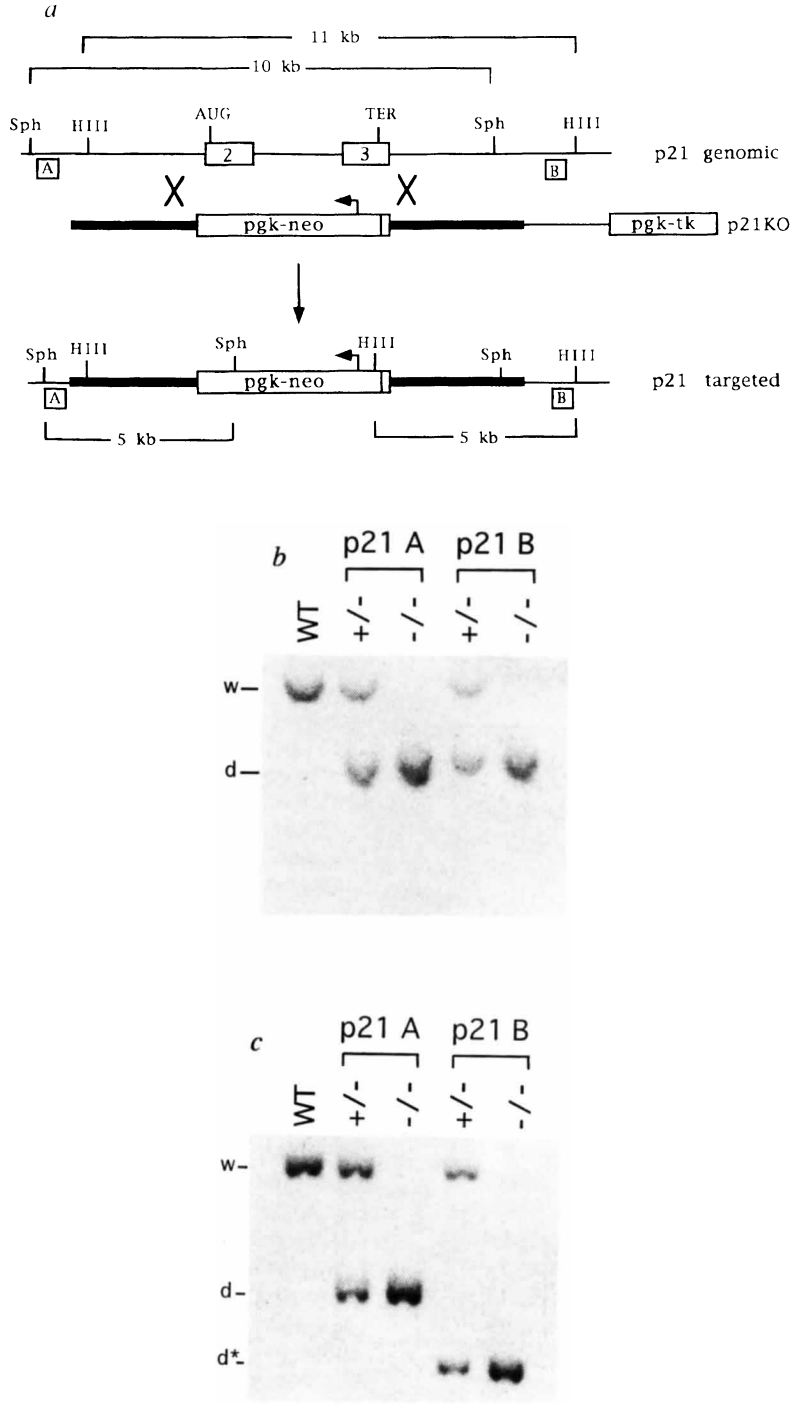


FIG. 1 p21 gene targeting in ES cells. *a*, Targeting scheme. The targeting vector, p21KO, contains ~8 kb of p21 genomic sequences (thick lines) flanking the pgk–neo expression cassette which is located in the opposite transcriptional orientation to p21. Recombination on the 5′-side of p21 was monitored by Southern blotting using an *Sph*I digest and probe A. The 3′ recombination junction was characterized by a *Hin*dIII digest of genomic DNA and probe B. Both probes lie outside the genomic regions present in the targeting vector. *b* and *c*, Southern blot analysis of ES cell clones. DNA from the parental D3 ES cells, two heterozygous mutant clones ($p21^{+/-}$ A and B) and two homozygous mutant clones that were derived from the heterozygous clones following exposure to increased G418 concentrations ($p21^{-/-}$ A and B) are shown. Using probe A on *Sph*I-digested genomic DNA (*b*), the $p21^{+/-}$ clones show a ~5 kb mutant-specific band (band 'd') in addition to the ~10-kb band (band 'w') corresponding to the wild-type *p21* allele. In the $p21^{-/-}$ cell lines the wild-type band is absent. At the 3′ recombination junction (*Hin*dIII-digested DNA and probe B, in c), $p21^{+/-}$A shows the expected mutant-specific band of ~5 kb (band d); the mutant band in clone B is ~3.6 kb (band d*). Extensive restriction mapping revealed that $p21^{+/-}$B contained a 1.4-kb deletion 3′ to exon 3 of p21. In both $p21^{-/-}$ lines, the band corresponding to the wild-type allele of p21 is again absent. Gene targeting was performed using D3 ES cells as described[19–21]. $p21^{-/-}$ ES cell clones were generated from $p21^{+/-}$ ES cells by selection with 1.8 mg ml^{-1} G418 (active weight) for 10–11 days.

carrying the random integration were substantially arrested in G1 following irradiation (Fig. 4*a*). The fraction of cells in S phase after irradiation was 39.5 or 39%, respectively, of that in non-irradiated cells. For $p53^{-/-}$ MEFs, the S-phase fraction after irradiation was 84.5% of the value in untreated cells, a result that is consistent with previous reports demonstrating that p53 is required for G1 cell-cycle arrest following DNA damage in fibroblasts[15]. The MEF populations derived from the two $p21^{-/-}$ ES cell clones showed an intermediate irradiation response, with post-irradiation S-phase fractions at 57.5 or 54.5% of the level in non-irradiated samples. Similar results were obtained in six independent experiments (Fig. 4*a*, *b*).

Several models could explain the intermediate phenotype of $p21^{-/-}$ MEFs. For example, $p21^{-/-}$ cells may differ from wild-type cells in the timing of the radiation response or in the amount of damage required to induce arrest. We therefore examined the radiation response of $p21^{-/-}$ MEFs at several times following treatment (14, 18 and 22 hours after a dose of 5.5 Gy) and at two different radiation doses (5.5 and 11 Gy, analysed 18 hours after irradiation). In each case, our results were similar to those shown in Fig. 4*a*, *b*. The intermediate phenotype of $p21^{-/-}$ cells could alternatively reflect the fact that primary MEFs are composed of a mixture of cell types, each of which may depend to a different extent on p21 for growth arrest in the presence of DNA damage. Finally, the position of cells in G1 phase could determine sensitivity to the p21-dependent mechanism of p53-induced cell-cycle arrest.

It is well established that, following irradiation, the activity of CDK2 kinase is reduced by ~3–5-fold in human fibroblasts[16]. The same is true in normal MEFs (Fig. 4*c*: ~5-fold reduction). However, inhibition of CDK2 is not observed upon irradiation

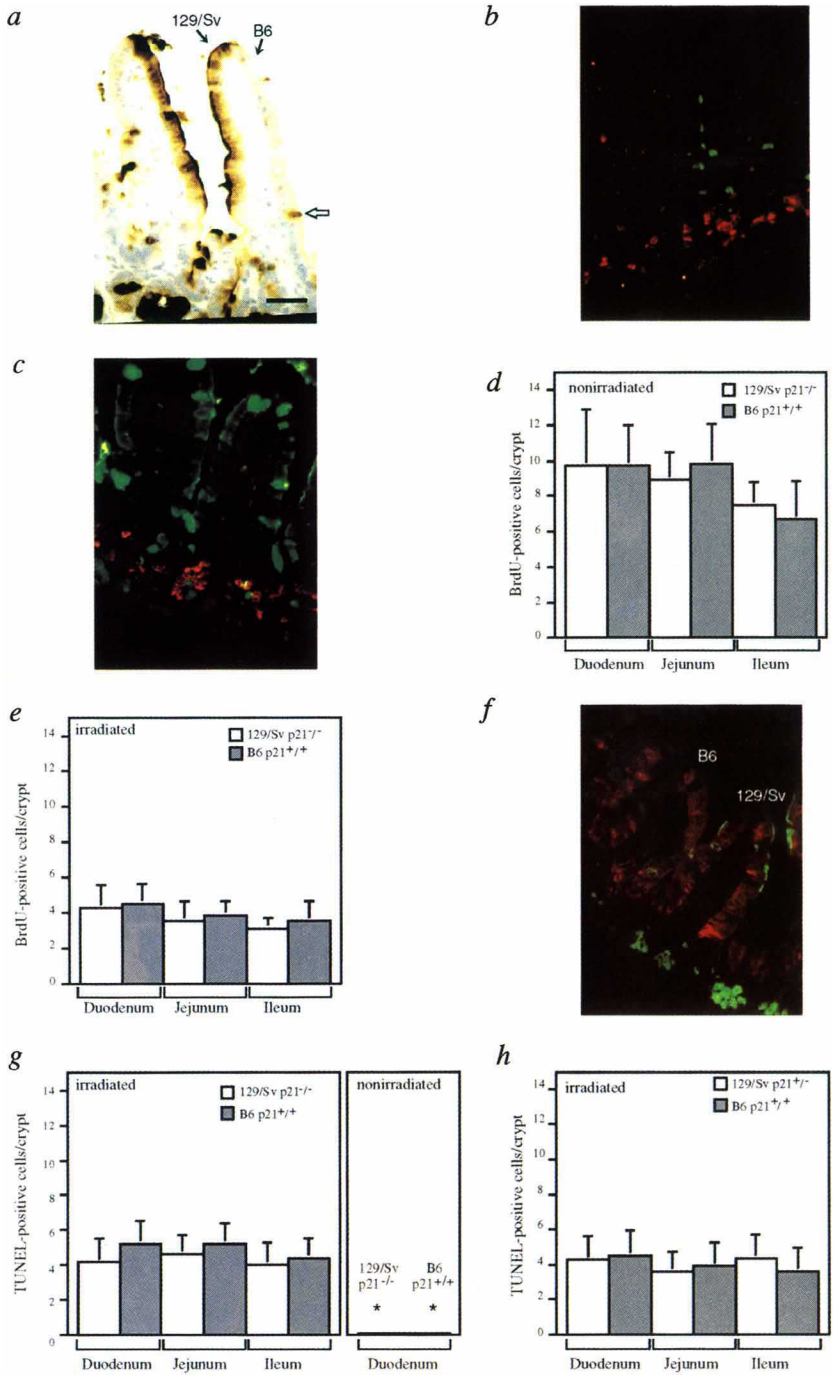


FIG. 2 Use of B6↔129/Sv $p21^{-/-}$ chimaeric mice to assess the role of p21 in proliferation, differentiation and death programs. *a*, A polyclonal jejunal villus from a chimaeric mouse stained with peroxidase-conjugated UEA-1. 129/Sv but not B6 enterocytes bind the lectin. A subset of B6 and 129/Sv goblet cells are also labelled (open arrow). *b*, *c*, Adjacent patches of $p21^{+/+}$ B6 and $p21^{-/-}$ 129/Sv jejunum (*b* and *c*, respectively) were stained with FITC-conjugated UEA-1 (green) and anti-BrdU (red). *d*, *e*, S-phase cells were quantitiated in crypts from irradiated and non-irradiated (panels *c* and *d*) chimaeras following staining with UEA-1 and anti-BrdU. 30 crypts were counted per section per region per mouse ($n = 3$ mice each). Mean values ± 1 s.d. are plotted. *f*, Adjacent B6 and 129/Sv villi show similar patterns of expression of liver fatty-acid-binding protein (L-FABP), a differentiation marker for the enterocytic lineage. The section was stained with FITC–UEA-1 (green) and rabbit anti-L-FABP sera (red). *g*, *h*, Quantitation of p53-dependent apoptosis in crypts from irradiated animals is shown (TUNEL assay). Asterisk denotes that non-irradiated age-matched B6↔129Sv $p21^{-/-}$ chimaeras contain on average only one TUNEL-positive cell per 30 crypts. Scale bar in *a*, 25 μm.

METHODS. Irradiated chimaeras were killed 4.5 h after receiving 6 Gy of total-body γ-irradiation. Fixation and multilabel immunohistochemical staining of chimaeric intestine were as described[11]. TUNEL assays were performed on periodate-lysine-paraformaldehyde-fixed tissues using the procedure described in ref. 22, except that slides were incubated in 0.5% Triton X-100/PBS for 20 min at room temperature.

of $p53^{-/-}$ or $p21^{-/-}$ cells (Fig. 4*c*). This demonstrates that DNA-damage-induced inhibition of CDK2 requires p21.

Since its discovery[17], p21 has been implicated in a broad spectrum of biological processes. Inhibition of cell proliferation by p21 has been linked to the permanent cell cycle withdrawal that accompanies senescence[6]. As a transcriptional target of p53, p21 has been proposed as an effector of cell-cycle arrest following DNA damage, as a component of the cell death program and as a tumour suppressor. Our data indicate that p21 is not required for p53-dependent apoptosis (at least in some cell types). However, the consequences of p21 disruption clearly demonstrate the involvement of this protein in p53-dependent cell-cycle arrest following DNA damage. Our data also indicate that additional p53-responsive growth controls exist. This may help to explain the lack of p21 mutations in human tumours[18]. We have followed a small number of chimaeric $p21^{-/-}$ mice (up to 80% $p21^{-/-}$, as judged by coat colour) for more than one year. The fact that none of these animals appears predisposed to cancer provides further evidence against a role for p21 as a major tumour suppressor. Several studies have also suggested a

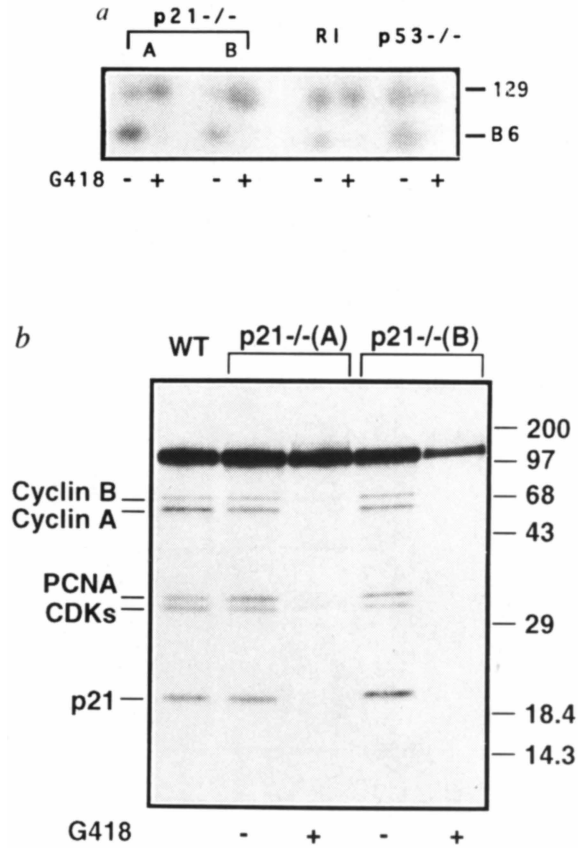
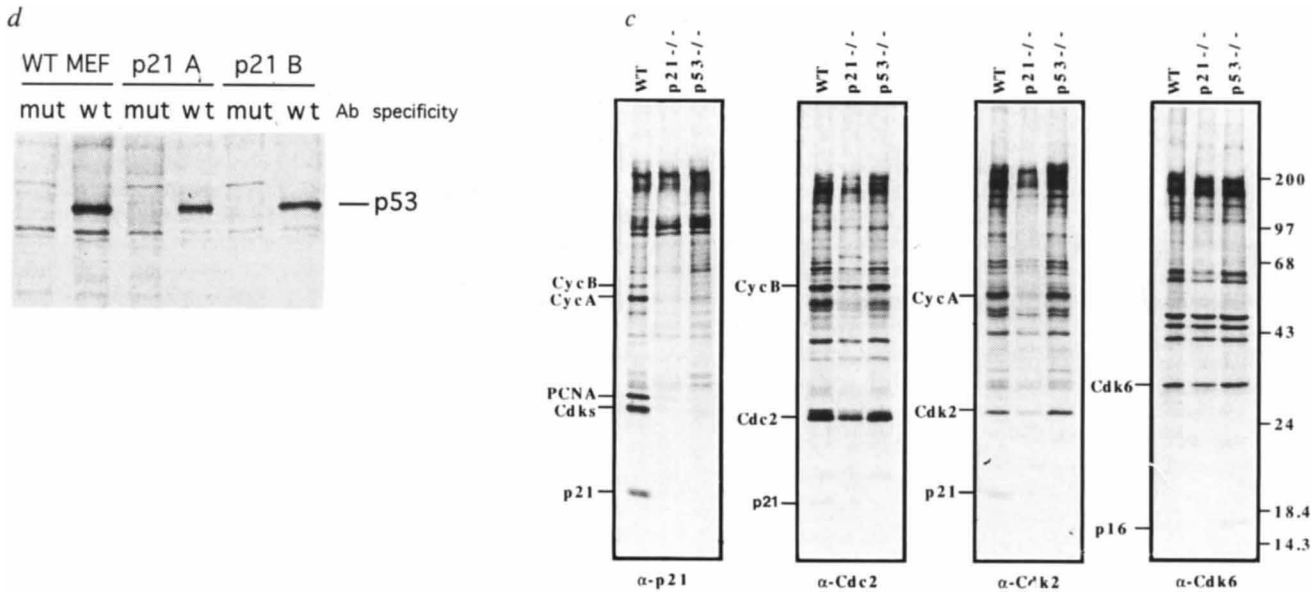
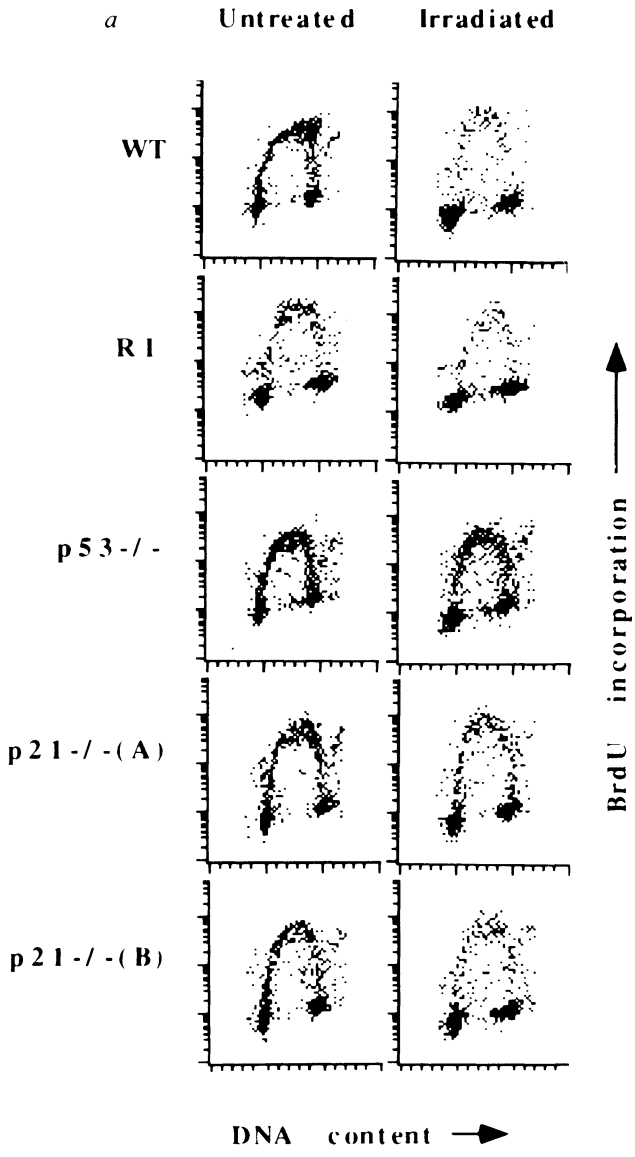


FIG. 3 Selection of targeted mouse embryo fibroblasts (MEFs). *a*, glucose-6-phosphate isomerase (GPI) isoenzyme analysis. Donor ES cells (strain 129/Sv) contain a GPI isoenzyme with a different electrophoretic mobility from that of the recipient C75BL/6 blastocysts. The ratio of these isoforms was used to estimate the contribution of the targeted and recipient cells to MEF populations. GPI analysis is shown for the initial MEF preparations from representative chimaeras generated from each ES cell clone (as indicated; RI, random integration) and for MEFs enriched for cells derived from targeted ES cells by selection with G418. *b*, $p21^{-/-}$ MEFs lack p21 protein. MEF populations (as indicated) were tested for the presence of p21 by immunoprecipitation. Note that p21 and associated proteins are present in the unselected $p21^{-/-}$ populations but are barely detectable following selection. *c*, Cyclin–CDK complexes in $p21^{-/-}$ cells. Cyclin–CDK complexes were recovered from the indicated cell populations using antisera against p21, CDC2, CDK2 and CDK6. The identities of co-precipitated proteins are indicated. *d*, Immunoprecipitation of p53 with conformation-specific antibodies. p53 protein was recovered from lysates of ^{35}S-labelled cells (indicated) by immunoprecipitation with antibodies specific for either the wild-type (WT; PAb 1620 from Oncogene Sciences) or the mutant (mut; PAb 240, Oncogene Sciences) conformations of p53.

METHODS. Chimaeras were generated by injection of 11–13 donor ES cells into C57BL/6 blastocysts[21]. 11 days after embryo injection and reimplantation ~E13.5 embryos were collected and MEFs isolated as described[23]. Passage-3 MEFs were selected with 300 μg ml^{-1} G418 (Gibco BRL) for 6–7 days. p21 antiserum was raised against a GST–p21 fusion and depleted of anti-GST antibodies by affinity purification. Immunoprecipitations were done as previously described[1].





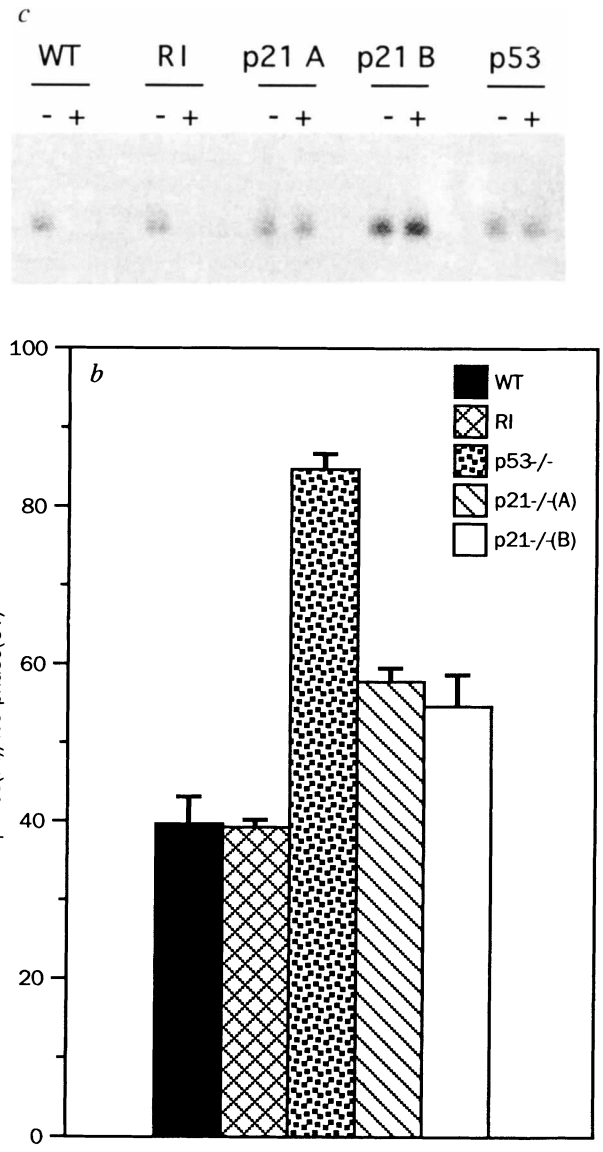


FIG. 4 Radiation response of $p21^{-/-}$ cells. *a*, Representative two-dimensional FACS analysis of untreated and irradiated samples of $p21^{-/-}$, $p53^{-/-}$, random integration (RI) and wild-type (WT) MEFs are shown. Asynchronous cultures were irradiated with a dose of 5.5 Gy and the percentage of cells synthesizing DNA was evaluated with a 4-hour BrdU pulse, beginning 14 hours after irradiation. Cells in the G0 or G1 phase of the cell cycle appear in the lower left quadrant. Cells in the G2 or M phases appear in the lower right quadrant. S-phase cells are positive for BrdU and are detected in the arch that connects G0/G1 and G2/M populations. *b*, Histogram showing the S-phase fraction in irradiated versus untreated samples. A quantitation of the number of cells remaining in S phase following irradiation compared to the number of S-phase cells in an untreated control is shown. The mean of 6 independent experiments is shown for all samples except the random integration (3 independent experiments). Error bars show standard deviations. *c*, Activity of CDK2 kinase following irradiation. CDK2 immunoprecipitates from either irradiated or non-irradiated MEF populations (as indicated) were prepared at 18 hours following treatment with 5.5 Gy. Precipitates were tested for the ability to phosphorylate histone H1.

METHODS. MEFs were grown to subconfluence in DME medium, 10% FCS, 5 mM glutamine with antibiotics. Cells were plated at a density of $5–6 \times 10^5$ cells per 10-cm plate and were irradiated between 24 and 30 h after plating. Cell cycle analysis was as described[15], except that histones were extracted in 0.7% Triton-X 100, 1.2 M HCl. Samples were analysed with a FACScan using CellQuest software (Becton–Dickinson). Immunoprecipitation and histone H1 kinase assays were done as described[1].

role for p21 during differentiation in different cell lineages[7–9]. Our data demonstrate that p21 is not essential for terminal differentiation in the small intestine. Furthermore, we have generated a germline, homozygous p21-deficient animal. The $p21^{-/-}$ mouse is viable and displays no apparent phenotype by seven weeks of age. Thus, the fact that p21 is not solely responsible for p53-dependent growth arrest or cell-cycle withdrawal following differentiation could indicate a general need for tight regulation of cell proliferation by several overlapping pathways.

Note added in proof. Following submission of this paper, Deng *et al.* (*Cell* **82,** 675–684, 1995) reported the generation and characterization of a p21-deficient mouse strain. They also observe that p21 deficient embryo fibroblasts are defective in the irradiation response, while p53-dependent apoptosis and overall embryonic and postnatal development were not affected by the absense of p21. □

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Requirement for TFIIH kinase activity in transcription by RNA polymerase II

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An array of tandem heptapeptide repeats at the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II constitute a highly conserved structure essential for viability[1–3]. Studies have established that the CTD is phosphorylated at different stages of the transcription cycle[4–7], and that it may be involved in transcriptional regulation[8–12]. The exact role of the CTD remains elusive, as *in vitro* reconstituted transcription using the adenovirus major late promoter does not require the CTD[13,14]. Previous studies[7,15,16] showed that transcription from the murine dihydrofolate reductase (DHFR) promoter can be only accomplished by the form of RNA polymerase II that contains the hypophosphorylated CTD (RNAPIIA), but not by the form that lacks it (RNAPIIB)[7]. Here we show that the CTD, but not its phosphorylation, is required for initiation of transcription. We also show that transcription requires CTD kinase activity provided by the CDK[7] subunit of TFIIH[17–19].

We examined the activity of the A and B forms of RNAPII on the murine dihydrofolate reductase (*DHFR*) promoter using a reconstituted transcription system. Both preparations of RNAPII (Fig. 1*a*) exhibited similar specific activities in a run-off transcription assay with the adenovirus major late (AdML) promoter (Fig. 1*b*, *c*). However, in agreement with previous studies[7,15,16], transcription from the *DHFR* promoter using the B form of RNAPII was tenfold less efficient in production of full-length transcripts (Fig. 1*b*, lanes 3 and 6, and *c*). To identify the step(s) in transcription impaired by the lack of the CTD, we analysed transcription initiation from the *DHFR* promoter. In the presence of ATP and [α-^{32}P]CTP, RNAPIIA forms the first phosphodiester bond at the transcriptional start sites on the *DHFR* and AdML promoters and releases the ApC dinucleotide as a product of multiple abortive cycles of transcription initiation (Fig. 2*a*). Analysis of initiation revealed a 15-fold reduction in efficiency of RNAPIIB from the *DHFR*, but not from the AdML promoter (Fig. 2*b*, lanes 2 and 4, *c*). Thus it appears that in the reconstituted transcription system, the requirement for the CTD is related to the steps preceding promoter clearance, these being preinitiation complex formation and/or initiation.

To identify which of these two steps requires the CTD, we used dinucleotide priming[20]. This technique circumvents the requirement for the formation of the first phosphodiester bond by incorporating dinucleotides corresponding to the transcription start site (Fig. 2*d*). Dinucleotide priming using the linearized *DHFR* promoter template resulted in a drastic increase in the activity of RNAPIIB, approaching half that of RNAPIIA (Fig. 2*e*, lanes 1 and 3, and *f*). As shown previously[20], abortive initiation under these conditions was highly specific. Indeed, it was dependent on TATA-box-binding protein (TBP) (Fig. 2*e*) and the other general transcription factors (data not shown). Moreover, when ApA was substituted with CpC, no initiation was observed (Fig. 2*g*). These results indicate that the formation of the first phosphodiester bond during transcription of the *DHFR* is dependent on the CTD.

Because the CTD is implicated in transcription initiation, a step apparently bypassed by dinucleotide priming, it raises the question of whether productive run-off transcription by RNAPIIB could also be rescued by priming with ApA. A titration of ApA into transcription reactions containing the B form of RNAPII resulted in stimulation of the run-off transcription, but the same titration was without effect on reactions containing the IIA form of RNAP (Fig. 3*a*). The RNAPIIB activity recovered by dinucleotide priming was only 15% of the RNAPIIA activity (Fig. 3*b*), which indicates that there must be other critical steps dependent on the CTD during productive transcription. One step may entail the phosphorylation of the CTD[21]. However, a previous study indicated that inhibition of the CTD-kinase activity with the ATP analogue H8 in reconstituted systems transcribing the AdML promoter does not result in transcription inhibition[14]. Transcription of the AdML promoter does not discriminate between the A and B forms of RNAPII, so this promoter may not have been the appropriate one with which to test phosphorylation requirements. Indeed, application of H8 resulted in a dose-dependent suppression of transcription from the *DHFR* gene, but not from the AdML promoter (Fig. 3*c*). Consistent with results presented above (Fig. 3*b*), RNAPIIB activity recovered by dinucleotide priming was resistant to H8 inhibition (Fig. 3*d*). Moreover, reactions containing the dinucleotide ApA and the IIA form of RNAPII were resistant to H8 inhibition (Fig. 3*c*). It is surprising, however, that under these conditions the levels of transcription were not diminished. It is possible that, in the presence of the dinucleotide, RNAPII enters an 'unusual conformation' which requires the CTD for optimal transcription but bypasses its phosphorylation[22]. Nonetheless, the above results suggest that transcription from the *DHFR* promoter requires not only the presence of the CTD, but is also subjected to regulation by phosphorylation.

A CTD kinase is associated with TFIIH[17,23,24], its activity residing in the CDK-activating kinase (CAK) composed of Cdk7 and cyclin H[17–19]. To analyse the role of CAK in transcription from the *DHFR* promoter, we first tested the effect of affinity-purified antibodies against cyclin H. Incubation of these antibodies with TFIIH before formation of the preinitiation complex abolished both transcription activity and phosphorylation of RNAPII (ref. 19). However, addition of antibodies after the formation of a complete preinitiation complex had no effect on transcription from the AdML promoter[19] (Fig. 4*a*). In contrast, titration of the cyclin H antibodies on preformed preinitiation complexes using the *DHFR* promoter strongly suppressed transcription (Fig. 4*a*). Analysis of the electrophoretic mobility of the preinitiation complex formed on the *DHFR* and AdML promoters shows that, as with the previous observation[19], cyclin

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