Cell cycle arrest following exposure of EBV-immortalised B-cells to gamma irradiation correlates with inhibition of cdk2 activity

Emma J. Cannell[a,b], Paul J. Farrell[a], Alison J. Sinclair[a,c,*]

[a] *Ludwig Institute for Cancer Research, Imperial College School of Medicine at St. Mary's Hospital, Norfolk Place, London W2 1PG, UK*
[b] *Institute for Cancer Research, Chester Beatty Laboratories, Fulham Road, London, SW3 6JB, UK*
[c] *School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK*

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Abstract The exposure of Epstein-Barr virus immortalised B cells (LCLs) to the genotoxic effects of gamma irradiation causes a decreased proliferation of the cells. The early events in this process have been investigated here. The induction of p53 expression correlates with a cell cycle arrest in the G1 and G2/M phases of the cell cycle within 24 h of exposure. The molecular mechanism governing the decreased proliferation appears to involve the induction of the cyclin dependent kinase (cdk) inhibitor p21^{CIP1} and its functional association with cdk2.

Key words: p53; Lymphoblastoid cell line; Gamma irradiation; Epstein-Barr virus; Cell cycle

1. Introduction

The p53 protein is intimately involved in the response of mammalian cells to genotoxic stresses, including gamma irradiation [1–3]. The activation of p53 is achieved by a variety of mechanisms including changes to the half-life of the protein, increased translation of p53 and altered post-translational modification (reviewed in [1]). Once p53 is activated, it is involved in a number of different cellular processes including cell cycle arrest, DNA repair and apoptosis [2,3]. Some of these functions can be accounted for by the transcriptional activity of p53; several cell genes are induced following p53 activation including p21^{CIP1}, mdm-2, gadd45, cyclin G, bax and IGF-BP3 [2]. However, the identification of a point mutation in the p53 gene that renders p53 transcriptionally inactive yet still promotes apoptosis demonstrates that p53 must also act via non-transcriptional routes [4].

We and others have observed that the expression of p53 is increased following the infection of primary human B-cells with the DNA tumour virus Epstein-Barr virus (EBV) [5,6]. The activation is manifested by an increase in the expression of p53 RNA and protein in infected cells [5] and an increase in the expression of the p53 responsive gene p21^{CIP1} [7]. It has been suggested that this is achieved via the activation of the transcription factor NFκB by two EBV genes, EBNA-2 and LMP-1 [8]. The increased expression of p53 following infection of quiescent primary B-cells with EBV was a surprising observation since EBV causes cells to enter into the cell division cycle and to proliferate indefinitely, resulting in the generation of immortal lymphoblastoid cell lines (LCLs) (reviewed in [9]). Thus, in the context of EBV infected B-cells, the elevated levels of p53 did not appear to be sufficient to activate either its cell cycle arrest or apoptosis programmes.

This leads to the hypothesis that the activation of the cell proliferation pathway by EBV primes infected cells to respond to any subsequent genotoxic stress by inducing the expression of basal levels of p53 and p21^{CIP1}. Consistent with this, we have shown that the level of p21^{CIP1} in LCLs remains below the threshold required to fully inhibit the activity of the cyclin dependent kinase, cdk2 [7], and we and others have shown that p53 levels are elevated still further in LCLs after exposure to genotoxic agents [5,10–14].

In this report, we characterise the downstream effects of the exposure of LCLs to the genotoxic agent gamma irradiation. Early in the response, increased expression of p53 and p21^{CIP1} were observed. This correlates with an association of p21^{CIP1} with cdk2 and an inhibition of cdk2 associated histone H1 kinase activity. Cell cycle arrest is observed within 24 h of the exposure, which involves cell cycle checkpoints in the G1, G2/M and possibly also S phases. This contrasts with the predominant outcome for LCLs exposed to another genotoxic agent, cisplatin, and to LCLs which are subject to the enforced expression of p53; in both of these situations, cell cycle arrest and apoptosis occur almost simultaneously within 24 h [5].

2. Materials and methods

2.1. Cell culture

Lymphoblastoid cell lines, IB4 [15] and LCL#3 [16], were maintained at a density of between 2 and 8×10^5/ml in standard growth medium [16].

The viability of the cells was determined using a trypan blue exclusion assay [17].

2.2. DNA synthesis assay

2×10^5 LCL cells, in 1 ml of standard culture medium, were incubated with 1 μCi of [^{3}H]thymidine for 2 h. After washing in PBS, the cells were lysed in 1 ml of 10% (w/v) trichloroacetic acid (TCA) and the precipitated material collected onto a GF/C (Whatman) filter. Following extensive washing with 10% (w/v) TCA, the filters were allowed to air dry. The [^{3}H]thymidine incorporation into acid insoluble material was determined by liquid scintillation counting. All experiments were performed in triplicate.

2.3. Western blot analysis

Cells were harvested and washed with PBS. Total protein extracts were prepared from the LCLs and fractionated on SDS-polyacrylamide gels as described previously [7]. Proteins were transferred to either nitrocellulose membrane or PVDF membrane by Western blotting and blocked with a 5% (w/v) solution of skimmed milk powder in PBS supplemented with 0.1% (v/v) NP40. The primary antibodies were diluted in the same buffer and incubated with the filters for between 1 and 24 h at 4°C. Primary antibodies included: anti-PARP (Boehringer Mannheim); anti p53 (DO1, [18]); anti-p21^{CIP1} [19]; and cdk2 (M2, Santa Cruz). This was followed by extensive

*Corresponding author. Fax: (44) (1273) 678 433.
E-mail: a.j.sinclair@sussex.ac.uk

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washing in PBS supplemented with 0.1% (v/v) NP40. To detect p21 and p53, a species specific secondary antibody (DAKO) was applied for 1 h at room temperature. Following a washing step, HRP-conjugated species specific secondary antibodies (Amersham or DAKO) were applied to the filters and incubated for 1 h at room temperature. Following further washing, an enhanced chemiluminescence (ECL) reaction was undertaken (Amersham) and the resulting signals were recorded by autoradiography.

2.4. Kinase assays

NP40 extracts were prepared by lysing the 1×10^7 cells/ml on ice in 150 mM NaCl, 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1% (v/v) NP40, 10% (v/v) glycerol, 0.1 mM PMSF, 2 μg/ml aprotinin, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate. Following a brief exposure to sonication (2 min), the lysates were clarified by centrifugation at 12 000 rpm in a microfuge and endogenous immunoglobulins collected by incubation with protein G-Sepharose beads. Antibodies were then added to the precleared supernatant for 120 min at 4°C. The antibody-protein complexes were collected on protein G-Sepharose beads for a further 30 min at 4°C. The complexes were washed 4 times in lysis buffer followed by 2 washes in 50 mM HEPES, pH 7.5, 1 mM DTT. The complexes were then incubated in 40 μl of kinase buffer [7] containing 1 μg of histone H1 (Boehringer Mannheim) and 10 μCi of [γ-^{32}P]ATP. Following a 30-min incubation at 30°C, the samples were heated to 95°C in the presence of total protein extraction buffer [7] and the proteins separated on SDS-polyacrylamide gels. The gels were fixed by incubation in 10% (v/v) ethanol, 10% (v/v) acetic acid, dried and phosphorylated proteins identified by autoradiography.

2.5. Complex assays

NP40 extracts were prepared and proteins precipitated as described for cdk2 kinase assays. The complexes were heated to 95°C in the presence of total protein extraction buffer and the proteins separated on a 15% SDS-polyacrylamide gel. Following Western blotting to nitrocellulose membrane, the filter was incubated with anti-p21^{CIP1} antisera [19] and signals detected by ECL.

2.6. FACs analysis

Cells were harvested, washed with PBS and fixed in 65% (v/v) ethanol at 4°C for at least 24 h. The cells were then incubated in 0.1% (v/v) Triton X-100, 3.7% (w/v) EDTA, 100 μg/ml propidium iodide, 6 μg/ml RNaseA for 30 min in the dark. The DNA content of the cells was analysed using a Coulter Epics fluorescence activated cell sorter (FACs). Human mononuclear lymphocytes were used to identify the peak of 2*n* DNA content. Gates were manually applied to the 2*n*, 4*n* and 2–4*n* populations of cells.

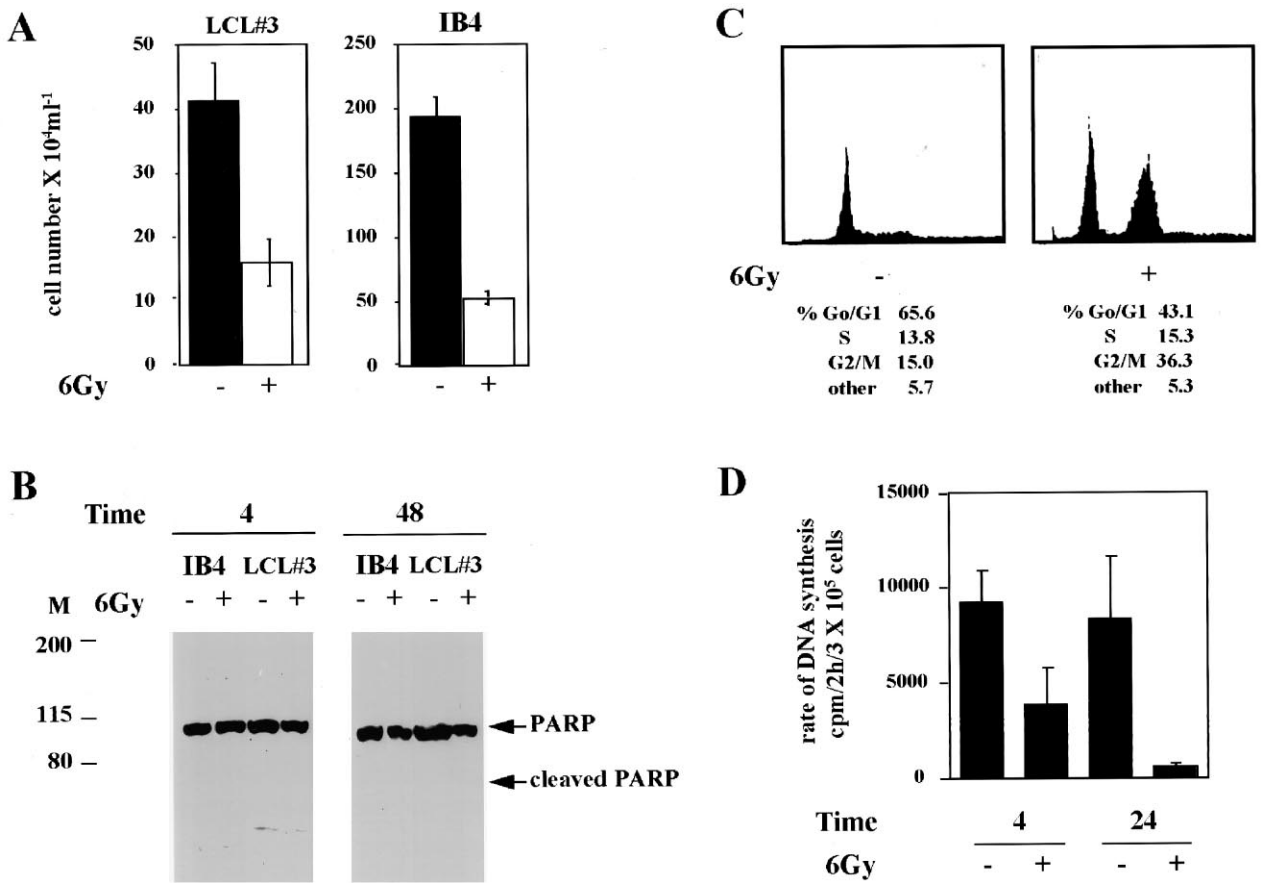

Fig. 1. The proliferation of LCLs is compromised by exposure to gamma irradiation. Exponentially growing LCLs were exposed to 6 Gy of gamma irradiation where indicated. The cells were then seeded at a density of 20×10^4/ml. A: After 48 h the number of viable cells were determined. The error bars represent the S.E.M. from 3 experiments. B: After 4 and 48 h, total protein extracts were prepared from the cells, fractionated on a 10% SDS-polyacrylamide gel, blotted onto PVDF membrane and exposed to anti-PARP antisera. The resulting signal was detected using ECL. The migration of the molecular weight standards (in kDa), full length PARP and the larger of the PARP-cleavage products are indicated. C: After 24 h LCL#3 cells were harvested and fixed. Subsequently, the cells were stained with propidium iodide and the DNA content analysed by FACs using a Coulter Epics flow cytometer. The DNA profile is shown in the upper panel with the analysis of the % of cells in particular phases of the cell cycle below. D: At the indicated times (in hours) the rate of DNA synthesis in LCL#3 cells was determined. [^{3}H]Thymidine was included for the final 2 h of each incubation. The [^{3}H]thymidine incorporation into acid insoluble material was determined in triplicate and the S.E.M. is represented as error bars.

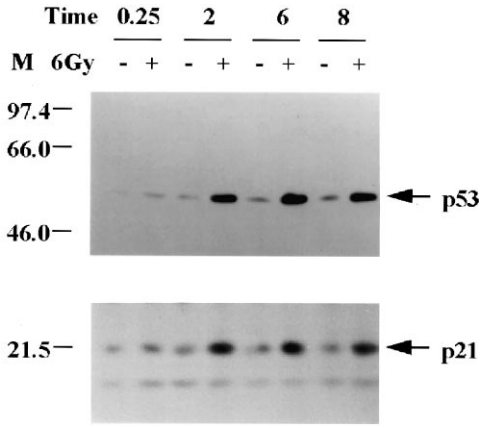

Fig. 2. Steady state levels of p53 and p21 are rapidly elevated. LCL#3 cells, were exposed to 6 Gy of gamma irradiation (−/+). At the indicated times (in hours) total protein extracts were prepared from the cells, fractionated on a 10% (upper panel) or a 15% (lower panel) SDS-polyacrylamide gel, blotted onto nitrocellulose membrane and exposed to anti-p53 or anti-p21 antisera. The resulting signals were detected using ECL. The migration of the molecular weight standards (in kDa) and the proteins of interest are shown.

3. Results and discussion

We characterised the response of two LCLs, LCL#3 and IB4, to 6 Gy (600 Rads) of gamma irradiation. Within 48 h, clear differences in the number of viable cells in the non-exposed compared to the exposed populations were apparent (Fig. 1A). This suggests that the exposure to gamma irradiation either initiated an arrest in cell proliferation or an increase in apoptosis. To distinguish between these possibilities, we asked whether we could detect evidence for apoptosis in these cells. An early event during apoptosis is the activation of ICE-like proteases, which specifically cleave the 113-kDa poly(ADP)ribose polymerase (PARP) protein generating two stable products of lower molecular weight. Since we were unable to detect either of these products during the first 48 h post-exposure (Fig. 1B), we suggest that an arrest of cell proliferation is the most likely outcome for cells at this stage. Supporting evidence that an arrest of proliferation predominates over apoptosis came from the analysis of changes to the rate of DNA synthesis and from the profile of the DNA content of LCL#3 cells 24 h after exposure. Fig. 1C demonstrates that exposed cells are present in the G1 and G2/M phases of the cell cycle. Concomitant measurements of the rate of DNA synthesis (Fig. 1D) show a 14-fold decrease suggesting that the G1 and the G2/M cells are arrested. This, together with the paucity of cells in the apoptotic window with a sub-G1 DNA content, allows us to conclude that the predominant early response to gamma irradiation exposure in LCLs is the activation of G1 and G2/M cell cycle check points.

In order to address the molecular mechanism by which this cell cycle arrest occurs, we investigated whether the expression of p53 and $p21^{CIP1}$ were affected. Both p53 and $p21^{CIP1}$ were elevated within 2 h in LCL#3 cells after exposure (Fig. 2). This confirms and extends previous investigations that demonstrate that the induction of p53 and $p21^{CIP1}$ are apparent within 4–6 h [10–14]. The timing of the induction of p53 and $p21^{CIP1}$ is consistent with a model suggesting that the induction of transcriptionally active p53, in response to DNA damage, activates the expression of $p21^{CIP1}$. However, the key question is whether the level of $p21^{CIP1}$ within the exposed cells has been raised above the threshold required to inhibit cyclin dependent kinases (cdks).

The association of cdk2 with $p21^{CIP1}$ was investigated in irradiated and non-irradiated LCL#3 cells. The overall level of cdk2 expression was not significantly altered following exposure to gamma irradiation (Fig. 3A). However, the amount of $p21^{CIP1}$ associated with cdk2 was dramatically increased (Fig. 3B, track 1 compared with track 2). We then assessed the functional significance of the $p21^{CIP1}$ association with cdk2 by comparing the kinase activity of cdk2 containing complexes, before and after exposure to gamma irradiation. As seen in Fig. 4, no activity was seen when mock complex isolations were undertaken. In contrast to this, when the cdk2 antibody was used in the absence of exposure a basal level of kinase activity directed against histone H1 was observed. Surprisingly, a reproducible increase in the cdk2 activity was observed after 7–8 h. This was likely to be a response to the

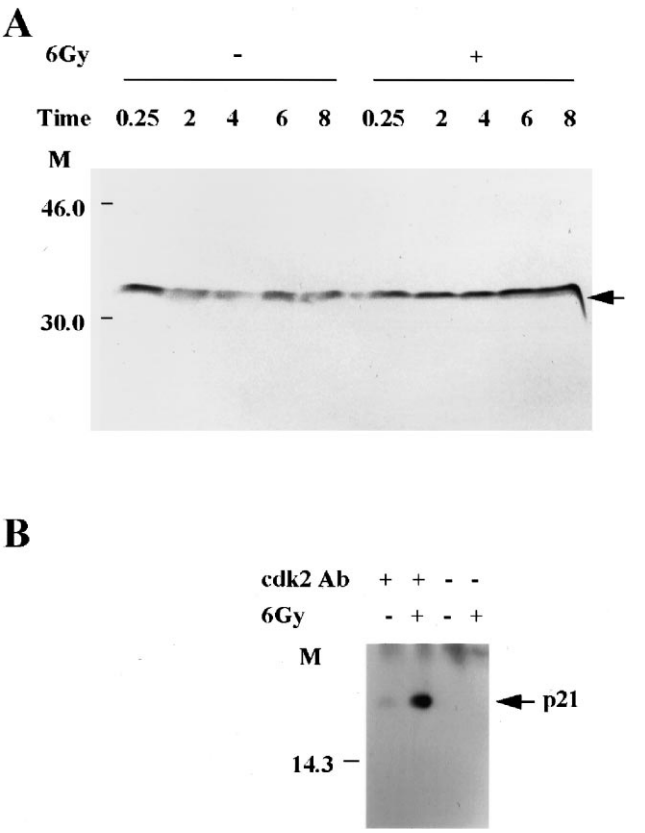

Fig. 3. The level of expression of cdk2 is unaltered, but its association with $p21^{CIP1}$ increases. LCL#3 cells were exposed to 6 Gy of gamma irradiation (−/+). A: At the indicated times (in hours) total protein extracts were prepared and fractionated on a 15% SDS-polyacrylamide gel, blotted onto nitrocellulose membrane and exposed to anti-cdk2 antisera. The resulting signal was detected using ECL. B: After 4 h NP40 extracts were prepared from the cells. Cdk2 and associated proteins were purified by immunoprecipitation (+). In parallel, mock precipitations were carried out using control sera (−). The resulting proteins were fractionated on a 15% SDS-polyacrylamide gel, blotted onto nitrocellulose membrane and exposed to anti-p21 antisera. The resulting signal was detected using ECL. The migration of the molecular weight standards (in kDa) is shown.

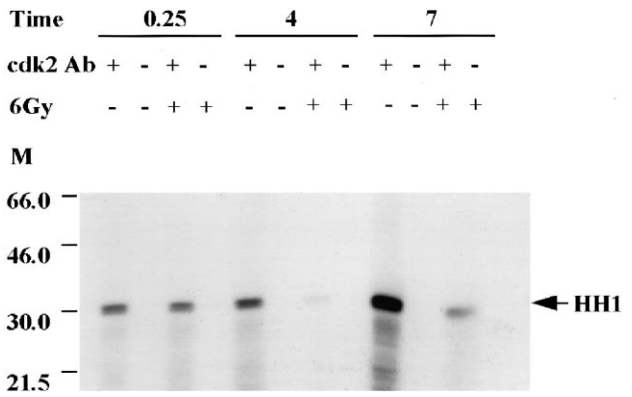

Fig. 4. cdk2 kinase activity is decreased within 4 h after exposure. At the indicated times (in hours) NP40 extracts were prepared from the cells. Cdk2 and associated proteins were purified by immunoprecipitation (+). In parallel, mock precipitations were carried out using control sera (−). In vitro kinase assays were undertaken and the resulting proteins were fractionated on a 15% SDS-polyacrylamide gel. After drying the phosphorylation of histone H1 (HH1) was determined after exposure of the gel to autoradiography. The migration of the molecular weight standards is shown (in kDa).

stimulation the cells received after dilution into fresh medium at the start of the experiment, potentially involving changes in cyclin/cdk expression or cdk phosphorylation. Despite this, within 4 h from the exposure, a significant reduction in cdk2 associated kinase activity was observed. A similar reduction was observed when retinoblastoma protein is used as the substrate for the in vitro kinase assay (data not shown).

In this report, we characterise the downstream effects of the exposure of LCLs to the genotoxic agent gamma irradiation. In agreement with previous investigations, it is apparent that the major early response of EBV immortalised cells is cell cycle arrest ([10–14,20] and Fig. 1). We confirm the observation that the expression of both p53 and the p53-responsive gene $p21^{CIP1}$ are induced above their basal levels following irradiation [10–14]. We show that these are early events, reaching maximal levels by 15 min post-exposure and being maintained at this level for at least 8 h (Fig. 2). Furthermore, we investigate the biochemical consequences of the increase in $p21^{CIP1}$ protein following irradiation. A dramatic decrease in cdk2 associated kinase activity is detected within 4 h of exposure, in the absence of changes to the basal level of expression of cdk2 (Figs. 3 and 4). This correlates temporally with the formation of complexes containing both cdk2 and the inhibitor $p21^{CIP1}$ (Fig. 4). Thus in EBV immortalised LCLs, at the basal (EBV induced) level of expression, $p21^{CIP1}$ does not prevent the formation of active cdk2 containing kinase complexes, whereas, following exposure to gamma irradiation, the induced $p21^{CIP1}$ associates with cdk2 in an inhibitory manner. This is consistent with current models describing the effects of elevated p53 and $p21^{CIP1}$ levels in other cell types [1–3]. In addition, since the p53 induced pathway appears to be fully functional in LCLs, this provides further support for the conclusion that the viral genes expressed in EBV immortalised LCLs do not disrupt the function of p53 [5,7,21].

However, it is interesting to note that the outcome of the enhanced expression of p53 in LCLs does not always follow the course detailed here. Indeed, the exposure of LCLs to another genotoxic agent, cisplatin, causes a rapid G1 cell cycle arrest combined with almost simultaneous apoptosis, resulting in the survival of few viable cells 24 h after the initial exposure [5]. Although some apoptosis is observed in LCLs between 24 and 72 h after exposure to gamma irradiation (20–30% of the population of cells) [13,14], the early responses to these two genotoxic agents are clearly distinct. The alternate responses of LCLs to gamma irradiation and cisplatin may be a reflection of the biochemical responses induced by the differences in DNA damage: gamma irradiation causes double-strand DNA breaks whereas cisplatin generates intra- and inter-strand crosslinks. However, the combined cell cycle arrest/apoptosis response, which is characteristic of cisplatin treated cells, appears to be mediated solely by the elevated levels of p53, since enforced expression of p53 in LCLs and some Burkitt's lymphoma cell lines stimulates the same outcome [5,22,23].

Since the experiments investigating gamma irradiation exposure, enforced p53 expression and cisplatin exposure have been undertaken in the same cell line (IB4), we can exclude the possibility that the genetic background of the cell directs the alternate outcomes. Therefore, we propose that one of the following scenarios accounts for the difference in the decision to instigate the cell cycle arrest or the cell cycle arrest plus apoptosis programmes. (i) The absolute level of expression of p53 within the cells may govern which branches of the p53 response pathway are activated, with cell cycle arrest being triggered by more modest changes in expression than apoptosis. (ii) Additional molecular changes in the gamma irradiated cells may modulate the function of the p53 initiated apoptosis pathway while allowing the p53 initiated cell cycle arrest pathway to proceed. Since the timing and degree of p53 induction are similar (Fig. 2, [5] and data not shown), the latter scenario is more likely.

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