KRÜPPEL-LIKE FACTOR 4 MEDIATES p53-DEPENDENT G1/S CELL CYCLE ARREST IN RESPONSE TO DNA DAMAGE

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Running Title: KLF4 and DNA damage

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The tumor suppressor p53 is required for the maintenance of genomic integrity following DNA damage. One mechanism by which p53 functions is to induce a block in the transition between G1 to S phase of the cell cycle. Previous studies indicate that the Krüppel-like factor 4 (KLF4) gene is activated following DNA damage and that such activation depends on p53. In addition, enforced expression of KLF4 causes G1/S arrest. The present study examines the requirement of KLF4 in mediating p53-dependent cell cycle arrest process in response to DNA damage. We show that the G1 population of a colon cancer cell line, HCT116, null for the p53 alleles (-/-), was abolished following γ irradiation as compared to cells with wild-type p53 (+/+). Conditional expression of KLF4 in irradiated HCT116 p53-/- cells restored the G1 cell population to a level similar to that seen in irradiated HCT116 p53+/+ cells. Conversely, treatment of HCT116 p53+/+ cells with small interfering RNA (siRNA) specific for KLF4 significantly reduced the number of cells in the G1 phase following γ irradiation as compared to the untreated control or those treated with a non-specific siRNA. In each case, the increase or decrease in KLF4 level due to conditional induction or siRNA inhibition, respectively, was accompanied by an increase or decrease in the level of p21\(^{\text{WAF1/CIP1}}\). Results of our study indicate that KLF4 is an essential mediator of p53 in controlling G1/S progression of the cell cycle following DNA damage.
INTRODUCTION

The mammalian cell cycle is operationally divided into five distinct phases: gap 1 (G1), DNA synthesis (S), gap 2 (G2), mitosis (M) and growth arrest phase (G0), also called quiescence (1). Complex networks of control mechanisms called “checkpoints” are responsible for the orderly progression of these events within the cell cycle. Defects in checkpoint control increase genetic instability, thereby contributing to uncontrolled proliferation (2). For example, damage to the DNA elicits a series of signal transduction pathways that result in an arrest of the cell cycle at various checkpoints (3). Much of the DNA damage-induced signals are funneled through p53, which directs further downstream actions that lead to inhibition of G1-to-S and G2-to-M transitions, among other events such as apoptosis (4). It is therefore not surprising that $p53$ is the most frequently mutated tumor suppressor gene in human cancers (5).

The arrest in the transition between G1 and S phase of the cell cycle elicited by p53 requires in part the transcriptional activation of the gene encoding the cyclin-dependent kinase (Cdk) inhibitor $p21^{WAF1/CIP1}$ (6, 7). $p21^{WAF1/CIP1}$ binds to several G1 cyclin/Cdk complexes and inhibits phosphorylation of the retinoblastoma susceptibility gene product Rb (8), a step required for the onset of DNA synthesis (9). Recent evidence suggests that $p21^{WAF1/CIP1}$ is also required to sustain G2 arrest after DNA damage (10). Here, $p21^{WAF1/CIP1}$ mediates the function of p53 in response to DNA damage by inhibiting Cdc2 (11), a Cdk required for the entry into mitosis (12). The proportion of cells that arrests in G1/S or G2/M depends on the cell type and status of checkpoint controls in each cell (13).
Although earlier studies indicate that expression of $p21^{\text{WAF1/CIP1}}$ is the result of direct binding of p53 to its promoter (14), it is now evident that a myriad of transcription factors under various physiologic conditions can also lead to the transcriptional activation of $p21^{\text{WAF1/CIP1}}$ (15). Among these is the zinc finger-containing transcription factor, Krüppel-like factor 4 (KLF4, also called gut-enriched Krüppel-like factor or GKLF) (16, 17). KLF4 is a member of a rapidly expanding family of mammalian Krüppel-like factors that exhibit homology to the *Drosophila* protein Krüppel (18). Expression of KLF4 is highly enriched in the post-mitotic, terminally differentiated epithelial cells of the intestine and epidermis (19, 20). In cultured cells, expression of KLF4 is associated with growth arrest resulted from serum deprivation or contact inhibition (19, 21). Conversely, enforced expression inhibits DNA synthesis and results in decreased cell proliferation (19, 22, 23). These studies suggest that KLF4 is a negative regulator of cell growth.

Recently, it was demonstrated that expression of KLF4 is also induced by DNA damage and that such induction is dependent on p53 (24). Importantly, KLF4 was shown to physically interact with p53, resulting in a synergistic activation of the $p21^{\text{WAF1/CIP1}}$ promoter. Moreover, antisense inhibition of KLF4 leads to a decreased level of $p21^{\text{WAF1/CIP1}}$ in response to DNA damage (24), suggesting that KLF is a potentially important mediator of p53-induced growth arrest. Indeed, recent studies using an inducible system for KLF4 indicate that its induction leads to arrest in the G1/S transition of the cell cycle (25). In the present study, we further characterize the role of KLF4 in mediating p53-dependent cell cycle arrest. By manipulating KLF4 expression, we show that KLF4 is essential for the G1/S cell cycle arrest resulted from DNA damage.
EXPERIMENTAL PROCEDURES

Cell Lines. The colon cancer cell lines wild-type and null for p53, HCT116 p53+/+ and HCT116 p53-/-, respectively, were generous gifts from Dr. Bert Vogelstein of Johns Hopkins University (10). The cells were cultured in McCoy’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. EcR116 p53-/- cells were established by stably transfecting pVgRXR (25), which contains VgEcR and RXR that form a receptor for the insect hormone, ecdysone, into the parental HCT116 cell line and selected with 100 µg/ml Zeocin (Invitrogen). The level of RXR expression was determined by Western blot analysis.

γ Irradiation. Gamma irradiation of cultured cells was performed using a 137Cs γ irradiator at 0.8 Gy/min for 15 min, for a total of 12 Gy. Cells were harvested at 24 hours after γ irradiation for subsequent assays.

Adenovirus Infection. The recombinant adenovirus containing GFP and KLF4 (AdEGI-KLF4) or GFP alone (AdEGI) were described previously (25, 26). EcR116 p53-/- cells were grown to 40% confluence in 10-cm dishes and replenished with fresh media containing 2% FBS followed by the addition of 10^6 plaque forming units of recombinant virus per dish. Infected cells were incubated at 37 °C for 6 hr, at which time cells were γ irradiated and the media changed. Cells were treated with 5 µM ponasterone A (Invitrogen) for 24 hr and then collected for further analysis.

Preparation of siRNA and Transfection. 23-nucleotide single-stranded RNAs (ssRNAs) were produced by Integrated DNA Technologies, Inc. (Coralville, IA). The small interfering RNA
(siRNA) sequences targeting KLF4 (Acc. No. XM 047517.1) correspond to the coding region between nucleotides 121-141 from the translation initiation site. The complementary ssRNAs were dissolved in 10 mM Tris- HCl and 1 mM EDTA (pH 7.0) and annealed in 25 mM KoAc, 10 mM Tris-HCl and 1 mM EDTA (pH 7.0) by briefly heating to 70 °C, then incubating for 20 min each at 37 °C and 23 °C. A non-specific double-stranded siRNA with identical length was also generated based on the sequence of an unrelated protein and used as a control.

HCT116 p53+/+ cells were grown to 40% confluence in 10-cm dishes, γ irradiated for a total of 12 Gy and transfected with annealed siRNA using DMRIE-C reagent (Invitrogen) for 6 hr as recommended by the manufacturer. McCoy’s media containing 20% FBS and 2% penicillin-streptomycin were added to each dish to a final concentration of 10% FBS and 1% penicillin-streptomycin. Cells were harvested 24 hr later for further assays.

**Western Blot Analysis.** Cell protein extraction and Western blot analyses were performed using standard procedures. Protein samples were mixed with loading buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiotheitol, 0.01% bromphenol blue and 10% glycerol), heated at 100 °C for 5 min, and loaded onto a SDS-polyacrylamide gel in electrophoresis buffer containing 25 mM Tris-HCl, pH 8.3, 250 mM glycine and 0.1% SDS. Protein was then transferred to nitrocellulose membranes using Trans-Blot Semi-Dry system (Bio-Rad). The membranes were immunoblotted with primary antibodies against KLF4 (19), p53, p21^{WAF1/CIP1} or β-catenin (Santa Cruz). Following incubation with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; 1:10000 dilution; Santa Cruz), KLF4, p53, p21^{WAF1/CIP1} or β-catenin were visualized with SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).
**Cell Cycle Analysis.** Cells were rinsed in Dulbecco’s phosphate-buffered saline (Mediatech, Inc.), trypsinized, re-suspended in McCoy’s media containing 10% FBS and 1% penicillin-streptomycin, collected by centrifugation, washed with Dulbecco’s phosphate-buffered saline, again collected by centrifugation, re-suspended in 70% ethanol and fixed at –20 °C overnight. Cells were pelleted again by centrifugation and re-suspended in a staining solution containing 50 µg/ml propidium iodide, 50 µg/ml RNase A, 0.1% Triton X-100 and 0.1 mM EDTA, for 30 min. Flow cytometry was performed on a FACSCalibur (Becton Dickinson) cytometer.
RESULTS

G1/S arrest is dependent on p53 in HCT116 cells following γ irradiation.

Both HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells exhibited comparable cell cycle profiles before irradiation (Figures 1 A, B and E). Following 12-Gy of γ irradiation, HCT116 $p53^{+/+}$ cells demonstrated a normal cell cycle arrest pattern, with ~15% of cells in G1, ~80% cells in G2 and a significantly reduced S population (Figures 1 C and F). HCT116 $p53^{-/-}$ cells, however, exhibited an abnormal cell cycle pattern after γ irradiation, with ~90% of the cells in G2 and few remained in either G1 or S (Figures 1 D and F). Consistent with the effect of γ irradiation on the cell cycle of HCT116 $p53^{+/+}$ cells, protein levels of KLF4 and p21$^{WAF1/CIP1}$ were both significantly increased in response to an increase in p53 protein level (Figure 2, lanes 1 and 2). In contrast, no induction in the level of either KLF4 or p21$^{WAF1/CIP1}$ was observed following γ irradiation in HCT116 $p53^{-/-}$ cells (Figure 2, lanes 3 and 4). These results suggest that at least part of the cell cycle arrest caused by γ irradiation is a result of p53-dependent activation of KLF4 and p21$^{WAF1/CIP1}$.

Inducible expression of KLF4 in HCT116 $p53^{-/-}$ cells restores G1 peak.

The failure of γ irradiation to induce expression of KLF4 and p21$^{WAF1/CIP1}$ in HCT116 $p53^{-/-}$ cells correlated with the reduction in G1 and S population (Figures 1 and 2). This suggests that activation of KLF4, with consequent activation of p21$^{WAF1/CIP1}$, maybe necessary for the accumulation of cells in G1. To test this hypothesis, we established a stable HCT116 $p53^{-/-}$ cell line that expressed the receptors for the insect nuclear hormone, ecdysone, and its partner, RXR (25). This cell line, called EcR116 $p53^{-/-}$, was infected with the recombinant adenovirus AdEGI or AdEGI-KLF4 (25) that contained enhanced green fluorescence protein.
(EGFP) as a control or EGFP plus KLF4, respectively. Following infection, cells were γ irradiated or not and then treated with the inducer, ponasterone A (PA), or vehicle alone for 24 hr before being harvested for cell cycle analysis. As seen in Figure 3, treatment of AdEGI-KLF4-infected cells with ponasterone A without irradiation resulted in a statistically significant increase in the G1 population and decrease in G2/M population (Figures 3C, D and J), whereas AdEGI-infected cells without irradiation and treated with ponasterone A had no effect on the cell cycle when compared to non-treated cells (Figures 3 A, B and I). Cells infected with AdEGI followed by irradiation showed G2/M arrest in the absence or presence of ponasterone A (Figures 3 E, F and K), as were cells infected with AdEGI-KLF4 and irradiated without any ponasterone A treatment (Figures 3 G and L). In contrast, upon the addition of ponasterone A, AdEGI-KLF4-infected and irradiated cells had a statistically significant increase in the G1 population (Figures 3 H and L). This finding is reminiscent of the G1/S arrest seen in HCT116 p53+/+ cells following irradiation (compare Figures 3H and 1C).

Figure 4 shows that only cells infected by AdEGI-KLF4 and induced with ponasterone A (lanes 11 and 12) had appreciable amounts of KLF4. The increase in KLF4 level correlated with an increase in p21WAF1/CIP1 level, a finding consistent with our previous observation that KLF4 is an activator of p21WAF1/CIP1 expression (25). The combined results of Figures 3 and 4 indicate that the inducible expression of KLF4 in irradiated cells lacking p53 restores the characteristic G1/S arrest in cells with wild type p53 following irradiation. This finding indicate that KLF4 is necessary and sufficient in mediating the G1 cell cycle effect of p53 following DNA damage.
Small interfering RNA targeting KLF4 mRNA abolishes G1 arrest in γ irradiated HCT116 p53+/+ cells.

Recently, Tuschl and colleagues demonstrated that RNA interference (RNAi) can be provoked in mammalian cell lines through the introduction of small interfering RNA (siRNA) (27, 28). The mediators of sequence-specific mRNA degradation are 21- to 23-nt siRNA duplexes that trigger specific gene silencing in mammalian somatic cells without activation of the unspecific interferon response (27-29). To determine whether we could “knockdown” KLF4 expression using siRNA, we synthesized 23-nt siRNA duplex specific for KLF4 with which to transfect HCT116 p53+/+ cells with or without irradiation. As seen in Figure 5, siRNA for KLF4 significantly reduced the level of KLF4 in response to γ irradiation when compared to untransfected or mocked transfected cells (lanes 2, 4, 6, and 8). In contrast, a control non-specific siRNA failed to abrogate the DNA damage-induced synthesis of KLF4 (lanes 10 and 12). Again, there was a corresponding reduction in the level of p21WAF1/CIP1 in response to γ irradiation in KLF4 siRNA-treated cells (lanes 6 and 8). Importantly, KLF4 siRNA but not non-specific siRNA abolished the G1 population in cells upon γ irradiation (Figure 6L; lanes F, G, H, I, and J). These results complement those from the preceding sections and provide strong evidence that KLF4 is an essential factor in mediating p53-dependent G1 arrest in response to DNA damage.
DISCUSSION

Cell cycle progression is regulated by checkpoint controls, which function to safeguard the integrity of the genome. Activation of DNA integrity checkpoints occurs through the detection of damaged or unreplicated DNA and is in effect until DNA damage has been repaired (30). The checkpoint that arises after DNA damage can activate during G1, S or G2 (3, 31). Arrest in G1 permits repair prior to replication, whereas arrest in S or G2 permits repair of the genome before mitotic segregation. The p53 tumor suppressor has been shown to be integral to both the G1 (32, 33) and G2 (34, 35) DNA damage machinery. This was supported by the results in Figure 1 showing that HCT116 p53+/+ cells arrested at either G1 or G2/M after γ irradiation, as expected for cells with intact checkpoint function. The resultant activation of p53 due to γ irradiation was accompanied by a significant increase in the level of KLF4 and p21<sup>WAF1/CIP1</sup> (Figure 2) in a manner similar to previously observed response of fibroblasts subjected to DNA damage caused by methyl methane sulfonate (24). HCT116 p53-/- cells, in contrast, showed no induction of either KLF4 or p21<sup>WAF1/CIP1</sup> by γ irradiation and arrested only in G2/M (Figure 1). The latter result was consistent with that from a previous study, which also demonstrated that p53 was necessary to sustain G2 arrest (10).

Recent studies indicate that the G1 checkpoint control after DNA damage is consisted of 2 steps (31). The first step is a rapid and p53-independent induction of the G1 checkpoint. It is a result of rapid redistribution of p21<sup>WAF1/CIP1</sup> form cyclin D1-Cdk4/6 complexes to cyclin E-Cdk2 complexes, which are inhibited by p21<sup>WAF1/CIP1</sup> (36, 37). The second step involves the post-translational modifications of p53 by upstream protein kinases including ATM/ATR and Chk1/Chk2 (38, 39), which results in p53 activation and subsequent transcriptional induction of...
Several lines of evidence suggest that KLF4 is involved in the p53-dependent induction of p21\textsuperscript{WAF1/CIP1}. First, p53 mediates the transcriptional induction of KLF4 in response to DNA damage (24). Second, the induction in KLF4 precedes that in p21\textsuperscript{WAF1/CIP1} following DNA damage (24). Third, KLF4 binds to a specific \textit{cis}-element in the proximal promoter of the p21\textsuperscript{WAF1/CIP1} gene and activates the promoter (24). Fourth, p53 and KLF4 physically interact and cause a synergistic induction in p21\textsuperscript{WAF1/CIP1} gene expression (24). The importance of KLF4 in mediating the transcriptional induction of p21\textsuperscript{WAF1/CIP1} is further demonstrated by the observation that p53 fails to activate the p21\textsuperscript{WAF1/CIP1} promoter if the KLF4-response element in the promoter is mutated (24).

In addition to the biochemical evidence supporting a crucial role for KLF4 in mediating the transcriptional induction of p21\textsuperscript{WAF1/CIP1} by p53, the present study provides the genetic evidence to further substantiate the significance of KLF4 in p53-mediated G1 arrest caused by DNA damage. Specifically, the conditional induction of KLF4 in \(\gamma\) irradiated HCT116 p53\textsuperscript{-/-} cells restored the G1 population of cells that are normally present in irradiated HCT116 p53\textsuperscript{+/-} cells (Figure 3). Conversely, inhibition of KLF4 expression in irradiated HCT116 p53\textsuperscript{+/-} cells resulted in an abolishment of the G1 peak, in a manner that resembles the consequence of \(\gamma\) irradiation of HCT116 p53\textsuperscript{-/-} cells (Figure 6). In each case, the induction or inhibition of KLF4 expression was accompanied by a corresponding increase or decrease, respectively, in the level of p21\textsuperscript{WAF1/CIP1} (Figures 4 and 5). Coupled with the findings from our previous study that demonstrated inducible expression of KLF4 causes a G1/S cell cycle arrest (25), it is highly likely that KLF4 serves a pivotal role in mediating the G1 checkpoint function of p53 in response to DNA damage.
In addition to its effect on the G1/S checkpoint, p53 also regulates the G2/M transition in response to DNA damage (11). Part of the mechanism by which p53 inhibits the G2 checkpoint involves inhibition of Cdc2, the cyclin-dependent kinase required to enter mitosis (12). Binding of Cdc2 to cyclin B1 is required for its activity, and repression of the cyclin B1 gene by p53 contributes to blocking of entry into mitosis (40, 41). p53 also represses expression of the Cdc2 gene (42, 43), to help ensure that cells do not escape from the initial block. Moreover, several of the transcriptional targets of p53 can inhibit Cdc2, including p21\(^{WAF1/CIP1}\), 14-3-3\(\sigma\) and Gadd45 (44-46). It is therefore of great interest to note that a recent analysis of KLF4 target genes by cDNA microarrays showed that KLF4 activates expression of 14-3-3\(\sigma\), in addition to p21\(^{WAF1/CIP1}\), and represses expression of Cdc2 (Chen and Yang, unpublished observations). Whether KLF4 is also involved in mediating the G2 checkpoint function of p53 in response to DNA damage needs to be empirically determined.
ACKNOWLEDGEMENTS

We thank Dr. Bert Vogelstein for kindly providing the HCT116 p53+/+ and -/- cell lines. This work was in part supported by grants from the National Institutes of Health (DK52230 and CA84197). V.W.Y. is the recipient of a Georgia Cancer Coalition Distinguished Cancer Clinician Scientist Award.
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ABBREVIATION FOOTNOTE

Cdk, cyclin-dependent kinase; EcR, ecdysone receptor; EGFP, enhanced green fluorescence protein; FBS, fetal bovine serum; GKLF, gut-enriched Krüppel-like factor; KLF4, Krüppel-like factor 4; PA, ponasterone A; RXR, retinoid X receptor; siRNA, small interfering RNA.
FIGURE LEGEND

Figure 1. The effect of γ irradiation on HCT116 p53+/+ and HCT116 p53−/- cells.

In panels A through D, flow cytometric analysis of HCT116 p53+/+ and HCT116 p53−/- were performed at 24 hr after 0 or 12-Gy γ irradiation. Cells were stained with propidium iodide and DNA content was analyzed by flow cytometry. The DNA content of haploid and diploid cells is designated 2n and 4n, respectively. Panels E and F show the means and standard deviations of percentage of G1, S, and G2/M population from 5 independent experiments in non-irradiated and irradiated cells, respectively. Open bars represent HCT116 p53+/+ and closed bars, HCT116 p53−/-. Fifteen thousand cells were analyzed in each experiment. *, p < 0.05 compared with HCT116 p53+/+ cells.

Figure 2. Western blot analysis of p53, KLF4 and p21WAF1/CIP1 in response to γ irradiation.

The levels of p53, KLF and p21WAF1/CIP1 were determined by Western blot analysis in HCT116 p53+/+ (lanes 1 and 2) and HCT116 p53−/− (lanes 3 and 4) without irradiation (lanes 1 and 3) and 24 hr after 12-Gy γ irradiation (lanes 2 and 4).

Figure 3. The effect of inducible KLF4 expression on cell cycle of p53−/− cells following γ irradiation.

EcR116 p53−/− cells were infected with AdEGI or AdEGI-KLF4 and then irradiated with 12-Gy γ radiation (panels E, F, G, and H) or not (panels A, B, C, and D). Cells were then treated with vehicle alone (panels A, E, C, and G) or ponasterone A (PA) (panels B, F, D, and H) for 24 hr before being harvested for flow cytometric analysis. Panels I-L show the means and standard deviations of percent cells in the three phases of the cell cycle in 5 independent experiments.
Fifteen thousand cells were analyzed per experiment. *, p < 0.05 compared with non-induced (-PA) cells.

**Figure 4. Western blot analysis of KLF4 and p21\textsuperscript{WAF1/CIP1} in EcR116 p53-/- cells.**

The levels of KLF4 and p21\textsuperscript{WAF1/CIP1} were determined by Western blot analysis in EcR116 p53-/- cells not infected with any virus (lanes 1-4), infected with AdEGI (lanes 5-8) or with AdEGI-KLF4 (lanes 9-12), followed by 0-Gy (lanes 1, 3, 5, 7, 9, and 11) or 12-Gy (lanes 2, 4, 6, 8, 10, and 12) γ irradiation and induced with ponasterone A (lanes 3, 4, 7, 8, 11, and 12) or vehicle alone (lanes 1, 2, 5, 6, 9, and 10).

**Figure 5. The effect of KLF4 siRNA on protein levels of p53, KLF4 and p21\textsuperscript{WAF1/CIP1} in HCT116 p53+/+ cells following γ irradiation.**

The levels of p53, KLF4 and p21\textsuperscript{WAF1/CIP1} were determined in γ irradiated (even lanes) or non-irradiated (odd lanes) HCT116 p53+/+ cells that were untransfected (lanes 1 and 2), mock-transfected (lanes 3 and 4), transfected with 2 (lanes 5 and 6) or 4 µg (lanes 7 and 8) KLF4-specific siRNA, or transfected with 2 (lanes 9 and 10) or 4 µg (lanes 11 and 12) non-specific siRNA. Cells were harvested 24 hr later for Western blot analysis of protein levels.

**Figure 6. The effect of KLF4 siRNA on the cell cycle in HCT116 p53+/+ cells following γ irradiation.**

Flow cytometric analyses were performed in HCT116 p53+/+ cells that were non-irradiated (panels A-E) or irradiated (panels F-J) followed by mock transfection (panels A and F), transfected with 2 (panels B and G) or 4 µg (panels C and H) KLF4-specific siRNA, or
transfected with 2 (panels D and I) or 4 µg (panels E and J) non-specific siRNA. Cells were studied 24 hr following irradiation. Panels K and L show the means and standard deviations of percent cells in G1 phase for each of the treatments in 5 independent experiments. Fifteen thousand cells were analyzed in each experiment. *, $p < 0.05$ compared with mock-transfected cells.
Figure 1
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- **p53**
- **KLF4**
- **p21**

Figure 2
Figure 3

**AdEGI**

PA

- AdEGI-KLF4

A

B

C

D

E

F

G

H

Cell Number

DNA Content

**AdEGI**

0-Gy

12-Gy

Cell Number

DNA Content

**AdEGI-KLF4**

0-Gy

12-Gy

**AdEGI (0-Gy)**

I

J

K

L

% Cells

% Cells

% Cells

% Cells

G1

S

G2/M

G1

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G2/M

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Figure 3
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Figure 4
Figure 5
Figure 6
Kruppel-like factor 4 mediates p53-dependent G1/S cell cycle arrest in response to DNA damage
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J. Biol. Chem. published online November 8, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M211027200

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