Radiation-induced cellular senescence results from a slippage of long-term G₂ arrested cells into G₁ phase

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Diploid cells undergoing senescence and mitotic slippage have been reported in the literature. However, the mechanisms triggering senescence in long-term G₂-arrested cells are currently unclear. Previously, we reported that the cell cycle of the human uveal melanoma cell line, 92-1, is suspended for up to 6 d upon exposure to 10 Gy ionizing radiation (IR), followed by senescence. In the current study, we initially distinguished senescence in long-term blocked 92-1 cells from mitotic slippage by confirming the blockage of cells in the G₂ phase. We subsequently showed that the genes essential for G₂-M transition are prematurely downregulated at both the transcriptional and translational levels. Furthermore, levels of the G₁-specific markers, Cyclin D1 and Caveolin-1, were distinctly increased, while S/G₂-specific markers, Cyclin B1 and Aurora A, were significantly downregulated. These findings collectively imply that long-term G₂-arrested cells undergo senescence via G₂ slippage. To our knowledge, this is the first study to report that the cellular process of G₂ slippage is the mechanism responsible for senescence of cells under long-term G₂ arrest.

Introduction

Cellular senescence is induced by several factors, such as telomere shortening below a crucial length,[1] constitutive activation of oncogenes,[2] tumor suppressor inactivation,[3] exposure to exogenous DNA damaging agents[4] and mitochondrial oxidative stress,[5] which are associated with activation of DNA damage response (DDR) pathways and irreversible loss of proliferative potential. Cellular senescence, as a naturally existing mechanism, was described to involve in organismal aging by terminating cellular proliferation in vivo.[6] Senescent cells usually contain diploid DNA, a typical characteristic of the G₁ phase in somatic or in vitro cultured cells.[7,8] Several earlier and recent studies have reported that replicatively senescent or DNA damage-induced senescent cells contain a sizeable subpopulation with tetraploid DNA content.[9-13] As a dogma, once a cell progresses past the restriction point, it becomes irreversibly committed to the S phase and subsequent division until the next G₁ phase, signifying that all intermediate phases are unstable, except G₀ and early G₁.[14-16] Upon completion of genome replication, G₂ cells accumulate significant amounts of mitotic enzymes for chromosome segregation and cytokinesis. These factors are degraded by the anaphase-promoting complex (APC/C) to promote exit of cells from mitosis,[17] and their unscheduled degradation is believed to result in long-term G₂ arrest. Indeed, in response to DNA damage, APC/C, normally inactive from late G₁ to metaphase, is prematurely activated in G₂.[12,18,19] Moreover, factors destroyed by APC/C cannot recover, and long-term G₂-arrested cells present a senescence-like phenotype.[12] However, the mechanisms by which cells in long-term G₂ arrest undergo senescence remain to be established.

When the spindle assembly checkpoint is activated by nocodazole (Noc), colcemid, taxol, monastrol, vinblastine or other cytotoxic reagents, cells exit mitosis via mitotic slippage accompanied by Cyclin B1 proteolysis, re-enter the G₁ phase and are subsequently arrested at this phase in association with senescence.[20,21] This phenomenon gives rise to the theory that long-term G₂ arrested cells undergo G₂ slippage, bypass the M phase, directly enter the G₁ phase via degradation of Cyclin B1, and consequently become senescent.

In this study, we found that after 92-1 cells exposed to 10 Gy X-rays, long-term G₂ arrest was induced accompanied by the downregulation of genes essential for G₂-M transition. Next, we proved that these cells skipped M phase, directly slipped into G₁ phase, and then underwent senescence. To our knowledge, this
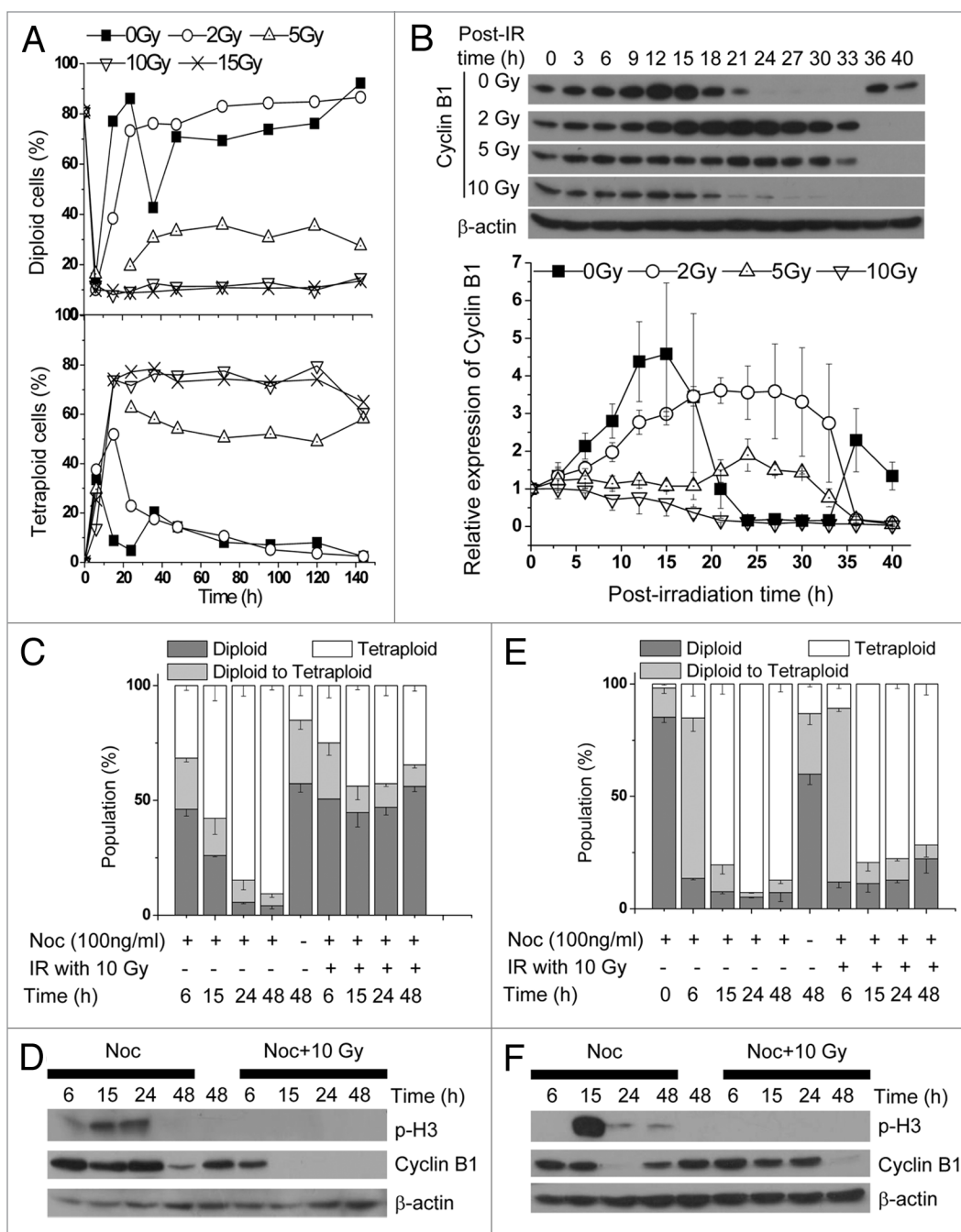
Figure 1. IR induces long-term G$_2$ arrest. 92-1 cells were synchronized in the G$_1$/S border with 1 mM thymidine for 24 h and released with fresh culture medium to allow re-entry into the cell cycle. After release for 1 h, cells were treated with the specified radiation doses. Between 0 and 144 h after X-ray treatment, cells were harvested and analyzed with a flow cytometer. (**A**) Kinetics of cell cycle distribution. Square, 0 Gy; circle, 2 Gy; up-triangle, 5 Gy; down-triangle, 10 Gy; cross, 15 Gy. (**B**) Kinetic variations in Cyclin B1 protein analyzed via immunoblot using anti-Cyclin B1 antibody. Protein levels of Cyclin B1 were calculated by averaging results from three independent experiments. Bars represent SEM. Square, 0 Gy; circle, 2 Gy; up-triangle, 5 Gy; down-triangle, 10 Gy. 92-1 cells in exponentially growing status with 70% confluence (**C and D**) or G$_1$/S border cells synchronized with thymidine and released for 1 h (**E and F**) were exposed to 10 Gy of X-rays, prior to administration of 100 ng/mL nocodazole (Noc). The percentage of cells in each cell cycle phase was shown in panel (**C and E**). Levels of Cyclin B1 protein and H3 phosphorylated at Ser 28 were shown in panel (**D and F**).

is the first study to present the current hypothesis on G$_2$ slippage and provide supportive experimental evidence using the highly radiosensitive cell line, 92-1. In this case, the cell cycle is suspended following treatment with 10 Gy IR, and cells undergo senescence 5 d after irradiation.

Results

Cells under long-term G$_2$ arrest undergo senescence. Previous studies have shown that upon exposure to 10 Gy IR, the cell cycle of exponentially growing 92-1 cells is suspended, and cells

Figure 2. Long-term G$_2$ arrested cells undergo senescence. (**A**) 92-1 cells in the exponentially growing phase and G$_1$/S border cells released for 1 h (S phase) or for 18 h (G$_1$ phase) were treated with 10 Gy X-rays. Cells were fixed and stained with the Senescence β-Galactosidase Staining Kit at the corresponding times. Ratios of SA-β-gal-positive cells were quantified by counting at least 1,000 cells. Cells in the exponentially growing phase were irradiated at a dose of 10 Gy, harvested at early (15 h) or late (48 h) time points and analyzed for Ki67 expression with immunofluorescence staining. Ratios of Ki67-positive cells were calculated for 92-1 (**B and C**) and OCM-1 (**D and E**) basing on the results obtained by counting at least 1,000 cells.

undergo senescence.[22] In the current investigation, we repeated the cell cycle assay with 92-1 cells synchronized at the G$_1$/S border using thymidine. As shown in **Figure 1A**, untreated G$_1$/S border cells showed typical cell cycle progression and successively entered S, G$_2$/M and the subsequent G$_1$ phase. Cells exposed to IR also resumed cell cycle progression, but failed to undergo cell division, and tetraploid cells accumulated in a dose-dependent manner.

Cyclin B1, a regulatory subunit of the M phase-promoting factor, is essential for G$_2$-M transition. Downregulation or over-expression of a non-degradable form of Cyclin B1 can lead to blockage of cells in the tetraploid stage.[23,24] Monitoring of the kinetics of Cyclin B1 expression in G$_1$/S border cells exposed to X-rays revealed similar threshold dose-dependent induction by Cyclin B1 to cell cycle progression. At doses above the threshold level, Cyclin B1 degradation was induced about 6 h after IR, and the cell cycle was not recovered (**Fig. 1B**).

Next, we wanted to distinguish G$_2$ block from the mitotic spindle checkpoint in exponentially growing 92-1 cells treated with 10 Gy X-rays by using phosphorylated histone H3 serine 28, a marker of mitotic cells. After the treatment with Noc, which blocks cells in prometaphase,[25] the percentage of tetraploid cells increased (**Fig. 1C**), with upregulated phosphorylation level of histone H3 at Ser 28 (**Fig. 1D**) indicating that these cells were in M phase. But we didn't observe the phosphorylation of H3 Ser 28 in the cells treated by IR (**Fig. 1D**). Similar results were obtained with G$_1$/S border cells (**Fig. 1E and F**). The phosphorylation of H3 Ser 28 was not observed, even though the proportion of tretraploid cells reached to 79.4 ± 0.06% 15 h after irradiation. These results demonstrate that tetraploid 92-1 cells induced by 10 Gy X-rays are actually in the G$_2$ phase.

To confirm whether long-term G$_2$-blocked cells undergo senescence, we repeated the senescence assay with 92-1 cells synchronized at the G$_1$/S border with thymidine. The rates of SA-β-gal-positive cells were not significantly different among G$_1$, exponentially growing and S phase cells, and displayed a dramatic increase on the day 5 after irradiation (**Fig. 2A**). As senescent cells could not undergo cell cycle recovery, cell proliferation was investigated. Ki67 is an accepted marker for determining cell growth fractions,[26] as it is preferentially expressed during the late G$_1$, S, G$_2$ and M phases but absent in resting cells (G$_0$) and the early G$_1$ phase. As shown in **Figure 2B and C**, at 48 h after IR, Ki67 was significantly decreased in 92-1, but not in OCM-1 cells, which were able to recover and re-enter the cell cycle (**Fig. 2D and E**).[22]
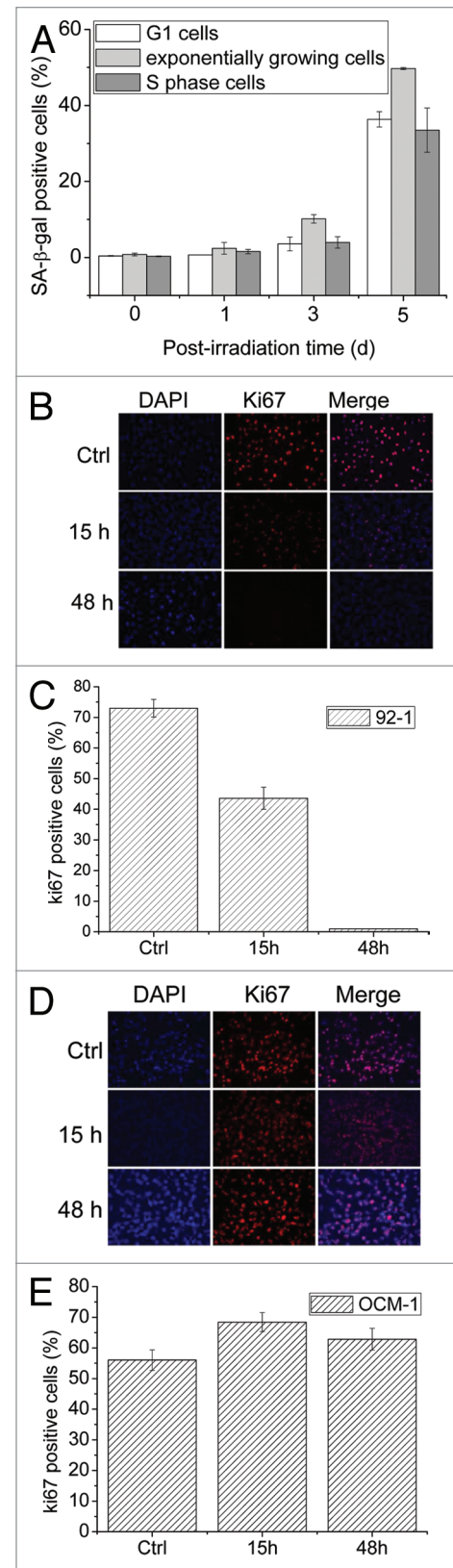
Genes functioning in G$_2$-M transition are downregulated upon long-term G$_2$ arrest. To establish the precise mechanism underlying long-term G$_2$ arrest induced by IR, we measured global genome expression profiles following exposure of cells to

Table 1. Spearman rank correlation for two independent replicate experiments

Repeat		Ctrl		15 h		48 h		Noc	
		1	2	1	2	1	2	1	2
Ctrl	1	1.000	0.997	0.991	0.991	0.981	0.983	0.991	0.992
	2	0.997	1.000	0.992	0.992	0.981	0.983	0.992	0.992
15h	1	0.991	0.992	1.000	0.997	0.985	0.987	0.990	0.990
	2	0.991	0.992	0.997	1.000	0.984	0.987	0.990	0.990
48h	1	0.981	0.981	0.985	0.984	1.000	0.993	0.981	0.982
	2	0.983	0.983	0.987	0.987	0.993	1.000	0.982	0.984
Noc	1	0.991	0.992	0.990	0.990	0.981	0.982	1.000	0.997
	2	0.992	0.992	0.990	0.990	0.982	0.984	0.997	1.000

*r^2 value for two independent replicate experiments. Four kinds of samples, "Ctrl," "15 h," "48 h" and "Noc" as described in Materials and Methods, were analyzed by Affymetrix Human Genome U133 Plus 2.0 Arrays. Two independent replicate experiments were measured by Spearman Rank Correlation.

10 Gy X-rays with Affymetrix Human Genome U133 Plus 2.0 Arrays containing more than ~54,000 probe sets representing ~38,500 different human genes. The mean transcript levels from two independent replicate experiments were highly reproducible, with r^2 values of 0.993 to 0.997 (**Table 1**).

As depicted in **Figure 3A**, 1,265 genes displayed a 2-fold or higher change following treatment, compared with sham control (Ctrl), among which 772 genes were annotated with a biological process using the PANTHER program (**Fig. 3B**) and 925 using the GOTERM program. Among the 925 genes, 206 in the cell cycle category were further annotated with a biological process using GOTERM program. Within this gene subgroup, 63 functioned in nuclear division, 39 in chromosome segregation and 26 in DNA replication (**Fig. 2C**). The majority of these genes were downregulated after irradiation (except 10 cases), and expression changes were markedly different from those following Noc treatment. Analysis of the significance of the functional categories revealed that changes were significant in genes associated with nuclear division and chromosome segregation among the 1,265 altered genes (**Fig. 3D**). Enrichment was additionally observed in mRNA transcription, DNA replication and chromatin packaging and remodeling categories.

A number of key factors, Cdc25, Cdk1, Cyclin B1, Plk1 and Aurora A, essential for G_2-M transition were significantly downregulated after IR exposure, but not Noc treatment (**Fig. 3E and F**). Notably, FoxM1, an essential factor in DNA damage checkpoint recovery, was decreased by 17-fold. Wiebusch and colleagues showed that long-term G_2 arrest is induced via p53/p21-dependent premature Cdh1 activation.[12] As shown in **Figure 3F**, IR induced stability of p53 and p21 accumulation. Cdc20 and Cdh1, E3 activators of APC/C, target several essential mitotic regulators for proteolysis. Moreover, Cdc20 is one of the substrates of Cdh1.[27] Notably, Cdh1 expression was stable, but not Cdc20, implying that Cdh1 promotes the degradation of targeted proteins by the proteasome in irradiated 92-1 cells. Based on these collective findings, we propose that IR triggers downregulation of genes essential for G_2-M transition at both the mRNA and protein levels, leading to long-term G_2 arrest.

Long-term G_2-arrested cells undergo G_2 slippage. Within the gene expression spectrum, 31 cell cycle-relevant genes were altered upon X-ray irradiation. Genes associated with the S/G_2 phase were downregulated, while those functioning in G_0/G_1 were upregulated after IR (**Fig. 4A**), implying possible slippage from the G_2 to G_1 phase. Therefore, we assessed the actual status of long-term G_2-arrested cells.

Initially, Cyclin B1 and Aurora A were validated as markers for G_2 phase cells. Meanwhile, Cyclin D1 and Caveolin-1 were identified as the markers for the G_1 phase, based on their corresponding cell cycle distribution (**Fig. 4B**) and expression patterns (**Fig. 4C**) of cells released from the G_1/S border synchronized with thymidine. Next, expression patterns of cell cycle-specific genes from G_1/S border cells after IR were examined. Notably, Cyclin B1 and Aurora A proteins disappeared, while the expression of Cyclin D1 and Caveolin-1 gradually increased during the post-irradiation incubation period (**Fig. 4D–F**). Alterations were confirmed in cells from G_2 to G_1 phases, with upregulation of NFκB and Akt that regulate Cyclin D1 expression (**Fig. 4G**).[28-30] These findings collectively indicate that 92-1 cells undergo mitotic-like slippage from G_2 directly to the G_1 phase.

Discussion

In the current study, we showed that long-term G_2-arrested 92-1 cells undergo senescence via G_2 slippage due to promoted degradation of numerous APC/C substrates. The microarray assay revealed significant downregulation of the mRNA levels of the majority of G_2-M transition genes. Furthermore, levels of the G_1-specific markers, Cyclin D1 and Caveolin-1, were significantly increased,[31-33] while those of S/G_2-specific markers, Cyclin B1 and Aurora A, were markedly decreased.[34,35] The data collectively provide insights into the mechanisms underlying long-term G_2 arrest induced by IR, and support the hypothesis that long-term G_2-arrested cells undergo senescence via G_2 slippage.

G_2 slippage describes a process whereby S or G_2 phase cells treated with DNA damage agents are arrested in the G_2 phase. Although most of the damage is repaired rapidly,[22] these cells cannot enter the M phase and display downregulation of the genes essential for G_2-M transition. Instead, they bypass the M phase and slip into the G_1 phase without mitosis. G_2 slippage is distinct from endocycle or endomitosis and represents a novel
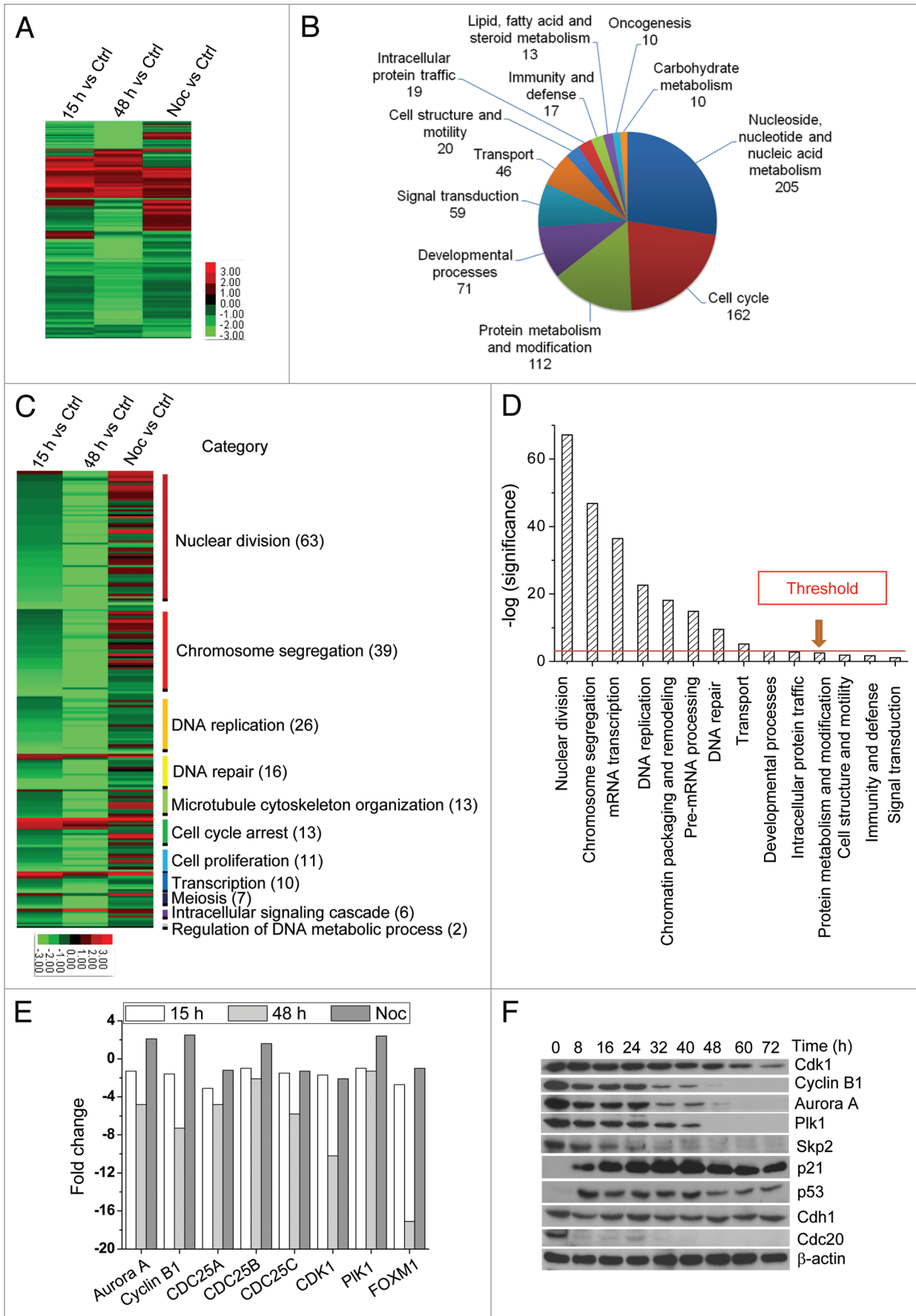
A

B
- Lipid, fatty acid and steroid metabolism 13
- Oncogenesis 10
- Intracellular protein traffic 19
- Immunity and defense 17
- Carbohydrate metabolism 10
- Cell structure and motility 20
- Transport 46
- Signal transduction 59
- Developmental processes 71
- Protein metabolism and modification 112
- Nucleoside, nucleotide and nucleic acid metabolism 205
- Cell cycle 162

C
Category
- Nuclear division (63)
- Chromosome segregation (39)
- DNA replication (26)
- DNA repair (16)
- Microtubule cytoskeleton organization (13)
- Cell cycle arrest (13)
- Cell proliferation (11)
- Transcription (10)
- Meiosis (7)
- Intracellular signaling cascade (6)
- Regulation of DNA metabolic process (2)

D
-log (significance)
Threshold

Nuclear division
Chromosome segregation
mRNA transcription
DNA replication
Chromatin packaging and remodeling
Pre-mRNA processing
DNA repair
Transport
Developmental processes
Intracellular protein traffic
Protein metabolism and modification
Cell structure and motility
Immunity and defense
Signal transduction

E
Fold change
15 h 48 h Noc
Aurora A Cyclin B1 CDC25A CDC25B CDC25C CDK1 PlK1 FOXM1

F
Time (h) 0 8 16 24 32 40 48 60 72
Cdk1
Cyclin B1
Aurora A
Plk1
Skp2
p21
p53
Cdh1
Cdc20
β-actin

Table 2. Fold change of gene expression in Notch signaling pathway category

Genes	Fold change (15 h vs. Ctrl)	Fold change (48 h vs. Ctrl)	Fold change (Noc vs. Ctrl)
DLL1	2.44	1.37	4.03
NRG1	2.57	2.05	1.16
CFD	1.33	2.27	1.15
JAG1	3.58	1.65	1.07

process of endoreplication. Endocycle is a form of endoreplication in which the cell cycle consists of discrete S and G phases. The duplicated chromatids remain physically associated, leading to the production of polytene chromosomes.[36,37] In contrast, long-term G_2-arrested cells exhibit obvious separate sister chromatids interspersed with dispersed centromeres.[13] Mitotic slippage, a canonical case of endomitosis, is another form of endoreplication in which cells contain early mitotic markers, such as phosphohistone H3, but do not undergo chromosome segregation and cytokinesis.[36-38] In long-term G_2-arrested cells, phospho-histone H3 and chromosome segregation were not detected in this study. Therefore, G_2 slippage appears to represent a third means of endoreplication with chromatid separation, but lack early mitotic markers and mitosis.

Although the mechanism of G_2 slippage is unknown at present, a number of findings have provided clues. First, the genes essential for G_2-M transition were downregulated at both transcriptional and translational levels. Most of these genes are substrates of Cdh1 and transcribed by FoxM1.[27,39] Stress-induced Cdh1 activation may be explained by at least three distinct mechanisms. Phosphorylated Cdc14B may be released from the nucleolus to nucleoplasm and induce activation of Cdh1 in response to genotoxic stress.[19] In addition, downregulation of Emi1 functions as a redundant mechanism to safeguard Cdh1 activation after DNA damage.[12] The Notch signaling pathway influences mitosis to endoreplication transition through modulating the expression of three important genes. This pathway induces expression of Cdh1, but suppresses the S-phase Cyclin-dependent kinase inhibitor, p27 and Cdc25.[40] Earlier studies demonstrated that Notch mutation prevents follicle cells from switching to endoreplication, but mitotic division is continued.[41,42] Activation of Notch signaling following exposure to IR has been reported.[43] Interestingly, the genes (CFD, DLL1, NRG1 and JAG1) participating in the Notch signaling pathway were significantly upregulated (**Table 2**), while Cdc25 expression was decreased, following X-ray irradiation. Second, the transcription factor network, in which transcription factors expressed in one cell cycle phase bind to the promoters of genes encoding transcription factors that function

in a subsequent phase,[44] may drive cells to undergo G_2 slippage and enter the G_1 phase regardless of the Cyclin-Cdk complex. Yeast cells lacking S phase and mitotic B-Cyclin genes showed oscillations and repeated G_1 events, despite failure of DNA replication, mitosis and cytokinesis.[45] Recent studies revealed that these events are driven by regulation of the transcription factor network, independently of the Cyclin-Cdk complex.[46] In conclusion, after X-ray irradiation, activation of ATM/ATR induces immediate G_2 arrest and premature activation of Cdh1, in turn, causing marked downregulation of substrate genes at both protein and mRNA levels and maintenance of cells in long-term G_2 arrest. Subsequently, the transcription factor network drives G_2 slippage and causes arrested cells to enter the G_1 phase. However, the detailed mechanisms require further investigation.

Replicatively senescent cells tend to remain at the G_1 or G_0 stage of the cell cycle.[8] However, a few studies to date have reported that populations of senescent cells contain a sizeable fraction of tetraploid cells.[9-11,13] G_2 slippage provides a reasonable explanation for why cells with 4N DNA content undergo cellular senescence. Indeed, our group and other researchers consistently observed that Cyclin B1, which promotes G_2/M transition, is absent in senescent cells with 4N DNA content, while Cyclin D1 promoting progression through G_1/S is elevated significantly within the process of senescence.[13] However, it is unclear why these cells become polyploid through G_2 slippage. One possibility is that polyploidization occurs as a precursor to aneuploidy that may contribute to oncogenesis.[47] Another hypothesis is that these cells use G_2 slippage as a means of survival during mitotic or genotoxic stress. Endopolyploidization in senescence is associated with re-replication of the genome in damaged G_2/M cells, which undergo meiotic-like division and become genome-reduced. Indeed, besides diploid and tetraploid, aneuploid cells are present as reverted cells that escape senescence.[48] Moreover, 0.3% of senescent cells in the G_2 phase can re-replicate the genome.[13]

Materials and Methods

Cell culture and radiation exposure. Cells were cultured and treated, as described previously.[22] Briefly, human choroidal malignant melanoma 92-1 cells were cultured in RPMI-1640 (Sigma) complemented with 10% fetal bovine serum (FBS, Hyclone), 100 μg/mL streptomycin and 100 units/mL penicillin in a humidified atmosphere of 5% CO_2. Cells were seeded in 12-well plates, φ35 mm culture dishes or 25 cm^2 culture flasks 2 d before irradiation, resulting in < 70% confluence at the time of irradiation at room temperature (100 kV, 5 mA, no additional filter; Faxitron RX650) and a dose rate of 1 Gy/min.
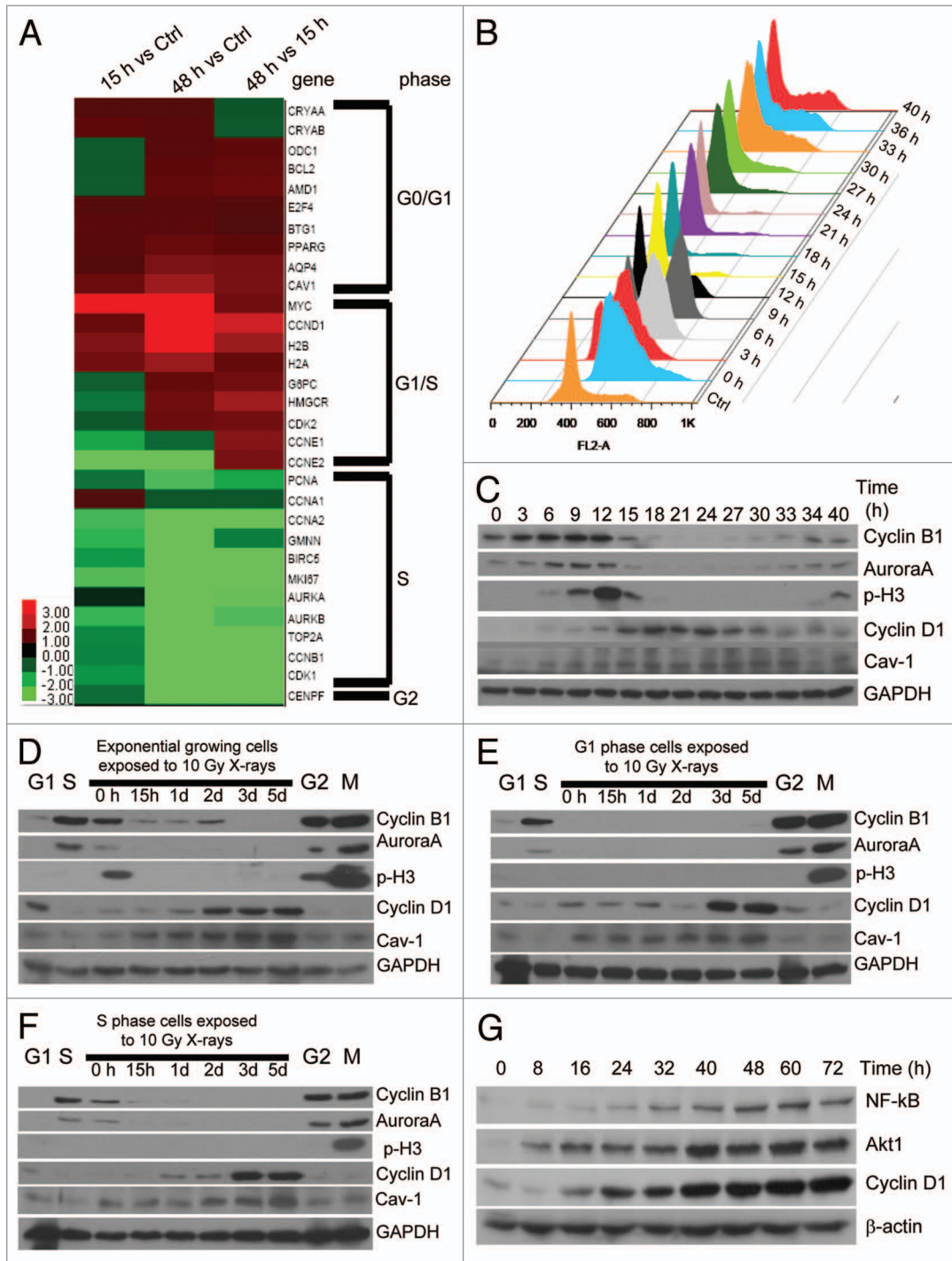
Figure 4. Long-term G$_2$ arrested cells undergo G$_2$ slippage. (**A**) Overall, 31 cell cycle-specific genes were analyzed and presented in the heatmap. Caveolin-1 (encoded by *CAV1*), Cyclin D1 (encoded by *CCND1*), H2B (encoded by *HISTIH2B*), Aurora A (encoded by *AURKA*), Ki67 (encoded by MKI67) and Cyclin B1 (encoded by *CCNB1*) are presented. Magenta, upregulated; green, downregulated. Cell cycle distribution (**B**) and protein expression of specific biomarkers (**C**) of 92-1 cells synchronized in the G$_1$/S border, as described in **Figure 1**, and released at the indicated time-points were analyzed. (**D–F**), Protein expression of specific biomarkers in 92-1 cells exposed to 10 Gy irradiation. Positive/negative controls, whose cell cycle distribution are displayed in (**B**), were loaded on each side of the western blot gel. G$_1$, 92-1 cells obtained by synchronization in G$_1$/S with thymidine and release for 18 h; S, 92-1 cells obtained by synchronization in G$_1$/S with thymidine and release for 6 h; G$_2$, 92-1 cells obtained by synchronization in G$_1$/S with thymidine and release for 9 h; M, 92-1 cells obtained by synchronization in the M phase, with Noc treatment for 15 h. Cav-1, Caveolin-1; p-H3, phosphorylated histone H3. (**D**) Exponentially growing 92-1 cells. (**E**) G$_1$ phase 92-1 cells obtained by synchronization in G$_1$/S with thymidine and release for 18 h. (**F**) S-phase 92-1 cells obtained by synchronization in G$_1$/S with thymidine and release for 6 h. (**G**) Expression of Cyclin D1, NFκB and Akt1 in exponentially growing cells exposed to 10 Gy X-rays. Whole-cell lysates were analyzed for expression of the indicated proteins via immunoblot analysis. Western blot experiments were independently repeated three times. A single gel for each protein is presented.

Cell synchronization. For cell synchronization, 92-1 cells were grown in medium containing 1 mM thymidine (Sigma) for 24 h, and released by culture in RPMI-1640 for different times to obtain the corresponding phase cells. G_1/S border cells were released for 0 h, S phase cells for 6 h and G_2 cells for 8 h. M phase cells were obtained by synchronization with 100 ng/mL nocodazole (Noc) for 15 h.

Cell cycle assay. Cells were harvested and fixed with 70% pre-chilled ethanol for over 24 h at –20°C. Prior to analysis, fixed cells were washed twice with PBS, treated with 100 μg/mL RNase A and 50 μg/mL propidium iodide (BD Biosciences) for 30 min at 37°C and analyzed using FACS Calibur flow cytometry (Becton Dickinson). Cell cycle distribution was analyzed with FlowJo. DNA content was measured with CellQuest (Becton Dickinson).

Immunofluorescence. Exponentially growing cells were plated on sterile coverslips at a density of 1×10^5 cells/well. After 48 h, cells were irradiated with X-rays, fixed in 4% paraformaldehyde for 10 min and methanol at –20°C for 5 min, permeabilized with 0.5% Triton X-100 for 1 h, blocked in buffer (5% non-fat dry milk with 0.5% Triton X-100) for 2 h and stained with primary anti-Ki67 antibody (1:2,000, Cell Signaling Technology) for 2 h. Bound antibody was visualized using Alexa Fluor 594 anti-rabbit antibody (1:2,000, Molecular Probes), and cell nuclei were counterstained with DAPI solution (Invitrogen). Images were obtained using a fluorescence microscope (Keyence). Ki67-positive cells were counted using fluorescence microscopy (Nikon BX50), and at least 500 cells scored for each sample.

cDNA synthesis and microarray hybridization. Four types of samples were prepared for microarray assay. Cells were synchronized for 24 h with 1 mM thymidine and released by culture in regular medium for 2 h. "Ctrl" represents the sham control group. Cells were harvested immediately after release. The "15 h" group represents G_2 blocked cells. Cells were treated with 10 Gy X-rays after release and harvested after 15 h. The "48 h" group signifies long-term blocked cells. Cells were treated with 10 Gy irradiation after release and harvested 48 h later. The "Noc" group represents M phase cells. Cells were administered 100 ng/mL Noc after release and harvested 15 h later. For harvest, cells were washed twice with PBS and collected by gentle centrifugation (300 g for 5 min). Pellets were frozen immediately in liquid nitrogen, and total RNA was extracted from cells as described previously.[20] RNA was purified with an RNAeasy MinElute Cleanup Kit (Qiagen). cDNA synthesis and fluorescent labeling were performed by the Gene Tech (Shanghai) Company using GeneChip One cycle Labeling (Affymetrix), and hybridized to Human 2.0 Expression array (Affymetrix). Hybridization and image collection were performed by the Gene Tech Company.

Western blot analysis. Western blotting was performed as described previously.[22] Briefly, cells were collected and lysed with RIPA solution (Beyotime). Samples were centrifuged at 10,000 *g* for 15 min at 4°C, and total protein concentrations determined from supernatants using the BCA protein assay kit (Pierce). Thereafter, samples were resolved with SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (GE healthcare). Membranes were blocked for 2 h in blocking buffer (5% non-fat dry milk) and incubated with primary antibodies for 2 h. Next, membranes were washed three times with PBS containing 0.1% Tween-20, and incubated with secondary antibody for 1.5 h. Following three further washes with PBS containing 0.1% Tween-20, protein bands were visualized using the enhanced chemiluminescence system (Amersham-Buchler) and exposed to X-ray medical film (Kodak). β-actin or GAPDH was used as the loading control. The antibodies employed in this study were: anti-β-actin (1:5,000, Santa Cruz), anti-Cdh1 (1:1,000, DCS-266, Abcom), anti-Cdc20 (1:1,000, H-175, Santa Cruz), anti-Skp2 (1:1,000, H-435, Santa Cruz), anti-Plk1 (1:1,000, 36–298, Abcam), anti-Aurora A (1:1,000, 35C1, Abcam), anti-Cyclin B1 (1:1,000, GNS1, Santa Cruz), anti-Cyclin D1 (1:1,000, A-12, Santa Cruz), anti-Akt1 (1:1,000, 9272, Cell Signaling), anti-NFκB (1:1000, 3037, Cell signaling) and Caveolin-1 (1:500, N-20, Santa Cruz).

SA-β-gal staining. 92-1 cells (1×10^5) were plated in 35 mm tissue culture dishes and incubated for 48 h before exposure to 10 Gy X-rays. At each indicated timepoint after treatment, cells were stained with the Senescence β-Galactosidase Staining Kit (C0602, Beyotime) following the standard protocol suggested by the manufacturer. Senescent cells were identified under a light microscope.

Computational and statistical analyses. All experiments were repeated at least three times, and data presented as means ± SEM.

Bioinformatic analysis. To compare transcript dynamics among control, 15 h, 48 h and Noc groups, data sets were systematically aligned using Perseus software. Bioinformatics analysis was performed using PANTHER or GOTERM. Similar gene ontology analysis data were obtained with both programs.

Conclusion

Both G_2 slippage and cellular senescence are responses of S or G_2 phase cells to genotoxic stress, which prevent those with DNA damage from dividing. The G_2 slippage mechanism explains why long-term G_2 arrested cells undergo cellular senescence. Based on the collective findings, we propose a model whereby G_2-arrested cells undergo G_2 slippage and subsequently become senescent, and additionally present evidence that cellular senescent cells are in the G_1 phase.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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