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13. ABSTRACT (Maximum 200 Words)

Clusterin is a protein that has been implicated in many normal physiological processes (tissue remodeling, sperm maturation) as well as many pathological processes (Alzheimer disease, atherosclerosis, cancer). Our laboratory became interested in clusterin when we identified it as an x-ray induced protein/transcript in human melanoma cells. The secretory form of clusterin (sCLU) has been shown to have cytoprotective effects after cellular stress and injury. Recently, Redondo *et. al* demonstrated that sCLU was overexpressed in breast cancer. sCLU over expression may provide a selective advantage in malignant cells. The most effective therapies for breast cancer after surgery include chemo- and radiation therapies. These therapies often fail as the tumor develops drug and radiation resistance. Our lab has shown that sCLU is induced by physiological doses of taxol, taxotere and radiation. Additionally, we have shown that sCLU is transcriptionally repressed by the tumor suppressor protein, p53, which is found mutated in approximately 20% of mammary tumors. Understanding the cellular and molecular responses of malignant and normal cells to these chemo- and radiation therapy would allow us to increase the efficacy of these treatments. Insight into the regulation of sCLU will allow us to better understand some of these processes.

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Introduction:

Clusterin (CLU) is a glycoprotein that has been implicated in a multitude of biological and pathological processes, including breast cancer (1). The function of clusterin is still unknown. Our laboratory identified CLU as a x-ray induced protein/transcript that could interact with the DNA double strand break repair protein, Ku70, implicating a possible role for CLU in DNA repair (2). This led us to propose the existence of a nuclear form of this protein (nCLU) (3). While secretory clusterin (sCLU) is thought to be cytoprotective, nCLU is cytotoxic(4,5).

The most effective therapies for breast cancer after surgery include chemo- and radio- therapies. These therapies often fail as the tumor develops drug and radiation resistance. Our lab has shown that sCLU is induced by physiological doses of taxol, taxotere and radiation (Criswell *et al.*, unpublished data). Understanding the cellular and molecular responses of malignant and normal cells to these therapies would allow us to increase the efficacy of these treatments. Insight into the regulation of sCLU will allow us to better understand these processes.

Determining the transcriptional regulation of sCLU will allow us to better understand its function after IR. As we began to investigate the regulation of sCLU, we noticed a correlation between sCLU expression and p53 status. p53 is a tumor suppressor protein that is found mutated in over 50% of all human cancers (6) and in 20% of all breast cancers (http://p53.curie.fr). The p53 protein is stabilized in response to genotoxic stress and acts as a transcription factor for genes resulting in either cell cycle arrest or apoptosis(7-9). Several lines of evidence suggest that sCLU is transcriptionally repressed by p53. (A) Wild-type p53 status in various breast cancer cell lines correlates with low basal levels of sCLU and, in general, no inducibility of sCLU after IR exposure. In contrast, breast cancer cell lines that contain mutant p53 or are null for p53 demonstrate high basal levels of sCLU; (B) HCT116 colon cancer cells that are p53 null show a dramatic induction of sCLU after IR as compared to cells that contain wild-type p53; and (C) MCF-7 cells and RKO colon cancer cells that contain the HPV-16 E6 protein have an earlier and greater induction of sCLU after IR as compared to cells without E6. Current work is focused on better understanding the mechanisms underlying p53 suppression of the gene, as well as transcription factors needed for IR induction. We have included the new statement of work that was submitted in the last update to cover these experiments.

Revised Statement of Work:

Aim 1: To investigate the transcriptional repression of secretory clusterin (sCLU) by the tumor suppressor protein, p53.

Task 1:

1. Screen various breast cancer cell lines for p53 and sCLU status before and after ionizing radiation (IR) exposure. This will allow us to examine sCLU basal levels and inducible levels after IR in breast cancer cells that contain either wild-type or mutant p53. We will use western blot analyses to examine sCLU protein levels.

Task 2:

1. Generate MCF-7 cells that stably express a clusterin promoter luciferase reporter construct that will allow us to monitor clusterin promoter activity in these cells before and after IR exposure.
    a. MCF-7 cells will be transfected with 1403 bp of the clusterin promoter that have been fused to a luciferase reporter (these cells will be referred to as MCF-7 1403 cells). Time course and dose response assays will be used to select a stable clone will be selected that mimics the behavior of the endogenous gene before and after IR exposure.
2. sCLU expression in MCF-7 cells that stably express the human papilloma virus E6 protein to abrogate p53 expression will be monitored via western blot and northern blot analyses. MCF-7 1403 cells will be stably transfected with the E6 protein and sCLU promoter activity will be monitored by luciferase assays.
3. p53 status will be further modulated in the MCF-7 1403 cells by stable expression of the dominant negative 273 mutant of p53. The effect of this mutant on sCLU expression will be monitored by luciferase assays, western blot and northern blot analyses.

Task 3:

1. Isogenically matched HCT116 colon cancer cell lines that differ only in their p53 status will be used as a genetic model to investigate the effect of p53 on sCLU (wild-type p53 versus p53 null). We will switch to this genetic system in colon cancer cells since no equivalent system currently exists in a breast cancer model. Western and northern blot analyses will be used to determine sCLU expression in these cell lines.
2. sCLU expression will be monitored in mice that either contain wild-type p53 or are heterozygous/homozygous null for p53 status. These mice will be irradiated with 10 Gy or mock irradiated and

major organs (heart, lung, spleen, colon, liver, kidney, brain, testes/ovaries) will be harvested 72 h later. These samples will be processed for protein and RNA for western and northern blot analyses respectively. Additionally, quantitative RT-PCR will be used to compare sCLU mRNA expression in the various tissues.

Aim 2: To determine the mechanism of p53 repression of sCLU transcription and to identify the transcription factors required for sCLU induction after IR exposure.

Task 1:

1. Deletion mutant analysis of the clusterin promoter luciferase reporter will be used to narrow down the region of the promoter required for CLU induction after IR. These constructs will be transiently transfected into HCT116 parental and p53 $^{-/-}$ cells and luciferase assays will be used to monitor promoter activity. These deletion mutants will allow us to define the site of the clusterin promoter that is required for p53 transcriptional repression as well as the transcription factors required for sCLU induction after IR exposure.
    a. Once the region required for sCLU induction is found, point mutations of the transcription factor binding sites within that specific region will be used to determine which transcription factors are required for induction.
    b. Electromobility shift assays (EMSA) will be performed to confirm the binding of the transcription factors to this site.
2. Chromatin immunoprecipitation assays (ChIP assays) will be used to demonstrate the physiological binding of these transcription factors after IR exposure and to determine whether p53 binds the clusterin promoter directly or causes the transcriptional repression indirectly.

Body of Grant Update:

Aim 1: To investigate the transcriptional repression of secretory clusterin (sCLU) by the tumor suppressor protein, p53.

Task 1: Screen various breast cancer cell lines for sCLU and p53 status.

Progress: We screened six breast cancer cell lines and looked for a correlation between p53 status and sCLU expression. In general, cells that contain wild-type p53 have low basal levels of sCLU protein as determined by

western blot analyses. In contrast, cells with mutant p53 have high basal levels of sCLU protein (see Criswell *et al., Cancer Biology and Therapy*, 2003, in appendix).

Task 2: Examine sCLU expression in MCF-7 breast cancer cells in which the p53 status has been modulated.

1. Generate MCF-7 cells that stably express 1403 bp of the clusterin promoter fused to a luciferase reporter.

Progress: We have generated a stable cell line expressing the clusterin promoter luciferase reporter (MCF-7 1403 cells). Time course and dose response experiments were performed to show that these cells behaved similarly to the endogenous gene after ionizing radiation (IR) exposure (see Criswell *et al., Cancer Biology and Therapy*, 2003, in appendix).

2. Monitor sCLU expression in MCF-7 cells that stably express the HPV-16 E6 protein. The HPV-16 E6 protein binds p53 and targets it for rapid degradation leaving these cells essentially p53 null.

Progress: Western and northern blot analyses show that the MCF-7:E6 cells have higher basal levels of sCLU protein and message as compared to parental MCF-7 cells, suggesting that p53 is repressing transcription of this gene. Western blot analyses were used to demonstrate a similar phenomena in RKO cells that stably express the E6 protein (see Criswell *et al., Cancer Biology and Therapy*, 2003, in appendix), suggesting that increased levels of sCLU expression is due to a decrease in functional p53, and not just an artifact of this system. We are currently in the process of developing MCF-7 1403 cells that stably express the E6 protein as well as clones that contain a mutation in the E6 protein (K11E) that abrogates its ability to bind to p53.

3. p53 status will be modulated in the MCF-7 1403 cells by stable expression of the 273 dominant negative mutant of p53.

Progress: We are currently in the process of generating this cell line.

Task 3: Monitor sCLU status in the genetically matched HCT116 parental and $p53^{-/-}$ colon cancer cell lines with and without IR treatment.

1. Western and northern blot analyses show that HCT116 parental cells that contain wild-type p53 have low basal and inducible levels of sCLU. In contrast, the $p53^{-/-}$ cells show a dramatic increase of sCLU after IR exposure (see Criswell *et al., Cancer Biology and Therapy*, 2003, in appendix).

2. We have been able to detect sCLU protein in various tissues from FVB/N mice that is inducible after 5Gy whole body irradiation. We are currently housing a $p53^{-/-}$ mouse colony obtained from Jackson Labs and are hoping to begin exploring sCLU expression in these mice with and without irradiation.

Aim 2: To determine the mechanism of p53 repression of sCLU transcription and to identify the transcription factors required for sCLU induction after IR exposure.

Task 1: Deletion mutant analysis of the CLU promoter to determine regions of the promoter required for p53 repression of transcription and transcription factors required for sCLU induction after IR exposure.

1. Deletion mutants of the CLU promoter luciferase vector will be made to identify regions of the promoter involved in p53 transcriptional repression and involved in CLU induction after IR.

Progress: Initial deletion mutants have been generated around various $NF_{\kappa}B$ and Sp1 sites within the CLU promoter (Fig. 1). We suspect $NF_{\kappa}B$ and/or Sp1 to be involved in the induction of sCLU after IR since our lab has previously identified the involvement of the transcription factors in the induction of other IR-inducible genes (10). This task has proven to be problematic, since endogenous sCLU as well as the exogenous promoter seem to be transcriptionally upregulated in response to various transfection methods. to overcome this obstacle, we are in the process of generating MCF-7, HCT116 parental and HCT116 $p53^{-/-}$ cells that contain a site for integration of exogenous genes (Flp-In System, Invitrogen). This will allow us to stably integrate each deletion mutant into the same site in the genome. Therefore, we will be able to screen our deletion mutants by stable expression without the problem of clonal variation.

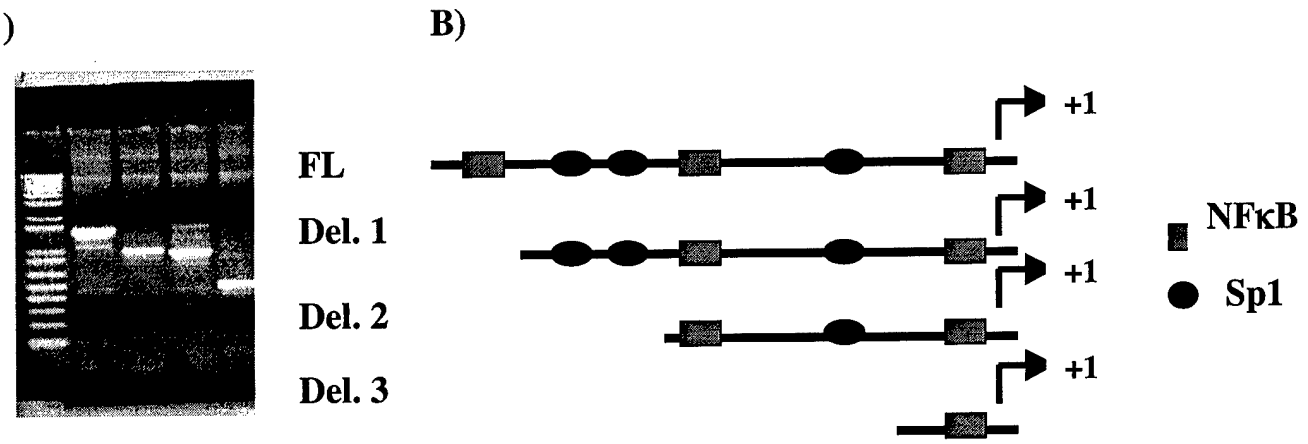

Figure 1: Preliminary data diagramming deletion mutants of the CLU promoter. (A) Agarose gel showing deletion mutants generated by PCR. (B) Schematic diagram illustrating location of each deletion within the CLU promoter (FL, full-length promoter; Del. 1, deletion 1; Del. 2, deletion 2; Del. 3, deletion 3).

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of **key** research accomplishments emanating from this research.

We have accomplished the following objectives of this grant. We have determined/generated that:

Aim #1:

1. There is a correlation between p53 status and secretory clusterin (sCLU) status in various breast cancer cell lines.
2. A MCF-7 breast cancer cell line that stably expresses the clusterin (CLU) promoter luciferase reporter vector that behaves similarly to the endogenous sCLU gene.
3. MCF-7 and RKO cells that stably express the HPV-E6 gene have high basal levels of sCLU protein and message and little inducibility after IR.
4. HCT116 colon cancer cells that contain wild-type p53 show little inducibility of sCLU after IR, whereas HCT116 $p53^{-/-}$ cells show strong sCLU inducibility after IR.

Aim #2:

5. Deletion mutants of the CLU promoter have been generated.

REPORTABLE OUTCOMES:

CURRENT LIST OF PUBLICATIONS RESULTING FROM THIS AWARD
PAPERS PUBLISHED IN PEER-REVIEWED JOURNALS (Enclosed):

Criswell, T., Klokov, D., Beman, M., Lavik, JP., and Boothman, D.A. Repression of IR-Inducible Clusterin Expression by the p53 Tumor Suppressor Protein. Cancer Biol and Ther. **2 (4)**, 2003.

ABSTRACTS AND PRESENTATIONS:

Poster Presentations:

1. DOD Era of Hope Meeting, Sept. 2003.
   Title: p53 repression of the secretory protein clusterin.

DEVELOPMENT OF CELL LINES, TISSUE OR SERUM REPOSITORIES:

-MCF-7 breast cancer cells that stably express the CLU promoter luciferase reporter vector (MCF-7 1403 cells.

CONCLUSIONS:

The main progress on this grant has been the identification of p53 as a transcriptional repressor of sCLU. Studies have begun that will allow us to identify the mechanism of p53 repression as well as the transcription factors required for the induction of this gene after IR.

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Repression of IR-inducible clusterin expression by the p53 tumor suppressor protein

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Running Title: *Suppression of sCLU by p53*

Criswell, T., Klokov, D., Beman, M., Lavik, J.P., Boothman, D.A.

Repression of IR-inducible clusterin expression by the p53 Tumor Suppressor Protein

Cancer Biology and Therapy

ABSTRACT

The clusterin (CLU) protein has been reported to have both cytoprotective and cytotoxic activities. Previous data from our lab suggest that the secretory form of CLU (sCLU) is cytoprotective and induced after very low, nontoxic doses of ionizing radiation (IR: $\geq$0.02 Gy), while a nuclear form is cytotoxic.[1] Cells must presumably suppress sCLU to stimulate cell death, however, factors regulating the stress-inducible expression of sCLU have not been elucidated. Here we demonstrate that p53 can suppress sCLU induction responses. A variety of cytotoxic agents stimulated sCLU expression and DNA damage was sufficient but not necessary for induction. IR-stimulated CLU promoter activity, with concomitant increases in CLU mRNA and protein, showed that CLU induction was delayed with maximal expression observed 48-96 h post-treatment. Expression of the human papillomavirus E6 protein in MCF-7 breast or RKO colon cancer cells enhanced basal CLU levels. Isogenically matched HCT116 colon cancer cell lines that differed only in p53 or p21 status, confirmed a role for p53 in the transcriptional repression of sCLU. Loss of functional p53 in HCT116:$p53^{-/-}$ cells augmented CLU *de novo* synthesis after IR exposure. Repression of sCLU protein levels by p53 may be important for the cascade of p53-mediated events leading to cell death after IR or other cytotoxic agent exposure.

ABBREVIATIONS: base pair, bp; CLU, clusterin; IR, ionizing radiation; ER, endoplasmic reticulum; nCLU, nuclear clusterin; DSBs, DNA double strand breaks; kb, kilobases; PI, propidium iodine; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase pump; sCLU, secretory clusterin; TPA, tetradecanoylphorbol acetate.

MAIN POINTS:

- sCLU is transcriptionally upregulated by low doses of ionizing radiation.
-sCLU is upregulated by a variety of cytotoxic agents.
-DNA damage appears to be sufficient, but not necessary for sCLU expression.
-sCLU is transcriptionally repressed by p53; loss of p53 augments stress-inducible sCLU expression.
- sCLU basal or IR-inducible levels are not cell cycle regulated.

INTRODUCTION

Clusterin (CLU) is a sulfated glycoprotein implicated in many physiological and pathological processes, including tissue remodeling,[2] complement inhibition,[3, 4] lipid transport,[5, 6] multiple sclerosis,[7] atherosclerosis,[8, 9] and Alzheimer's disease.[10-12] Two different forms of the CLU protein exist: an 80 kDa secretory form composed of 40 kDa alpha and beta subunits and an ~55 kDa nuclear form. Our laboratory identified CLU as a x-ray inducible protein/transcript (xip8).[13] We showed that a nuclear form of CLU (nCLU) associated with the DNA double strand break (DSB) repair protein, Ku70, and was a pro-death factor.[14, 15] However, the secretory form of CLU (sCLU) did not associate with Ku70, and this form of CLU was induced by much lower, nontoxic doses of IR. In fact, sCLU was induced at ~0.02 Gy, a dose found to be growth-stimulatory and cytoprotective in many human cancer cells.[14] The regulation of sCLU remains poorly understood.

Elevated levels of sCLU protein and mRNA were noted in several different types of human malignancies,[16, 17] and forced over-expression of sCLU in transformed cell lines resulted in an increased resistance to various chemotherapeutic agents.[18, 19] In addition, abrogation of CLU mRNA expression following antisense expression lead to modest chemo- and IR-sensitizations in various cell lines.[20-23] These data support a cytoprotective role for sCLU.

The p53 tumor suppressor gene is mutated in over half of all human tumors.[24] Wild-type p53 protein is stabilized after cellular stress and acts as a transcription factor for various downstream genes, including Bax, p21 and GADD45, resulting in either cell cycle arrest or apoptosis.[25-27] p53 can also act as a repressor of transcription, although exact mechanisms of transcriptional suppression still remain to be elucidated. Examples of p53-repressed genes include presenillin,[28] hsp70,[29] cyclins A[30] and B[31] and cdc2.[32]

Our laboratory identified CLU as a x-ray inducible protein/transcript (xip8).[13] We showed that a nuclear form of CLU (nCLU) associated with the DNA double strand break (DSB)

repair protein, Ku70.[14, 15] However, sCLU did not associate with Ku70, and this form of CLU was induced by much lower, nontoxic doses of IR. In fact, sCLU was induced at ~0.02 Gy, a dose found to be growth-stimulatory and cytoprotective in many human cancer cells.[14]

Although regulation of sCLU gene expression following estrogen and testosterone exposures has been investigated,[33, 34] the regulatory control of sCLU synthesis after IR or other cytotoxic agents has not been elucidated. We show that sCLU mRNA and protein synthesis in human cells is induced after various cytotoxic stresses, including exposure to many anti-tumor agents. IR-induction studies of CLU promoter activity, CLU mRNA accumulation, and sCLU protein synthesis confirm that sCLU expression occurs in a delayed fashion, with initial IR-activation of the CLU promoter occurring 24 h post-exposure, and mRNA and protein levels maximally accumulating 48-96 h post-IR. The low levels of IR (>0.02 Gy) that induce sCLU and the dramatic accumulation of sCLU protein following taxol, TPA or thapsigargin (a sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump inhibitor that causes dramatic alterations in intracellular calcium homeostasis) exposures, suggest that DNA damage may not be required for CLU gene expression in MCF-7 breast cancer cells. Expression of the human papillomavirus (HPV) E6 protein, as well as isogenically matched cell lines that differ only in their p53 status, were used to demonstrate a role for p53 in the transcriptional repression of sCLU in unirradiated as well as IR-exposed cells. Loss of functional p53 results in elevated basal levels of sCLU in some cells, and augmented IR-induced gene expression in all cells examined. These data strongly suggest that sCLU mRNA production and protein synthesis are repressed by the tumor suppressor protein, p53.

EXPERIMENTAL PROCEDURES

Chemical Reagents- Camptothecin, etoposide, colcemid, nocodazole, taxol, mimosine, TPA, and thapsigargin were obtained from the Sigma Chemical Co (St. Louis, MO) and

dissolved in either PBS or DMSO. Topotecan was generously provided by Glaxo SmithKline (Research Triangle Park, NC). ß-Lapachone was prepared for us by Dr. William G. Bornmann (Synthetic Preparatory Core Facility, Memorial Sloan Kettering, NY, NY). Taxotere was generously provided to us by Aventis Pharmaceuticals (Bridgewater, NJ).

Cell Culture- MCF-7:WS8 human breast cancer cells (MCF-7) were obtained from Dr. V. Craig Jordan (Northwestern University; Evanston, IL). MCF-7 cells were transduced by retroviral transfer with a CMV-driven papillomavirus E6 vector by Dr. Jordan's lab, and subsequently subcloned by our lab into cell lines with varying E6 expression. The E6-D MCF-7 cell line showed no p53 expression, even after IR exposure. Human colorectal carcinoma HCT116 parental, $p53^{-/-}$, and $p21^{-/-}$ cell lines were developed[35] and generously provided by Dr. Bert Vogelstein (Johns Hopkins University; Baltimore, MD). These cell lines were confirmed by our laboratory to be null for p53 and p21, respectively, by western blot analyses. MCF-7, ZR-75-1, T47-D, BT474, MDA-MB-231 and MDA-MB-468 cell lines were grown in RPMI 1640 cell culture media supplemented with 5% fetal bovine serum (FBS) at 37°C in a humidified incubator with a 5% CO_2-95% air atmosphere as described.[36] MCF-7:E6 cells were maintained in 0.4 mg/ml geneticin (Life Technologies; Carlsbad, CA). RKO:neo and E6-expressing RKO cell lines were obtained from the American Type Culture Collection, maintained in G418, and experiments performed in the absence of antibiotics. HCT116 and RKO cell lines were grown in DMEM supplemented with 10% FBS at 37°C in a humidified incubator with a 10 % CO_2-90% air atmosphere. All experiments were initiated by seeding 5 x 10^5 log-phase cells per 10-cm^2 tissue culture dish in the appropriate medium in the absence of any antibiotics (e.g., geneticin). All cell lines were free from mycoplasma contamination.

IR and Chemical Treatments- Cells were irradiated as described.[13] Briefly, cells were irradiated with ^{137}Cs gamma rays at a dose rate of 0.87-0.92 Gy/min, using a Shepard Mark Irradiator. Untreated cells were mock-irradiated as described.[13] MCF-7 cells were treated with

topotecan, camptothecin, mimosine, colcemid, nocodazole, TPA, thapsigargin, taxotere, taxol or etoposide using drug exposures at the indicated doses as described in Table 2. Cells were treated with ultraviolet radiation as described.[37] ß-Lapachone and hypoxic exposures[38] of log-phase MCF-7 cells were performed as indicated in Table 2.

Northern Blot Analyses- Total RNA was extracted from control or irradiated MCF-7 or HCT116 cells as indicated using Trizol (Life Technologies; Carlsbad, CA) as per the manufacturer's instructions. Ten - twenty micrograms (10 - 20 μg) total RNA was separated on a denaturing formaldehyde gel, transferred to a Hybond membrane (Amersham Pharmacia; Sunnyvale, CA), and probed with ^{32}P-labeled full-length CLU or 36B4 cDNAs as described;[37] 36B4 levels are not affected by cell stress, or cell cycle status.[37] Corresponding transcript signals were quantified using ImageQuant software version 4.1 (Molecular Dynamics; Sunnyvale, CA) on a Molecular Dynamics phosphoimager. CLU mRNA levels were normalized to untreated control levels, and to 36B4 mRNA levels for X-fold induction calculations as described.[37]

Luciferase Assays- All luciferase assays were performed using the Luciferase Assay System (Promega; Madison, WI). MCF-7 cells were stably transfected with a 1403 bp fragment of the human CLU promoter in a luciferase reporter plasmid using a standard liposome transfection protocol (Effectene, Qiagen, Valencia, CA). The plasmid was a generous gift from Dr. Martin Tenniswood (University of Notre Dame; Notre Dame, IN). These cells (MCF-7:1403 cells) were seeded in 6-well plates at approximately 50% confluency. Cells were irradiated at the indicated dose(s) and harvested at various times in 1X reporter lysis buffer (Promega; Madison, WI). Each dose/time point was completed in triplicate and a Student's T-Test was performed to determine statistical significance.

Western Blot Analyses- Whole cell extracts from control or irradiated cells were extracted in RIPA buffer (0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris pH 8.0) and separated on a 10% gel by SDS-PAGE western blot analyses as described.[15] Proteins were

transferred to Immobilon-P (Millipore; Bedford, PA) and probed with the B-5 human sCLU monoclonal antibody, the DO-1 human p53 monoclonal antibody, and the human C-19 Ku70 polyclonal antibody. All antibodies were obtained from Santa Cruz and used as per manufacturer's instructions. Ku70 was used as a control for equal loading of protein, since its levels remain unaltered after IR or cell cycle status under the time-frame of our experiments. Western blots shown are representative of experiments performed at least three times. For sCLU protein analyses in cells before and after IR, we routinely use the 60 kDa form, since nearly all cell lines produce this 60 kDa protein, which is a precursor to the mature glycosylated α- and β- ~40 kDa polypeptides of sCLU.

Cell Cycle Analyses- HCT116 parental, $p53^{-/-}$ or $p21^{-/-}$ cells were synchronized by allowing them to grow to 100% confluence on 10-cm^2 tissue culture dishes as described.[13, 39] Cells were then maintained for 48 h in serum-free medium to maximize G_0-G_1 arrest. Cells were released from the dual confluence and serum-free cell cycle arrest by trypsinization (using 0.05% trypsyn with 0.53 mM EDTA) and replated at 1:8-1:10 dilution in DMEM containing 10% FBS under conditions described above. For IR treatments, cells were exposed to 10 Gy, 10 h after release from the cell cycle. Concurrent flow cytometric and western blot analyses were performed as indicated. At various times after mock- or IR-exposures, cells were dissociated by scraping into 1X PBS, collected by centrifugation (500 x g), fixed in 90% ethanol, and stored at –20 ^{0}C until analyzed. Cells were then stained with 33 mg/ml propidium iodide (PI) (Sigma; St. Louis, MO), 1.0 mg/ml RNase A (Sigma), and 0.2% NP-40 (Calbiochem; La Jolla, CA) at 4 ^{0}C overnight. Stained nuclei were then analyzed for DNA content by PI fluorescence using a Coulter Epics XL (Beckman Coulter Electronics; Miami, Fl) flow cytometer. Data were analyzed using ModFit LT, version 2.0 software (Verify Software House; Topsham, ME). Western blot analyses were completed simultaneously with flow cytometry, and results shown represent experiments performed at least three times.

RESULTS

sCLU is transcriptionally upregulated after IR. Our laboratory previously showed that CLU was an x-ray-induced protein (xip8),[37] however, regulation of this gene was not elucidated. To further characterize induction of sCLU after IR exposure, northern blots were used to determine if sCLU protein accumulation in log-phase MCF-7 human breast cancer cells after IR exposure was due to increased transcription, or a result of protein stabilization (Figs. 1A,B). Log-phase MCF-7 cells were mock-irradiated or exposed to 10 Gy and harvested at various times post-IR to analyze the temporal kinetics of CLU gene expression. Maximal induction of sCLU mRNA (7- to 10-fold) over untreated cells occurred 72 to 96 h after 10 Gy (Fig. 1A). Induction of CLU transcripts in MCF-7 cells after IR was confirmed using RNase protection assays (data not shown). IR dose-response experiments in log-phase MCF-7 cells were performed, and CLU mRNA accumulations 72 h after exposure were examined (Fig. 1B), since maximal protein and mRNAs were noted at this time post-IR (Fig. 1A). As previously reported for sCLU protein induction at 72 h post-IR,[15] sCLU mRNA was induced 2-fold after as little as 2 cGy, with maximal induction of 22-fold in MCF-7 cells after 5 Gy (Fig. 1B). Steady state CLU mRNA accumulation corresponded well with previously described sCLU protein accumulations in MCF-7 cells after IR in terms of temporal and dose-response kinetics.[15]

To determine if IR-induced CLU transcriptional increases were due to *de novo* mRNA synthesis, or to decreases in mRNA degradation (i.e., via post-transcriptional modifications), we examined CLU promoter activity in time-course and dose-response studies after IR. For these experiments, we generated an MCF-7 cell line containing a stably integrated copy of a plasmid containing a 1403 bp fragment of the human CLU promoter directing expression of a downstream luciferase reporter as described in 'Experimental Procedures'. Transient transfections with the CLU reporter plasmid were problematic, since all transfection methods examined to date affected the regulation of the CLU promoter-luciferase construct in MCF-7 cells, as well as

endogenous sCLU gene/protein expression (data not shown); induction of sCLU may be triggered by cell membrane insult.[40] Dose-response (Fig. 1C) and time-course (Fig. 1D) assays of exogenous CLU promoter activation in MCF-7:1403 cells were performed to show that this clone behaved similarly to the endogenous CLU gene before and after IR exposure. The CLU promoter was activated in a time- and dose-dependent manner similar to that previously shown for sCLU protein and mRNA (Figs. 1A and B). CLU promoter activity was stimulated by as low as 0.5 Gy.

sCLU is a stress protein induced by a variety of cytotoxic agents. Table 1 lists various cytotoxic agents that induce sCLU protein expression in MCF-7 cells. These agents included ultraviolet radiation (UV), topoisomerase I and IIα poisons, microtubule stabilizers/destabilizers, as well as other agents that do not cause direct damage to DNA (e.g., TPA, thapsigargin). Treatment of MCF-7 cells with hypoxic conditions or various doses of β-lapachone (2-10 μM, 4h), a novel apoptotic drug that quickly depletes cellular NAD(P)H and ATP in NQO1-expressing MCF-7 cells,[41] did not induce sCLU protein expression. These data suggest that damage to DNA may be sufficient, but is not required for sCLU induction. Alterations in calcium homeostasis (indicated by thapsigargin induction of sCLU, Table 1) or ER stress responses may play a common role in triggering CLU gene induction.

Correlation of sCLU expression and loss of functional p53. Various human breast, colon and prostate cancer cell lines with known mutations in p53 were examined for basal and IR-inducible sCLU levels as monitored by western blot analyses and described in 'Experimental Procedures'. With one exception, cells expressing mutant p53 exhibited increased basal levels of sCLU (Table 2). Mutant p53-expressing MDA-MB-231 cells appear to lack basal or IR-inducible CLU protein expression, and have no detectable CLU mRNA levels by Northern blot analyses (data not shown). In contrast, cells expressing wild-type p53 expressed low or no detectable basal levels of sCLU (Table 2). With the exception of MCF-7 cells, we also noted that

cells expressing wild-type p53 did not greatly induce sCLU expression after various doses of IR to the same extent as null or mutant p53-expressing cells.

HPV-16 E6-expressing MCF-7 cells have high basal levels of sCLU. The data in Table 2 indicate an inverse correlation between sCLU expression and expression of wild-type p53. To further elucidate the effect of p53 on sCLU expression, we compared vector alone-transfected parental MCF-7 cells to isogenically matched cells stably transfected with the HPV-16 E6 protein, as described in 'Experimental Procedures'. The E6 protein binds to p53 and targets it for rapid degradation through the proteasome pathway, leaving these cells deficient (i.e., null) for p53 expression.[42] Protein and RNA from log-phase MCF-7:parental and MCF-7:E6 cells were harvested at various times after exposure to 10 Gy. Consistent with the mRNA changes shown in Fig. 1, sCLU protein was induced in parental MCF-7 cells starting at 24 h, and levels peaked at 72 h post-10 Gy (Fig. 2A). The 60 kDa band in Fig. 2A is a precursor form of sCLU (psCLU) expressed in the ER and is cleaved at an α/β cleavage site resulting in two 40 kDa peptides that heterodimerize through five disulfide bonds to form mature 80 kDa sCLU protein. Western blots performed under denaturing conditions result in the appearance of a 40 kDa smeared band consisting of glycosylated α- and β-peptides of sCLU. sCLU basal levels were higher in mock-irradiated MCF-7:E6 cells compared to parental MCF-7 cells. As expected, p53 protein levels were not detected in MCF-7:E6 cells at various times before or after IR. Furthermore, induction of sCLU protein was enhanced in MCF-7:E6 cells after IR compared to levels in parental MCF-7 cells after 10 Gy (Fig. 2A). Since MCF-7:E6 cells have a higher basal level of sCLU, IR-induction of sCLU in these cells was more difficult to quantify, and Northern blot analyses indicate this fact (see below). Finally, we noted a similar dramatic increase of sCLU in the medium of IR-treated MCF-7 cells, and a significantly higher basal level of sCLU in the medium of MCF-7:E6 compared to MCF-7:neo vector alone parental cells (Klokov *et al.*, unpublished data).

Northern blot analyses confirmed that basal CLU mRNA levels were 3-fold higher in mock-treated MCF-7:E6 cells compared to MCF-7:parental cells (Fig. 2B). Furthermore, CLU mRNA levels were only modestly induced in MCF-7:E6 cells (~3-fold from 4h to 96 h post-IR), compared to IR-treated vector alone MCF-7:parental cells, in which a 10-fold increase in CLU mRNA level was noted (Fig. 2B).

RKO:neo and RKO cells stably expressing the E6 protein (RKO:E6) were mock-irradiated or treated with 5 Gy. Protein was harvested 72 h after exposure and analyzed by western blotting (Fig. 2C). As with many other cells examined, RKO cells showed low levels of the mature 40 kDa glycosylated form of sCLU, presumably because this protein is secreted from the cell. Wild-type p53-expressing RKO:neo cells expressed low basal levels of sCLU protein, with a measurable IR-inducible expression of the 60 kDa sCLU protein form after IR. In contrast, RKO:E6 cells expressed high basal levels of the 60 kDa sCLU protein, similar to that seen in MCF-7:E6 cells. As in MCF-7 cells, sCLU induction was more dramatic in RKO:E6 cells compared to RKO:neo cells, suggesting that loss of p53 function relieves IR-induction responses of sCLU.

Somatic deletion of p53 in HCT116 colon cancer cells results in greater IR-inducible sCLU levels. Since E6 expression may have additional unknown 'gain of function' properties, and to confirm the ability of p53 to repress sCLU expression after IR, we used isogenically matched human HCT116 colon cancer cell lines that differed only in their p53 or p21 status. As with RKO cells, we were not able to observe intracellular mature 40 kDa sCLU levels in HCT116 cells and IR-induced sCLU induction responses were monitored via the ~60 kDa sCLU precursor protein (psCLU). Protein and RNA from log-phase HCT116:parental and HCT116:p53$^{-/-}$ cells were harvested at various times after exposure to 10 Gy. Western blot analyses showed that IR-treated HCT116:parental cells stabilized and accumulated p53 (i.e., expressed wild-type p53), but increases in steady state levels of sCLU were minimal to non-

detectable (Fig. 3A). In contrast, HCT116:p53$^{-/-}$ cells dramatically induced sCLU after 10 Gy. As found with RKO:E6 and MCF-7:E6 cells, HCT116:p53$^{-/-}$ cells expressed higher basal levels of sCLU compared to the low levels noted in HCT116:parental cells. IR dose-response analyses of sCLU responses in HCT116:p53$^{-/-}$ cells demonstrated induction of sCLU protein at doses as low as 1 Gy (Fig. 3B), a dose of IR that caused minimal loss of clonogenic survival.[43] Northern blot analyses confirmed induction (6- to 7-fold) of steady state sCLU mRNA in HCT116:p53$^{-/-}$ cells (Fig. 3C), whereas p53$^{+/+}$ HCT116:parental cells showed little or no induction of sCLU mRNA at various times (up to 96 h) after 10 Gy. To demonstrate that induction of sCLU in p53 null cells was specific for the absence of p53 and not a gene downstream from p53, we utilized HCT116 cells that were somatically knocked out for the p21 gene (i.e., HCT116:p21$^{-/-}$ cells).[35] As in p53$^{+/+}$ HCT116:parental cells, sCLU protein and mRNA levels were only minimally induced in HCT116:p21$^{-/-}$ cells after 10 Gy compared to mock-treated cells, as determined by western and northern blot analyses (Fig. 4A, B).

sCLU is not cell cycle regulated. An alternative explanation for sCLU induction and subsequent repression by p53 could be that the sCLU gene is cell cycle regulated, and that wild-type p53-expressing cells suppress sCLU expression by arresting cells in a particular phase of the cell cycle. Recent reports suggested that sCLU may be expressed exclusively in quiescent normal cells.[44] To address this issue in cancer cells, HCT116:parental, HCT116:p53$^{-/-}$ and HCT116:p21$^{-/-}$ cells were arrested in the G_0/G_1 phase of the cell cycle by dual serum-starvation and confluence-arrest conditions, released by replating and irradiated 10 h later as described in 'Experimental Procedures'.[45] The cell cycle profiles of untreated and irradiated isogenic HCT116 cells were then monitored (Fig. 5). Untreated HCT116:parental cells subsequently entered S-phase 14-16 h after release from low serum and confluence arrest, with concomitant decreases in G_0/G_1 cells. As previously noted with this synchronization technique,[46] one synchronous cell

division was achieved, and mock-irradiated isogenic HCT116 cells returned to a log-phase cell cycle distribution after 58 h post-release. There were significant differences in synchronized mock-irradiated HCT116:parental, HCT116:p53$^{-/-}$ and HCT116:p21$^{-/-}$ cells, particularly in the time of entry into S-phase, with both p53- and p21-deficient HCT116 cells entering S-phase sooner than wild-type p53 HCT116:parental cells (compare the cell cycle distributions in Figs. 5A, C and E). Since p53 exerts its G_1 cell cycle checkpoint responses through, in part, induction of p21, mock-irradiated and IR-treated HCT116:p53$^{-/-}$ and HCT116:p21$^{-/-}$ cell cycle distributions were very similar (Figs. 5C, E).

As expected, p53$^{+/+}$ HCT116:parental cells treated with 10 Gy at 10 h post release resulted in a significant delay in the progression of synchronized cells into S-phase (a function of the IR-induced G_1 cell cycle checkpoint response, Fig. 5A, B) as described.[46] For example, at 36 h IR-treated HCT116:parental cells demonstrated >45% G_2 cells compared to less than 18% in mock-irradiated cells (compare Figs. 5A, B). In contrast, HCT116 cells with somatic deletions of p53 (Fig. 5C, D) or p21 (Fig. 5E, F), entered S-phase earlier, with accumulation of S-phase cell populations occurring at 12-16 h, accompanied by concomitant decreases in G_0/G_1 cells. At 18 h after release (8 h after 10 Gy IR exposure), only 14% and 28% of p53$^{-/-}$ and p21$^{-/-}$ cells, respectively, remained in G_1, while 80% and 66% of cells, respectively, proceeded into S phase. As expected, IR-treated HCT116:p53$^{+/+}$ cells arrested in G_1 and exhibited delayed S or G_2/M phase entry compared to IR-treated HCT116:p53$^{-/-}$ or HCT116:p21$^{-/-}$ cells. HCT116:p53$^{-/-}$ and HCT116:p21$^{-/-}$ cells responded similarly to IR treatment. Although responses to 10 Gy are shown, near identical responses to 2-5 Gy were also observed in other studies.[46]

Western blot analyses of non-irradiated synchronized HCT116:parental, HCT116:p53$^{-/-}$ and HCT116:p21$^{-/-}$ cell populations indicated that the levels of sCLU did not change relative to basal levels throughout the cell cycle (Fig. 5). Interestingly, sCLU was induced only in IR-

exposed synchronized HCT116:p53$^{-/-}$ cells with similar induction kinetics (maximal accumulation observed between 24-72 h) as noted in IR-treated asynchronous log-phase HCT116:p53$^{-/-}$ cells (Fig. 3). In contrast, only minimal sCLU induction responses were noted in synchronized IR-treated HCT116:parental or HCT116:p21$^{-/-}$ cells, even though irradiated HCT116:p21$^{-/-}$ cells exhibited nearly identical cell cycle distribution changes as IR-exposed HCT116:p53$^{-/-}$ cells. These data strongly suggested that: (a) sCLU induction was genetically programmed after IR stress resulting in a 48 – 72 h delay before sCLU accumulation is noted after IR exposure; and (b) sCLU was transcriptionally repressed by functional p53 independent of the cell cycle. Loss of functional p53 appears to relieve negative regulation on the IR-induction responses of CLU gene expression in a variety of cell types.

DISCUSSION

Our laboratory previously demonstrated that sCLU was an x-ray induced protein (i.e., xip8).[15] In this study, we further investigated the induction of sCLU by IR. We showed that sCLU was induced by doses of IR as low as 2 cGy (Fig. 1A & B). This low-dose IR induction is seen at both the transcript and protein levels, with promoter activation noted after 0.5 Gy in MCF-7 cells containing a stably integrated 1403 bp human CLU promoter directing expression of firefly luciferase. Induction of the CLU promoter was noted only after 0.5 Gy as analyzed by luciferase assays using a luminometer (Fig. 1C & D), however, induction of this promoter after IR doses <50 cGy has been noted when analyzed by bioluminescent imaging (Klokov *et al.*, unpublished data). We also showed that DNA damage appears to be sufficient, but not required for sCLU induction (Table 1).

We demonstrated that the basal level and IR induction of sCLU after IR exposure was repressed by p53. MCF-7 and RKO cells stably expressing the HPV E6 protein (both exhibiting loss of functional p53) have high basal levels of sCLU compared to parental cells that express

functional p53 (Fig. 2). Additionally, HCT116:parental and RKO:neo cells that express wild-type p53 minimally induce sCLU after IR, whereas HCT116:p53$^{-/-}$ and RKO:E6 greatly induced sCLU at the protein and transcript level after IR (Fig. 3). Finally, the effect of p53 on IR-inducible sCLU expression was not dependent on the cell cycle, but was delayed in its induction, requiring at least 48 h post-IR in all cells examined (Fig. 5). Since the relationship between p53 status and sCLU expression and p53 repression of IR-induced sCLU expression was observed in cells of different origins, p53 repression of this gene appears to be a general phenotype and not unique to specific cell lines. A limited screen of cancer cell lines indicated an inverse regulatory relationship between p53 status and sCLU expression (Table 2). To directly explore the role of p53 in basal and IR-inducible levels of CLU gene expression, we used three model cell line systems from breast and colon cancer origins to investigate the role of p53 in the transcriptional regulation of sCLU. All three cell lines confirmed that p53 exerted negative regulation on sCLU expression.

The effect of IR exposure on sCLU expression in MCF-7 cells was different from that found in HCT116 parental cells, even though both cell lines express wild-type p53. HCT116:parental cells did not induce sCLU after IR exposure. In fact, MCF-7 cells are the only wild-type p53-expressing cell line examined to date that strongly induced sCLU after IR. It may be that MCF-7 cells overexpress the IR-activated transcription factors required for induction of CLU gene expression, while HCT116 cells maintain lower levels, which are in turn efficiently suppressed by wild-type p53 even after IR exposures. It appears that these as yet unknown transcription factors may be constitutively expressed in MCF-7 cells, since E6 expression greatly enhanced sCLU expression in MCF-7 cells without IR exposure, whereas loss of functional p53 in HCT116 cells did not cause an appreciable increase in basal levels of sCLU protein expression. The factors needed for sCLU induction have not been elucidated. Analyses of the

transcription factors and DNA elements within the CLU promoter that regulate the IR inducibility of this gene are currently being performed in our laboratory.

The signaling pathway(s) that regulate sCLU induction and expression after IR exposure is(are) unknown. Our laboratory identified CLU as a Ku70 binding protein using yeast-two-hybrid analyses.[14] Through our screen of cytotoxic agents, we noted that DNA damage was not required for sCLU induction. This was best demonstrated by the induction of sCLU after thapsigargin (TG) exposures, and at doses of TG (2 nM, 1 h) that are not lethal to exposed MCF-7 cells (Table 1). TG is an inhibitor of the SERCA pump in the ER. Treatment of MCF-7 cells with TG resulted in a transient release of intracellular calcium[47] and an induction of sCLU mRNA and protein, suggesting that calcium changes may be an upstream signaling event mediating sCLU induction. It is possible that calcium, as a signaling molecule, may be a triggering event common to all the agents in Table 1 that elicit sCLU induction responses. The exact signal transduction processes that result in CLU gene expression after DNA damaging agents compared to non-DNA damaging agents is being elucidated in our laboratory.

The mechanism of sCLU repression by p53 also remains to be elucidated. There are several proposed models of p53 transcriptional repression. In the first model, p53 binds to its putative DNA binding sequence and sterically inhibits the binding of transcription factors required for induction. This model was proposed to account for repression of Bcl-2,[48] α-fetoprotein[49] and HBV[50] genes by p53. In the second model, p53 binds and sequesters transcription factors required for upregulation. For example, p53 can directly bind several transcription factors including Sp-1,[51, 52] AP-1,[53] NF-Y,[54, 55] Brn-3a[48] and C/EBPβ,[56] that may be responsible for upregulated CLU promoter activity after IR. Additionally, it was shown that p53 can bind the TATA binding protein (TBP) *in vitro* and inhibit transcription by disrupting formation of the TFIID complex.[57] Alternatively, Johnson *et al.* have proposed a novel putative DNA binding sequence for p53 that is strictly involved in transcriptional repression.[58]

Collectively, our data strongly suggest that the CLU gene is transcriptionally repressed by p53, although the mechanism of this repression still remains to be elucidated. The cell models used in this study will allow us to further investigate the mechanism(s) of p53 repression of sCLU, as well as the signaling pathways required for sCLU induction after IR exposure. Understanding the cellular responses to ionizing radiation exposure, in normal and tumor tissue, is vital for improving the efficacy of radio-therapy in the clinic.

The data presented in this paper provide a first examination of how a cell may regulate the clusterin molecular switch, turning on the cytoprotective sCLU gene at low doses of IR (0.02 - 0.1 Gy), while at the same time allowing p53 responses after high doses of IR (≥ 1.0 Gy) to shut down this cytoprotective protein to allow for cell cycle checkpoint responses and for cell death in severely damaged cells. For example, we are exploring the possibility that functional p53 is responsible for mediating CLU alternative splicing that produces nCLU protein expression.[1] We previously demonstrated that nCLU, and not sCLU, could associate with Ku70 and cause apoptotic cell death responses. In this way, p53 would down-regulate the cytoprotective sCLU protein, while simultaneously stimulating the synthesis and possibly activation of nCLU. Expression, and nuclear translocation, of nCLU after >1 Gy of IR would then result in a cascade of events leading to cell death and apoptosis. Understanding the regulatory events affecting the relative levels of different forms of the CLU protein after IR should allow elucidation of ways to modulate death responses in tumor cells, while possibly sparing the survival of normal cells.

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FIGURE LEGENDS

Figure 1. sCLU is transcriptionally upregulated after IR exposure in MCF-7 human breast cancer cells. CLU mRNA levels were monitored in asynchronous MCF-7 cells after 10 Gy by northern blot analyses and luciferase assays. In *A,* log-phase growing MCF-7 cells were irradiated with 10 Gy and 10 μg of total RNA was analyzed by northern blot analyses as described in *Experimental Procedures*. In *B,* MCF-7 cells were irradiated with various doses of IR and total RNA was harvested 72 h after exposure. Total RNA (10 μg) was used for northern blot analyses. In *C,* time-course of sCLU induction after 10 Gy exposure was analyzed by luciferase assays in MCF-7 cells stably transfected with 1403 base pairs of the CLU promoter (i.e., MCF-7 1403 cells) using the Luciferase Assay System (Promega). In *D*, an IR dose-response was performed on the MCF-7 1403 cells 72 h after IR exposure. Each dose/time point was performed in triplicate and a Student's T-Test was performed to determine statistical significance.

Figure 2. sCLU basal levels are elevated in MCF-7 and RKO cells that overexpress the HPV E6 protein. MCF-7:parental and MCF-7:E6 cells were exposed to 10 Gy and protein was harvested at various time points. In *A,* protein (100 μg) was loaded for each sample and separated by standard 10 % SDS-PAGE. Blots were probed for sCLU, p53 and Ku70 using western blot analyses as described in *Experimental Procedures*. Ku70 was used as a loading standard as described. In *B,* total RNA (10 μg) was analyzed using standard northern blot techniques as described in Fig. 1. Shown are representative blots from experiments performed at least three times. In *C,* RKO:neo and RKO:E6 cells were exposed to 5 Gy and protein was harvested at 72 h. Protein (100 μg) was loaded for each sample and separated by standard 10 %

SDS-PAGE. Blots were probed for sCLU, p53 and Ku70 using western blot analyses as described in *Experimental Procedures.*

Figure 3. sCLU is induced in HCT116:p53$^{-/-}$ cells, but not in p53$^{+/+}$ HCT116:parental cells. Asynchronous HCT116:parental and HCT116:p53$^{-/-}$ cells were exposed to 10 Gy and protein harvested at various times. In *A,* western blot analyses were performed as in Fig. 2, and probed for sCLU, p53 and Ku70 as described in *Experimental Procedures*. In *B,* HCT116:p53$^{-/-}$ cells were treated with various doses of IR and protein was harvested 72 h later. Western blot analyses were performed as in Fig. 2. In *C,* total RNA (10 μg) was analyzed using northern blot techniques as described in Fig. 1 and *Experimental Procedures*. Shown are representative blots from experiments performed at least three times.

Figure 4. sCLU is not induced in HCT116:p21$^{-/-}$ cells. HCT116:p21$^{-/-}$ cells were exposed to 10 Gy and protein harvested at various times. In *A,* western blot analyses were performed as in Fig. 2. Blots were probed for sCLU, p53 and Ku70 by western blot analyses as described in *Experimental Procedures*. In *B,* total RNA (20 μg) was analyzed using standard northern blot techniques as described in Fig. 1. Shown are representative blots from experiments performed at least three times.

Figure 5. sCLU is not cell cycle regulated. HCT116:parental, p53$^{-/-}$ and p21$^{-/-}$ cells were synchronized by serum starvation and confluence-arrest. Synchronized cells were released by low density seeding in 10% FCS-DMEM medium and cells were allowed to proceed through the cell cycle for 10 h and then mock-irradiated (Fig. 5A, C, E), or exposed to 10 Gy (Fig. 5B, D, F). Cells were allowed to progress through G_1 (●), S (▼) and G_2/M (○) phases of the cell cycle. Protein was harvested for flow cytometric or western blot analyses at the indicated times as

described previously in *Experimental Procedures*. Western blots were probed for CLU, p53 or Ku70 expression as described in *Experimental Procedures*. Shown are data for HCT116:$p53^{+/+}$ parental (A, B), HCT116:$p53^{-/-}$ (C, D) and HCT116:$p21^{-/-}$ (E, F) cells. Western blots and cell cycle analyses are representative of experiments performed at least three times.

Table 1: Induction of sCLU protein expression in MCF-7 cells[1] by various cytotoxic agents.

Agent	Dose Range for Induction[2]	Level of Induction[3]
DNA Damaging Agents		
Ionizing Radiation (IR)	0.02 - 10 Gy	+++
Ultraviolet Radiation (UV)	12 J/m^2	+++
Topotecan	50 nM	+++
Camptothecin	100 nM	+++
Etoposide (VP-16)	15 uM	+++
Non-DNA Damaging Agents		
Photodynamic Therapy (PDT)[4]	200 nM PC-4/200 mJ/cm^2	++
Colcemid	70 ng/ml	++
Nocodazole	150 ng/ml	+++
Taxol	1 - 50 nM	+++
Taxotere	1 - 10 nM	+++
Mimosine	0.5 mM	++
TPA	100 nM	+
Thapsigargin	10 - 500 nM	++
Non-Inducing Agents		
β–Lapachone	2 - 10 μM	NA
Hypoxia[5]	(<0.1% O_2)	NA

[1]Log phase MCF-7 cells were seeded at approximately 5X10^5 cells per 10 cm plate.

[2] Topotecan, camptothecin, TPA and thapsigargin were continuous treatments. Cells were treated with colcemid, nocodazole and mimosine for 24 hours, washed with PBS and replated into fresh media. Cells treated with taxol and taxotere for 4 hours, washed with PBS and replated into fresh media. Cells were treated with VP-16 for 1 hour. Protein was harvested at least 48 hours after drug addition/ irradiation.

[3] Level of induction of sCLU protein as compared to sCLU protein derived from 10 Gy irradiated MCF-7 cells.

[4]Photosensitizing drug used was Phthalocyanine 4 (PC-4). Induction of sCLU protein was only seen after addition of drug and light exposure. No induction was observed with light alone or PC-4 alone.

[5] Hypoxia was induced as previously described.[37]

Table 2: Effect of p53 status on sCLU basal and IR inducible expression.

		sCLU expression		
Cell Line	p53 Status	Basal[1]	IR Inducibility[2]	RNA[3]
Breast cancer cell lines				
MCF-7:parental	wild-type (wt)	low	yes	+
MCF-7:E6D	wt (no expression)	high	yes	+
ZR-75-1	wt	low	no	+
T47-D	mutant (194)	high	no	+
BT474	mutant (275)	high	no	+
MDA-MB-231	mutant (280)	ND	ND	ND
MDA-MB-468	mutant (273)	high	no	+
Colon cancer cell lines				
HCT116:parental	wt	low	minimal	+
HCT116:p21 $^{-/-}$	wt	low	minimal	+
HCT116:p53 $^{-/-}$	null	low	yes	+
RKO:neo	wt	low	yes	NP
RKO:E6	wt (no expression)	high	yes	NP
Prostate cancer cell lines				
LNCaP	wt	low	minimal	NP
DU-145	mutant (275)	high	minimal	NP

[1]Basal levels determined as compared to log-phase growing untreated MCF-7 parental cells.
[2]Log-phase growing cells were treated with 10 Gy IR and protein was harvested 48 hours after exposure. MCF-7 parental cells were used as the standard for "high" IR inducibility.
[3]RNA status was determined by RT-PCR using primers designed to full length CLU DNA.
ND: Not detected NP: Not performed

Figure 1

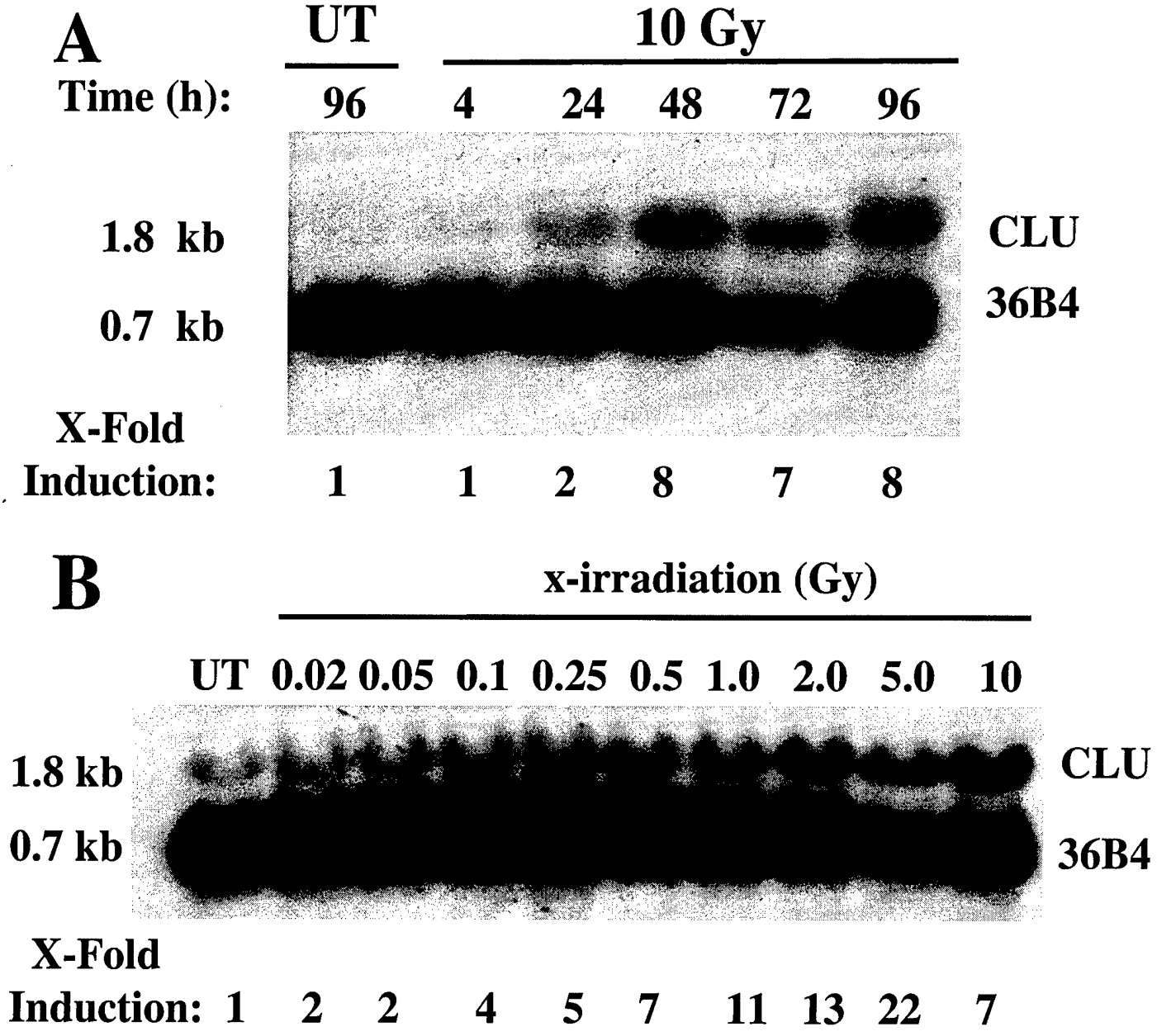
A
UT
10 Gy
Time (h):
96
4
24
48
72
96
1.8 kb
0.7 kb
CLU
36B4
X-Fold
Induction:
1
1
2
8
7
8
B
x-irradiation (Gy)
UT
0.02
0.05
0.1
0.25
0.5
1.0
2.0
5.0
10
1.8 kb
0.7 kb
CLU
36B4
X-Fold
Induction:
1
2
2
4
5
7
11
13
22
7

Figure 1

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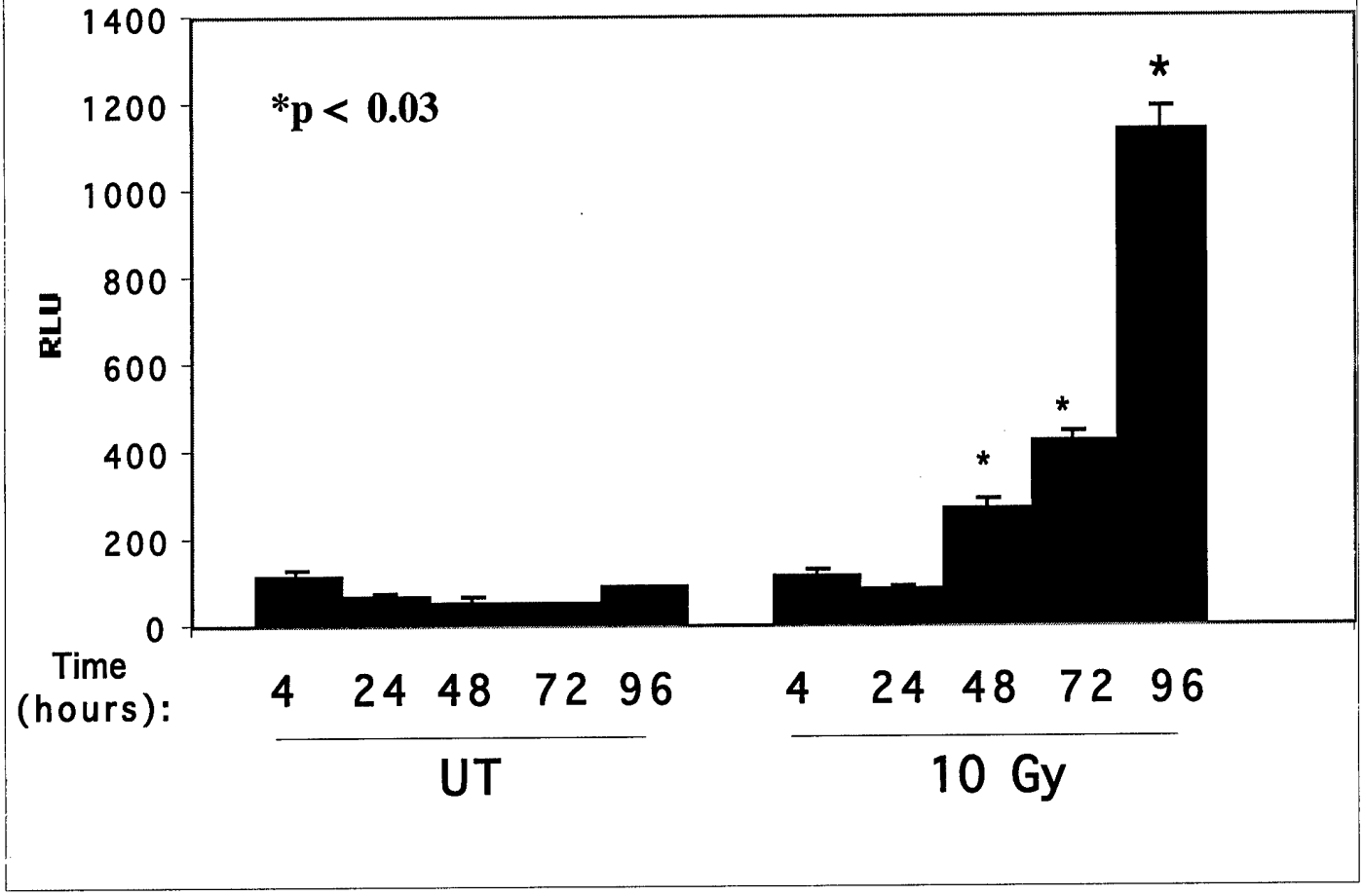


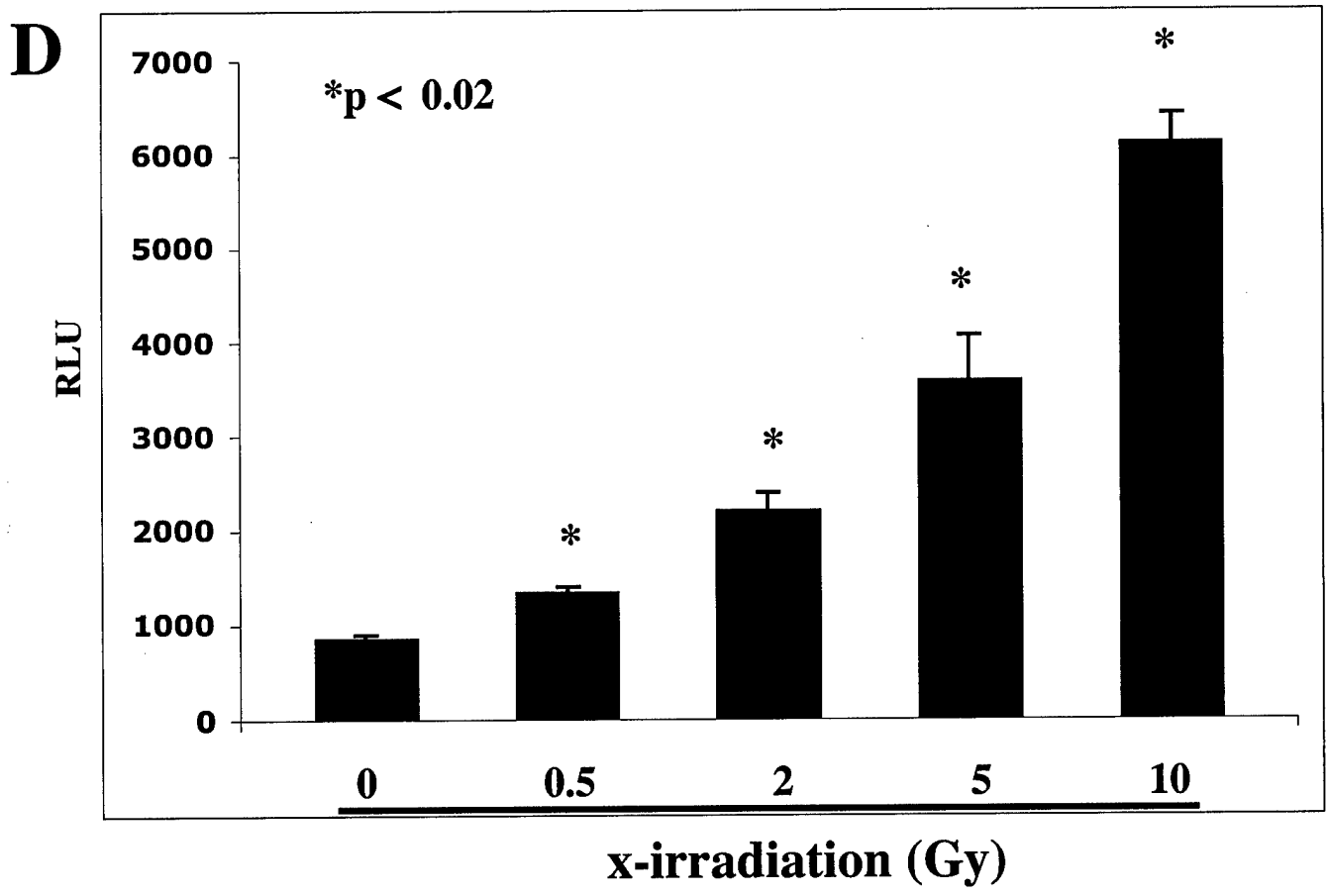


Figure 2

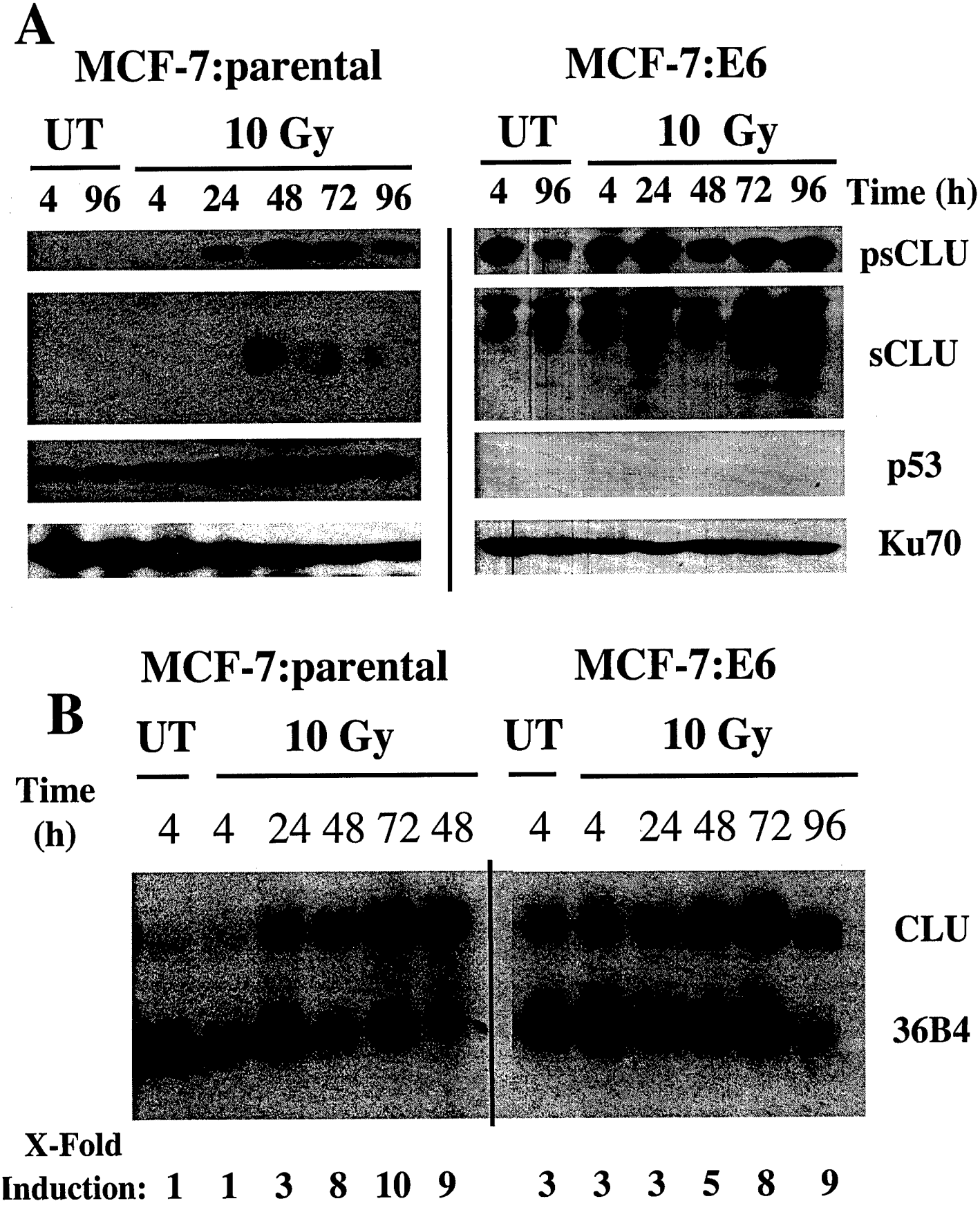

A
MCF-7:parental
MCF-7:E6
UT
10 Gy
UT
10 Gy
4 96 4 24 48 72 96
4 96 4 24 48 72 96 Time (h)
psCLU
sCLU
p53
Ku70
B
MCF-7:parental
MCF-7:E6
UT
10 Gy
UT
10 Gy
Time (h)
4 4 24 48 72 48
4 4 24 48 72 96
CLU
36B4
X-Fold Induction: 1 1 3 8 10 9
3 3 3 5 8 9

Figure 2

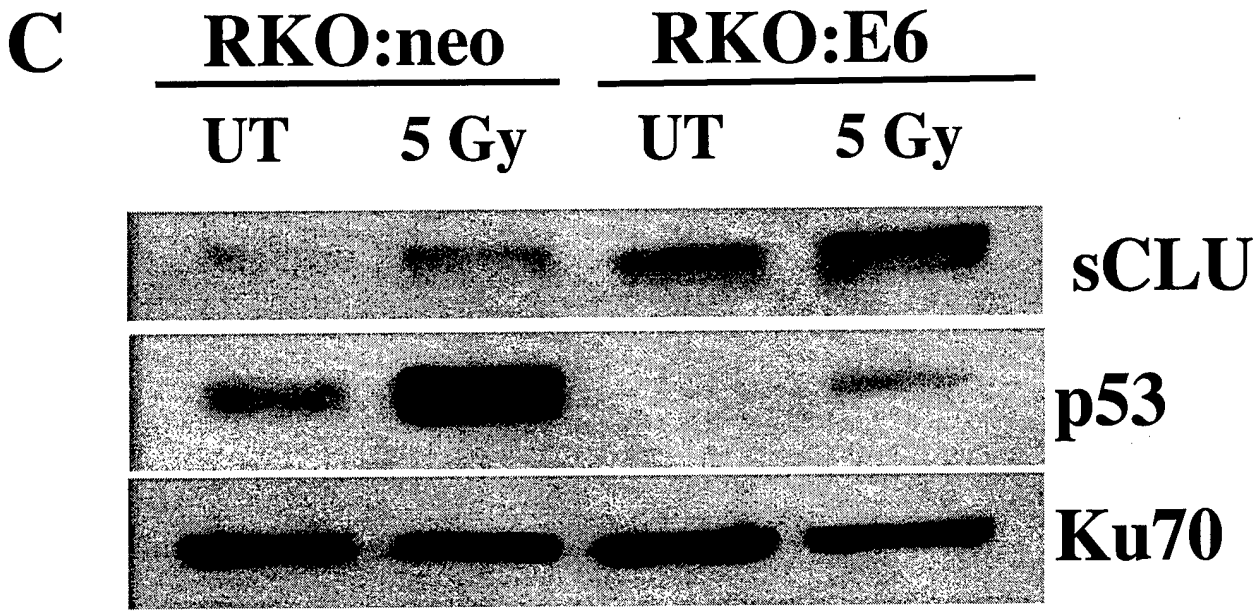
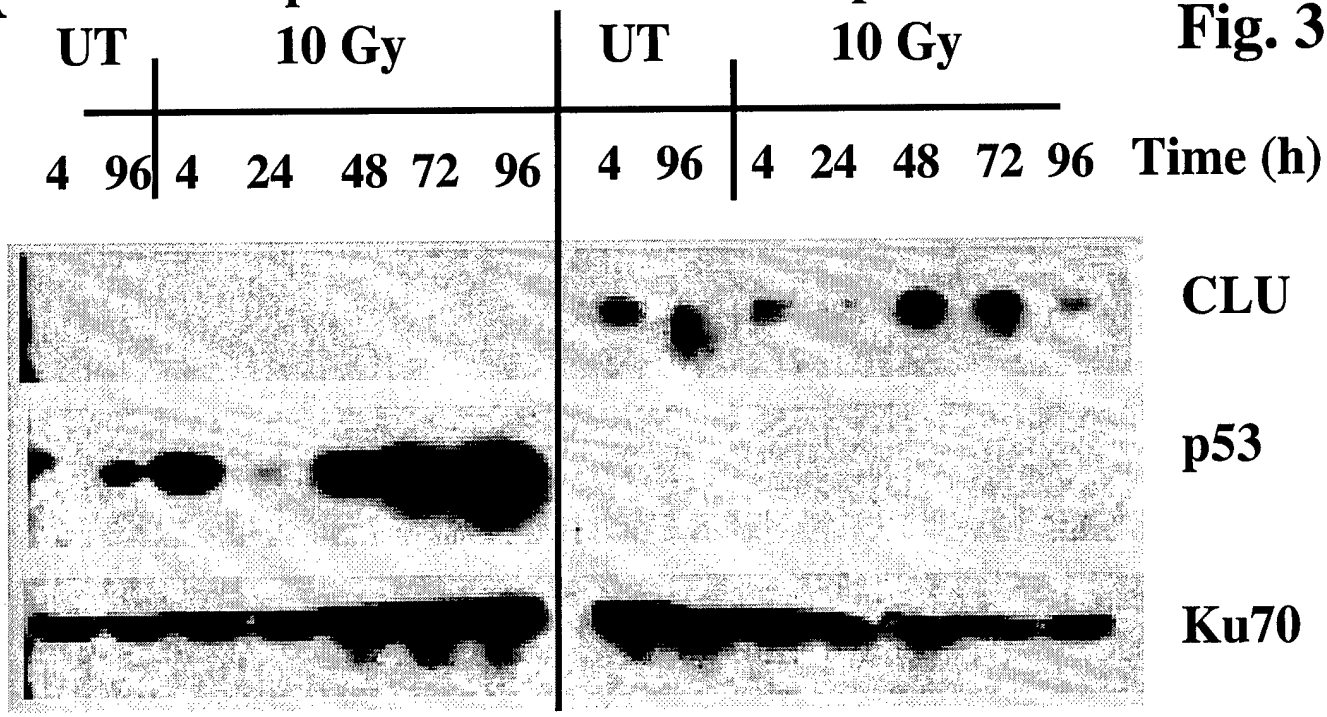
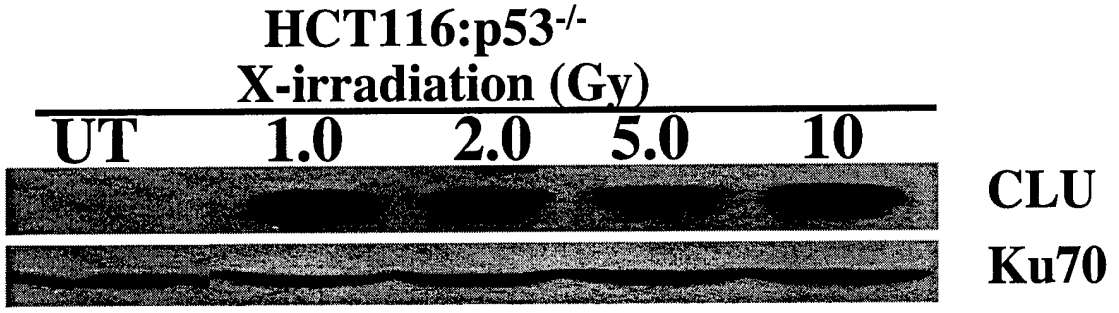


Fig. 3

A


HCT116:parental
HCT116:p53 -/-
UT
10 Gy
UT
10 Gy
4 96 4 24 48 72 96
4 96 4 24 48 72 96
Time (h)
CLU
p53
Ku70

B


HCT116:p53-/-
X-irradiation (Gy)
UT 1.0 2.0 5.0 10
CLU
Ku70

C

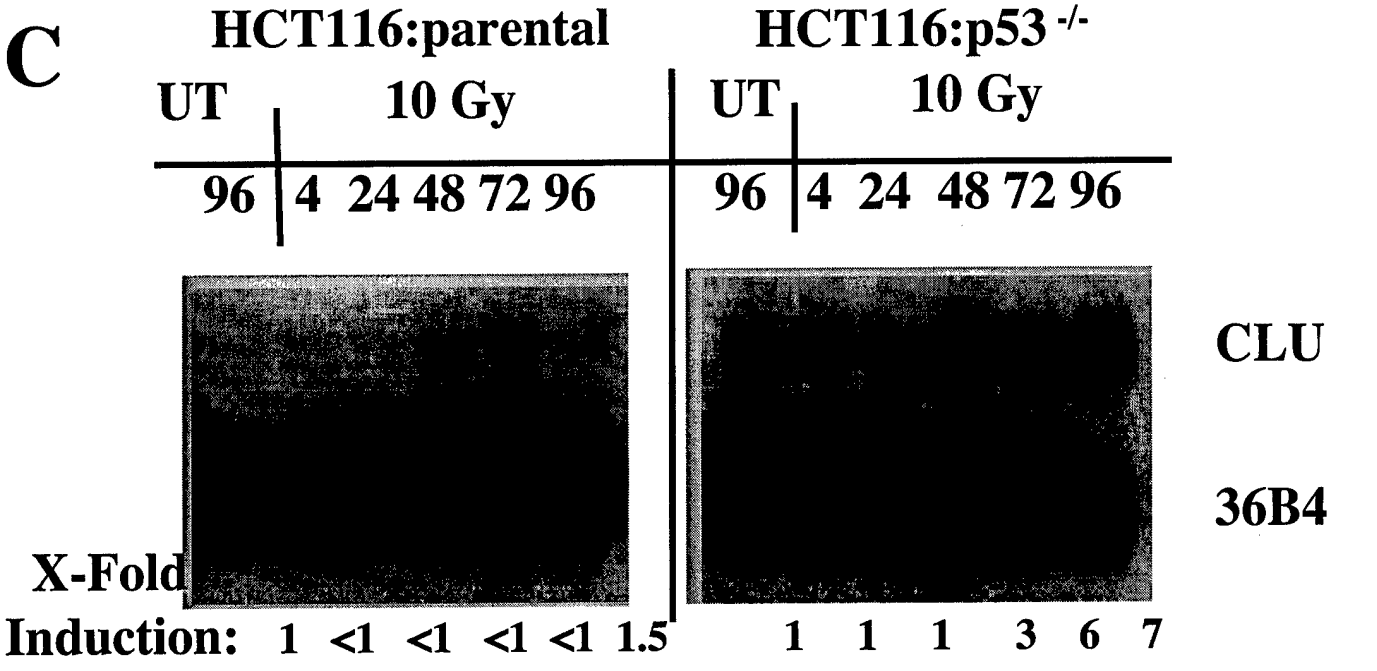
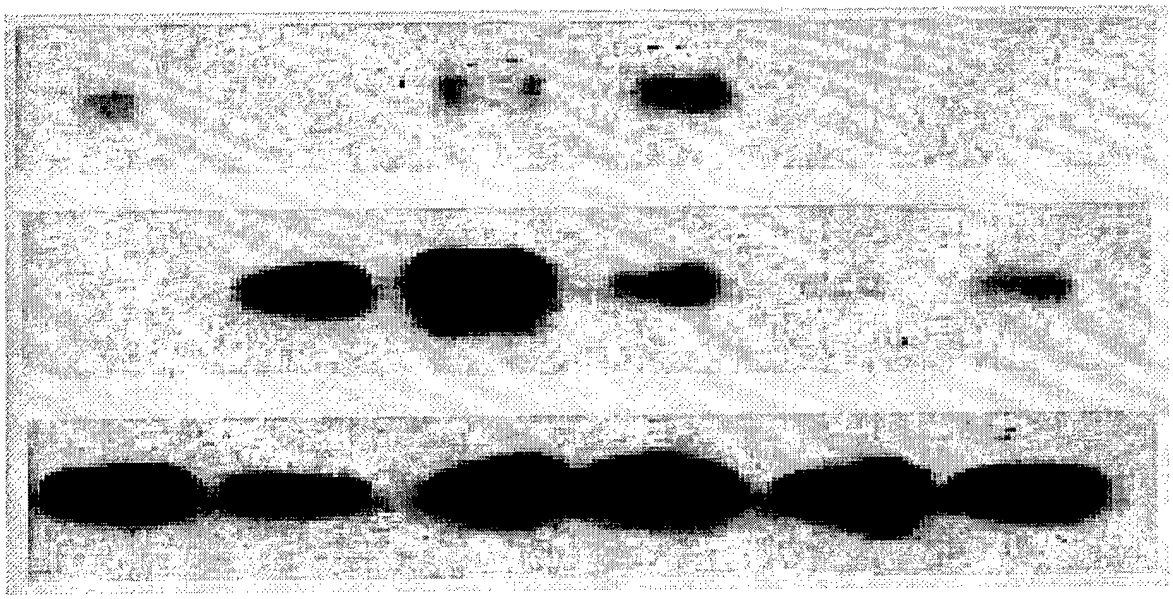

HCT116:parental
HCT116:p53 -/-
UT
10 Gy
UT
10 Gy
96 4 24 48 72 96
96 4 24 48 72 96
CLU
36B4
X-Fold
Induction: 1 <1 <1 <1 <1 1.5
1 1 1 3 6 7

Fig. 4

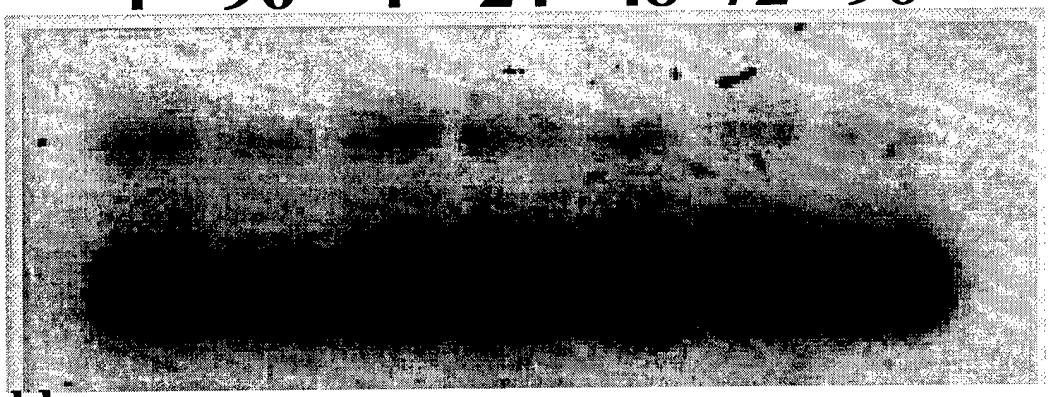
A **HCT116:p21$^{-/-}$**

UT	10 Gy				
96	4	24	48	72	96



B **HCT116:p21$^{-/-}$**

UT		10 Gy				
4	96	4	24	48	72	96



X-Fold Induction:	1	<1	<1	<1	<1	<1	<1

Fig. 5

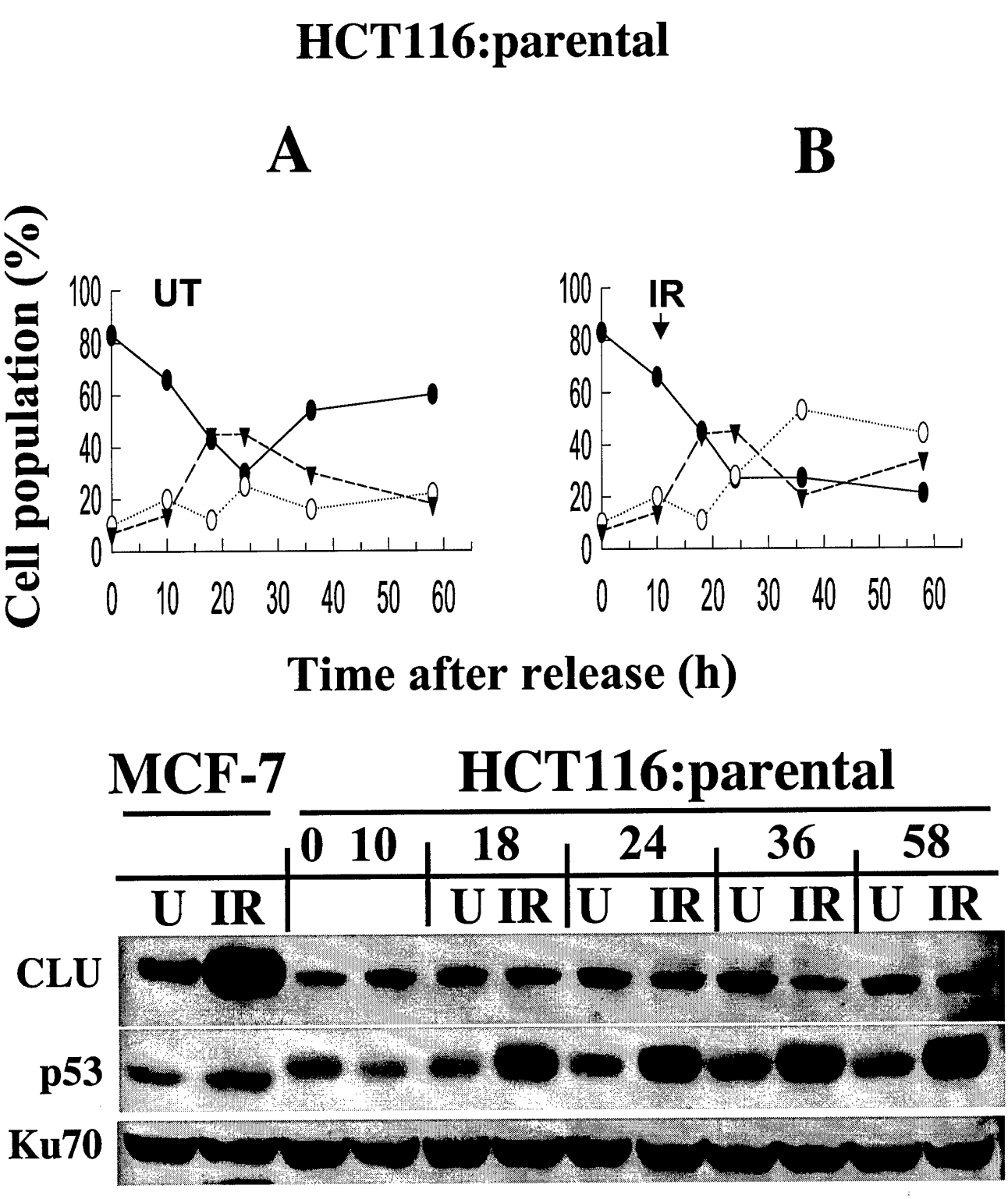
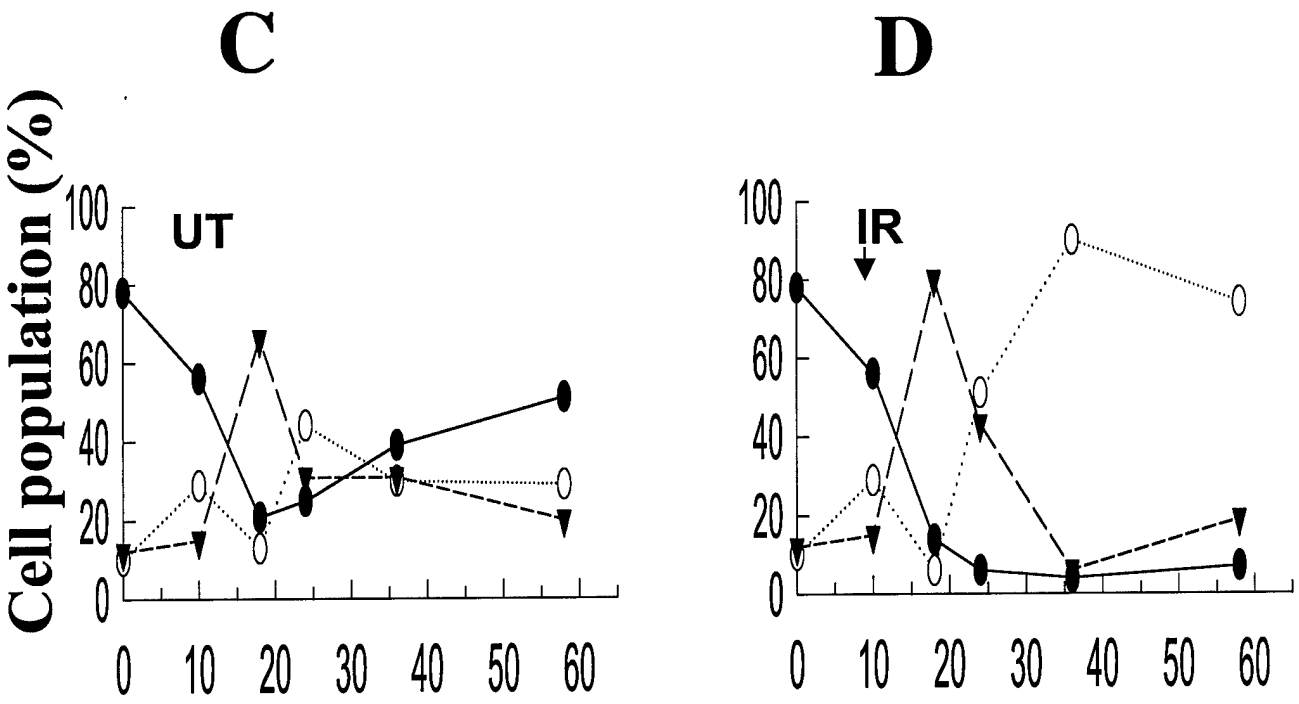

HCT116:parental
A
B
Cell population (%)
UT
IR
100
80
60
40
20
0
0 10 20 30 40 50 60
Time after release (h)
MCF-7
HCT116:parental
0 10 18 24 36 58
U IR U IR U IR U IR U IR
CLU
p53
Ku70

Fig. 5

HCT116:p53$^{-/-}$



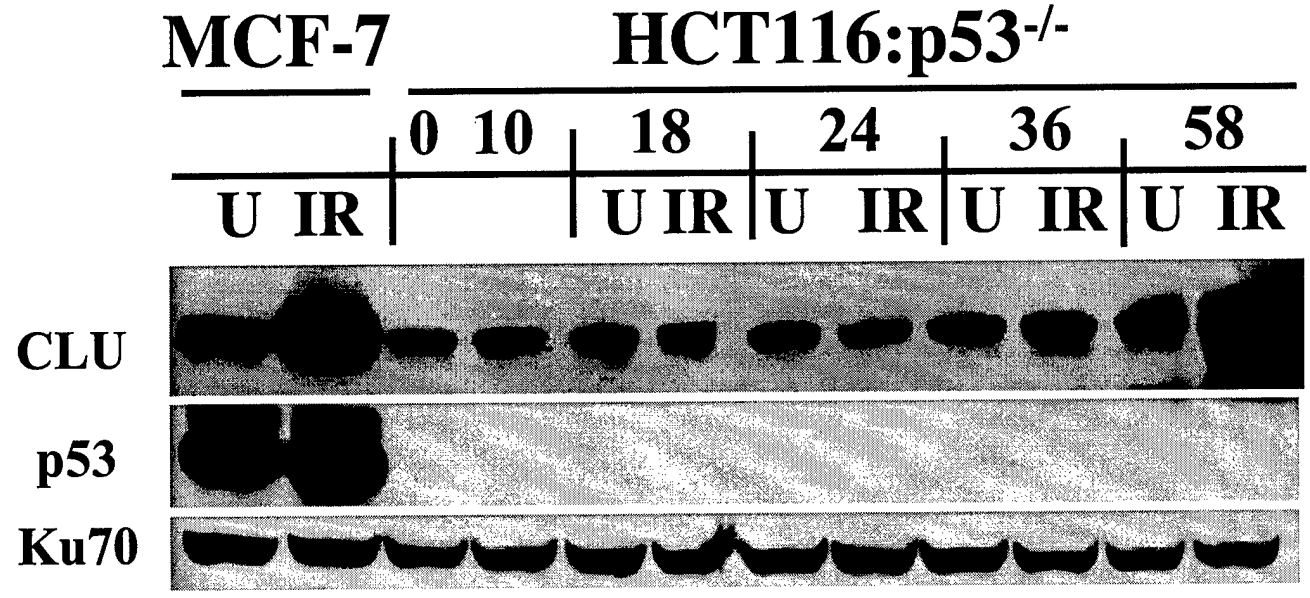


Fig. 5

HCT116:p21$^{-/-}$