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TITLE: Investigating the Role of Nuclear Clusterin (nCLU) in Lethality and Genomic Instability in Paclitaxel (taxol)-treated Human Breast Cancer Cells

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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 <i>et. al</i> demonstrated that sCLU was over-expressed 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 identify the signaling pathway and transcription factors required for sCLU induction after IR.

Task 1:

1. Identify the signaling pathway required for sCLU induction after IR. Western blot analyses will be used to determine the activation of Src, Raf, Mek and Erk after IR. Phospho-antibodies to each of the proteins will be used to determine activation.
 - a. Once we identify the signaling cascade that is activated after IR, chemical inhibitors (PP1-src, U0126-Mek) and dominant negative (src kinase dead (KD), dn Mek-1, dn erk-1/2) and over-expression studies (src constitutively active(CA)) will be done to determine which proteins are involved in the activation of sCLU after IR. sCLU status will be monitored in MCF-7 cells via western blot analyses and in MCF-7 1403 cells, which stably contain 1403 bp of the CLU promoter linked to a firefly reporter, by luciferase assays.
2. It has previously been shown that the epidermal growth factor receptor (EGFR) and the insulin like growth factor receptor-1 (IGF-1R) can both be activated by IR (10). We will determine if either of these receptors are upstream of sCLU induction after IR selective chemical inhibitors of the receptors (AG1478-EGFR, AG1024-IGF-1R). sCLU induction will be monitored by western blot analyses and luciferase assays.

Task 2:

1. Identify the transcription factors required for sCLU induction after IR. Only a few transcription factors are known to be induced by IR (11). We have identified several Egr-1 binding sites within the *CLU* promoter.
 - a. Over expression of Egr-1 in MCF-7 1403 cells will be done to investigate a potential role for Egr-1 in sCLU induction. Luciferase assays will be used to monitor *CLU* promoter activity.
 - b. DNA pull-down assays will be performed to determine if Egr-1 can bind to the *CLU* promoter. These experiments will be done in the presence of AG1478 (selective EGFR inhibitor), AG1024 (selective IGF-1R inhibitor), PP1 (selective src inhibitor) and U0126 (selective Mek-1 inhibitor). If *CLU* transcription is dependent on any of these pathways, treatment with the inhibitor should abrogate transcription factor binding to the promoter.
 - c. siRNA specific to Egr-1 will be used to knock-down Egr-1 expression in MCF-7 cells. sCLU

expression in these cells will be monitored via luciferase assays.

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 identify the signaling pathway and transcription factors required for sCLU induction after IR.

Task 1: Identify the upstream receptor and signaling pathway required for sCLU induction after IR.

1. Identify the signaling cascade that results in sCLU induction after IR.

Progress: Western blot analyses of protein harvested from MCF-7 cells at various times after 5 Gy (0, 0.25, 0.5, 1, 2, 4, 8, 24, 48 and 72 h) demonstrate the Src, Raf, Mek and Erk are activated after quickly IR, as measured by phosphorylation status, as has been previously reported (10). We report a novel reactivation of the Src-MAPK cascade 24-72 h after IR that corresponds to sCLU induction (see Criswell *et al.*, submitted to *Molecular Cell*, 2004, in appendix). We also demonstrate that treatment with PP1 (selective Src inhibitor) and U0126 (selective Mek-1 inhibitor) can abrogate *CLU* promoter activity after IR. In addition, over expression of a constitutively active src (Src CA) in MCF-7 cells can increase the basal activity of the *CLU* promoter as well as the IR-inducibility of the *CLU* promoter as compare to mock-transfected cells. In contrast, over expression of a kinase dead Src (Src KD), a dominant negative Mek-1 (dn Mek-1) or a dominant negative Erk-1/2 (dn Erk-1/2) in MCF-7 cells abrogated *CLU* promoter induction after IR (see Criswell *et al.*, submitted to *Molecular Cell*, 2004, in appendix). All of this data demonstrates a role for the MAPK cascade in the induction of sCLU after IR.

2. Identify the membrane receptor that is upstream of sCLU induction after IR.

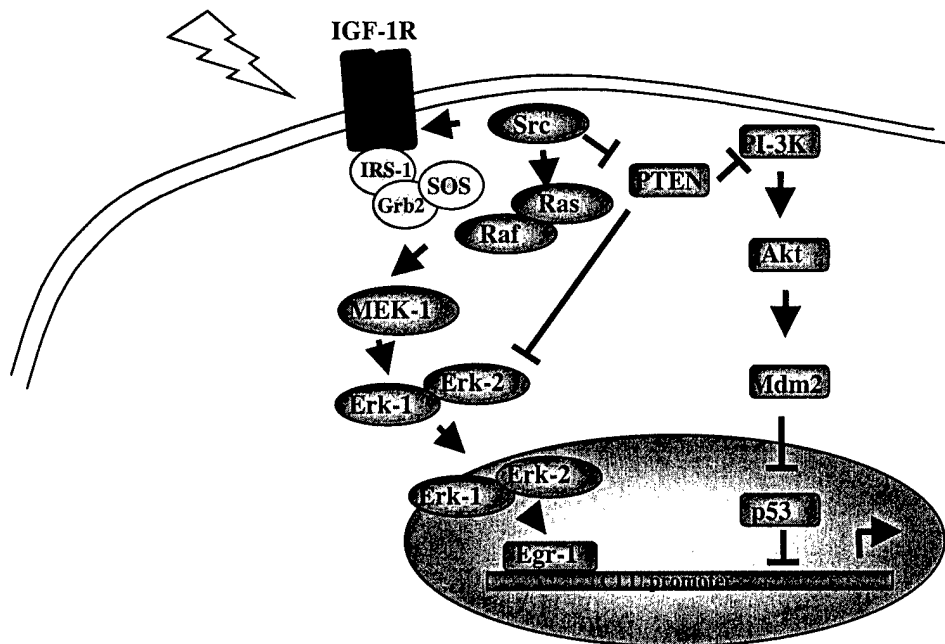
Progress: We have found that EGFR does not play a role in sCLU induction after IR by using AG1478, a selective inhibitor of EGFR (see Criswell *et al.*, submitted to *Molecular Cell*, 2004, in appendix). Additionally, we show that EGF is not able to activate the *CLU* promoter or induce sCLU protein expression. In contrast, we demonstrated that IGF-1R did play a role in sCLU induction after IR using AG1024, a selective inhibitor of IGF-1R. We also demonstrate that treatment of MCF-7 cells with IGF can induce sCLU expression. Furthermore, treatment of MCF-7 cells with insulin-like growth factor binding protein-3 (IGFBP3), an endogenous IGF-1R inhibitor, can inhibit *CLU* promoter activity after IR. We also demonstrate that the IGF-

IR is upregulated after IR as well as IGF secretion, suggesting a putative autocrine feedback loop in these cells (see Criswell *et al.*, submitted to *Molecular Cell*, 2004, in appendix).

Task2:

1. Identify the transcription factor(s) required for sCLU induction after IR.

Progress: Over-expression of Egr-1 in MCF-7 1403 cells increased basal *CLU* promoter activity as well as IR-inducible promoter activity as compare to mock-transfected cells, suggesting a role for Egr-1 in sCLU induction after IR. DNA pull-down assays were performed to determine if Egr-1 was capable of binding to the *CLU* promoter. We found that Egr-1 did bind to the *CLU* promoter and that binding was enhanced after IR up to 72 h post-IR exposure in a temporal fashion that matched sCLU induction (see Criswell *et al.*, submitted to *Molecular Cell*, 2004, in appendix). Additionally, when cells were treated with an inhibitor to IGF-1R (AG1024), Src (PP1) or Mek-1 (U0126), Egr-1 binding was abrogated (see Criswell *et al.*, submitted to *Molecular Cell*, 2004, in appendix). In contrast, treatment of cells with an EGFR inhibitor (AG1478) had no effect on Egr-1 binding to the *CLU* promoter. Finally, we used siRNA specific to Egr-1 to knock-down Egr-1 expression in MCF-7 cells. *CLU* promoter activity after IR was significantly decreased in cells expressing Egr-1 siRNA as compared to cells expressing a scrambled siRNA (see Criswell *et al.*, submitted to *Molecular Cell*, 2004, in appendix). This data demonstrated the requirement for Egr-1 in sCLU induction after IR. See model below (Figure 1).



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. The src-MAPK cascade is upregulated after IR in a temporal sequence that corresponds to sCLU induction (24-72 h post-IR).
6. IGF-1R, not EGFR, is activated after IR and is required for sCLU induction after IR.
7. The Src-MAPK cascade is upstream of sCLU induction after IR.
8. The Egr-1 transcription factor binds to the *CLU* promoter and is required for sCLU induction after IR.

REPORTABLE OUTCOMES:

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

- Criswell, T., Klovov, 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.
- Criswell, T., Beman, M., Araki, S., Leskov, K., Cataldo, E., Mayo, L., and Boothman, D.A. Induction of Clusterin, a Pro-Survival Factor, Requires the Delayed Activation of IGF-1R/MAPK Signal Cascade after IR. *Mol Cell.* submitted, 2004. (Included, appendix)

ABSTRACTS AND PRESENTATIONS:

Poster Presentations:

1. DOD Era of Hope Meeting, Sept. 2002.
Title: p53 repression of the secretory protein clusterin.
2. International Congress of Radiation Research, Aug. 2003.
Title: The Signaling Network Responsible for Clusterin Induction after Ionizing Radiation

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:

This grant has identified p53 as a transcriptional repressor of sCLU. Additionally, we have identified a role for IGF-1R, Src, MAPK and Egr-1 in the induction of sCLU 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*

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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: ≥ 0.02 Gy). 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, show that CLU induction is 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 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

Secretory clusterin (sCLU) is a sulfated glycoprotein that has been implicated in many physiological and pathological processes, including tissue remodeling,¹ complement inhibition,² lipid transport,^{4, 5} multiple sclerosis,⁶ atherosclerosis,^{7, 8} and Alzheimer's disease.⁹⁻¹¹ Elevated levels of sCLU protein and mRNA were noted in several different types of human malignancies,^{12, 13} and forced over-expression of sCLU in transformed cell lines resulted in an increased resistance to various chemotherapeutic agents.^{14, 15} In addition, abrogation of CLU mRNA expression following antisense expression lead to modest chemo- and IR-sensitizations in various cell lines.¹⁶⁻¹⁹ These data suggest a cytoprotective role for sCLU.

The p53 tumor suppressor gene is mutated in over half of all human tumors.²⁰ 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.²¹⁻²³ 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,²⁴ hsp70,²⁵ cyclins A²⁶ and B²⁷ and cdc2.²⁸

Our laboratory identified CLU as a x-ray inducible protein/transcript (xip8).²⁹ We showed that a nuclear form of CLU (nCLU) associated with the DNA double strand break (DSB) repair protein, Ku70.^{30, 31} 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.³⁰

Although regulation of sCLU gene expression following estrogen and testosterone exposures has been investigated,^{32, 33} 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). β -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 developed³⁴ 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% CO₂-95% air atmosphere as described.³⁵ 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 % CO₂-90% air atmosphere. All experiments were initiated by seeding 5 x 10⁵ log-phase cells per 10-cm² 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.²⁹ Briefly, cells were irradiated with ¹³⁷Cs 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.²⁹ 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.³⁶ β -Lapachone and hypoxic exposures³⁷ 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 μ g) 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;³⁶ 36B4 levels are not affected by cell stress, or cell cycle status.³⁶ 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.³⁶

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.³¹ 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-cm² tissue culture dishes as described.^{29, 38} Cells were then maintained for 48 h in serum-free medium to maximize G₀-G₁ arrest. Cells were released from the dual confluence and serum-free cell cycle arrest by trypsinization (using 0.05% trypsin 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 °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 °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),³⁶ 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,³¹ 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.³¹

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.³⁹ 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 II α 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 μ M, 4h), a novel apoptotic drug that quickly depletes cellular NAD(P)H and ATP in NQO1-expressing MCF-7 cells,⁴⁰ 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.⁴¹ 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.⁴² 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).³⁴ 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.⁴³ To address this issue in cancer cells, HCT116:parental, HCT116:p53^{-/-} and HCT116:p21^{-/-} cells were arrested in the G₀/G₁ 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'.⁴⁴ 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 G₀/G₁ cells. As previously noted with this synchronization technique,⁴⁵ 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 G₁ 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 G₁ cell cycle checkpoint response, Fig. 5A, B) as described.⁴⁵ For example, at 36 h IR-treated HCT116:parental cells demonstrated >45% G₂ 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 G₀/G₁ 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 G₁, while 80% and 66% of cells, respectively, proceeded into S phase. As expected, IR-treated HCT116:p53^{+/+} cells arrested in G₁ and exhibited delayed S or G₂/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.⁴⁵

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.³¹ In this study, we further investigated the induction of sCLU by IR. We have shown that sCLU is 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 have shown that DNA damage appears to be sufficient, but not required for sCLU induction (Table 1).

We have shown 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 is not dependent on the cell cycle, but appears to be 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 exerts 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 appear to be 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.³⁰ 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 calcium⁴⁶ 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.

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 (\geq 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.⁴⁷ 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 μ g 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 μ g) 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 μ g) 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 μ g) 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 μ g) 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 G₁ (●), S (▼) and G₂/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: Cytotoxic agents inducing sCLU in MCF-7 cells¹

Agent (doses tested²)

DNA damaging agents

Ionizing radiation (0.02-10 Gy)

Ultraviolet radiation (12 J/m²)

Topotecan (50 nM)

Camptothecin (100 nM)

Etoposide (VP-16, 15 μ M)***Non-DNA damaging agents***Photodynamic Therapy³ (200 nM PC-4/200 mJ/cm²)Colcemid (70 μ g/ml)Nocodazole (150 μ g/ml)

Taxol (1-50 nM)

Taxotere (1-10 nM)

Mimosine (0.5 mM)

TPA (100 nM)

Thapsigargin (10-500 μ M)***Non-Inducing agents*** β -Lapachone (2-10 μ M, 4 h)Hypoxia⁴ (<0.1% O₂)

¹ Log phase MCF-7 cells were seeded at approximately 5X10⁵ cells per 10 cm plate and exposed to agents at the doses indicated above 24 h later.

² Topotecan, camptothecin, TPA, and thapsigargin were given continuously. Cells were exposed to colcemid, nocodazole and mimosine for 24 h, washed with PBS and replated into fresh media. Cells were treated with taxol and taxotere for 4 h, washed with PBS, and replated into fresh media. Cells were treated with VP-16 for 1 h. After treatment, cells were harvested at least 48 h post-exposure, and protein extracted for western blot analyses as described in 'Materials and Methods'.

³ The photosensitizing drug used was Phthalocyanine 4 (PC-4), which incorporates into mitochondria and results in free radicals at this organelle only after red light exposure. Induction of sCLU protein was only observed after co-addition of drug and light exposure. No induction was observed with red light or PC-4 alone.

⁴ Hypoxia was induced as previously described.³⁷

Table 2: Correlation of p53 status and sCLU basal and IR-inducible protein expression.

Cell Line	p53 Status	sCLU Expression		
		Basal ¹	IR-Inducibility ²	RNA ³
<i>Breast cancer cell lines</i>				
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	+
<i>Colon cancer cell lines</i>				
HCT116:parental	wt	low	minimal	+
HCT116:p21 ^{-/-}	wt	low	minimal	+
HCT116:p53 ^{-/-}	null	low	yes	NP
RKO:neo	wt	low	no	NP
RKO:E6				
<i>Prostate cancer cell lines</i>				
LNCaP	wt	low	minimal	NP
PC-3	null	high	no	NP
DU-145	mutant (275)	high	minimal	NP

¹ Basal levels were determined in untreated, log-phase MCF-7 parental cells.

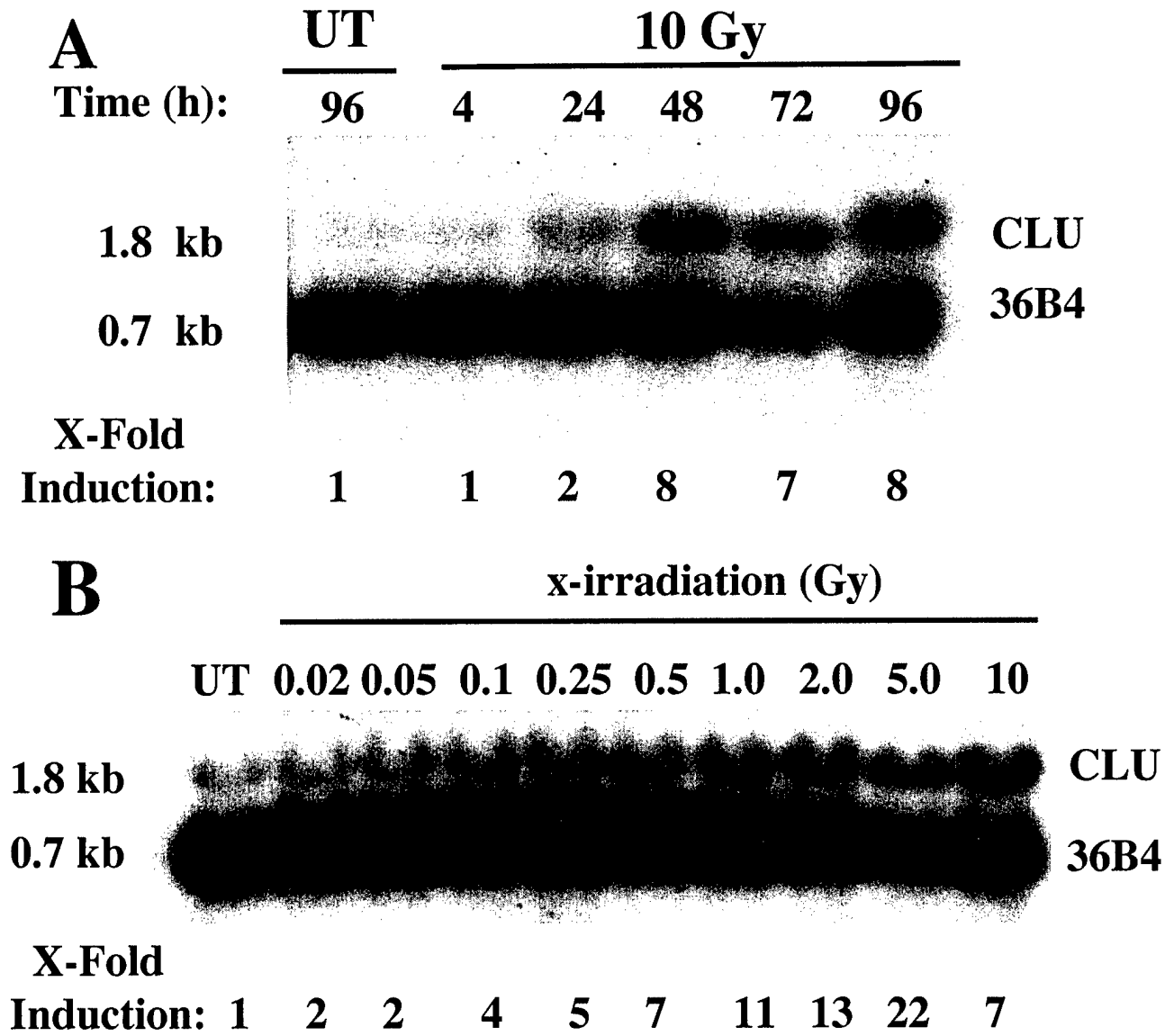
² Log-phase MCF-7 cells were treated with 10 Gy, cells were harvested 48 h post-exposure, and protein analyzed by Western blotting as described in Materials and Methods. MCF-7 parental cells were used as the standard for "high-level" IR inducibility.

³ RNA status was determined by RT-PCR using primers to full-length CLU DNA.

ND: Not detected; NP: Not performed.

Figure 1

Criswell *et al.*



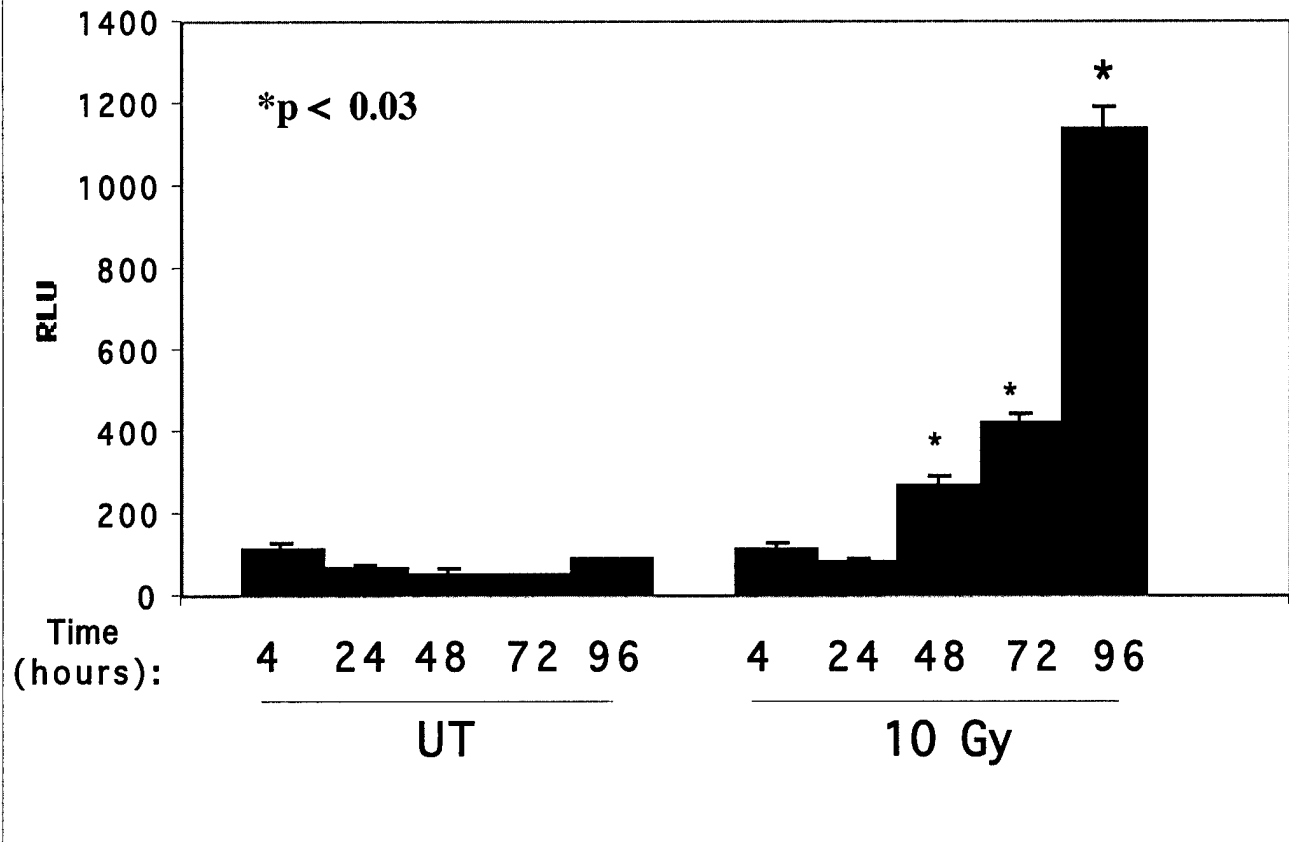
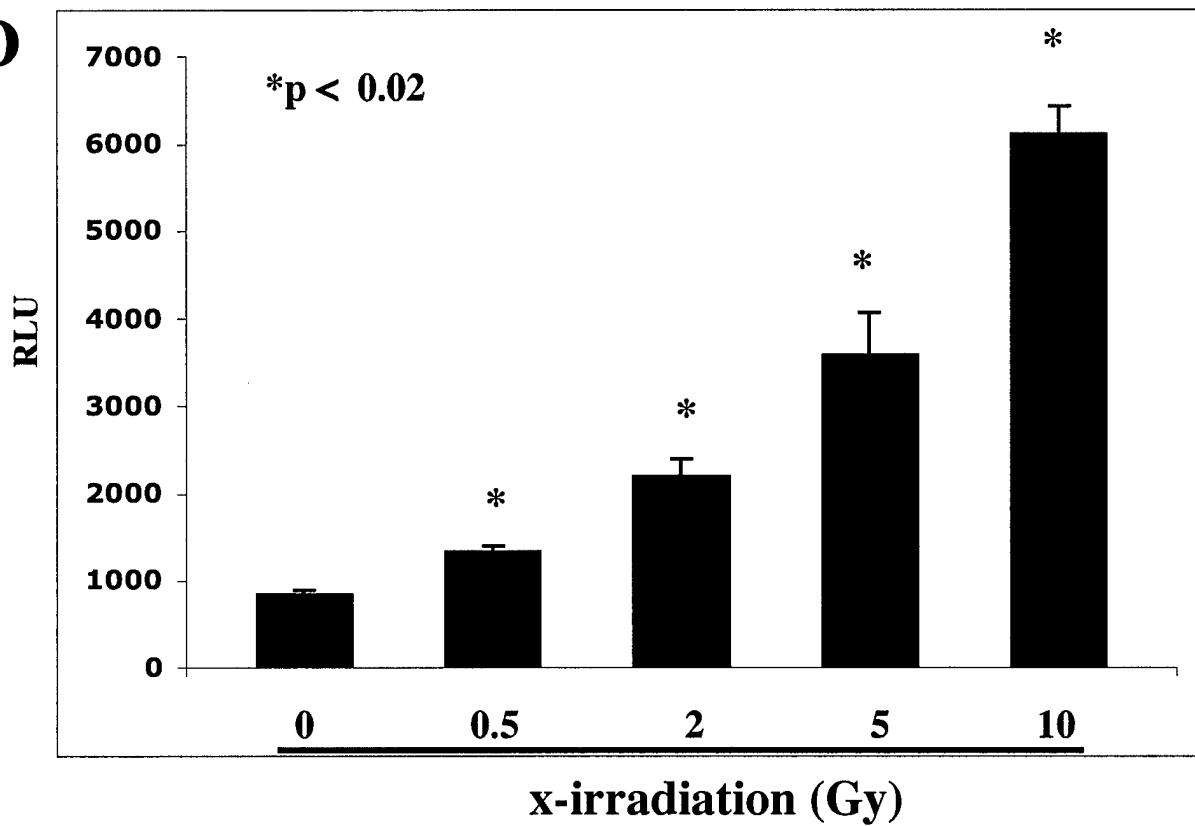
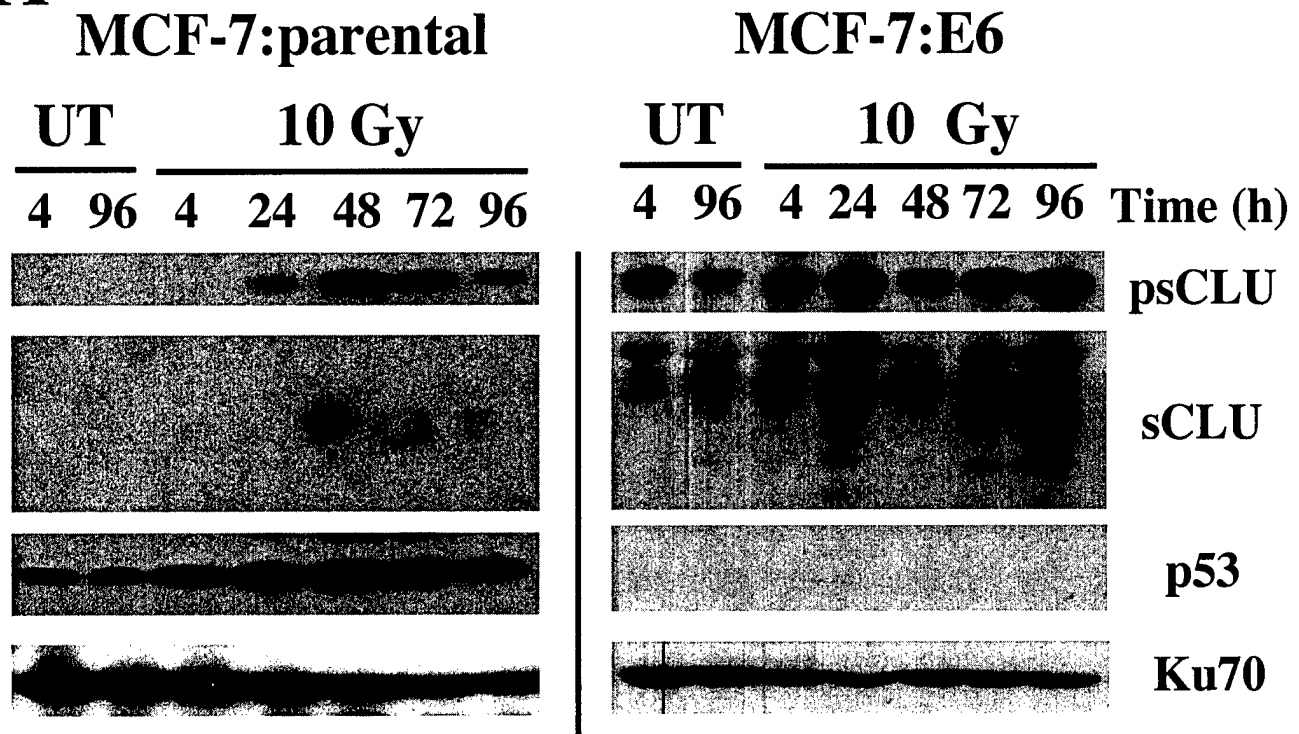
C**Figure 1**Criswell *et al.***D**

Figure 2

Criswell *et al.*

A



B

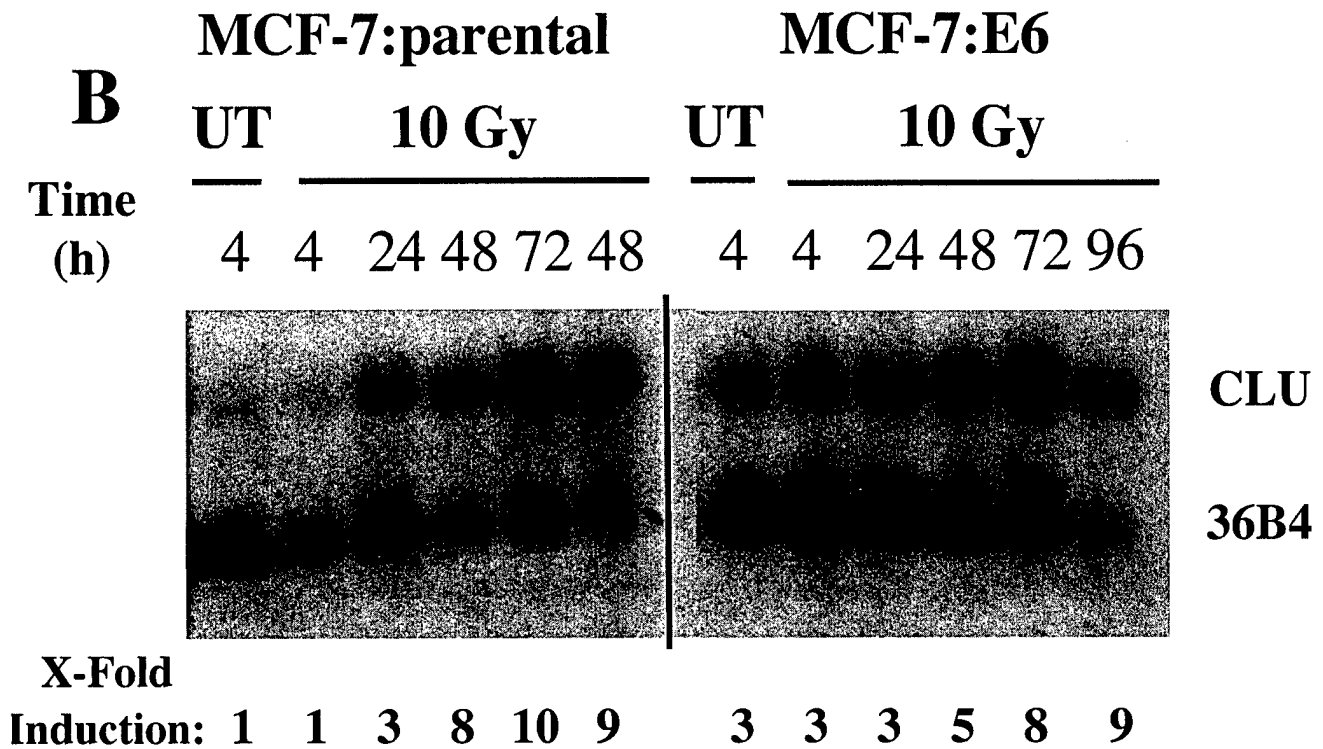


Figure 2

Criswell *et al.*

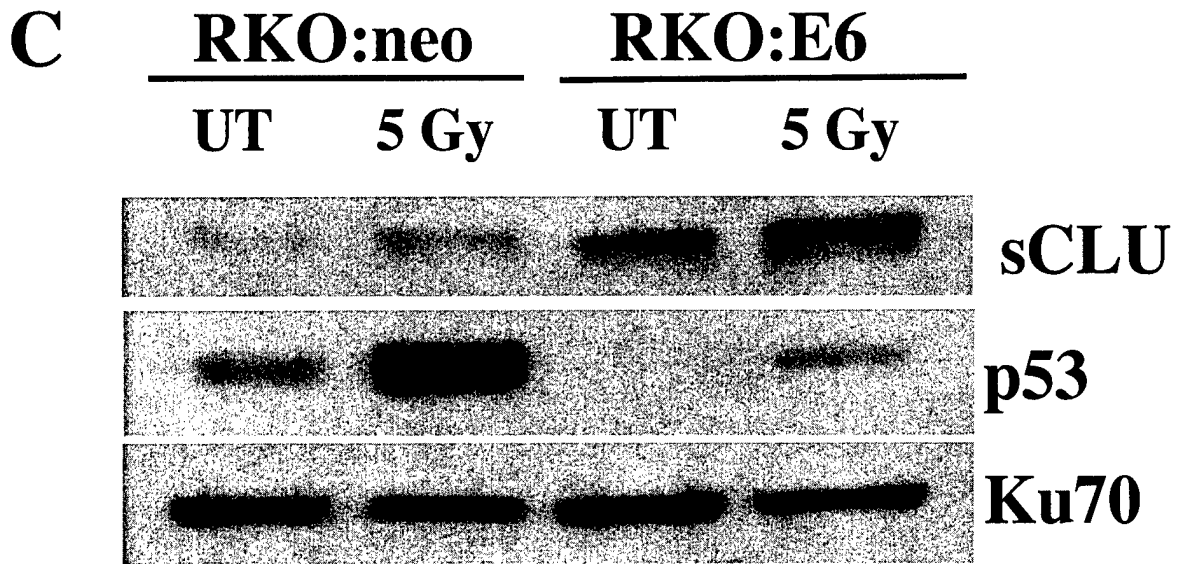


Fig. 3

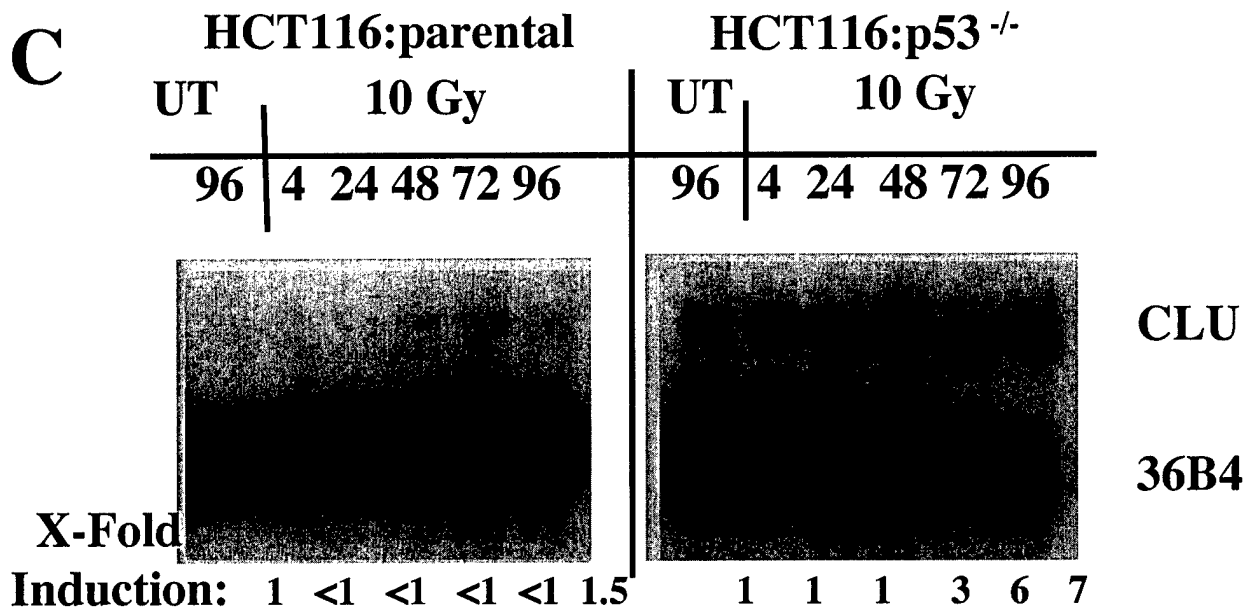
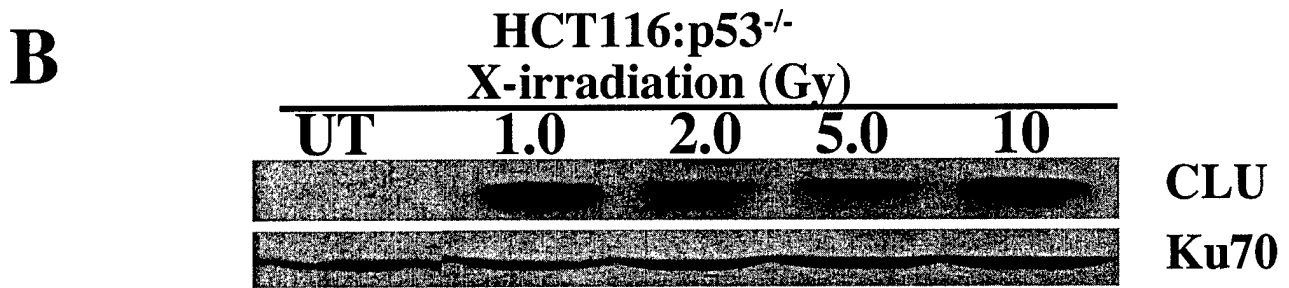
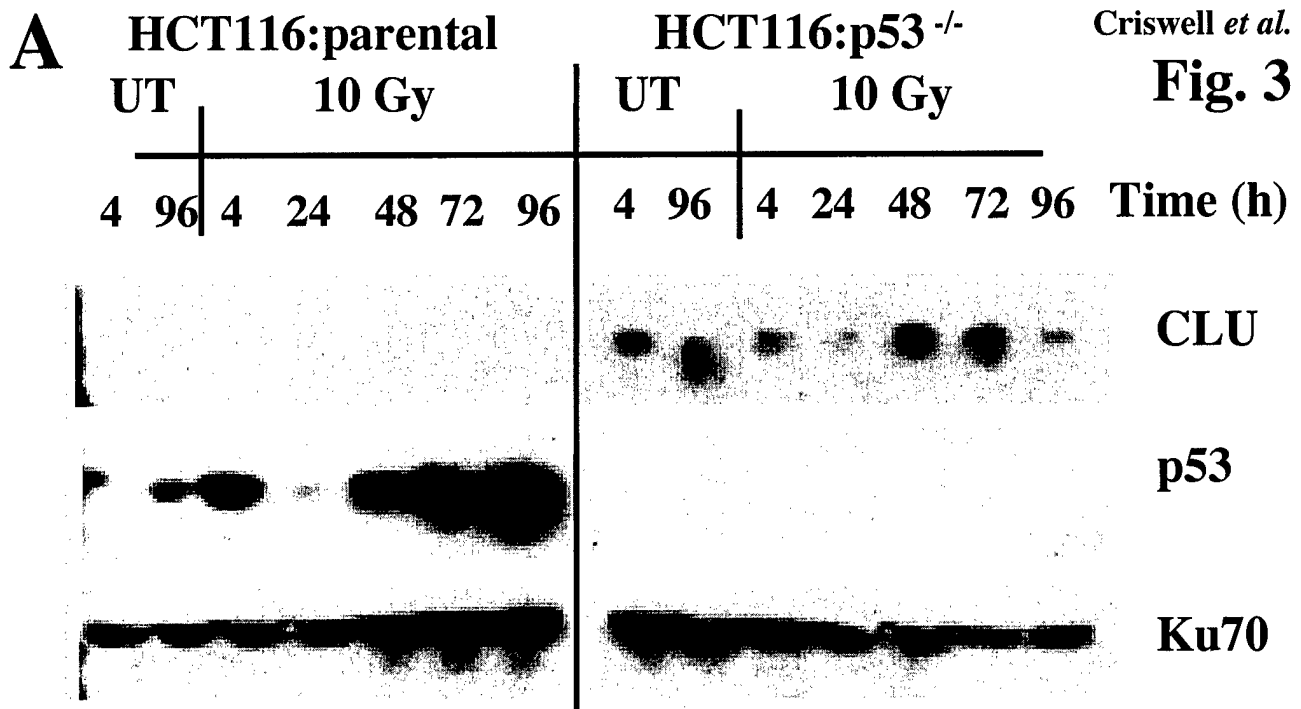
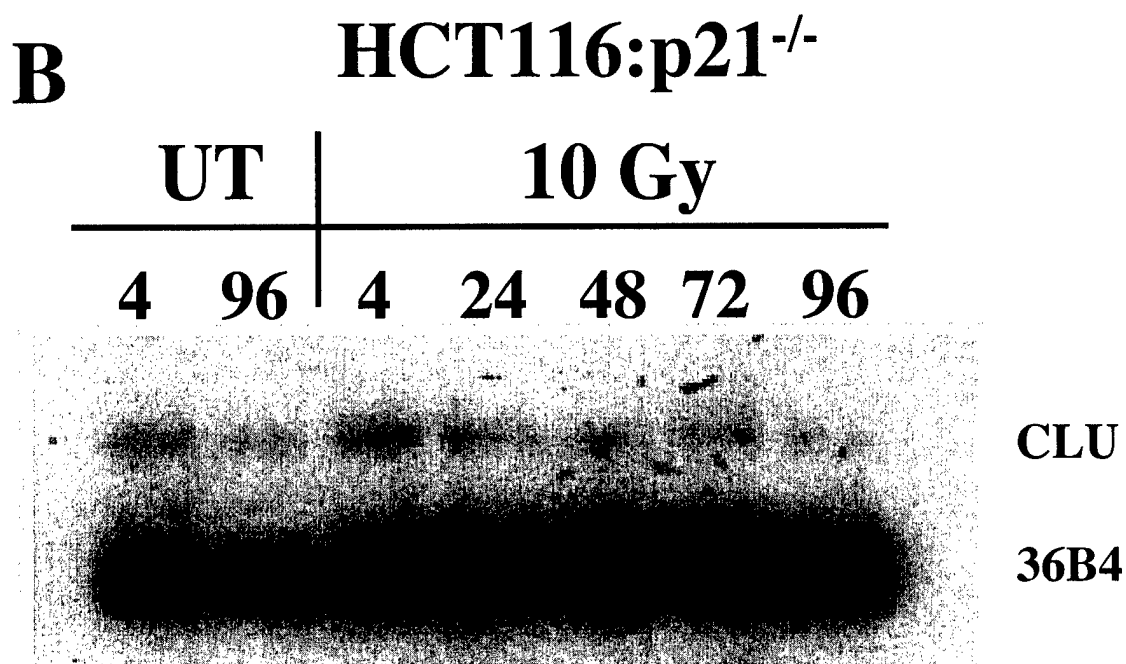
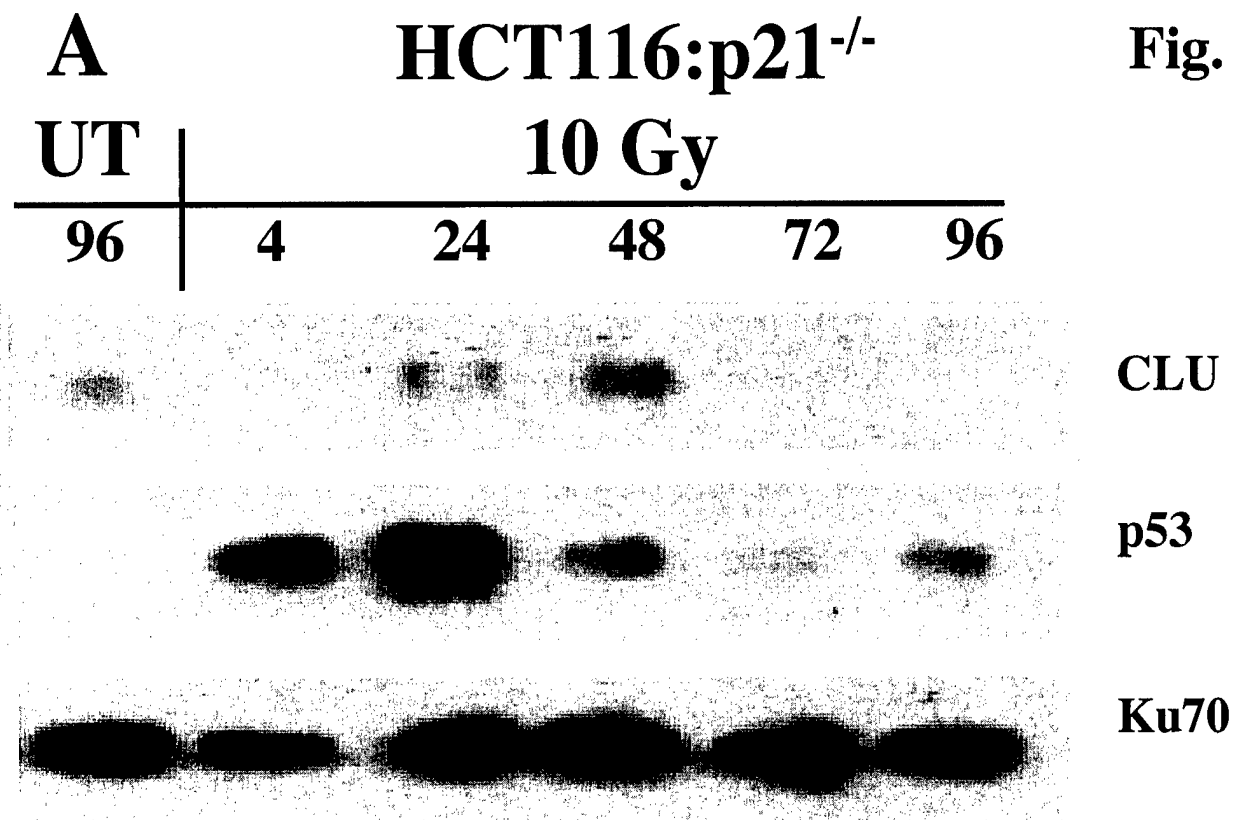


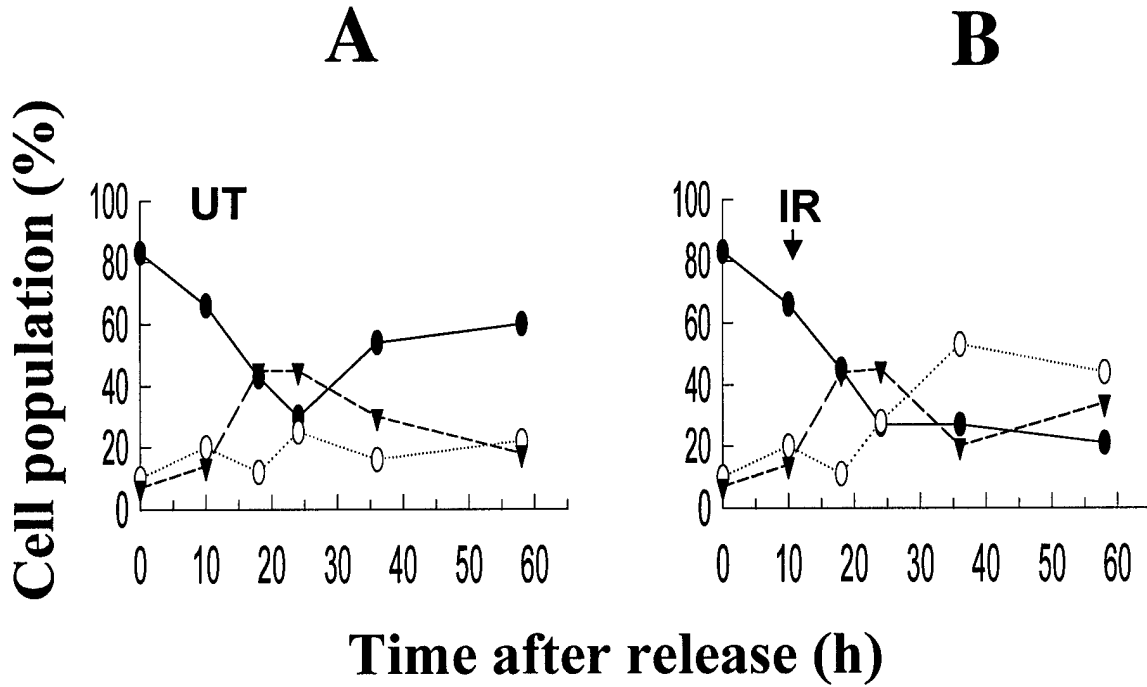
Fig. 4



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

Fig. 5

HCT116:parental



MCF-7

HCT116:parental

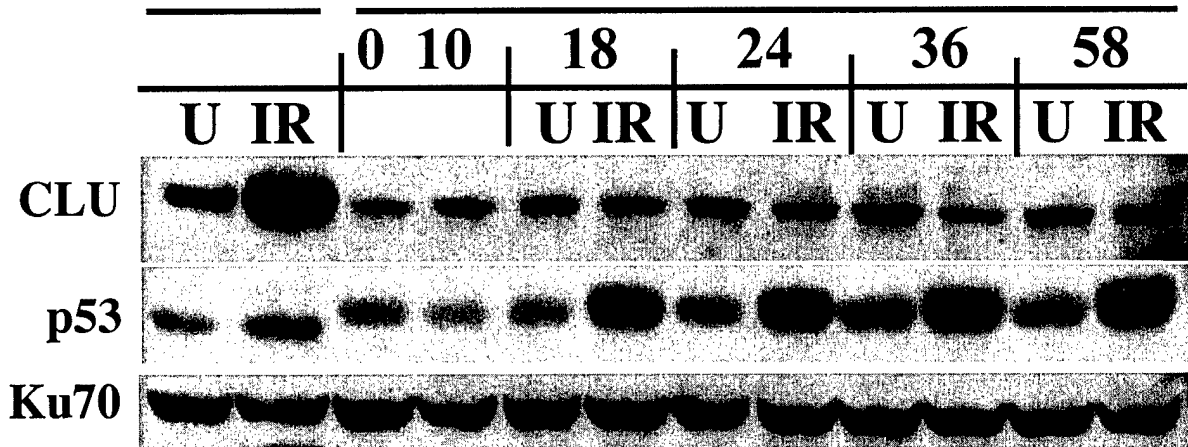


Fig. 5

HCT116:p53^{-/-}

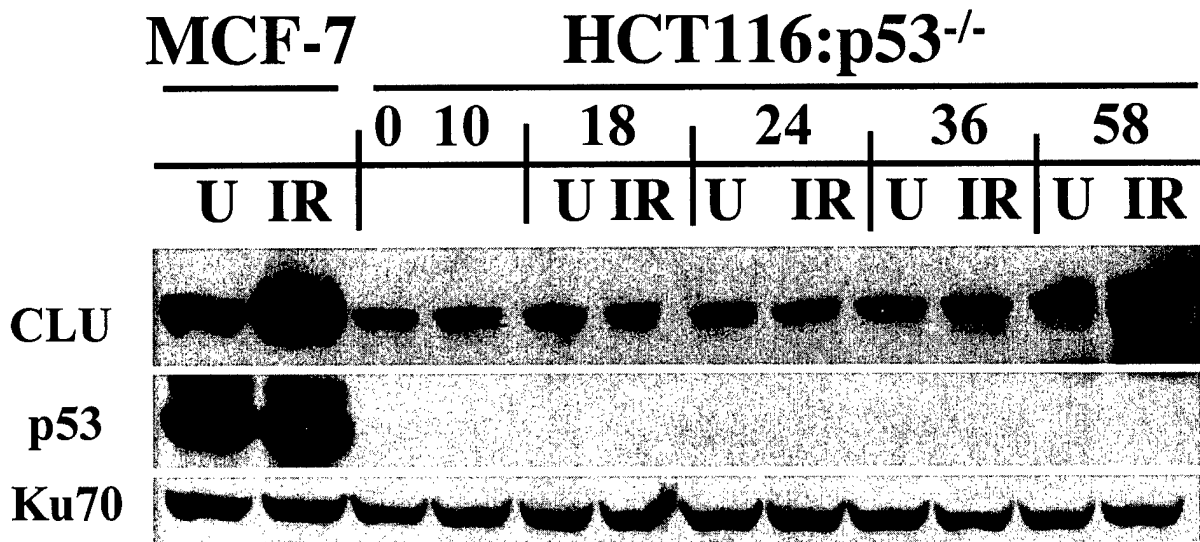
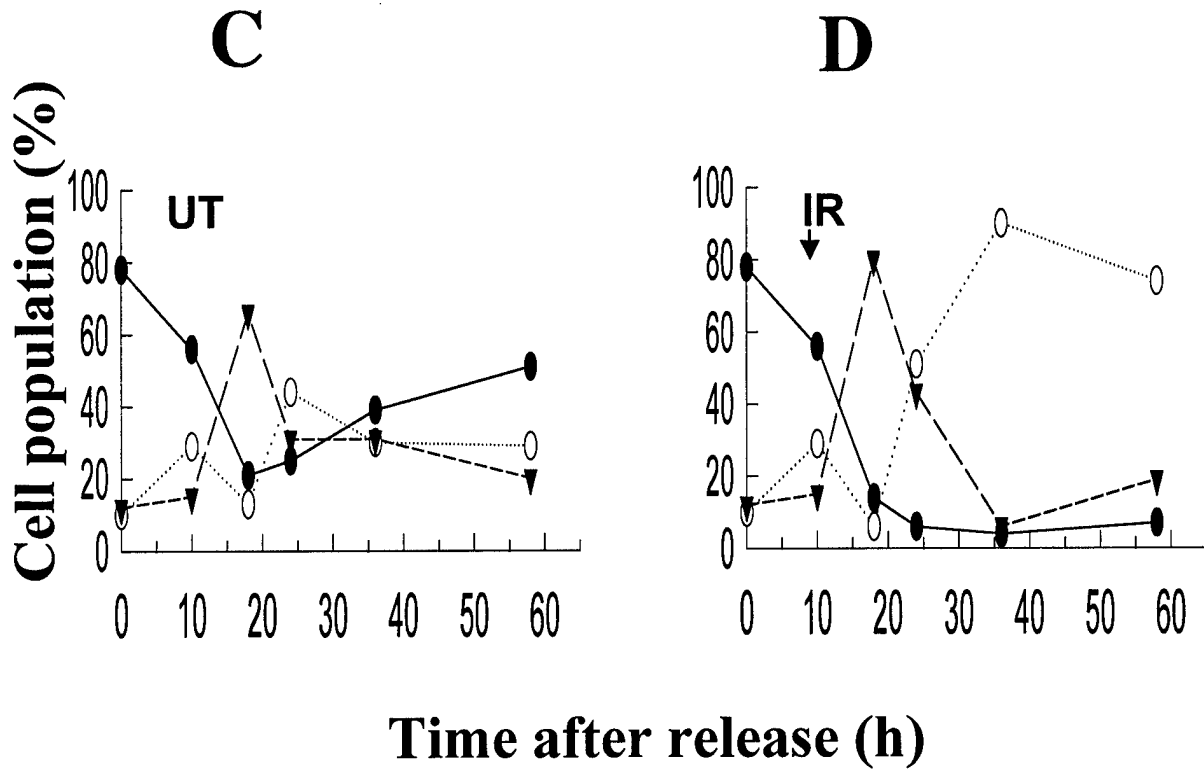
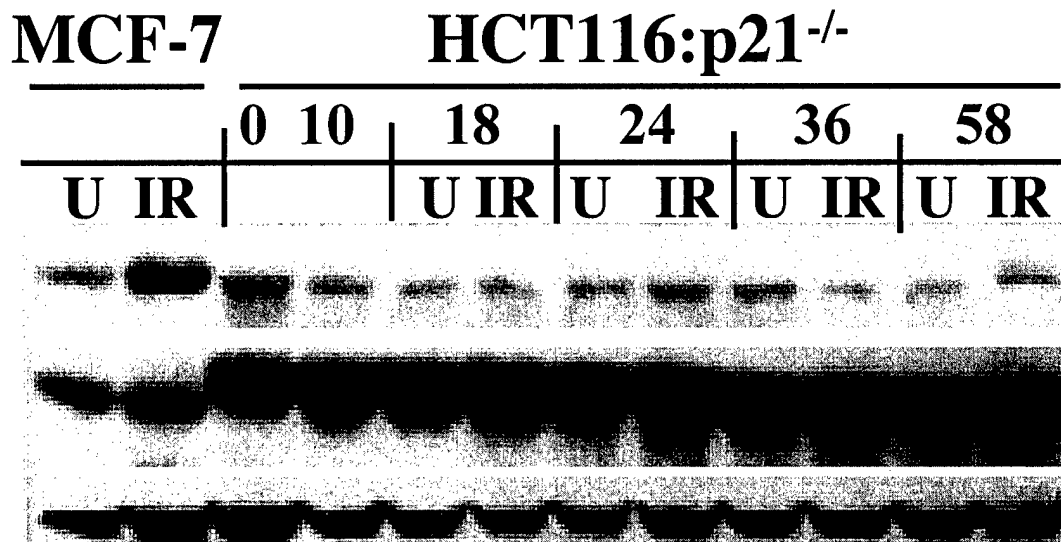
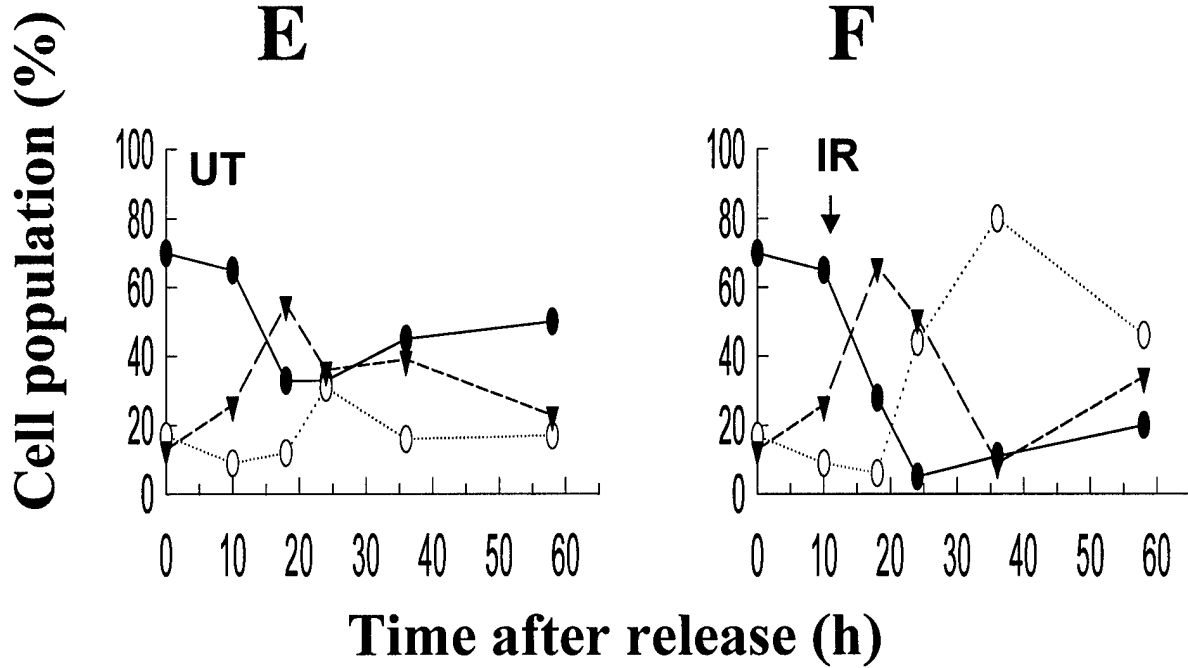


Fig. 5

HCT116:p21^{-/-}



Induction of Clusterin, a Pro-Survival Factor, Requires Delayed Activation of the IGF-1R/MAPK Signal Cascade after IR

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Summary

Secretory clusterin (*sCLU*), an ionizing radiation (IR)-induced gene, is a pro-survival protein implicated in aging, obesity, heart disease, as well as cancer biology and therapy. Induction of *sCLU* is delayed, reaching maximal levels 72 hrs after low doses of IR. We show that delayed induction is dependent on the up-regulation of the insulin-like growth factor-1 receptor and its ligand (IGF-1R and IGF-1, respectively), and stimulation of the downstream Src-Raf-Mek-Erk signaling cascade. This activates the early growth response-1 (Egr-1) transcription factor required for *sCLU* induction. Our data suggest that the IGF-1R-Src-Mek-Erk-Egr-1-*sCLU* signal transduction cascade is a general pro-survival pathway important for radiation resistance in cancer therapy, as well as implications for aging, diabetes, and heart disease.

Running Title: IGF-1R and MAPK Involvement in IR-Induced *sCLU*

Introduction

Ionizing radiation (IR) is a common therapy for many types of cancers, and elucidation of refractory responses to clinically relevant doses of IR are under intense investigation. Understanding these cellular processes, especially the contribution of signal transduction pathways that precede gene expression after IR, will lead to interventions that bring about a more favorable clinical outcome. Although it was once thought that the only important cellular responses to IR originated from DNA damage, it is clear that IR creates many different lesions in macromolecular targets within the cell that set in motion cascades of responses in both irradiated, as well as in neighboring non-irradiated cells. For example, the epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF-1R) and tumor necrosis factor- β (TNF- β) receptor can be activated after IR resulting in intracellular signaling cascades that lead to gene expression that mediate resistance to IR-induced cell death (Dent et al., 2003). IR also stimulates production of ligands for these receptors (e.g., EGF, TGF- β , IGF-1, and TNF- β) that, in turn, activate downstream signaling (Dent et al., 1999; Grana et al., 2003; Kim et al., 2003). Since these signaling cascades can enhance survival of cancer cells, their stimulation can limit radiotherapy efficacy in the treatment of various human malignancies that over-express specific receptors. A better understanding of these signal transduction mechanisms is needed to improve radiotherapy.

Clusterin is a highly glycosylated secretory protein (i.e., sCLU) that provides cytoprotection after various cell stresses, possibly due to its role as a molecular chaperone (Bartl et al., 2001; Wilson and Easterbrook-Smith, 2000). CLU plays a direct role as a cellular stress sensor, since a variety of cytotoxic agents (e.g., topotecan, nocodazole,

taxol and thapsigargin) induce *CLU* gene and protein expression (Criswell et al., 2003a) at doses far below their cytotoxic effects.

CLU has been implicated in many pathological states, including Alzheimer disease (DeMattos et al., 2001; Harr et al., 1996), atherosclerosis (Ishikawa et al., 1998; Jordan-Starck et al., 1994), prion diseases (Chiesa et al., 1996), rheumatoid arthritis (Newkirk et al., 1999), glomerulonephritis (Ghiggeri et al., 2002) and cancer (Bettuzzi, 2003; Chen et al., 2003; Hough et al., 2001; Saffer et al., 2002; Xie et al., 2002). Over-expression of endogenous s*CLU* correlates with higher tumor grade and poor prognosis in prostate and breast cancer (Bettuzzi, 2003; Redondo et al., 2000; Steinberg et al., 1997; Xie et al., 2002). Furthermore, over-expression of exogenous s*CLU* results in resistance to paclitaxel (Miyake et al., 2000a; Miyake et al., 2000b), doxorubicin (Cervellera et al., 2000), cisplatin (Chung et al., 2004) and radiation therapy (Zellweger et al., 2002), while antisense *CLU* expression enhances the chemosensitivity of various cell lines (Gleave et al., 2001; Zellweger et al., 2001). Small interfering RNA (siRNA) to *CLU* was used to show a cytoprotective role for this protein after genotoxic and oxidative stress (Trogakos et al., 2004), suggesting that s*CLU* provides a survival advantage in cancer cells.

We identified *CLU* as an IR-induced protein/transcript (Boothman et al., 1993). s*CLU* was induced by ≥ 2 cGy in various breast and colon cancer cells and peak *CLU* promoter activity, transcript, and protein levels occurred 24-72 h after IR (Criswell et al., 2003a). Although several transcription factors were implicated in s*CLU* induction after various stresses, including c-fos (Jin and Howe, 1999), c-myb (Cervellera et al., 2000),

and TGF- β (Jin and Howe, 1997; Jin and Howe, 1999; Wegrowski et al., 1999), the signaling pathways involved in its induction after IR have not been elucidated.

Using siRNA, we demonstrate a role for *sCLU* in cell survival after IR. Furthermore, the activation of the Src/Raf/Mek/Erk signaling cascade, by clinically relevant doses of IR, in MCF-7 breast cancer cells is required for *sCLU* induction. MAPK signaling was detected in cells minutes after IR as reported (Dent et al., 1999; Dent et al., 2003; Schmidt-Ullrich et al., 1997). We discovered a dramatic re-activation of MAPK signaling, 24-72 h after IR that was required for *CLU* promoter activation, and elevated *sCLU* protein levels. A role for the insulin-like growth factor-1 receptor and its ligand (IGF-1R/IGF-1) in *sCLU* induction after IR was elucidated. Since *sCLU* is a cytoprotective protein, these data act to define radio-resistant tumor cell phenotypes that over-express the IGF-1 receptor (Dent et al., 2003), and offer specific mechanisms for therapeutic intervention.

Results

siRNA to *sCLU* in MCF-7 Cells Causes Radio-sensitization

Induction of *sCLU* by various cancer cells in response to chemotherapeutic agents provides cytoprotection (Cervellera et al., 2000; Gleave et al., 2001; Zellweger et al., 2001). To determine if *sCLU* provides a similar protective role after IR, siRNA oligomers specific to exon II in *sCLU* mRNA was transiently transfected into MCF-7 cells. *sCLU*-siRNA transfected MCF-7 cells demonstrated a significant increase in clonogenic lethality with increasing IR doses compared to mock-transfected cells (Fig. 1A). Transfection with scrambled siRNA oligomers (scr-siRNA) did not alter the survival of irradiated MCF-7 cells (Fig. 1A), or *sCLU* protein levels (Fig. 1B). *sCLU* protein levels were decreased 70% in MCF-7 cells using siRNA specific to *sCLU* (Fig. 1B), whereas little change was observed in nuclear *CLU* (n*CLU*) protein levels; n*CLU* lacks exon II (Leskov et al., 2003). Thus, decreased *sCLU* levels significantly decreased the survival responses of MCF-7 cells.

Activation of MAPK by IR

Since *sCLU* is expressed late after IR, with induction occurring 48-72 h after exposure (Criswell et al., 2003a), it was not understood how early signaling events could result in the late activation of this gene. We, therefore, examined the temporal activation of Src, Raf, Mek and Erk in MCF-7 cells after 5 Gy (Fig. 2). Increases in P-Src (Tyr⁴¹⁶), P-Raf (Ser³³⁸), P-Mek-1/2 (Ser^{218/222}) and P-Erk-1/2 (Tyr²⁰⁴) protein levels were detected after IR at 0.25 h, 0.5 h, 0.5-1.0 h, and 0.25-2 h, respectively. Thus, as previously reported after high doses of IR (Schmidt-Ullrich et al., 1997), the Src/Raf/Mek cascade was induced

immediately after IR exposure. However, sCLU induction at these early times was not observed. Interestingly, a dramatic re-activation of the Src/Raf/Mek/Erk1/2 pathway was noted 24-72 h after IR correlating well with sCLU induction (Fig. 2). These data suggested that a delayed functional reactivation of the MAPK cascade correlated with sCLU protein induction in irradiated MCF-7 cells.

EGFR is Not an Upstream Activator of Clusterin

Prior work demonstrated that IR can activate the epidermal growth factor receptor (EGFR), resulting in activation of the Raf-Mek-Erk MAPK cascade (Dent et al., 1999). We, therefore, examined the role of EGFR in mediating CLU expression after IR, using MCF-7 cells transiently transfected with 4250 bp of the *CLU* promoter linked to a firefly reporter gene. Increasing doses of AG1478, a selective EGFR inhibitor, did not affect IR-induction of the *CLU* promoter in serum-starved MCF-7 cells monitored 48 h post-IR (Fig. 3A). Stimulation of these cells with increasing amounts of EGF had no effect on *CLU* promoter activity suggesting that EGFR does not play a role in CLU induction.

These results were confirmed by western blot analyses (Fig. 3B), where sCLU was not induced by 1 ng/ml EGF, with or without 1 μ M AG1478 (Lane 4). Since AG1478 attenuated EGFR activation (Fig. 3C) by IR, as monitored by phosphorylation of Y1068 of EGFR, but did not alter IR induction of sCLU, we conclude that EGFR does not play a role in the induction of sCLU after IR.

Inhibition of Insulin-Like Growth Factor-1 Receptor Abrogates Clusterin Induction after IR

Stimulated IGF-1R is a potent activator of MAPK (Burgaud and Baserga, 1996), however, involvement of IGF-1R after IR has not been explored. The involvement of insulin-like growth factor receptor 1 (IGF-1R) in IR-mediated CLU induction was then examined. Serum-starved MCF-7 cells transiently transfected with 4250 bp of the *CLU* promoter fused to a firefly luciferase reporter were treated with AG1024, a selective inhibitor of IGF-1R. Exposure of cells with AG1024 significantly reduced *CLU* promoter activity compared to DMSO controls (Fig. 4A). Furthermore, increasing doses of IGF directly induced *CLU* promoter activity, which was blocked by pretreatment with 2.5 μ M AG1024 (Fig. 4B). IR-inducible and IGF-inducible *CLU* promoter activity were also abrogated by addition of insulin-like growth factor binding protein-3 (IGFBP-3), an endogenous inhibitor of IGF (Fig. 4C). These results were confirmed at the protein level by western blot analyses (Fig. 4D). Furthermore, we noted that phosphorylation of IGF-1R increased dramatically 48 h after IR, and IGF-1R levels were slightly induced in irradiated MCF-7 cells (Fig. 4E). Treatment of cells with AG1024 and 5 Gy dramatically decreased IGF-1R levels (Lane 3). Addition of AG1024 also lowered basal levels of IGF-1R in unirradiated, serum starved cells, suggesting a possible autocrine feedback loop induced by IR, where irradiated cells up-regulate IGF-1R. AG1024 also repressed sCLU levels in PC-3 prostate cancer cells that constitutively express IGF-1R (S1). No induction of sCLU after exposure to 5 Gy or 10 ng/ml IGF was seen at earlier time points (0-24 h) (S2). It is of interest to note that in MCF-7 cells that stably express 1403 bp of

the *CLU* promoter linked to a firefly reporter (MCF-7 1403 cells), serum-starvation alone was enough to raise basal *CLU* promoter activity and the addition of 1 μ M AG1024 was able to lower this basal level to background (S3).

Since IGF-1R levels were elevated and phosphorylated in MCF-7 cells after IR, we investigated whether IGF-1 production was also induced after 5 Gy. Media collected from serum-starved MCF-7 cells at 0, 24, 48 and 72 h post-IR showed elevated (~20%) levels of IGF-1 24 h after IR, and sustained increases were noted 72 h post-IR (Fig. 4F). Thus, MCF-7 cells have high basal levels of IGF-1 as reported (Freed and Herington, 1989), with increased levels of both IGF-1R and IGF noted 24-72 h post-IR, forming a putative autocrine feedback loop.

c-Src is an Upstream Activator of sCLU Induction After IR

It is known that c-Src, but not other Src family members, can activate and be activated by IGF-1R (Boney et al., 2001; Gruden et al., 2003; Lebrun et al., 1998; Sekharam et al., 2003). Accordingly, PP1, a selective inhibitor of c-Src, abrogated *CLU* promoter induction after low doses of IR and partially blocking *CLU* induction (by 50%) after higher doses of IR (Fig. 5A). As previously noted (Criswell et al., 2003a), sCLU was induced by 0.25-5 Gy in DMSO-treated MCF-7 cells, and this induction was blocked by 20 μ M PP1 (Fig. 5B).

We then determined the lowest dose of PP1, given as a continuous 72 h treatment, that could inhibit *CLU* promoter activity after 5 Gy. PP1 (5 μ M) significantly decreased the basal activity of the *CLU* promoter and effectively blocked IR-activated *CLU* promoter activity (Fig. 5C). IR-inducible sCLU protein levels were also decreased (Fig.

5D). Phosphorylation of Fak, a known c-Src substrate (Y925), decreased with increasing doses of PP1 (Fig. 5E). Thus, PP1 effectively blocked IR-inducible c-Src activity at doses that also prevented sCLU induction.

To confirm a role for c-Src as an upstream regulator of sCLU induction after IR, we transiently over-expressed increasing amounts of a constitutively active Src plasmid (Y529F) (Src CA) or a kinase dead Src plasmid (K297R) (Src KD) in MCF-7 1403 cells. Increasing levels of Src CA stimulated basal and IR-inducible *CLU* promoter activities (lanes 1-6, Fig. 5F). In contrast, increasing amounts of Src KD repressed IR-induced *CLU* promoter activity. These data, using PP1 and over-expression of Src CA or Src KD, illustrated a role for c-Src in sCLU induction in MCF-7 cells after IR.

Activation of the MAPK cascade is required for sCLU Induction After IR

c-Src phosphorylates the downstream signaling kinase, Raf, that in turn activates Mek-1 through phosphorylation (Kyriakis et al., 1992). U0126 (1 μ M), a selective Mek-1 kinase inhibitor, completely abrogated IR-inducible *CLU* promoter activity (Fig. 6A) and protein (Fig. 6B) in MCF-7 cells.

The Erk 1/2 kinases are downstream substrates for Mek-1 (Dent et al., 1999). To demonstrate involvement of Erks in *CLU* gene induction, we over-expressed dominant-negative Erk-1 (dn Erk-1) or dominant-negative Erk-2 (dn Erk-2) in 1403 MCF-7 cells. Both dn Erk-1 and dn Erk-2 completely suppressed *CLU* promoter activity after 5 Gy (Fig. 6C).

Log-phase MCF-7 cells were co-transfected with the 4250 bp *CLU* promoter-luciferase reporter and Src CA, Src KD, dominant-negative Mek-1 (dn Mek-1 K97A) or

dominant-negative Erk-1 (dn Erk-1) (Fig. 6D). In response to 5 Gy, *CLU* promoter activity was induced ~4-fold in MCF-7 cells. Over-expression of Src CA resulted in significant elevation (4-fold) of *CLU* promoter basal levels compared to control. After IR, a further increase in *CLU* promoter activity was noted in Src CA transfected MCF-7 cells. Over-expression of Src KD, dn Mek-1 and dn Erk-1 abrogated IR-induction of the *CLU* promoter. Western blot analyses confirmed over-expression of the corresponding proteins from the plasmids used above (Fig. 6E). Collectively, these data demonstrate a role for MAPK signaling in *sCLU* induction in MCF-7 cells after IR.

The Egr-1 Transcription Factor is Required for *CLU* Promoter Activation After IR

Several transcription factors are activated by the IGF-1R/MAPK signaling cascade, but only a few are known to be stimulated after IR (Criswell et al., 2003b). The *CLU* promoter contains three potential Egr-1 binding sites. Egr-1 is a known downstream target of the MAPK cascade (Lo et al., 2001), and can be activated by high doses of IR in lymphoid tumor cells (Meyer et al., 2002). Over-expression of Egr-1 cDNA increased *CLU* promoter activity ~2-fold over basal levels, and an ~3-fold increase was noted in MCF-7 cells after IR (5 Gy) (Fig. 7A). Western blot analyses confirmed the over-expression of the Egr-1 protein in these cells (Fig. 7B). These data suggested that the Egr-1 protein is an important transcription factor that mediates induction of *CLU* gene expression after IR.

DNA pull-down assays were then performed to determine if Egr-1 directly binds the 1403 *CLU* promoter and if IR exposure of MCF-7 cells enhanced Egr-1 binding. Egr-1 DNA binding activity was minimal in control mock-irradiated MCF-7 cells. An

increase in Egr-1 binding was noted beginning at 4 h after IR. More robust increases in Egr-1 DNA binding were noted at 24 h, with sustained levels at 72 h post-IR. In contrast, IR-treated MCF-7 cells exposed to either 5 μ M PP1 or 5 μ M U0126 showed no increase in Egr-1 DNA binding to the *CLU* promoter DNA after IR. Nuclear extracts (10% input) were separated by SDS-PAGE and probed for PCNA as a loading control (Fig. 7C).

Since we were able to show that the MAPK pathway was involved in sCLU induction, we examined whether IGF-1R or EGFR inhibition could block IR-inducible Egr-1 binding to the *CLU* promoter. Exposure of IR-exposed MCF-7 cells with 1 μ M AG1478 did not affect Egr-1 DNA binding activity to the *CLU* promoter (Fig. 7D). In contrast and consistent with our observations of IGF-1R mediating sCLU induction after IR, Egr-1 DNA binding activity was blocked with 1 μ M AG1024. Egr-1 specific binding to the *CLU* promoter was demonstrated by competition assays using non-biotin labeled *CLU* promoter DNA (Fig. 7E).

A role for Egr-1 in sCLU induction was confirmed using Egr-1-specific siRNA (siRNA-Egr-1). IR exposure of mock-transfected or 1403 MCF-7 cells transfected with scrambled siRNA (scr-siRNA) resulted in an ~6-fold induction of the 1403 *CLU* promoter (Fig. 7F). In contrast, *CLU* promoter activity was not stimulated after 5 Gy in 1403 MCF-7 cells transfected with 20 nM Egr-1 siRNA. The efficacy of knockdown was confirmed by dramatic decreases in Egr-1 protein levels only after transfection with *Egr-1* siRNA (Fig. 7G). Thus, decreased Egr-1 protein levels abrogated induction of *CLU* promoter activity at 72 h in MCF-7 1403 cells after IR.

Discussion

In response to IR, we have demonstrated that the delayed induction of sCLU, which plays a role in survival after IR (Fig. 1), requires activation of IGF-1R and the MAPK cascade. IR-induced sCLU was dependent on the novel reactivation of the Src/Raf/Mek/Erk signaling cascade 24-72 h after IR exposure. This signal transduction pathway culminates in the activation of the Egr-1 transcription factor (model, Fig. 7H).

Much attention has been given to EGFR inhibitors for cancer therapy. EGFR is over-expressed or constitutively activated in many types of tumors including colorectal, breast, pancreatic and ovarian cancers (Milas et al., 2004), and is a known mediator of radio-resistance in various tumor types, including glioblastoma multiforme and breast cancer cells through the activation of Erk-1/2 (Krishnan et al., 2003; Zhou et al., 2004). As a result, therapies to specifically target EGFR have been proposed, including use of monoclonal antibodies and small molecule inhibitors that target the kinase domain (Arteaga, 2003). Interestingly, the selective EGFR inhibitor, AG1478, did not block *CLU* promoter induction or regulate sCLU protein levels after IR (Fig. 3).

In contrast, IGF-1R is another membrane receptor that has been far less studied as a target for radiotherapy. IGF-1R activation resulted in mitogenic growth and cell survival (Ahmad et al., 2004) and treatment of cells with IGF-1 provided protection from doxorubicin and taxol induced apoptosis (Gooch et al., 1999). AG1024, a selective inhibitor of IGF-1R, blocked sCLU induction after IR (Fig. 4). Exposure to IR also activated IGF-1R (Fig. 4) and was required for delayed sCLU expression. These data appear to be consistent with a previous report that AG1024 treatment of MCF-7 cells

enhanced cell death after IR (Wen et al., 2001). The specific cytoprotective role of sCLU after IR (Fig. 1) strongly suggests that activation of IGF-1/IGF-1R signaling is a survival pathway is an important process to manipulate for improved radiotherapy.

MCF-7 cells produce and secrete IGF-1 (Freed and Herington, 1989), and IGF-1R is often over-expressed in breast cancer (Dricu et al., 1999). Peripheral lymph node stromal cells produce and secrete EGF and IGF-1 that can increase the growth and survival of breast cancer cells (LeBedis et al., 2002). Thus, EGF and IGF secretion by lymph nodes may be a factor in the tumorigenesis of neighboring breast tissue, especially for cells that have upregulated expression of EGFR or IGF-1R, through a paracrine mechanism. Consistent with a previous report (Ahmad et al., 2004), we demonstrated induction of IGF-1R after IR (Fig. 4D), as well as an increase in IGF-1R phosphorylation and a 20% increase in secretion of IGF-1, 24-72 h post-IR (Fig. 4E). Our data suggest a possible autocrine feedback loop induced by IR, where irradiated cells not only up-regulate IGF-1R, but also increase production of the ligand, IGF-1 (Fig. 4). Importantly, induction of IGF-1R and its ligand provide a plausible explanation for the late induction of CLU after IR.

We show, for the first time, that the Src-Raf-Mek-Erk-1/2 pathway is required for IR-induced sCLU activation. This cascade culminates in the transactivation of the Egr-1 transcription factor. As previously shown, IR does result in activation of MAPK, however, a novel re-activation of the MAPK cascade after IR that correlates with the temporal induction of sCLU. The physiological relevance of the biphasic activation of MAPK is unknown, and possible links between these pathways are being explored, but at least for sCLU the delayed pathway appears to be most important. The importance of

this delayed IR-stimulated pathway is highlighted by the fact that (Lu et al., 2001) IGF-1R production in MCF-7 cells can cause increased resistance to Herceptin (a monoclonal antibody to the Her2/neu receptor)-induced cell death. Our data strongly suggest a role for IGF-1R signaling in resistance to therapy. Thus, tumor cells that survive an initial phase of radiotherapy may develop resistance to EGFR inhibitors, as a result of delayed MAPK induction using signaling by IGF-1R and sCLU upregulation. These data suggest that current Herceptin therapies could be augmented by small molecule IGF-1R pathway inhibitors. These inhibitors would allow combinatorial therapies to overcome the IGF-1R survival pathway, leading to a down-regulation of sCLU, as well as possibly other downstream factors.

Our data also suggest that therapies specifically focusing on down-regulating sCLU levels should augment various cancer therapies. sCLU provides cytoprotection against doxorubicin, taxol and cisplatin in many cancer cells (Cervellera et al., 2000; Chung et al., 2004; Gleave et al., 2001; Zellweger et al., 2001), and we have shown that sCLU provided cytoprotection for IR-exposed MCF-7 cells (Fig. 1). sCLU expression is elevated in many tumor types, including prostate, colorectal, renal, and breast cancers (Chen et al., 2003; Redondo et al., 2000; Steinberg et al., 1997; Xie et al., 2002). IGF-1R and IGF-1 production are also elevated in many tumor types. In a recent review, CLU and insulin-like growth factor binding proteins (IGFBPs) were suggested as targets for antisense therapy against prostate cancer (Gleave et al., 2001), although a connection between IGF-1R signaling and sCLU induction was not examined. Our data strongly suggest that such a connection could be exploited for improved therapy.

IGF-1/IGF-1R/c-Src/MAPK signaling leading to expression of sCLU, a pro-survival protein, may have implications beyond cancer biology and therapy. Increased sCLU expression was noted in Alzheimer's patient tissues, after heart attacks, and during senescence in aging. Thus, the pro-survival, IGF-1/IGF-1R/c-Src/MAPK/Egr-1 signal transduction pathway may be involved in these processes to regulate sCLU expression needed for debris clearing and cell survival. The recent development of the rapidly aging, p44 knockout mice (Maier et al., 2004) and their recent reported increase in IGF-1/IGF-1R signaling may support this theory, since sCLU over-expression during senescence (Petropoulou et al., 2001) and after loss of p53 function (Criswell et al. 2003a) were reported.

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Experimental Procedures

Plasmids

Src CA, Src KD, dn Erk-1, dn Erk-2 and Egr-1 plasmids were cloned into the pcDNA3 mammalian expression plasmid. Dr. Jeff Milbrandt generously provided the Egr-1 expression plasmid. The dn Mek-1 plasmid was a kind gift from Dr. Jeff Holt at Vanderbilt University (Abbott and Holt, 1999). The 4250 CLU promoter was cloned into the pA₃luc plasmid (a gift from Dr. Richard G. Pestell, Georgetown University, Washington, DC) and co-expressed with RVS- β -galactosidase for standardization.

Cell Culture

MCF-7 breast and PC-3 prostate cancer cells were grown in RPMI 1640 with 5% FBS at 37°C in a humidified incubator with 5% CO₂-95% air atmosphere.

Transfections and Inhibitor Treatments

PP1 was obtained from BioMol (Plymouth Meeting, PA) and U0126 was obtained from Cell Signaling Technology (Beverly, MA). AG1478 and AG1024 were obtained from Calbiochem (La Jolla, CA). EGF and IGF were obtained from Sigma or Calbiochem, respectively. EGF/IGF treatments were performed on cells plated at 5×10^5 cells per 10 cm dish. Twenty-four hours later, normal growth media was removed and replaced with media containing no serum. Cells were then pretreated for 1 h with AG1478, AG1024 or DMSO at indicated doses and either mock-irradiated or exposed to 5 Gy as previously described (Criswell et al., 2003a). Cells were also treated with IGF or EGF in medium

containing no serum. One hour after IR or IGF/EGF treatments, medium containing 1% serum was added, and cells were maintained in medium with 1% serum until harvested at various times post-treatment. AG1478 and AG1024 were removed 24 h after IR or IGF/EGF treatments.

PP1 and U0126 treatments were performed on cells with similar density as noted above. Cells were pretreated for 1 h with either PP1, U0126 or DMSO and then mock-irradiated or exposed to 5 Gy as described (Boothman et al., 1989). In Figs. 5A and 5B, medium containing 20 μ M PP1 was removed 24 h after IR. In all other experiments continuous exposures of PP1 or U0126 were used, and new PP1 or U0126 in fresh media were added to cells every 24 h until harvest at 72 h unless otherwise indicated.

MCF-71403 cells were generated that stably express the -1403 to +1 region of the human *CLU* promoter linked to luciferase (Criswell et al., 2003a). Where applicable, MCF-7 cells were transiently transfected using Effectene (Qiagen; Valencia, CA) or Lipofectamine Plus (Invitrogen; Carlsbad, CA) as directed by the manufacturer.

Luciferase Reporter Assays

Luciferase reporter assays using a co-transfection with a -4250 to +1 bp region of the human *CLU* promoter with or without an RSV- β -gal constitutive reporter were performed with the Luciferase Assay System (Promega; Madison, WI) as described (Criswell et al., 2003a). MCF-7 cells co-transfected with the 4250 *CLU* promoter and Src CA, Src KD, dn Mek-1 or dn Erk-1/2 plasmids were mock- or IR-treated and assessed for *CLU* promoter-driven luciferase activity using the Dual Luciferase System (Promega; Madison, WI). MCF-7 cells were transfected 24 h before treatment with inhibitors and/or

5 Gy and harvested in 1X reporter lysis buffer at indicated times. β -galactosidase assays were used to control for transfection efficiency in all other experiments using the 4250 *CLU* promoter. All experiments were equalized for protein using Bradford Assays (Bio-Rad Laboratories; Hercules, CA). Each dose/time point was completed in triplicate and a Student's T-Test was performed to determine statistical significance.

Western Blot Analyses and Co-Immunoprecipitations

Whole cell extracts were extracted in RIPA buffer, proteins separated by 10% SDS-PAGE, and western blot analyses performed as described (Criswell et al., 2003a). Antibodies to human sCLU (B5), Ku70 (C-19), P-Raf-1 (Ser³³⁸), Raf-1 (C-12), P-Mek1/2 (Ser^{218/222}), Mek 1 (C-18), P-Erk (E-4), Erk 1 (K-23), P-Fak (Tyr⁹²⁵), Egr-1 (588) and Sp1 (PEP2) were obtained from Santa Cruz (Santa Cruz, CA). IGF-1 receptor β , P-EGF receptor (Tyr¹⁰⁶⁸), EGF receptor, and c-Src (Tyr⁴¹⁶) antibodies were obtained from Cell Signaling Technology (Beverly, MA). β -Tubulin and c-Src (Tyr⁴¹⁶) antibodies were obtained from Calbiochem (La Jolla, CA).

EGFR was immunoprecipitated from 250 μ g total protein using 2 μ g antibody at 4°C overnight. Complexed lysates and antibodies were incubated with protein G/agarose beads at 4°C for 1 h. Complexes were washed three times with RIPA buffer, resuspended in SDS loading buffer, boiled for 5 mins and separated by 12% SDS-PAGE.

Enzyme-Linked Immunosorbent Assays (ELISAs)

ELISAs for IGF-1 were performed following manufacturer's directions (R&D Systems, Minneapolis, MN). Briefly, $\sim 1.0 \times 10^5$ MCF-7 cells in 6-well plates were grown in

medium with or without whole serum, and 24 h later cells were mock- or IR-treated with 5 Gy in 2 ml. At various times (0, 24, 48 and 72 h), one ml medium was removed and stored at -80°C until analysis. Samples (50 μl) were compared to an IGF-1 standard curve to determine concentrations. Experiments were performed in triplicate.

siRNA and Clonogenic Survival Assays

siRNAs against the sCLU mRNA leader peptide (sCLU-siRNA) and a scrambled sequence (scr-siRNA) were synthesized by Dharmacon, Inc. (Lafayette, CO):

sCLU-siRNA	5'- GCG UGC AAA GAC UCC AGA AdTdT-3'
	3'-dTdTTCGC ACG UUU CUG AGG UCU U-5'
scr-siRNA	5'-GCG CGC UUU GUA GGA UUC GdTdT-3'
	3'-dTdTTCGC GCG AAA CAU CCU AAG C-5'

sCLU-siRNA or scr-siRNA was transfected into MCF-7 cells (5 μg to 5×10^5 cells/60 mm dish) using Lipofectamine Plus (Invitrogen, Inc.; Carlsbad, CA) according to the manufacturer's instructions. Mock-transfected cells were used as a control. Two days after transfection, cells were trypsinized, plated onto 60 mm dishes (500 cells per dish) in triplicate and 24 h later mock- or IR-treated. Ten days later, cells were fixed and stained using crystal violet. Colonies containing >50 normal appearing cells were counted.

Egr-1 siRNA was generated by Dharmacon, Inc. (Lafayette, CO) as a Custom SMARTpool that contained four siRNA sequences against *Egr-1*. MCF-7 cells were transfected with 20 μM *Egr-1* siRNA, 20 μM scr-siRNA or mock-transfected using Oligofectamine as instructed (Invitrogen, Inc; Carlsbad, CA). Twenty-four hours after transfection, cells were exposed to 5 Gy or mock-irradiated. Cells were harvested in 1 X reporter lysis buffer for luciferase assays or RIPA buffer for western blot analyses.

DNA Pull-Down Assays

A biotinylated 1403 bp human *CLU* promoter was amplified from the 4250 pA₃luc plasmid using primers ordered from Integrated DNA Technologies (Coralville, IA):

5' GAT CCA TTC CCG ATT CCT 3'

5' /5Bio/ AGC CAA GCT TCC TGT GCC 3'.

Nuclear extracts were harvested from DMSO, PP1 or U0126 treated, mock or IR-exposed MCF-7 cells as described (Schreiber et al., 1989). The biotinylated *CLU* promoter (3 µg) was incubated with 10 µl streptavidin beads (Oncogene Research Products; Boston, MA) for 1 h at rm. temp. Complexes were washed in binding buffer (50 mM Tris-HCL pH 7.5, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 0.25 µg/µl poly (dI-dC)·poly (dI-dC) and 20% glycerol) and 100 µg nuclear extract added at rm temp. for 20 mins. Binding buffer (1 ml) was added and complexes incubated at 4°C overnight. Complexes were washed twice 24 h later in binding buffer, and associated proteins separated by 12% SDS-PAGE.

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Figure Legends:

Figure 1. siRNA knock-down of *sCLU* in MCF-7 cells increases radiation sensitivity.

(A) MCF-7 cells were transfected with scrambled or siRNA oligomers specific to the endoplasmic reticulum (ER) leader peptide to *sCLU* 24 h prior to IR treatment. Cells were then assessed for clonogenic survival. Experiments were performed in triplicate and standard deviations determined using the Student's t-test.

(B) Western blot analyses of *sCLU* and nuclear clusterin (nCLU), an alternative splice form of the gene, levels in MCF-7 cells harvested 60 h later. Relative *sCLU* levels were determined compared to mock-transfected control cells. Blots are representative of experiments repeated three times.

Figure 2. Activation of MAPK by IR.

Activation of the MAPK signal transduction pathway in irradiated (5 Gy) MCF-7 cells in whole cell extracts harvested at various times (h) using Western blot analyses. Relative protein and phospho-protein levels were determined as a ratio of total phospho-protein to total protein. Relative phosphorylation and protein levels were calculated from 0 h control levels. Actin was used as a loading control for protein levels. Blots are representative of experiments performed three or more times.

Figure 3. EGFR is not an upstream activator of *sCLU*.

(A) MCF-7 cells were transiently transfected with 4250 bp of the *CLU* and pRSV β -gal transfection efficiency control vector and treated with 0, 1 and 3 μ M of AG1024, either

alone (white bars), 5 Gy (gray bars) or EGF (black bars). An asterisk denotes significant statistical difference between irradiated samples and cells treated with AG1478 alone.

Experiments were performed independently three times and standard deviation determined by Student's t-test.

(B) MCF-7 cells were serum starved for 24 h. Cells were pretreated with 1 μ M AG1478 1 h prior to exposure to 5 Gy or treatment with 10 ng/ml EGF. Media containing 0.1% serum was added to the cells 1 h after exposure to 5 Gy or EGF. AG1478 was removed 24 h later and media with 0.1% serum and DMSO or EGF was added to the cells. Protein was harvested for western analyses 48 h after IR or EGF treatment. Relative levels were determined as treated samples compared to mock-treated control.

(C) MCF-7 cells were treated as described above (Fig. 3B). EGFR was immunoprecipitated from total protein lysates using an antibody directed to EGFR. Samples were separated by SDS-PAGE and probed for P-EGFR and total EGFR levels.

Figure 4. Inhibition of Insulin-Like Growth Factor-1 Receptor abrogates sCLU induction after IR

(A) MCF-7 cells were transiently transfected with 4250 bp of the *CLU* and pRSV β -gal transfection efficiency control vector. After transfection (24 h), cells were incubated in serum free media for 24 h and then pretreated for 1 h with either DMSO or increasing doses of AG1024 before exposure to 5 Gy. Protein was harvested 72 h after IR for *CLU* promoter luciferase assays. A single asterisk denotes significant statistical difference between irradiated samples and mock-irradiated controls. Experiments were performed independently three times and standard deviation determined by Student's t-test.

(B) MCF-7 cells were transiently transfected with 4250 bp of the *CLU* and pRSV β -gal transfection efficiency control vector. After transfection (24 h), cells were incubated in serum free media for 24 h and then pretreated for 1 h with either DMSO or 2.5 μ M AG1024 before treatment with increasing doses of IGF (0, 10, 50 ng/ml). AG1024 was removed 24 h after treatment and the cells were allowed to grow in serum free media supplemented with IGF for another 48 h. Protein was harvested 72 h after IGF treatment for *CLU* promoter luciferase assays. Single asterisks denote a significant statistical difference between control sample and samples treated with IGF. Double asterisks denote a significant statistical difference between sample treated with AG1024 and IGF (black bars) and samples treated with IGF alone (white bars). Experiments were performed independently three times and standard deviation determined by Student's t-test.

(C) MCF-7 cells were transiently transfected with 4250 bp of the *CLU* and pRSV β -gal transfection efficiency control vector. After transfection (24 h), cells were either exposed to 5 Gy or treated with increasing amounts of IGFBP3. Protein was harvested 72 h later for *CLU* promoter luciferase assays. Asterisk denotes significant statistical difference between the irradiated sample and mock-irradiated control. Experiments were performed independently three times and standard deviation determined by Student's t-test.

(D) MCF-7 cells were serum starved for 24 h. Cells were pretreated with 1 μ M AG1024 1 h prior to exposure to 5 Gy or treatment with 10 ng/ml IGF. Media containing 0.1% serum was added to the cells 1 h after exposure to 5 Gy or IGF. AG1024 was removed 24 h later and media with 0.1% serum and DMSO or IGF was added to the cells. Protein

was harvested for western analyses 48 h after IR or IGF treatment. Relative levels were determined as treated samples compared to mock-treated control.

(E) MCF-7 cells were set up and treated as described above in 'D'. Protein was harvested for western analyses 48 h after IR or IGF treatment. Relative levels were determined as treated samples compared to mock-treated control.

(F) MCF-7 cells were grown in media with no serum and 24 h later were either mock-irradiated or exposed to 5 Gy. The total volume of media on each well was maintained at 2 ml. Samples (50 μ l) were compared to an IGF-1 standard curve to determine concentrations. All samples were performed in triplicate.

Figure 5. c-Src is an upstream activator of sCLU after IR

(A) MCF-7 1403 cells were pretreated for 1 h with 20 μ M PP1 or vehicle alone (0.01% DMSO) and exposed to increasing IR doses. PP1 was removed 24 h after IR, cells were washed with PBS and fresh medium added. Protein was harvested 48 h later for CLU promoter luciferase activities. Experiments were performed independently three times and standard deviation determined by Student's t-test.

(B) MCF-7 cells were treated with PP1, with or without IR as described in 'A'. PP1 was removed 24 h after IR, cells were washed with PBS and fresh medium added. Protein was harvested 48 h later and whole cell lysates analyzed by Western blot analyses. Ku70 was used for loading, and X-fold induction determined as DMSO-irradiated or PP1-irradiated samples compared to DMSO-unirradiated control.

(C) MCF-7 1403 cells were pretreated with increasing doses (0 – 15 μ M) of PP1 1 h prior to IR (5 Gy). Media containing fresh PP1 was added to cells every 24 h and proteins

harvested 72 h after IR for *CLU* promoter-luciferase assays. Experiments were performed three times and standard deviation determined by Student's t-tests. Asterisk indicates a significant statistical difference between unirradiated PP1-treated versus DMSO-treated cells (white bars), or irradiated PP1-treated versus DMSO-treated cells (black bars).

(D) MCF-7 cells were pretreated for 1 h with increasing doses of PP1 and then mock-irradiated or exposed to 5 Gy. Media containing fresh PP1 was added to cells every 24 h. Protein was harvested 72 h after IR for western blot analyses. X-fold induction was determined as PP1-treated and irradiated samples compared to DMSO-treated unirradiated control.

(E) PP1 inhibits phosphorylation of Fak, a c-Src downstream substrate, at doses that abrogate s*CLU* protein induction after 5 Gy. MCF-7 cells were treated as in 'D', and X-fold induction determined as PP1-treated and irradiated samples compared to DMSO-treated control cells.

(F) MCF-7 cells transiently transfected with a plasmid expressing a constitutively active c-Src protein (Lanes 1-6) increases basal and IR-inducible *CLU* promoter activities. Cells transiently transfected with a kinase dead c-Src protein (Lanes 7-12) inhibits IR-inducible *CLU* promoter activity. Lanes 1, 4, 7 and 10 were transfected with vector only DNA. Lanes 2, 5, 8 and 11 were transfected with 0.2 μg of the appropriate DNA and lanes 3, 6, 9 and 12 were transfected with 0.3 μg of the appropriate DNA. All transfections contained a total of 3 μg DNA. Experiments were performed three times and standard deviation determined by Student's t-test. Asterisks in lanes 2 and 3 indicate a significant statistical difference in *CLU* promoter activity between unirradiated c-Src CA transfected versus unirradiated mock-transfected cells. Asterisks in lanes 5 and 6 indicate a

significant statistical difference of *CLU* promoter activity in irradiated c-Src CA transfected versus irradiated mock-transfected cells. The asterisk in lane 12 indicates a significant statistical difference of *CLU* promoter activity in irradiated c-Src KD transfected versus irradiated mock-transfected cells.

Figure 6. Activation of the MAPK cascade is required for s*CLU* induction after IR

(A) MCF-7 1403 cells were pretreated with increasing doses of U0126, 1 h prior to exposure to 5 Gy. New media containing fresh U0126 was added to cells every 24 h. Protein was harvested 72 h after IR for luciferase assays. Experiments were performed three times and standard deviation determined by Student's t-test.

(B) MCF-7 cells were pretreated for 1 h with increasing doses of U0126 and mock-irradiated or exposed to 5 Gy. Medium containing fresh U0126 was added every 24 h. Protein was harvested 72 h after IR for western blot analyses, and X-fold induction determined as irradiated compared to DMSO-treated unirradiated control cells.

(C) MCF-7 cells transiently expressing a dominant-negative Erk-1 (dn Erk-1), a dominant-negative Erk-2 (dn Erk-2) or both were exposed to 5 Gy. Protein was harvested 72 h after IR for *CLU* promoter-luciferase assays. Experiments were performed three times and standard deviation determined by Student's t-test. Asterisks indicate significant statistical difference between transfected irradiated versus mock-transfected irradiated cells.

(D) MCF-7 cells were transiently co-transfected with 4250 bp of the *CLU* promoter in the pA₃luc vector with either vector only (VO), constitutively active Src (Src CA), kinase dead c-Src (Src KD), dn Mek-1, or dn Erk-1 24 h prior to exposure to 5 Gy. Protein was

harvested 72 h after IR for *CLU* promoter-luciferase assays. Relative *CLU* promoter activities were calculated using RSV TK- β -gal reporter levels. Experiments were performed three times and standard deviations determined by Student's t-tests. Asterisks indicate a significant statistical difference between transfected versus vector only (VO) transfected cells.

(E) Western blots confirmed over-expression of c-Src, Mek-1 or Erk-1 in MCF-7 cells. Dominant-negative Erk-1 over-expression was confirmed using an antibody directed to its HA-tag.

Figure 7. The Egr-1 transcription factor can bind to the *CLU* promoter and is required for promoter induction after IR

(A) MCF-7 cells were transiently co-transfected with the 4250 *CLU* promoter-luciferase, vector only (VO), or the Egr-1 cDNA. Transfected cells were exposed to 5 Gy and protein harvested 72 h later. Luciferase assays were used to monitor *CLU* promoter activities. Experiments were performed three times and standard deviations determined using Student's t-tests.

(B) Western blot confirming Egr-1 over-expression in MCF-7 cells.

(C) MCF-7 cells were pretreated for 1 h with DMSO (lanes 1-6), 5 μ M PP1 (lanes 7-12) or 5 μ M U0126 (lanes 13-18) before exposure to 5 Gy. Nuclear extracts were harvested at indicated time points for DNA pull-down analyses. Relative levels were determined as irradiated compared to control (0 h time point) samples. PCNA was used as a loading control and experiments were performed at least three times.

(D) MCF-7 cells were pretreated with DMSO (lanes 1-2), 1 μ M AG1024 (lanes 3-4) or 1 μ M AG1478 (lanes 5-6) for 1 h before exposure to 5 Gy. Nuclear extracts were harvested 72 h after IR for DNA pull-down analyses. Relative levels were determined as irradiated samples compared to unirradiated controls (UT). PCNA was used as a loading control.

(E) Egr-1 binding to the biotin-labeled 1403 *CLU* promoter was competed away by increasing amounts (0.0, 0.1 or 5.0 μ g) of non-biotin labeled 1403 *CLU* promoter.

(F) MCF-7 1403 cells were transfected with siRNA oligomers specific to Egr-1 or to a scrambled siRNA sequence and exposed to 5 Gy. Protein was harvested 72 h later for *CLU* promoter-luciferase assays. Experiments were performed three times and standard deviation determined by Student's t-test.

(G) Transfection with siRNA specific to Egr-1 diminished transcription factor protein levels in MCF-7 cells after. Relative protein levels were determined as Egr-1 protein levels in siRNA- compared to mock-transfected samples. Blots are representative of experiments performed three times.

(H) Model depicting delayed IR activation of the IGF-1R and c-Src/Raf/Mek/Erk cascade, culminating in activation of Egr-1 and s*CLU* gene and protein induction.

Supplemental Figure Legends:

Figure S1: AG1024 blocks sCLU induction after IR in PC-3 prostate cancer cells.

PC-3 cells were serum starved for 24 h. Cells were pretreated with 1 μ M AG1024 1 h prior to exposure to 5 Gy or treatment with 10 ng/ml IGF. Media containing 0.1% serum was added to the cells 1 h after exposure to 5 Gy or IGF. AG1024 was removed 24 h later and media with 0.1% serum and DMSO or IGF was added to the cells. Protein was harvested for western analyses 72 h after IR or IGF treatment. Relative levels were determined as treated samples compared to mock-treated control.

Figure S2: sCLU is induced 24-72 h after IR and IGF treatment, and is not blocked by pretreatment with AG1478.

MCF-7 cells were serum starved for 24 h. Cells were pretreated with 1 μ M AG1478 1 h prior to exposure to 5 Gy, or treated with DMSO for other samples (UT, IGF, EGF or IR alone). Media containing 0.1% serum was added to the cells 1 h after exposure to 5 Gy or IGF. AG1024 was removed 24 h later and media with 0.1% serum and DMSO or IGF was added to the cells. Protein was harvested for western analyses at indicated (0, 4, 8, 24, 48, 72 h) time points after treatment. Relative levels were determined as treated samples compared to mock-treated control.

Figure S3: MCF-7 1403 cells have high basal *CLU* promoter activity due to serum starvation.

(A) Increasing doses of AG1478 are not able to abrogate *CLU* promoter activity after IR. MCF-7 1403 cells were serum starved for 24 h and then pretreated with increasing doses

of AG1478 1 h prior to exposure to 5 Gy. Protein was harvested for luciferase assays 48 h after IR.

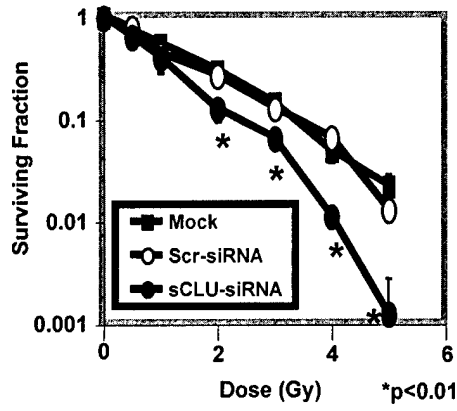
(B) Increasing amounts of EGF, with and without AG1478, have no effect on *CLU* promoter activity. MCF-7 1403 cells were serum starved for 24 h and then pretreated with 2.5 μ M AG1478 1 h prior to treatment with increasing amounts of EGF. Protein was harvested for luciferase assays 48 h after IR.

(C) Increasing doses of AG1024 are able to abrogate high basal *CLU* promoter activity. MCF-7 1403 cells were serum starved for 24 h and then pretreated with increasing doses of AG1024 1 h prior to exposure to 5 Gy. Protein was harvested for luciferase assays 48 h after IR.

(D) IGF cannot induce *CLU* promoter activity in MCF-7 1403 cells due to high basal levels, but treatment with AG1024 is able to abrogate promoter activity. MCF-7 1403 cells were serum starved for 24 h and then pretreated with 2.5 μ M AG1024 1 h prior to treatment with increasing amounts of IGF. Protein was harvested for luciferase assays 48 h after IR.

Figure 1

A



B

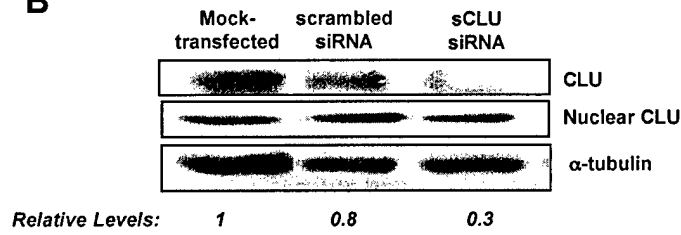


Figure 2

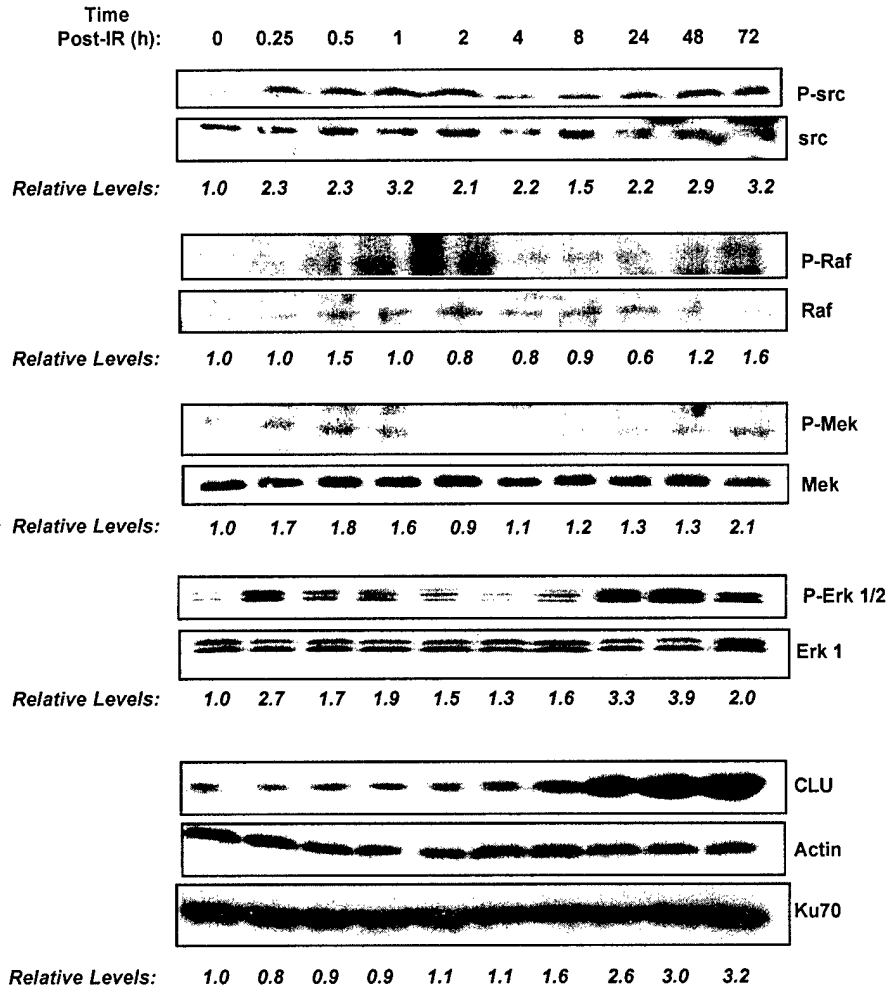
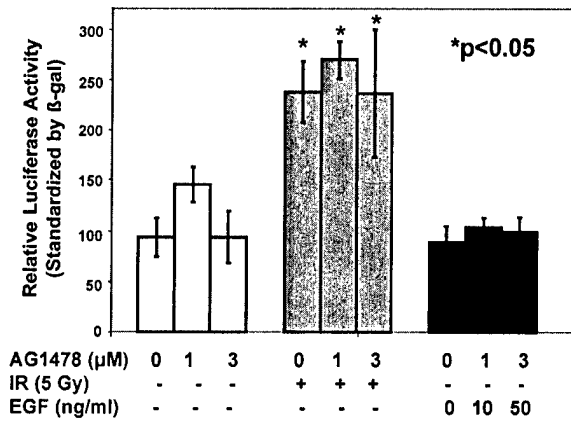
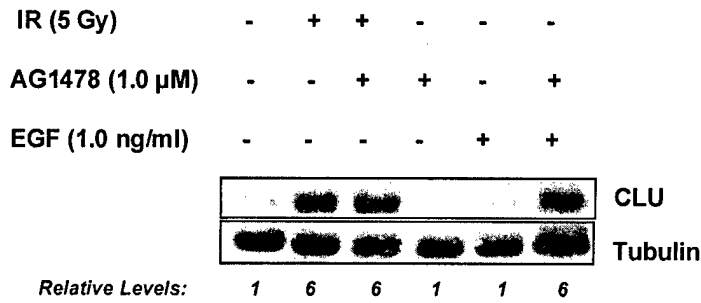


Figure 3

A



B



C

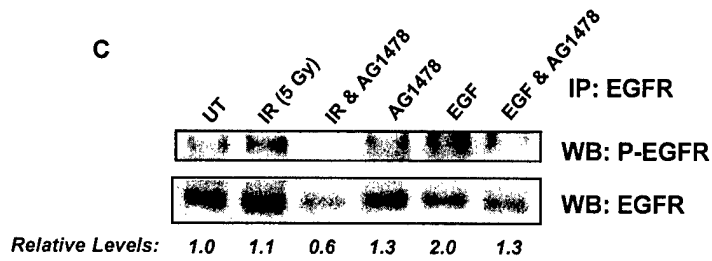


Figure 4

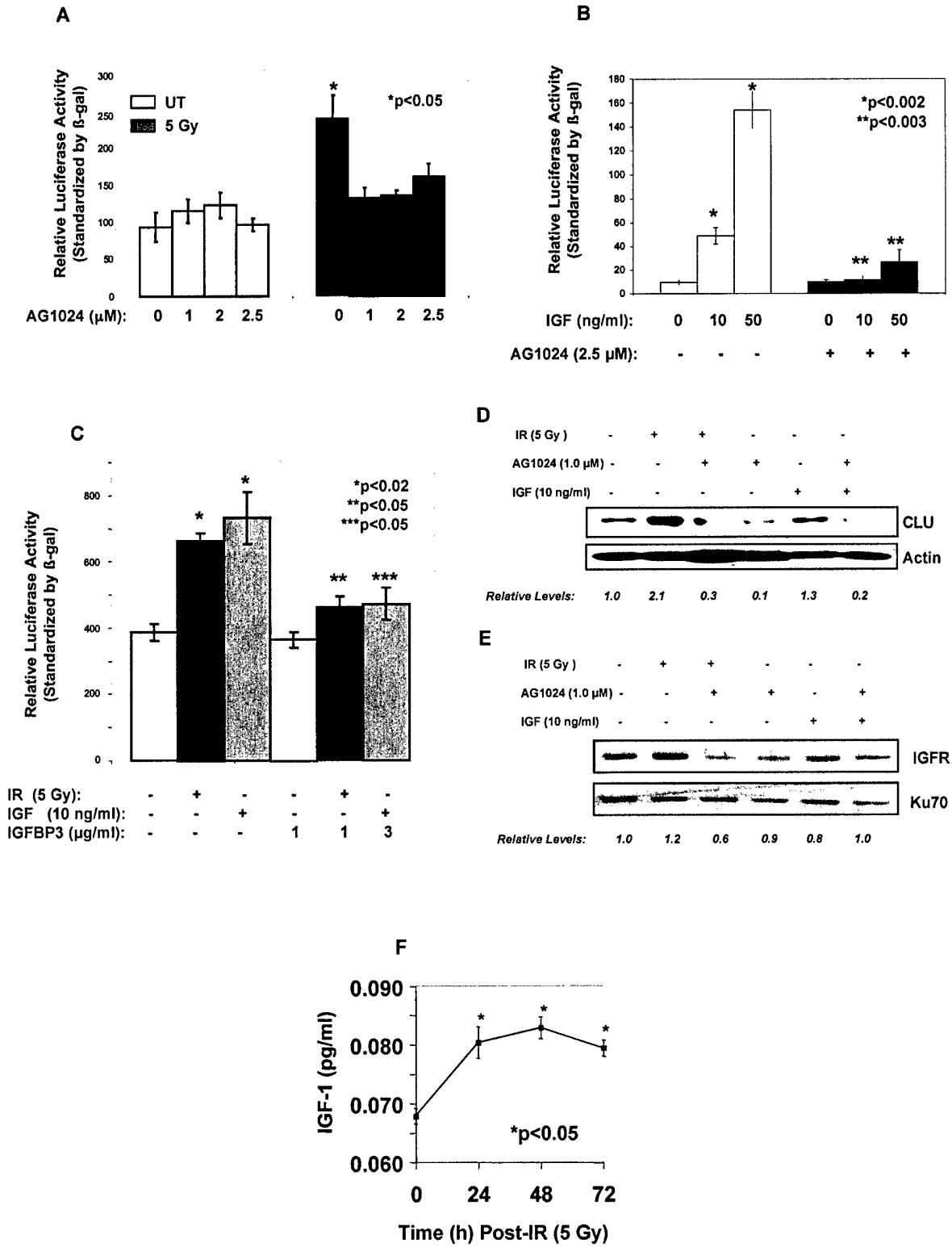


Figure 5

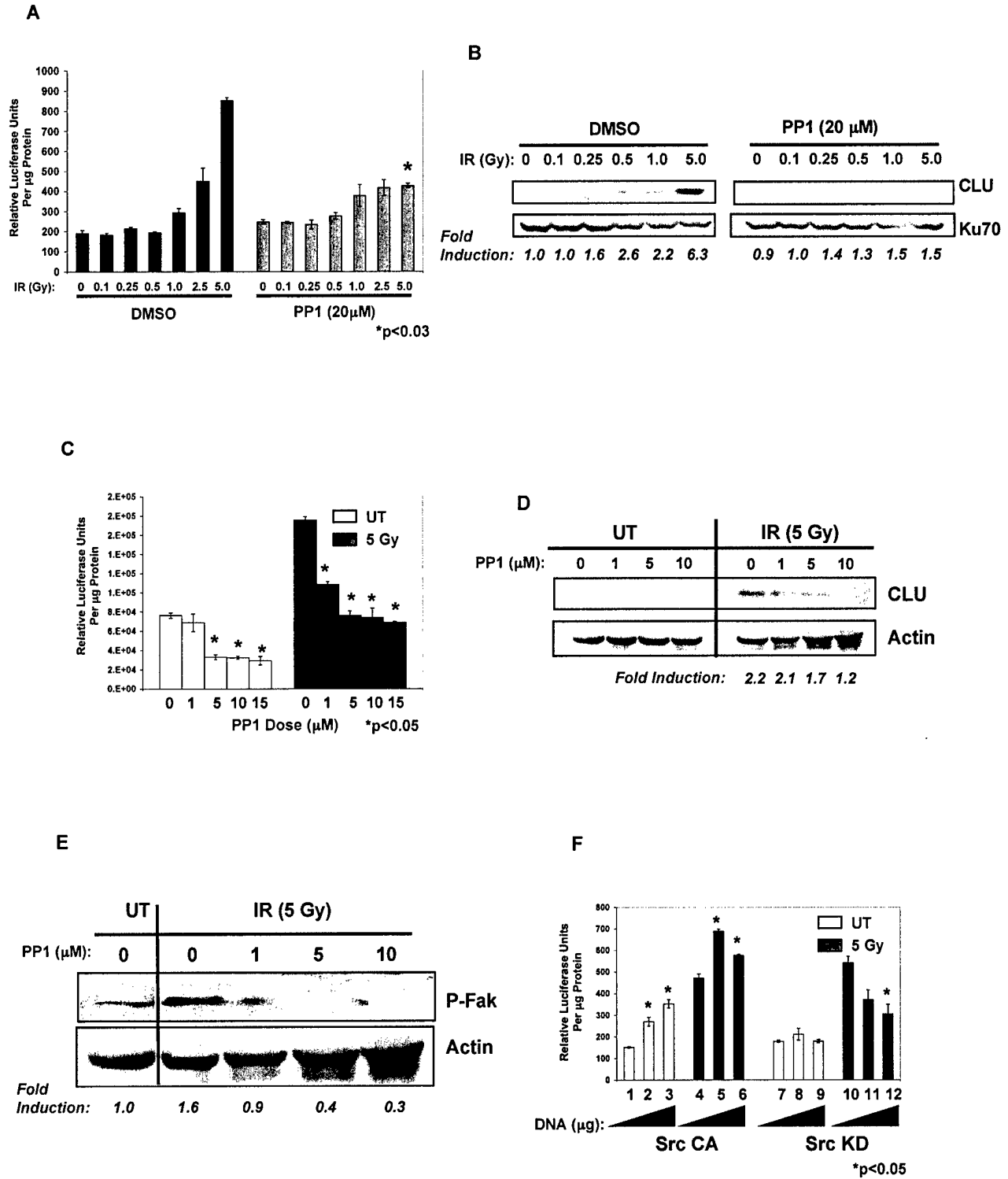
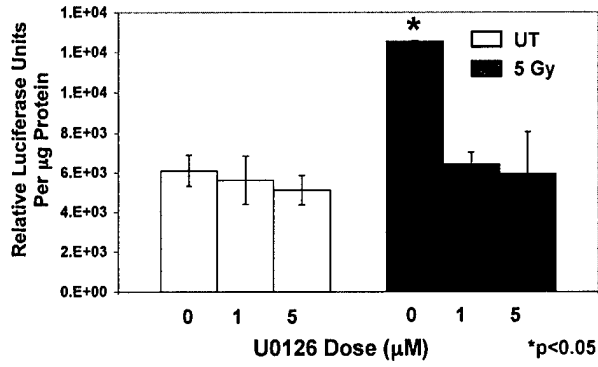
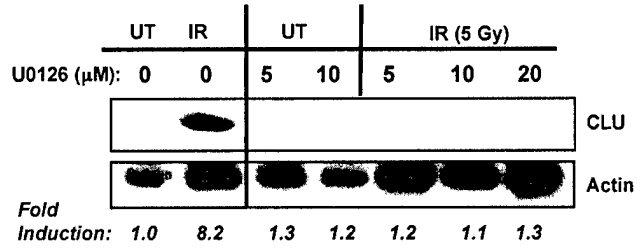


Figure 6

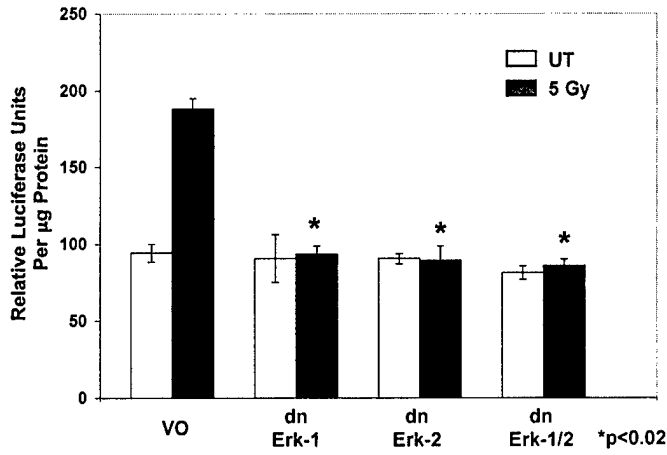
A



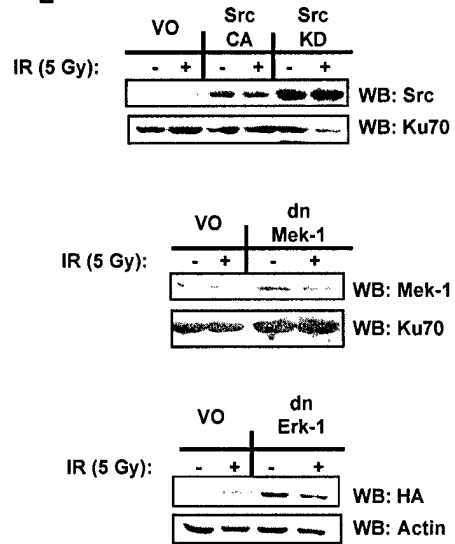
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E



D

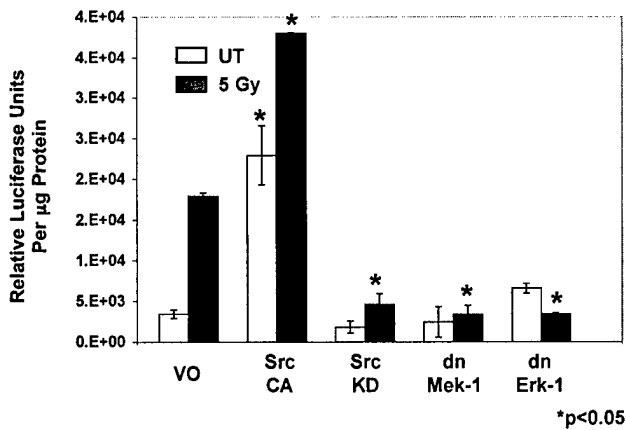
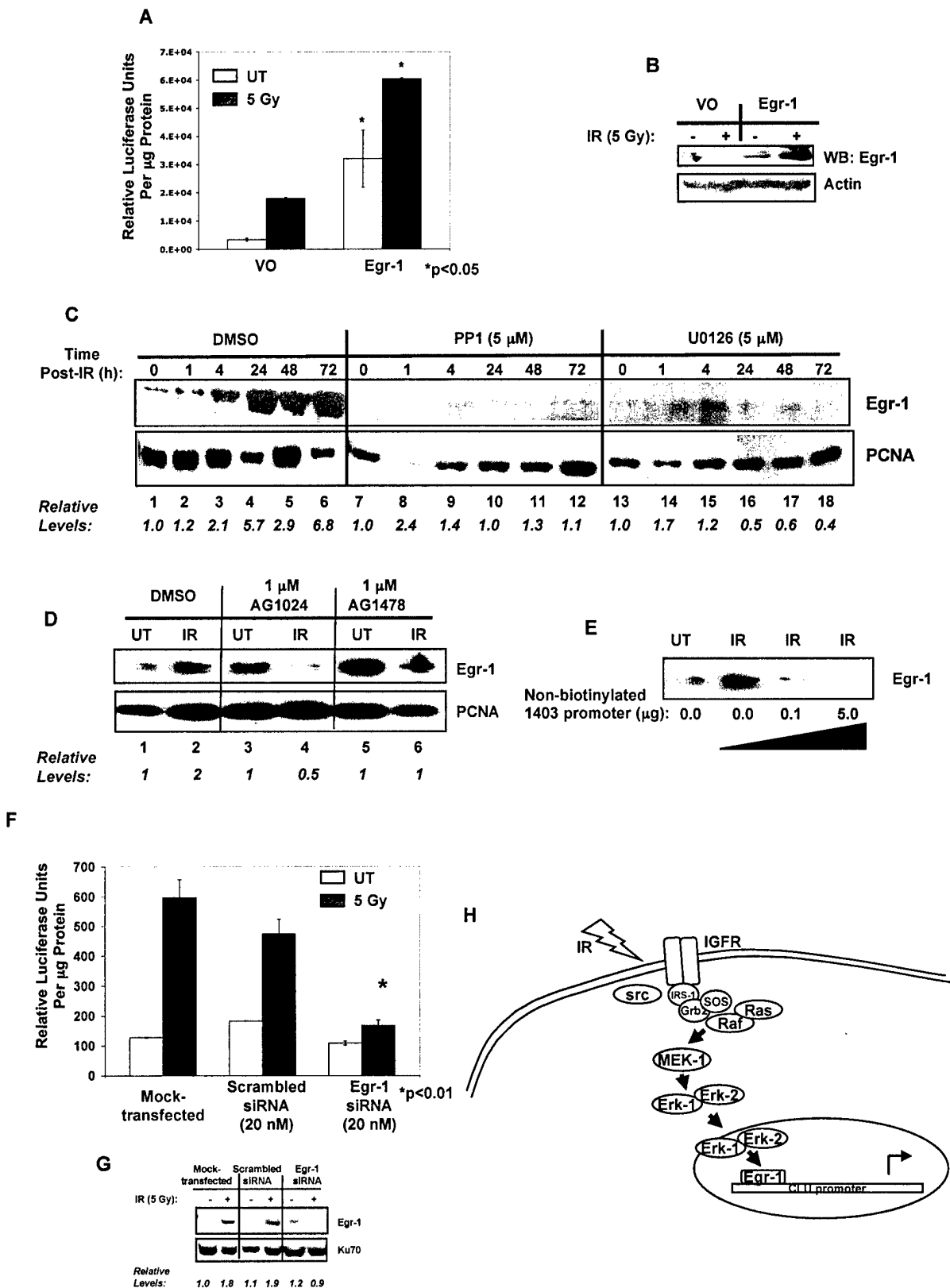


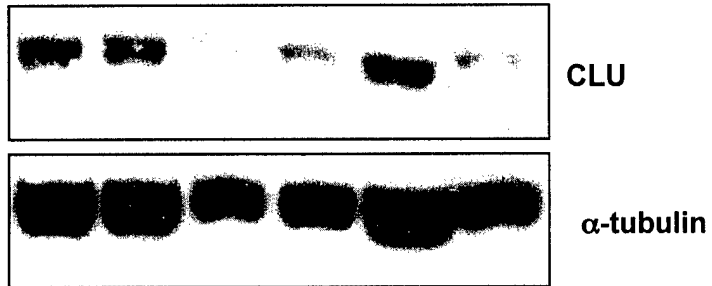
Figure 7



S1

PC3 Cells

IR (5 Gy)	-	+	+	-	-	-
AG1024 (1.0 μ M)	-	-	+	+	-	+
IGF (10ng/mL)	-	-	-	-	+	+



Relative sCLU levels: 1.0 0.9 0.2 0.5 1.0 0.4

S2

