REGULATION OF THE INSULIN-LIKE GROWTH FACTOR 1-SECRETORY CLUSTERIN EXPRESSION AXIS IN GENOMIC INSTABILITY AND CELL STRESS

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DEDICATION

First, I would like to thank my parents, Patricia Keith and Robert Cataldo, for their continued support and encouragement during all of my academic endeavors.

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REGULATION OF THE INSULIN-LIKE GROWTH FACTOR 1-SECRETORY CLUSTERIN EXPRESSION AXIS IN GENOMIC INSTABILITY AND CELL STRESS

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DISSERTATION

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Secretory clusterin (sCLU) is a pro-survival factor that is up-regulated in human tumors and after exposure to cell stress. Understanding the regulation of sCLU expression in cancer, and after exposure to therapeutic agents, could reveal new therapeutic targets for cancer treatment. A DNA damage induced signaling cascade leading from ATM to sCLU expression mediated by IGF-1/IGF-1R/MAPK activation was uncovered. IGF-1 ligand promoter activity, mRNA, and protein expression induced after exposure to ionizing radiation (IR), hydrogen peroxide, or topoisomerase I and IIα poisons matched sCLU expression. Elevated basal IGF-1-sCLU signaling was noted in genomically unstable cells, whether they were deficient in DNA repair factors or telomerase function. ATM function was necessary for induction of sCLU after IR, and for maintaining elevated expression of

sCLU in genomically unstable cells. p53 suppressed IGF-1 promoter activity, leading to decreased mRNA and protein expression, and abrogated induction of IGF-1 and sCLU by IR. Loss of p53 by knockdown or knockout enhanced IGF-1 and sCLU induction. Mutations in the p53 DNA binding domain found in cancer did not repress IGF-1 and sCLU. An NF-Y binding site in the IGF-1 promoter was essential for p53 suppression, and both p53 and NF-YA bound to the IGF-1 promoter. Nutlin-3, an Mdm2-p53 inhibitor, stabilized p53 expression, leading to dramatically decreased sCLU expression. Nutlin-3 treatment sensitized wild-type p53 cells to IR exposure. Finally, exogenous IGF-1 exposure led to serine 1981 auto-phosphorylation of ATM, and enhanced DNA damage repair and abrogated cell death after IR exposure. These studies uncovered key molecules important for the regulation of IGF-1-sCLU expression axis after IR exposure, and supported the use of IGF-1 or sCLU expression inhibitors for cancer chemotherapy.

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7. **Goetz EM**, Zou Y, Shankar BS, Boothman DA. p53 suppression of the IGF-1-sCLU expression axis. In preparation.

8. Zou Y, Araki S, **Goetz EM**, Shankar BS, Leskov KS, Thakur V, Klokov D, Danielpour D, Jackson MW, Mayo LD, Boothman DA. Regulation of the IGF-1-secretory clusterin expression axis in response to TGF-β1 in colon cancer. In preparation.

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LIST OF ABBREVIATIONS

AIB1: amplified in breast cancer 1

Aph: aphidicolin

ATM: *Ataxia telangiectasia mutated*

ATR: ATM and/or RAD3 related

ChIP: chromatin immunoprecipitation

CIN: chromosomal instability

CLU: clusterin

CLU-LUC: clusterin promoter fused to luciferase

CD: catalytically dead

β-Gal: beta galactosidase

βME: β-Mercaptoethanol

BER: base excision repair

DDR: DNA damage response

DNA-PK: DNA-dependent protein kinase

DNA-PKcs: DNA-dependent protein kinase catalytic subunit

DSB: double strand break

Egr-1: early growth response 1

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FAP: familial adenomatous polyposis

FBS: fetal bovine serum

H2AX: histone variant 2AX

HNPCC: hereditary non-polyposis colon cancer

HR: homologous recombination

IGF-1: insulin-like growth factor 1

IGF-1-LUC: IGF-1 promoter fused to lucifearse

IGF-1R: insulin like growth factor 1 receptor

IR: ionizing radiation

KD: kinase dead

LUC: luciferase

MAPK: mitogen activated protein kinase

MDC1: mediator of checkpoint response 1

MEFs: murine embryonic fibroblasts

MEK: MAP kinase or ERK kinase

MIN: microsatellite instability

MMR: mismatch repair

MRN: Mre11, Rad50, NBS1

MSI: microsatellite instability

mTR: RNA component of mouse telomerase

MUT: mutation

NER: nucleotide excision repair

NF-Y: nuclear factor Y

NHEJ: non-homologous end joining

PI3K: phosphoinositide-3-kinase

PIKK: PI3K related kinase

PLDR: potential lethal damage repair

psCLU: pre-secretory clusterin

sCLU: secretory clusterin

SD: standard deviation

SEM: standard error of the mean

shp53: small hairpin targeted to p53

SIPS: stress-induced premature senescence

SRC-3: steroid receptor co-activator 3

SSB: single strand break

TGF-β1: transforming growth factor β-1

TGFβRII: TGF-β receptor II

TPT: topotecan

UT: untreated

UV: ultraviolet

WT: wild-type

XIP: x-ray induced transcript leading to protein

CHAPTER 1: Introduction

Our laboratory first became interested in x-ray induced proteins, when the phenomenon of γ-ray host cell reactivation was discovered in human cells (Jeeves and Rainbow 1979). Host cell reactivation was originally observed in *E. coli* approximately 25 years earlier (Weigle 1953). The phenenomon is highlighted by the process of improved growth of ultraviolet (UV)-damaged bacteriophage when infected into UV-irradiated host cells, compared to infection in untreated bacterial cells. Besides increased survival of the phage, phage infected in irradiated cells had higher frequencies of mutations. This led to the idea that repair of phage in irradiated bacteria was 'error prone'. These studies were extended to mammalian viruses in mammalian cells, and finally in human cells with human viruses (ie, typically SV40), using a variety of DNA damaging agents to damage the virus and to 'induce' host cell reactivation, and similar effects were observed.

The study most pertinent to our lab was the Jeeves and Rainbow study (Jeeves and Rainbow 1979), where an adenovirus exposed to UV radiation was infected into cells, and the host cell reactivation capacity of the cell was determined by immunofluorescent detection of a viral protein, Vag, instead of by survival. When then cell repaired the virus, the viral structural protein, Vag, was detected. Moreover, when the cell was γ-irradiated before virus infection, Vag was detected in more cells, showing host cell reactivation in human cells after exposure

to ionizing radiation (IR) for the first time (Jeeves and Rainbow 1979). This phenomenon also occurred in human cells that were exposed to UV before virus infection (Lytle, Benane et al. 1974; Abrahams and Van der Eb 1976). In addition, it was noted that host cell reactivation was dependent on time after exposure to UV; the longer the time between UV exposure and virus infection, the greater the reactivation, seen up to five days after exposure (Bockstahler, Lytle et al. 1976). Finally, DasGupta and Summers (DasGupta and Summers 1978) treated cells with cycloheximide, a protein synthesis inhibitor, after UV exposures and removed it before viral infection. This allowed for protein synthesis during infection, but blocked all protein synthesis after radiation. Cycloheximide blocked host cell reactivation in mammalian cells, indicating that protein synthesis was necessary for host cell reactivation (DasGupta and Summers 1978). This result inspired our lab to look for x-ray-induced proteins (XIPs).

To examine proteins induced by IR that may be involved in potentially lethal DNA damage repair (PLDR), two-dimensional (2-d) gel electrophoresis was performed, and twelve x-ray-induced proteins (XIPs) were detected (Boothman, Bouvard et al. 1989). Once proteins were known to be induced after IR exposure, a search for their transcripts was peformed using subtractive hybridization (Boothman, Meyers et al. 1993). This study led to the discovery of secretory clusterin (sCLU) as x-ray-induced transcript leading to protein 8 (xip8)

(Boothman, Meyers et al. 1993). Since then, there have been many additional studies on sCLU, including the discovery of its functions in apoptosis and debris clearance (Humphreys, Carver et al. 1999; Zhang, Kim et al. 2005), its involvement in human disease (Jones and Jomary 2002), as well as its induction in stressed cells undergoing apoptosis (Ledda-Columbano, Coni et al. 1992). Nevertheless, there are still many remaining questions regarding the regulation and function of sCLU within normal compared to human cancer cells.

This dissertation serves to answer several questions concerning the regulation of sCLU after DNA damage. sCLU induction after IR involves IGF-1R to MAPK signaling (Criswell, Beman et al. 2005). p53 was reported to suppress sCLU after IR, but the mechanism of suppression remained unknown (Criswell, Klokov et al. 2003). These observations lead to new questions, such as how IGF-1R is activated after IR? Is the IGF-1/IGF-1R pathway induced by other DNA damaging agents or cell stressors? What is the DNA damage sensor kinase that triggers sCLU induction, and can the other PIKKs compensate for each other? What is the mechanism by which p53 suppresses CLU transactivation? Why does it take 48 to 96 hours to induce sCLU after cell stress; given that most cell stressing agents result in the same temporal kinetics of sCLU induction, what is the common mechanism?

In our previous studies, phosphorylation was of IGF-1R was observed after IR. The activation of IGF-1R signaling after IR was biphasic. Activation was first observed within 15 min after IR, and this was temporary, with normal levels observed within four hours. However, the pathway was reactivated 24 hours later, coinciding with sCLU induction. Therefore, we hypothesized that the late induction of IGF-1R signaling was probably due to an increase in IGF-1 synthesis. If true, the regulation of IGF-1 transcription, translation and release from cancer cells would need to be elucidated. We also hypothesized that this signaling pathway would be conserved after all types of DNA damage, since all DNA damaging agents examined to date induce sCLU. ATM is the major DNA damage sensor kinase induced after IR, so we hypothesized that ATM may be the link between DNA damage and IGF-1 signaling. The studies testing these hypotheses are described in Chapter III.

Wild-type p53 cells generally have lower expression of sCLU, and p53 suppresses induction of sCLU after IR, however, when p53 expression is lowered or becomes nonfunctional in the cells by knockdown, knockout, or expression of E6, sCLU is dramatically induced by IR exposure. It was originally thought that p53 may directly suppresses sCLU, however, a p53 binding site was not found in the CLU promoter and p53 was not found to bind the CLU promoter by pulldown assays. Therefore, we hypothesized that p53 suppressed factors required for sCLU

induction, such as IGF-1 signaling. Also, if IGF-1 ligand expression is induced after IR, then induction may also be suppressed by p53, like sCLU. I tested these hypotheses in Chapter IV.

There are several reasons why our laboratory is interested in the regulation of sCLU. First, sCLU is induced in cancer cells after chemotherapy treatment and is a pro-survival factor. Therefore, its induction could be a primary determinant of the resistance of cancer cells to therapy. Understanding and knowing the signaling molecules involved in this process could reveal new targets for therapy. Furthermore, since sCLU is induced by very low doses of IR ($\leq$ 2 cGy in most cells) it is a potential biomarker for harmful exposures to radiation from the environment. Indeed, the late induction of sCLU after exposure to IR could be used as a biomarker for unexpected human exposure to radiation, such as from a 'dirty bomb. Currently, there are few methods to detect human exposure to radiation in uncontrolled environments. Many of the direct methods to measure DNA damage are transient, such as congregation of DNA damage repair factors (or foci). These foci are formed within minutes of exposure and only last several hours at most. It would be very difficult to analyze all exposed persons within this period, so the ability of sCLU to be used as a marker of cellular exposure to IR, by examining blood serum or tissue biopsied levels of sCLU, may be useful for determining exposure. Consistently, increased levels of sCLU was found in the

serum of whole body irradiated mice both 2 and 7 days after exposure (Rithidech, Honikel et al. 2009). In conclusion, up-regulation of sCLU in cancer, and the late and sustained induction of sCLU after IR are very important to understand, not only in the development of cancer therapeutics, but as a marker of exposure to IR.

CHAPTER II: Review of Literature

Ionizing Radiation

Natural background low-dose radiation is a constant source of exposure to all organisms. In addition, ionizing radiation (IR) is used during diagnostic medical procedures including x-rays, CT scans, and as radioactive tracers. It is also used for medical therapy, specifically in cancer. Exposure to ionizing radiation produces DNA double strand breaks (DSBs), single strand breaks (SSBs), base damage, and DNA-protein crosslinks. These lesions are created through direct action of photons accelerating electrons that directly attack the DNA, or by indirect photolysis of water, in which photons interact with other atoms or molecules in the cell to create free radicals. Consequently, when photons interact with water, free hydroxyl radicals are formed that can also cause DNA damage. For x-rays, indirect action is the more dominant form of DNA damage. To cope with this damage, the cell has well conserved DNA repair pathways that include two distinct methods for repairing DSBs, the most lethal event after IR: non-homologous end joining (NHEJ) and homologous recombination (HR).

Repair of IR-induced DNA lesions

The two pathways for repair of IR-induced DSBs include NHEJ and HR, however, it is not completely clear how a secific pathway is chosen. There is evidence that NHEJ and HR compete for repair of DSBs, and it is also known that one pathway or the other is more dominant during different phases of the cell cycle (Roth and Wilson 1985; Chen, Nastasi et al. 1997). Cells in late S and G2 are more likely to stimulate HR, presumably due to the increased accessibility of templates in sister chromatids (Kadyk and Hartwell 1992). However, NHEJ can be stimulated in all phases of the cell cycle. Both NHEJ and HR have several DNA repair factors in common, and some factors are specific to each pathway. The common repair factors include, ATM, BRCA1, DNA-PKcs, RAD18, PARP-1, H2AX and the MRN complex. After DNA damage, the lesion is sensed by the MRN complex (MRE11-RAD50-NBS1) that leads to activation and recruitment of ATM and DNA-PKcs, and phosphorylation of H2AX (formation of γ-H2AX). Finally, MDC1 and 53BP1 congregate at DSBs, along with factors involved in NHEJ or HR. The factors involved in HR include RAD51, BRCA2, XRCC2, XRCC3, RPA, and others, while NHEJ requires DNA-PKcs, Ku70, Ku80, DNA ligase IV, XRCC4, XLF and artemis. ATM, along with other possible DNA damage sensors, can, in turn, activate other proteins, including p53, Chk2, and others to stall the cell cycle to allow time for repair. In all, many different factors are

involved in the sensing DNA damage and, in turn, stimulating repair of DSBs. NHEJ and HR, as their names suggest, repair DNA by very different mechanisms.

Non-homologous end joining (NHEJ)

NHEJ is generally thought of as an error-prone DNA repair process, but can accurately repair DNA that has 'clean' DSBs, such as complementary overhangs, 5' phosphates, and 3' hydroxyl groups, however, this is a relatively rare occurrence. When ends cannot be joined cleanly, NHEJ must 'process' the ends and use micro-homology between bases to combine DNA strands, leading to small deletions or insertions. In severely damaged DNA, misrepair by NHEJ commonly leads to large deletions and additions after IR. The MRN complex is the first complex at the break, and then DNA-PKcs, Ku70, and Ku80 are recruited and bind to DNA ends. Ku70 and Ku80 form a doughnut-shape structure around the DNA (Walker, Corpina et al. 2001), and this stable complex has helicase activity and recruits and activates DNA-PKcs (Chan, Ye et al. 1999; Singleton, Torres-Arzayus et al. 1999). Next, broken DNA ends are processed to remove non-ligatable ends and lesions, however, damage from IR can be widely varied and complex, so different processing molecules must be recruited depending on the type and extent of damage or lesion. One of the known nucleases, Artemis, is phosphorylated by DNA-PK and ATM, promoting its end processing activity (Ma, Pannicke et al.

2005; Goodarzi, Yu et al. 2006). Other known processing enzymes include DNA polymerases μ and λ, PNK, APLF, and WRN (Li and Comai 2001; Nick McElhinny, Havener et al. 2005; Bernstein, Karimi-Busheri et al. 2008; Macrae, McCulloch et al. 2008). Finally, DNA ligase IV, with XRCC4 and XLF, seal the break (Critchlow, Bowater et al. 1997; Ahnesorg, Smith et al. 2006).

Homologous Recombination (HR)

HR uses a template for repair of DNA involving DNA exchange, therefore, repair is more likely to be free from errors. Acceptable templates include sister chromatids, homologous chromosomes, or repeated regions on the same or other chromosomes. At the site of the break, the damaged DNA is removed 5' to 3' by the MRN complex (Paull and Gellert 1998), leaving a 3' overhang that is immediately bound by RPA (Gasior, Wong et al. 1998). RAD proteins, now replace RPA at the 3' ends, and form the RAD51 nucleoprotein filament (Sugiyama and Kowalczykowski 2002). The RAD complex finds and invade a homologous sequence of DNA, where the free 3' ends invade and bind complementary to the template DNA (Sugawara, Ivanov et al. 1995). Finally, DNA polymerases fill in the gap, and the break is sealed by DNA ligase. During the repair process, when DNA polymerase is filling the gap, the 3' free end can be connected to the other free end, called synthesis-dependent strand annealing, or the 3' free end can be

included into a double holiday junction that could result in a crossover of one DNA strand with another.

Along with NHEJ and HR, cells repair other types of damage by DNA mismatch repair (MMR) (Wildenberg and Meselson 1975), base excision repair (BER) (Lindahl 1979), nucleotide excision repair (NER) (Grossman, Riazuddin et al. 1979), and translesion synthesis (an error-prone escape of stalled replication forks that would otherwise be lethal) (Thomas and Kunkel 1993).

DNA Damage Signaling

After DNA damage sensing, and during the first steps of DNA repair, the cell simultaneously enacts a signaling cascade to presumably halt the cell cycle to preserve the integrity of the cell and genome, as well as perform DNA repair. If the damage is too extensive or complicated to repair, however, cells can also use this signaling program to induce cell death or stress-induced premature senescence (SIPS). There are many signaling molecules that participate in this cascade, however, activation of many downstream effectors can be attributed to *Ataxia telangiectasia mutated*, or ATM. ATM is activated immediately after DNA damage, and activates both DNA repair factors and cell cycle checkpoint proteins. ATM phosphorylates various DNA repair proteins, including H2AX, MDC1, 53BP1, NBS1, Artemis, DNA-PKcs, MRE11, RPAp34 and BRCA1(Cortez, Wang

et al. 1999; Dong, Zhong et al. 1999; Bao, Tibbetts et al. 2001; Burma, Chen et al. 2001; Ward, Minn et al. 2003; Chen, Morio et al. 2005; Chen, Uematsu et al. 2007). Phosphorylation of these factors usually results in functional activation, but can also aid in retention of proteins at DNA breaks or result in recruitment of proteins involved in processes to promote DNA repair.

ATM also phosphorylates factors involved in cell cycle arrest, senescence and telomere maintenance, and apoptosis. The tumor suppressor, p53, is directly phosphorylated by ATM, and by Chk1 and Chk2, that are activated by ATR and ATM (Canman, Lim et al. 1998; Hirao, Kong et al. 2000; Matsuoka, Rotman et al. 2000). p53 transcriptionally induces proteins involved in G1-S checkpoint arrest, apoptosis, and cell senescence, such as p21, 14-3-3σ, PUMA, and others. (el-Deiry, Tokino et al. 1993; Nakano and Vousden 2001). ATM-activated Chk2, SMC1, and FANCD2 promote arrest of the other phases of the cell cycle (Matsuoka, Huang et al. 1998; Nakanishi, Taniguchi et al. 2002; Yazdi, Wang et al. 2002). Arrest of the cell cycle after DNA damage is an important step to allow time to repair damage and prevent DNA replication over damaged bases or DNA breaks. These studies indicate that ATM is a very important signaling molecule activated after DNA damage, which is illustrated by the increased sensitivity of AT cells (ATM-/-) to IR (Taylor, Harnden et al. 1975).

Genomic Instability

It is understood that most tumors are genetically unstable. Nevertheless, whether this is a prerequisite for cancer development or a defect that occurs during the uninhibited proliferation of cancer cells is still under debate. Either way, cancer cells accumulate mutations and chromosome alterations during the transformation from a normal cell to a tumor cell. There are four categories of genetic alterations in cancer, and these include: sequence changes, changes in chromosome number, chromosomal translocations, and gene amplifications or deletions (Lengauer, Kinzler et al. 1998).

Sequence changes in tumors are probably due to defects in DNA repair rather than the fidelity of DNA polymerases involved in replication, as a consistent pattern of mutation has not been observed in cancer (Lengauer, Kinzler et al. 1998), and mutations in polymerases principally involved in replication would probably be lethal to the cell. Defects in NER and MMR have been shown to promote skin tumors after UV exposure (Cleaver 1968) and hereditary colon cancer (Bronner, Baker et al. 1994), respectively. Additionally, approximately 20% of sporadic colon and ovarian cancers can be linked to loss of MMR.

Chromosomal instabilities (CIN) result in changes in chromosome number and can be due to defects in proteins involved in the cell cycle, and more

specifically, proteins involved in checkpoint control and proper condensation and segregation of chromosomes during mitosis (Paulovich, Toczyski et al. 1997; Rajagopalan, Jallepalli et al. 2004). Besides random chromosomal translocations that may or may not lead to cancer, there are several common chromosomal translocations in leukemia and lymphoma. These translocations generally lead to activation of an oncogene, such as translocation of BCR and ABL, in chronic myelogenous leukemia (Kurzrock, Gutterman et al. 1988) and PML-RARα in acute promyelocytic leukemia (de The, Lavau et al. 1991). The last type of genetic instability is gene amplification and deletion. In cancer, gene amplifications are usually of oncogenes, while deletions are of tumor suppressors. Nevertheless, the basis behind gene amplifications and deletions are unknown. Most cases of genetic instability occur at the chromosome level, however changes in DNA sequence, chromosomal translocations, and gene alterations are still important aspects of tumor formation and progression. Understanding these types of genetic alterations may lead to therapies where genomic instability can be avoided.

Colon Cancer

One of the most obvious cases of genomic instability causing cancer is the loss of MMR in colon cancer. The American Cancer Society lists colorectal cancer as the third most common cause of cancer death for both men and women. On

average, 1 in 18 men or 1 in 19 women will develop colorectal cancer in their lifetimes. Overall, the five-year survival rate has been increasing since 1975, probably due to the prevalence of screening methods for colon polyps. Besides sporadic colon cancer, there are two types of hereditary colon cancer: hereditary nonpolyposis colon cancer (HNPCC) and familial adenomatous polyposis (FAP). Most individuals with HNPCC have germline mutations in *hMLH1* (51%), *hMSH2* (48%) , *hPMS2* (<1%), or *hMSH6* (<1%), genes that code for proteins involved in MMR (Fishel, Lescoe et al. 1993; Leach, Nicolaides et al. 1993; Bronner, Baker et al. 1994; Nicolaides, Papadopoulos et al. 1994; Papadopoulos, Nicolaides et al. 1994; Akiyama, Sato et al. 1997). As mentioned above, besides HNPCC patients, individuals with sporadic colon cancer can also have deficiencies in MMR leading to microsatellite instability (MSI+ or MIN). These cases result from hypermethylation of the hMLH1 promoter, causing silencing (Kane, Loda et al. 1997). In the 80-85% of the colon cancers without MSI, the predominant phenotype is chromosomal instability, however, defects that cause these phenotypes are much more varied than for MSI.

Loss of MMR promotes MSI because of a defect in repairing base mismatches and small loops resulting from inaccurate replication of long tracts of repeat sequences (microsatellites) (Fishel, Ewel et al. 1994). The loss of repair of defective repeat sequences is a major reason for the common mutation in *TGFBR2*

gene in MMR-defective tumors. The *TGFBR2* gene has a tract of 10 adenines which undergoes a frameshift mutation in 85% of MMR defective cells (Markowitz, Wang et al. 1995). Cells with a mutation in the *TGFBR2* gene are resistant to the growth suppressive affects of the TGF-β1 ligand, promoting tumorigenesis. Other genes that commonly have frameshift mutations in MSI and are related to cancer progression include IGFR2, BAX, and others (Souza, Appel et al. 1996; Rampino, Yamamoto et al. 1997; Yamamoto, Sawai et al. 1997).

Secretory clusterin was found to be a marker in a mouse model of colon cancer, Apc^{Min} (Chen, Halberg et al. 2003). The Apc^{Min} model carries the multiple intestinal neoplasia (Min) mutation in the adenomatous polyposis coli (APC) gene, and is an experimental model for FAP (Dove, Cormier et al. 1998). Chen et al (Chen, Halberg et al. 2003) performed subtractive hybridization, and found that sCLU was upregulated in all intestinal neoplasms regardless of stage, location, or mode of tumor initiation in the Apc^{Min} model. They also found higher sCLU expression in human colorectal cancers of all stages, and even in normal epithelia near tumors (Chen, Halberg et al. 2003). These data demonstrate a consistent role for sCLU in tumorigenesis.

Clusterin

Secretory clusterin was originally discovered in 1983 as the major glycoprotein in ram rete testis fluid that caused cellular aggregation (Blaschuk, Burdzy et al. 1983). Since then, clusterin has been rediscovered by many different labs, giving rise to its many names: sulfated glycoprotein-2 (SP-2), apolipoprotein J (ApoJ), testosterone repressed prostate message 2 (TRPM2) and x-ray inducible protein 8 (XIP8) discovered by our lab (Leger, Montpetit et al. 1987; Bettuzzi, Hiipakka et al. 1989; de Silva, Stuart et al. 1990; Boothman, Meyers et al. 1993). sCLU is induced after various forms of cell stress, including exposure to chemotherapeutic agents (Miyake, Nelson et al. 2000), IR (Boothman, Meyers et al. 1993), her2/neu blockade (Biroccio, D'Angelo et al. 2005), estrogen (Kyprianou, English et al. 1991) and androgen withdrawal (Kyprianou, English et al. 1990). Clusterin is associated with many diseased states, such as Alzheimer's disease (Lidstrom, Bogdanovic et al. 1998; Calero, Rostagno et al. 2000), lupus erythematosus (Newkirk, Apostolakos et al. 1999), retinitis (Jones, Meerabux et al. 1992; Wong, Borst et al. 1994), and cancer. sCLU is elevated in many cancers, including prostate (Steinberg, Oyasu et al. 1997; Miyake, Hara et al. 2003), breast (Redondo, Villar et al. 2000), lung (July, Beraldi et al. 2004), colorectal (Chen, Halberg et al. 2003), lymphomas (Wellmann, Thieblemont et al. 2000), and ovary (Hough, Cho et al. 2001). sCLU overexpression is responsible for increased

resistance to doxorubicin, cisplatin, and taxol in cancer cells (Miyake, Nelson et al. 2000; Miyake, Hara et al. 2003) and knockdown of sCLU leads to IR- and chemo-sensitization (Miyake, Chi et al. 2000; Criswell, Beman et al. 2005). These observations strongly suggest that elevated levels of sCLU in cancer, as well as induction of sCLU after cytotoxic agent exposure, may result in consequent resistance to therapy. Therefore, antisense constructs specific for sCLU (OGX-011) were developed and are now in phase II clinical trials for different cancers (Chi, Zoubeidi et al. 2008). OGX-011 has been shown to decrease sCLU expression in target tissue including human prostate, breast, and lung cancers, and increase the time to androgen independent prostate cancer growth in mice (Gleave and Chi 2005).

sCLU has been reported to have many functions, including lipid transport, tissue remodeling, reproduction, and apoptosis (Rosenberg and Silkensen 1995), mirroring the alternative names for the protein. Clusterin gene expression has both pro-death (Yang, Leskov et al. 2000; Leskov, Klokov et al. 2003) and pro-survival functions (Miyake, Chi et al. 2000) within the cell. These contradictory functions can be explained by different forms of the clusterin protein, including nuclear, secreted, and cellular/cytoplasmic. Translation of full-length CLU mRNA results in synthesis of a 60 kDa peptide that is localized in the ER by a peptide localization sequence (cytoplasmic/pre-secretory CLU/psCLU) (Jones and Jomary 2002). The

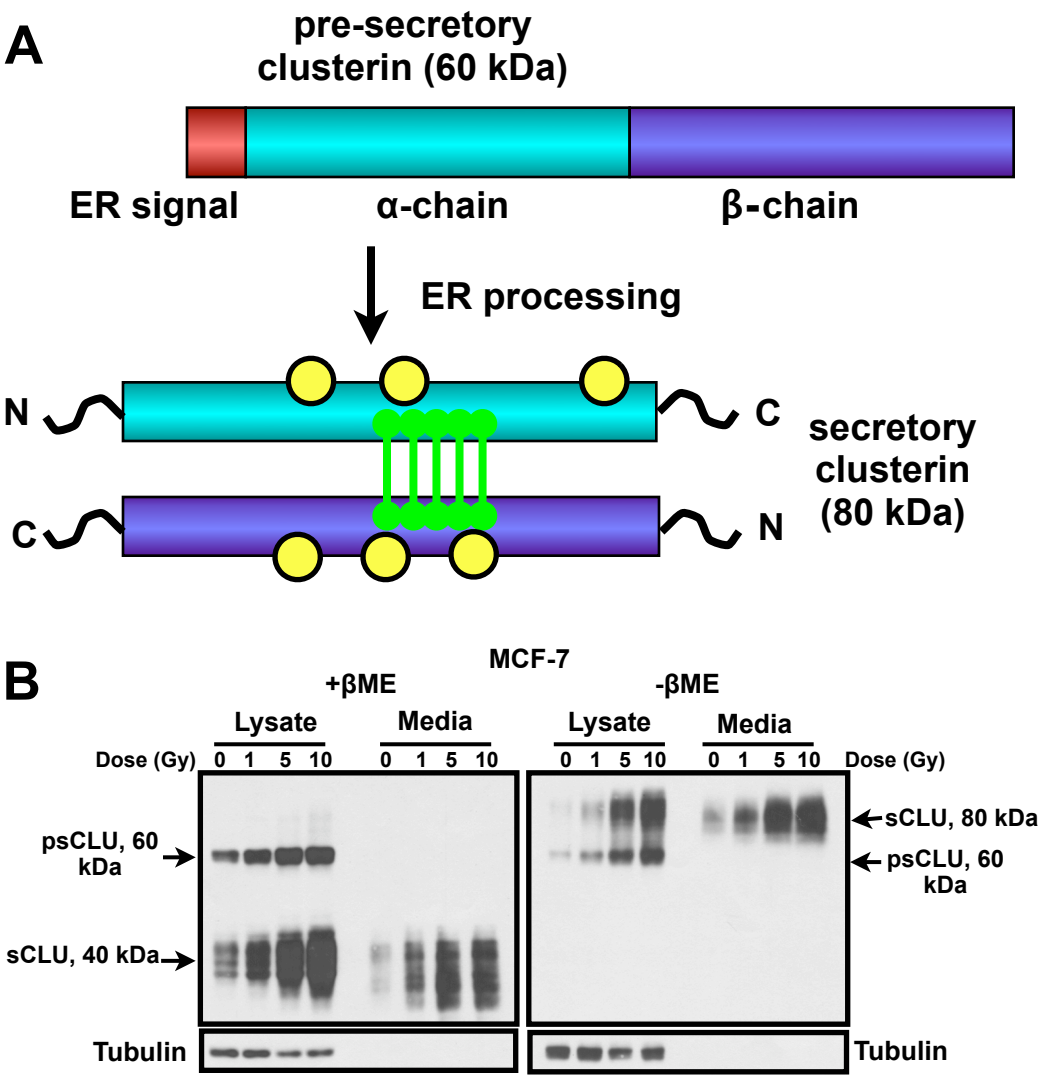

Figure 2.1 Processing of secretory clusterin. ***A***, the precursor form of sCLU contains an ER signal peptide, and is synthesized as a 60 kDa peptide. This peptide is processed by the ER, where the signal peptide is cleaved off, and the peptide is cleaved into two peptides, α and β. The two half-peptides are linked by five disulfide bonds (green), and then heavily glycosylated (yellow). The mature form is secreted from the cell as an 80 kDa glycoprotein. ***B***, Western blot of MCF-7 cell lysates or conditioned media from MCF-7 cells with or without β-mecaptoethanol (βME). Whole cell lysates show both the 60 kDa pre-secretory clusterin (psCLU) and the 80 kDa mature secretory clusterin (sCLU), while media only contains sCLU. Samples incubated with βME shows 80 kDa sCLU running at 40 kDa due to reducing-equivalents in the SDS-gel, breaking the disulfide bonds.

60 kDa psCLU is then cleaved into α and β strands that are cross-linked by five disulfide bonds, then heavily glycosylated, and ultimately secreted from the cell as a mature 80 kDa protein, sCLU (Figure 2.1).

In contrast, there is an alternatively spliced form of clusterin, named precursor nuclear clusterin (pnCLU), that is approximately 49 kDa. The alternative splicing occurs at exons 1 and 3, effectively removing the ER signal peptide (Yang, Leskov et al. 2000). This pnCLU form contains a functional nuclear localization sequence (NLS) and one nuclear export sequence (NES) that acts to shuttle the protein in and out of the nucleus, respectively. The 49 kDa cytoplasmic clusterin protein is 'activated' after cell stress, resulting in post-translational modification, creating a 55 kDa, pro-apoptotic protein (Leskov, Klokov et al. 2003) that remains in the nucleus (nCLU) and promotes cell death. The manner in which nCLU causes cell death has not been completely determined, but the 55 kDa protein can bind Ku70/Ku80 (Yang, Leskov et al. 2000), inhibits Ku end binding activity, and liberates Bax. Liberated Bax then translocates to the mitochondria and causes apoptosis (Sawada, Sun et al. 2003). Recent studies by our lab have suggested that nCLU mediates cell death through a Bax-dependent pathway (Leskov et al., unpublished observations). Bax-deficient, but not wild-type or heterozygote Bax murine embryonic fibroblasts (MEFs) are resistant to nCLU-induced cell death. Indeed, cells grown in the presence of exogenously

overexpressed nCLU that are resistant to nCLU-stimulated cell death lacked Bax expression (Leskov et al., unpublished observations). Specific siRNA knockdown of nCLU caused resistance to IR, and a dramatic reduction of apoptosis, supporting its role as a pro-death protein.

Originally the function of sCLU was not clear. sCLU expression was up-regulated in a variety disease states, and was able to bind to many different molecules. Due to the diversity of sCLU binding partners, sCLU function was attributed to the biological function of its partner. Therefore, sCLU was reported to function in phagocyte recruitment, cell-cell interactions, complement inhibition, apoptosis, membrane remodeling, and lipid transport [reviewed in (Wilson and Easterbrook-Smith 2000)]. However, the function of sCLU was determined to be as an extracellular molecular chaperone, explaining its previously perceived functions (Humphreys, Carver et al. 1999). Now, sCLU is thought of as an extracellular heat shock protein. As a chaperone, sCLU bound to unfolded proteins and prevented protein precipitation, however, did not appear to protect proteins from stress-induced loss of function (Humphreys, Carver et al. 1999). Also, extracellular radiolabeled sCLU bound to the gp330 endocytic receptor and was taken up by the cell (Kounnas, Loukinova et al. 1995). These data suggested that sCLU may bind to stress-induced unfolded proteins to promote uptake by the endosomes, and thereby, prevent inflammation.

To make matters more complicated, the 60 kDa psCLU can also found be found within the cytoplasm. The cytoplasmic sCLU was found to bind Bax, and prevented Bax translocation to the mitochondria, thereby protecting cells from apoptosis (Zhang, Kim et al. 2005). It is unknown how sCLU might escape the ER to exert its anti-apoptotic effect, however, sCLU may bind to Bax at the ER, like Bcl-2-Bax (Thomenius, Wang et al. 2003). The presence of intracellular clusterin also suggests a role for sCLU in prevention of the unfolded protein response (accumulation of unfolded proteins in the ER). Overall, these studies to date strongly indicate a pro-survival role for sCLU, *in vitro*.

To further study the function of sCLU, knockout mice were created by McLaughlin et al. (McLaughlin, Zhu et al. 2000). Overall, the mice do not have an obvious phenotype, however, differences are revealed when the mice are exposed to stress. When exposed to a challenge of cardiac myosin, inflammation was more severe in the CLU knockout compared to wild-type mice (McLaughlin, Zhu et al. 2000). In another study, CLU knockout animals were more likely to undergo apoptosis than wild-type animals when exposed to heat stress (Bailey, Aronow et al. 2002). Additionally, it was noted that there are less motor neurons in CLU knockout animals during development (Charnay, Imhof et al. 2008), and after transection of the hypoglossal nerve (Wicher and Aldskogius 2005). In addition, unpublished studies in our laboratory have show that CLU deficient mice have

more severe interstitial fibrosis during renal injury induced by unilateral ureteral obstruction (Zou et al, unpublished observations). Unexpectedly, the survival of IR-exposed CLU knockout animals are the same as wild-type littermates (Yang et al., unpublished observations). However, the targeting vector in the CLU knockout mouse effectively removes both the nuclear pro-death clusterin, as well as the pro-survival sCLU. Overall, these data indicate that sCLU acts as a pro-survival factor *in vivo,* however differences may be muted due to the lack of expression of both isoforms.

When the CLU knockout mouse was crossed with the Alzheimer disease mouse model, PDAPP, amyloid deposits were increased in the CLU wild-type mouse compared to the CLU knockout, PDAPP transgenic mouse (DeMattos, O'Dell M et al. 2002). Similarly, CLU wild-type mice infected with bovine spongiform encephalopathy have an increase in PrP depositions in the medulla (Sasaki, Doh-ura et al. 2006). These two studies strongly suggest a normal chaperone-like activity of sCLU, similar to *in vitro* studies.

IGF-1

Insulin-like growth factor 1 (IGF-1) treatment can induce sCLU expression, indicating that cell stress is not necessary for sCLU induction (Criswell, Beman et al. 2005). Furthermore, inhibitors of IGF-1 signaling,

AG1024 (tyrosine kinase inhibitor of IGF-1R), as well as, IGFBP3 (natural binding protein of IGF-1), prevented sCLU gene expression (Criswell, Beman et al. 2005). Like sCLU, IGF-1 was upregulated in many neoplasms including cancers of the lung (Minuto, Del Monte et al. 1986), prostate (Harman, Metter et al. 2000), colon (Tricoli, Rall et al. 1986), and breast (Hankinson, Willett et al. 1998), and during prostate cancer progression (Kaplan, Mohan et al. 1999). Marelli et al, published that IGF-1 promotes migration of androgen-independent prostate cancer cells (Marelli, Moretti et al. 2006), and others have published that IGF-1 can stimulate proliferation and migration of intestinal fibroblasts (Simmons, Pucilowska et al. 1999), explaining its role in tumor progression. In addition, IGF-1 activates gene transcription that is associated with poor breast cancer prognosis (Creighton, Casa et al. 2008).

The IGF-1 receptor (IGF-1R) also plays a role in cancer growth and progression, and drugs have been developed to efficaciously target the IGF-1R (Miller and Yee 2005), There are at least six IGF-1R antibodies or kinase inhibitors currently in clinical trials against numerous cancer types (Garber 2005; Pollak 2008). Many receptor tyrosine kinase inhibitors are aimed at blocking kinase activity through ATP analogs, so many of these are nonspecific. This highlights the importance of examining the regulation of IGF-1 to determine new targets to block IGF-1 signaling, and supports the use of blocking antibodies over kinase inhibitors.

Blocking either IGF-1R or IGF-1 ligand with antibodies or inhibitors should prevent its growth promoting activities and decrease sCLU levels, thereby potentially enhancing IR and chemotherapeutic sensitivities through decreased pro-survival functions.

The regulation of IGF-1 within cells is not completely understood. Within the body, IGF-1 is made in the liver due to endocrine regulation of growth hormone (GH). GH made in the pituitary, then travels to the liver where Jak signaling is stimulated. Activated Jak signaling induces phosphorylation of Stat5b that binds to, and stimulates, the IGF-1 promoter (Woelfle, Billiard et al. 2003). Nevertheless, mice with liver-specific IGF-1 deletion, only have a 75% decrease in circulating IGF-1 levels, indicating that cells and tissues can produce IGF-1 independent from the liver (Yakar, Liu et al. 2001). Additionally, the 25% IGF-1 levels were enough to allow for normal growth and development. The regulation of IGF-1 by individual cells is not clear, but has shown to be under the control of other hormones (Murphy and Friesen 1988; Penhoat, Naville et al. 1989; Hofbauer, Rafferzeder et al. 1995).

Two studies published in the early 1990's describe IGF-1 ligand knockout mice, and the most obvious phenotype is death shortly after birth. The frequency of death was dependent on the mouse background (Liu, Baker et al. 1993; Powell-Braxton, Hollingshead et al. 1993). The death rate varied between 32%-95%, with

most death being seen in C57BL/6 J mice and the least in a MF1 and 129/sv mix (Powell-Braxton, Hollingshead et al. 1993; Liu and LeRoith 1999). IGF-1-deficient mice that survive have significant growth retardation, and weigh 45% less than wild-type litter mates at birth, and this difference gradually increases, where deficient mice weigh 70% less than their wild-type litter mates at 8 weeks (Baker, Liu et al. 1993; Liu and LeRoith 1999). The changes over time could be due to IGF-1 that the fetus receives from the mother during prenatal development that allows for intrauterine growth (Liu, Yakar et al. 2000). There are also other phenotypes in these mice and include growth hormone resistance, infertility, and organ enlargement (Powell-Braxton, Hollingshead et al. 1993; Baker, Hardy et al. 1996; Liu and LeRoith 1999). Nevertheless, in mice engineered to contain only a liver-specific IGF-1 knockout, the defects observed in the complete knockout have been abrogated. Growth and development is normal despite a decrease of 75% in circulating IGF-1 levels (Yakar, Liu et al. 1999). Overall, these studies reveal that IGF-1 is essential for growth and development.

In the blood, IGF-1 is bound to IGF-1 binding proteins (IGFBP1-6). IGFBPs prolong the half-life of IGF-1, allowing IGF-1 to be delivered throughout the body, while also modulating the availability of IGF-1 to bind to its receptor and mediate its proliferative effect (Jones and Clemmons 1995). IGF-1 has 50x higher affinity for IGFBPs than its receptor. Proteases can cleave IGFBPs causing reduced

affinity for IGF-1. IGFBP-3 is the most common circulating IGFBP, and is responsible for binding to 90% of circulating IGF-1.

Along with IGF-1's pro-mitogenic effects, the growth factor has been shown to protect cells from death, including apoptosis. IGF-1 protected cells from death due to C2-ceramide (Kondo, Kitano et al. 2002), osmotic stress (Matthews and Feldman 1996), serum starvation, etoposide (Sell, Baserga et al. 1995), paclitael, brefeldin, and other exposures to cytotoxic agents. Many have shown that this protection is dependent on PI3K and MAPK signaling (Parrizas, Saltiel et al. 1997; Chung, Seo et al. 2007). Recently, Limesand et al. demonstrated that IGF-1 protected against IR-induced apoptosis in salivary acinar cells and completely preserved salivary gland function (Limesand, Said et al. 2009). These responses are due to IGF-1 induced activation of Akt. Similarly, IGF-1 increased the ability of cells to repair UV-damaged DNA (Heron-Milhavet, Karas et al. 2001). Another study indicated that IGF-1 can promote Rad51, a DNA repair factor, to translocate to the nucleus after DNA damage by regulating the activity of IRS-1. This allows Rad51 to promote DNA repair by HR (Trojanek, Ho et al. 2003). In more recent papers, Urbanska et al. found that IRS-1 and Rad51 colocalized at foci after cisplatin treatment (Urbanska, Pannizzo et al. 2008), and Jeon et al. found IRS-1 interacted with ATM, to affect DNA repair (Jeon, Kim et al. 2008). Besides directly influencing the DNA repair capacity of cells, Heron-

Milhavet and LeRoith have shown that IGF-1 can promote p53 degradation after DNA damage by inducing Mdm2-dependent E3 ligase ubiquitin-mediated degradation of p53 (Heron-Milhavet and LeRoith 2002). This could also promote DNA repair by protecting cells from p53-induced apoptosis that allows time for the cells to repair their DNA. In conclusion, these studies indicate an important role for IGF-1 signaling in DNA repair and cell cycle regulation.

The p53 tumor suppressor

p53 is a tumor suppressor that regulates many cellular functions, including the cell cycle, DNA repair, cellular senescence, and cell death. It exerts many of its affects by acting as a transcription factor to activate proteins involved in stopping the cell cycle or inducing apoptosis. Activation of p53 by cellular stress induces cell cycle arrest by p53-dependent induction of proteins involved in halting the cell cycle, such as p21, GADD45, and 14-3-3σ (Kastan, Zhan et al. 1992; el-Deiry, Tokino et al. 1993; Hermeking, Lengauer et al. 1997). If the stress or damage is too great or too prolonged, p53 can also induce cell death, including apoptosis, by activating the death genes, such as APAF-1, Bax, Puma, Bid, and part of the TNF-R super family (Selvakumaran, Lin et al. 1994; Moroni, Hickman et al. 2001; Nakano and Vousden 2001; Yu, Zhang et al. 2001; Henry, Thomas et al. 2002; Michalak, Villunger et al. 2005). Since p53 induces cell cycle

checkpoints at G1 and possibly S and G2, and induces cell death, it can be easily understood why function of this protein is commonly eliminated in cancer. In colon cancer, approximately 45% of patients have a mutation in p53 and the mutation is most commonly observed in the DNA binding domain of the p53 protein. In the majority of the remaining colon cancers, p53 function is likely compromised by elevated Mdm2, Akt, or other factors that regulate p53 expression. Also, the data are somewhat muted due to the lower incidence of p53 mutation (15-30%) in individuals with MSI colon cancer, compared to individuals with stable microsatellites and chromosomal instability (60-70%).

There are four major domains within the p53 protein. The N-terminal domain includes two transactivation domains, and a proline-rich domain. The DNA binding domain is responsible for binding to promoter DNA where p53 acts as a transcription factor. The tetramerization domain is where p53 oligmerizes to form a functional complex. The C-terminal domain contains three NLSs. During carcinogenesis, the DNA binding domain is the most mutated domain in p53, indicating the importance of this domain in p53 function to suppress tumor formation (Hjortsberg, Rubio-Nevado et al. 2008). Nevertheless, amino acids outside of the DNA binding domain can also be important for activity.

p53 is extensively post-translationally modified by phosphorylation, acetylation, sumoylation, ubiquitination, methylation, and neddylation [review,

(Olsson, Manzl et al. 2007)]. It is generally thought that stabilization of p53 after cell stress is not due to an increase in transcription of the gene, but to post-translational modification. Nevertheless, a recent study showed that translation of p53 is increased after DNA damage through a complex between nucleolin and L26, a ribosomal protein (Takagi, Absalon et al. 2005). Either way, post-translational modification of p53 is very important to its activity as a transcription factor. The negative regulation of p53 by Mdm2 is dependent on p53 phosphorylation. Mdm2 is an E3-ubiquitin ligase that binds p53 and attaches ubiquitins, promoting degradation by the proteasome (Honda, Tanaka et al. 1997). Phosphorylation on serine 20 and threonine 18, by Chk2 and CK2, respectively, after DNA damage has been shown to inhibit binding of p53 and Mdm2 (Chehab, Malikzay et al. 1999; Unger, Sionov et al. 1999), promoting stabilization of p53, however, there is conflicting data as to whether phosphorylation of serine 15 by ATM/ATR is important for Mdm2 binding (Shieh, Ikeda et al. 1997; Dumaz and Meek 1999). In addition, it was suggested that phosphorylation on serines 15 and 20 enhanced the transcriptional activity of p53 (Unger, Sionov et al. 1999), however, new studies indicate that these phosphorylation sites are dispensable for transactivation (Thompson, Tovar et al. 2004). The Thompson et al. study was performed using Nutlin-3, an inhibitor of the interaction between Mdm2 and p53. Treatment of wild-type p53 cells with Nutlin-3 promoted stabilization of p53 without

phosphorylation, and downstream genes were induced to the same degree as cells with p53 stabilized by DNA damaging agent treatment. Overall, these studies try to explain how phosphorylation of p53 acts to regulate gene transcription. However, the absolute affect of these sites cannot be determined unless they are mutated in an animal model.

Along with *in vitro* studies, mutations of the mouse equivalent of serines 15 and 20 to alanine caused different effects in the animal. Mutation of serine 18 (equivalent to serine 15 in humans) to a non-phosphorylatable mutant impairs p53 induced apoptosis (Chao, Hergenhahn et al. 2003; Sluss, Armata et al. 2004), and mutation of serine 23 (equivalent to serine 20 in humans) in the same manner, impairs the ability of p53 to suppress tumor formation (Wu, Earle et al. 2002; MacPherson, Kim et al. 2004), among other effects. Nevertheless, these sites are rarely, if ever, mutated in cancer (Hjortsberg, Rubio-Nevado et al. 2008), indicating that these residues may not be as important as others to the function of p53 as a tumor suppressor.

p53 is acetylated in the C-terminal domain by several histone acetyl transferases, including CBP/p300, PCAF, and p300(Gu and Roeder 1997; Liu, Scolnick et al. 1999). Generally, acetylation on these residues promotes p53 transactivation, especially after DNA damage (Barlev, Liu et al. 2001), and deacetylation is associated with loss of transactivation (Luo, Su et al. 2000).

However, the exact mechanism of enhanced transactivation by acetylated p53 is under debate. Methylation at lysine residues in p53 can also alter p53 function. Methylation at L372 increased p53 stability whereas methylation at L370 repressed transcriptional activity (Chuikov, Kurash et al. 2004; Huang, Perez-Burgos et al. 2006). Overall, these studies reveal the importance of post-translational modifications for p53 function(s).

p53 suppression of sCLU

Our lab showed that sCLU mRNA and protein expression were suppressed by p53, but the exact mechanism by which p53 suppresses CLU transcription was not elucidated (Criswell, Klokov et al. 2003). We discovered that modulation of signaling molecules upstream of p53 can alter sCLU expression. Transfection of 4250 base pairs of the CLU promoter fused to luciferase (CLU-LUC) with either catalytically dead PTEN (CD), kinase dead Akt (KD), wild-type p53 or HPV viral protein E6 causes altered p53 levels, and consequent changes in CLU-LUC activity. PTEN CD prevented suppression of the PI-3K pathway, activating p53 degradation, and preventing suppression of the CLU promoter. Akt KD prevented phophorylation and nuclear localization of Mdm2, causing p53 levels to increase, resulting in lowered CLU promoter activity. Similarly, transfection of wild-type p53 abrogated CLU induction after IR. HPV-viral protein, E6, degrades p53,

causing CLU-LUC activity to increase (Criswell, Klokov et al. 2003). Mdm2 knockdown using small hairpin RNA (shMdm2) stabilized p53 levels, and caused suppression of basal and IR-induced sCLU expression. These data indicate that modulation of p53 levels by altering upstream signaling will alter sCLU expression.

A p53 consensus sequence within the CLU promoter at position -3631 bp was noted, although mutation of this site did not alleviate the ability of p53 to repress CLU promoter activity (Leskov et al., unpublished observations). This is not surprising since there are few reports of p53 directly suppressing transcriptional activity at its consensus site, and none that used an intact p53 transactivation consensus site. p53 is known to suppress transcription by i), Interfering with activation by DNA-binding transcription factors; ii), Stabilizing a transcriptional repressor protein; iii), Interfering with basal transcription machinery; and iii), Recruitment of chromatin modifying proteins such as histone deactylase (Ho and Benchimol 2003). If p53 directly suppresses the CLU promoter, it is possible that p53 can directly interact with the TATA-binding protein (TBP) or some other transcription factor, such as Egr-1, and inhibit clusterin transcription in this manner. Nevertheless, it seems more likely that p53 inhibits some upstream factor that is important in clusterin gene induction such as IGF-1/IGF-1R or MAPK signaling.

In contrast to induction by IR, sCLU upregulation in response to TGF-β1 treatment was not dependent on the expression status of p53. Interestingly, after exposure to TGF-β1, sCLU was induced in p53 wild-type, as well as p53 null or mutant cells. MMR-deficient, HCT116 cells do not contain the TGFβRII receptor, and correction of these cells by fusion of chromosome 3 (HCT116:3-6) caused them to be responsive to TGF-β1, and therefore, induce sCLU. This induction was not dependent on p53; HCT116:3-6 cells contain wild-type p53 and TGF-β1 can still induce sCLU. This is due to a novel mechanism of Mdm2 induction after TGF-β1 treatment that leads to ubiquination and subsequent degradation of p53 (Araki et al., unpublished observations). Release of p53 suppression (through degradation) on the IGF-1 promoter leads to sCLU expression by the same IGF-1R/MAPK pathway (Zou et al, unpublished observations). Thus, there appears to be separate mechanisms of induction of sCLU by TGF-β1 compared with IR.

Clusterin Regulation

Our lab has shown that after IR, sCLU is induced from 5- to 40-fold within 24-72 h after treatment. This induction process is mediated through IGF-1/IGF-1R/ MAPK signaling that ultimately activates the Egr-1 transcription factor to bind to, and transactivate, the human CLU promoter (Figure 2.2). MAPK signaling is

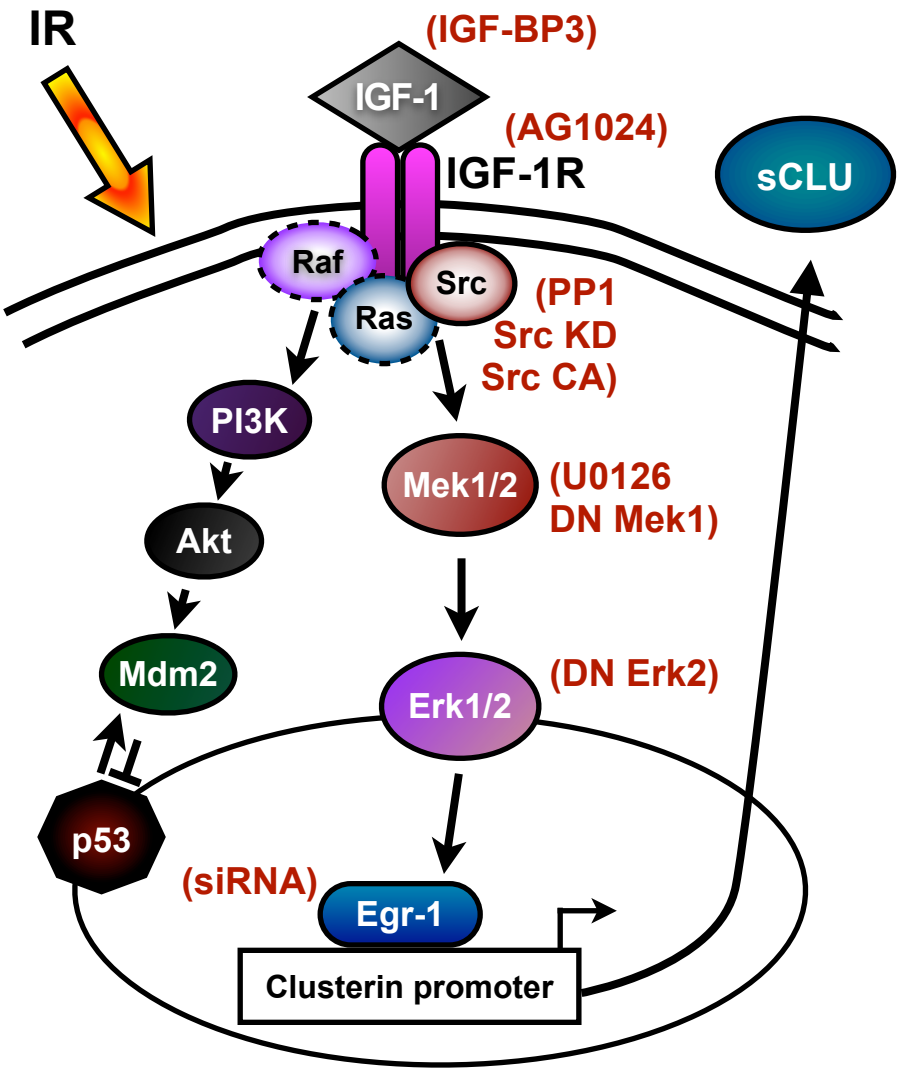

Figure 2.2 Summary of IGF-1 mediated induction of sCLU after IR. After IR, IGF-1R is activated and leads to activation of Src, and transmits a signal possibly through Ras to Mek1/2. Mek1/2 activates Erk1/2 translocation into the nucleus, where it activates the transcription factor Egr-1 to bind to the CLU promoter. Clusterin is synthesized and secreted from the cell. IGF-1R signaling can also activate Akt. Akt can phoshorylate and activate Mdm2 to degrade p53, and p53 can transcriptionally regulate Mdm2. In red, inhibitors, plasmids, or method used to characterize the IGF-1 to sCLU signaling pathway. KD is kinase dead. CA is constitutively active. DN is dominant negative

induced within minutes of IR, but 24 h after IR we noted a dramatic reactivation of MAPK signaling, which we have shown to be responsible for sCLU induction (Criswell, Beman et al. 2005). In addition, blockade of IGF-1R, Src, MEK, ERK, and Egr-1 using specific inhibitors, dominant negative proteins, or siRNA blocked sCLU induction after IR. Transfection of a constitutively-active Src increased basal sCLU expression. We showed that Egr-1 binding to the CLU promoter was linked to sCLU expression (Criswell, Beman et al. 2005). We theorize that DNA damage induction by other agents will also activate sCLU expression through the same pathway.

CHAPTER III : Secretory clusterin is a sensitive measure of genomic instability

Abstract

sCLU is a stress-induced pro-survival glycoprotein that is expressed in almost all tissues and bodily fluids. Here, the DNA-damage sensor, *Ataxia-telangiectasia mutated*, regulated sCLU expression by controlling IGF-1 ligand expression, and subsequent IGF-1-dependent signal transduction leading to sCLU expression. ATM activation, minimally originating from DSBs, regulated the IGF-1-sCLU expression axis. Genomically unstable cells commonly have persistent DSBs and endogenous sCLU expression was up-regulated in a series of genetically matched cell systems, including H2AX, MDC1, NBS1, mTR, and DNA mismatch repair deficient cells. Blockade of ATM or IGF-1 receptor signaling downregulated endogenous sCLU expression in all genomic instability syndromes examined. Importantly, cells lacking ATM failed to induce IGF-1-sCLU expression and ATR does not appear to compensate. Our results strongly suggest that IGF-1-sCLU expression is a sensitive indicator of genomic instability.

Introduction

Prior to cellular transformation from normal to tumor cells there is a re-programming of inherent cellular gene expression. These changes typically result in the up-regulation of proteins that provide pro-survival signals and down-regulation of cell-death signals. One pro-survival commonly and constitutively up-regulated in human tumors is the IGF-1/IGF-1R tyrosine kinase signaling cascade (Ryan and Goss 2008). IGF-1R is activated by dimerization after binding IGF-1 (Miller and Yee 2005). We previously found that sCLU, a pro-survival factor, is up-regulated by stress-induced signaling mediated by the IGF-1R signaling pathway. We noted that after IR, cells activated IGF-1R, leading to MAPK/ERK and Egr-1 induction, where Egr-1 bound to and transactivated the CLU promoter (Criswell, Beman et al. 2005).

sCLU is induced after various forms of cell stress including exposure to chemotherapeutic agents, IR, and other cellular stresses (Boothman, Meyers et al. 1993; Miyake, Nelson et al. 2000; Trougakos and Gonos 2006). sCLU was identified by our lab as X-ray-induced transcript leading to protein 8 (XIP8), whose expression was extremely sensitive to low doses of IR, an agent that introduces a spectrum of DNA lesions in the genome, however, the exact DNA lesion required for induction of this gene was not elucidated (Yang, Leskov et al. 2000). Besides being upregulated in response to external stressors, sCLU is

constitutively elevated in many cancers, during replicative senescence, in Alzheimer's disease, stroke victims, and in the pericardia of patients who suffered heart attacks (McGeer, Kawamata et al. 1992; Steinberg, Oyasu et al. 1997; Redondo, Villar et al. 2000; Trougakos, Poulakou et al. 2002; Chen, Halberg et al. 2003). Overexpression of sCLU is responsible for increased resistance to various cell stresses, including doxorubicin, cisplatin, and taxol in cancer cells (Miyake, Nelson et al. 2000). Knockdown of sCLU leads to sensitization of cancer cells to paclitaxel and IR (Criswell, Beman et al. 2005; So, Sinnemann et al. 2005)**.** These observations strongly suggest that elevated levels of sCLU in cancer, as well as induction of sCLU after antitumor agent exposure, may result in consequent resistance to therapy. Based on these findings, antisense to sCLU (OGX-011) was developed and is in phase II clinical trials in combination with chemotherapeutic agents. OGX-011 administration decreased sCLU expression in tumor and normal tissue in patients (Chi, Siu et al. 2008) and chemo-sensitized cancer cells in a mouse tumor model (So, Sinnemann et al. 2005).

The functions of sCLU are not completely understood, although sCLU induction after cell stress fits with its role as an extracellular molecular chaperone to remove cell debris (Humphreys, Carver et al. 1999; Wilson and Easterbrook-Smith 2000). Additionally, it was reported that a cytoplasmic form of sCLU binds

Bax at the ER, preventing Bax from translocating to the mitochondria, thereby blocking apoptosis (Zhang, Kim et al. 2005).

The introduction of DNA lesions, particularly DNA double strand breaks (DSBs) initiates the DNA-damage response (DDR) by activating phospho-inositide 3-like protein kinases (PIKKs), that includes *Ataxia-telangiectasia* mutated (ATM), *ATM- and RAD3-related* (ATR), and the DNA dependent protein kinase catalytic subunit (DNA-PKcs). ATM detects the formation of radiation-induced DSBs (Zhou, Chaturvedi et al. 2000) and chromatin relaxation caused by increased histone acetylation, stress, or hypotonic treatments (Bakkenist and Kastan 2003). ATM phosphorylates several important proteins involved in the DDR, such as i) histone variant H2AX at serine 139; ii) 53BP1 at serine 25; iii) Chk 2 at threonine 68 (Matsuoka, Huang et al. 1998; Ahn, Schwarz et al. 2000); iv) BRCA1 at serine 1524 (Cortez, Wang et al. 1999); v) p53 at serine 15 (Canman, Lim et al. 1998); and vi) itself, by auto-phosphorylation at serine 1981 (Bakkenist and Kastan 2003). Loss of ATM in humans, (ie, *Ataxia telangiectasia*, AT), leads to impaired immunological development, micro-enchephaly, and a predisposition to tumor formation, in-particular lymphomas and breast carcinomas (Lavin and Shiloh 1996). At the cellular level, loss of ATM leads to increased levels of DSBs, impaired phosphorylation and function of the p53 tumor suppressor, and loss of both intra-S and G_2/M DNA damage cell-cycle checkpoints (Xu and Baltimore

1996). ATM has been implicated in the amplification of IGF-1R signal cascades and loss of ATM leads to decreased IGF-1R expression (Peretz, Jensen et al. 2001). The loss of IGF-1R, while having no affect on ATM protein levels, abrogated ATM kinase activity (Macaulay, Salisbury et al. 2001), suggesting a reciprocal functional link between ATM and IGF-1R activation. The IGF-1R signaling pathway can also be activated by IR (Tezuka, Watanabe et al. 2001; Criswell, Beman et al. 2005). A link between DNA damage sensing and expression of IGF-1, as well as its downstream IGF-1R target, sCLU, remains unknown.

Here, we show that ATM is a link between DNA damage recognition and sCLU expression, with the IGF-1-signaling pathway as an intermediary step. Genomically unstable cells, or cells deficient in γ-H2AX, MDC1, mTR, NBS1, and hMLH1 displayed constitutive ATM auto-activation with concomitantly higher levels of sCLU expression than their corresponding genetically matched wild-type cells. In all cells examined, both basal and stress-induced sCLU expression levels were severely decreased by inhibiting ATM or IGF-1R signaling. Consistently, AT fibroblasts that are defective for ATM, were deficient in sCLU induction following stress compared to genomically stable matched wild-type cells. These data strongly suggest that sCLU up-regulation after DNA damage by the IGF-1/IGF-1R pathway is mediated by ATM kinase activity. Furthermore, the ATM-IGF-1-sCLU expression axis appears common to all genomically unstable cells examined,

consistent with a prior observation that sCLU over-expresion was noted in APC^{min} mice (Chen, Halberg et al. 2003).

Materials and Methods

Cell lines H2AX-/- MEFs were generously provided by Dr. Andre Nussenzweig (NIH, MD). MDC1-/- MEFs were a kind gift from Dr. Junjie Chen (Yale University, CT). ATM-deficient AT fibroblasts and those complemented with ATM were from Dr. Y. Shiloh (Tel Aviv University, Israel). NBS1-/- MEFs were from Dr. J. Petrini (Sloan–Kettering Institute, NY). mTR-/- MEFs were kindly provided to us by Dr. R. DePinho (Harvard University, MA). hMLH1-deficient MMR-deficient human HCT116 (parental) colon cancer cells and an isogenic MMR-corrected HCT116:3-6 derivative (corrected for hMLH1 expression by microcell transfer of an extra chromosome 3) were provided by Dr. C.R. Boland (Baylor College, TX). hMLH1-deficient RKO cells were corrected for MMR with full length hMLH1 (RKO7) by us (Wagner, Li et al. 2008). MCF-7 cells were purchased from ATCC. All cells were free from mycoplasma infection.

Cell culture and cell treatments All cell lines were cultured in DMEM (BioWhittacker; Walkersville, MA) containing 5% fetal bovine serum (FBS, HyClone, Utah) and 2 mM glutamine at 37 ˚C in a 10% CO_2-90% air atmosphere. AG1024 was obtained from EMD Biosciences (San Diego, CA). IGF-1 was

obtained from R&D Systems (Minneapolis, MN). Etoposide (VP16), Topotecan (TPT), Aphidicolin (Aph) and AAI (CGK733) were from Sigma Chemical Co. (St. Louis, MO). For treatments, 2 x 10^6 cells were plated overnight in 10 cm dishes and were treated with TPT, H_2O_2, or VP16 for 5 h. Sera-free medium were replaced with media containing 5% serum. For IGF-1 treatments, 2 x 10^6 cells in 10 cm dishes were serum-starved (0.5% FBS) overnight, and then exposed to IGF-1 for one hour. Exposure to irradiation was from a ^{137}Cs Mark I-68 irradiator (JL Shepherd & associates, CA) at a dose rate of 3.87 Gy/min. Cells were pretreated with N-acetyl cysteine (NAC) or IGF-1R inhibitor AG1024 overnight and Aph was administered for 1 h before treatment with DNA damaging agents and then 4 h post treatment. Cells were pretreated for 1 h with AAI before exposure to IR, or exposed for 48 h. Mock treated and Me_2SO (DMSO) treated cells served as controls (UT).

Luciferase reporter assays Cells were plated in a density of 1 x 10^5/well in 12 well plates and transfected with PA3-4250 clusterin luc (CLU-LUC, a 4250-bp region of *CLU* promoter controlling the firefly luciferase reporter enzyme (Criswell, Beman et al. 2005)) or pGL2b-IGF-1-LUC (IGF-1-LUC, a -1630 to +322 bp of the *IGF-1* promoter fused to luciferase, a kind gift from Dr. P. Rotwein, (Oregon Health and Science University, (Mittanck, Kim et al. 1997))) and RSV-β-gal. Cells were transfected using Effectene reagent (Qiagen, Valencia, CA). Cells transfected

with vector alone served as controls. After transfection (24 h), cells were treated with various DNA damaging agents, including IR, H_2O_2, TPT, or VP16 as stated above. *CLU* promoter driven luciferase assays were assessed 48 h after exposure. Cells were harvested in 1X reporter lysis buffer and luciferase activity was analyzed using Luciferase Assay Reagent (Promega, Madison, WI). β-galactosidase assays were performed using Galacto-Star reagent (Applied Biosystems, CA) as a control for transfection efficiency. Each sample was assessed in triplicate and paired Student's t-tests were performed for analysis of statistical significance. Results are graphed as the means +/− standard deviation (SD) of the replicates.

Enzyme-linked Immunosorbent Assays (ELISA) IGF-1 ligand in the media was detected using capture and detection antibodies (MAB291 and BAF291), and reagents from R & D Systems. Briefly, MCF-7 and RKO7 cells were grown in 10 cm dishes and media was collected 48 h after induction of DNA damage. Mock-treated cells served as controls. Samples (100 μl) were compared to an IGF-1 standard curve to determine concentrations. The concentration of IGF-1 in the media was normalized by cell number. Each experiment was performed three times. Results are means +/- SD of the replicates, and statistical significance determined by paired Student's t-test.

RT-PCR for IGF-1 MCF-7 and RKO7 cells were treated with DNA damaging agents as described above and total RNA was isolated using RNeasy mini kit (Qiagen). cDNA was synthesized using the high capacity cDNA archive kit (Applied Biosystems). RT-PCR for IGF-1 and actin was performed using the specific primers IGF-1 forward: AACACCATCCATTTGGGAAA, backward: TGACATATTGCCCCCATTTT, PCR product size is 290 bp; β-actin forward: GGACTTCGAGCAAGAGATGG, backward: AGCACTGTGTTGGCGTACAG, PCR product size is 234 bp. Experiments were repeated at least three times, and representative images are shown.

Flow cytometry Anti-IGF-1 (H70), (G-17) and G-17 blocking peptides were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pATM (S1981) antibody was from Rockland Immunochemicals (Gilbertsville, PA), and anti-γ-H2AX was from Millipore (Billerica, MA). Cells (5×10^5) were fixed with 1% formaldehyde and permeabilized with 100% ethanol. Cells were stained with specific primary antibodies and FITC-tagged secondary antibodies, and counterstained with propidium iodide. Cells were assessed for IGF-1, pATM, or γ-H2AX staining, and cell cycle distribution. Experiments were repeated in triplicate, and either a representative dot plot was shown, or graphed to represent all data. Results are means +/- SD of the replicates, and statistical significance was calculated using paired Student's t-tests.

Western blotting Western blotting of IGF-1-sCLU expression and signaling were previously described (Criswell, Beman et al. 2005). Antibodies to human sCLU (B-5), mouse sCLU (M18), Chk1, Mdm2, β-actin and α-tubulin were from Santa Cruz Biotechnology. Antibodies to phosphorylated Akt (S473), Akt, phosphorylated ERK (Y204), ERK, phosphorylated Chk1 (S317 and S345) were from Cell Signaling Technology (Beverley, MA). Antibodies against phosphoryated IGF-1R and IGF-1R were from Abcam Inc. (Cambridge, MA) and Millipore, respectively. GAPDH antibody was from EMB Biosciences. Blots were visualized by chemiluminescence and probed with antibodies against GAPDH, β-actin or α-tubulin as the loading control. All western blots were repeated at least three times, and representative images are presented. Relative expression was calculated from x-ray films using NIH Image J, by comparing the relative density of experimental conditions to a loading control (GAPDH, β-actin or α-tubulin). Control values were set to 1.

Results

Clusterin is induced by DNA damage

To investigate the specific DNA lesions that are required for IGF-1-sCLU induction, MCF-7 cells were treated with different DNA damaging agents, and analyzed for sCLU protein expression or activity of 4250-bp of the CLU promoter fused to luciferase (CLU-LUC). sCLU protein expression and CLU promoter activity were induced in MCF-7 and RKO7 cells after treatment with IR, H_2O_2, and topoisomerase I and IIα poisons (Figures 3.1 A-D). We observed an increase in both the 60 kDa psCLU and 40 kDa sCLU protein. We previously showed that IGF-1R/Src/MAPK/Egr-1 signaling was upstream of sCLU expression (Criswell, Beman et al. 2005), so we examined whether ERK was phosphorylated in response to DNA damaging agents. As expected, we observed ERK phosphorylation after treatment with all the DNA damaging agents tested (Figure 3.1E).

To evaluate the role of reactive oxygen species (ROS) in DNA damage induced sCLU expression in human breast and colon cancer cells, MCF-7 and RKO7 cells were pre-treated or not with N-acteyl cysteine (NAC) for 24 h followed by exposure to various DNA damaging agents. NAC exposure up-regulates glutathione levels in cells and acts as a free radical scavenger itself (Bentle, Reinicke et al. 2006). NAC abrogated H_2O_2-induced sCLU protein

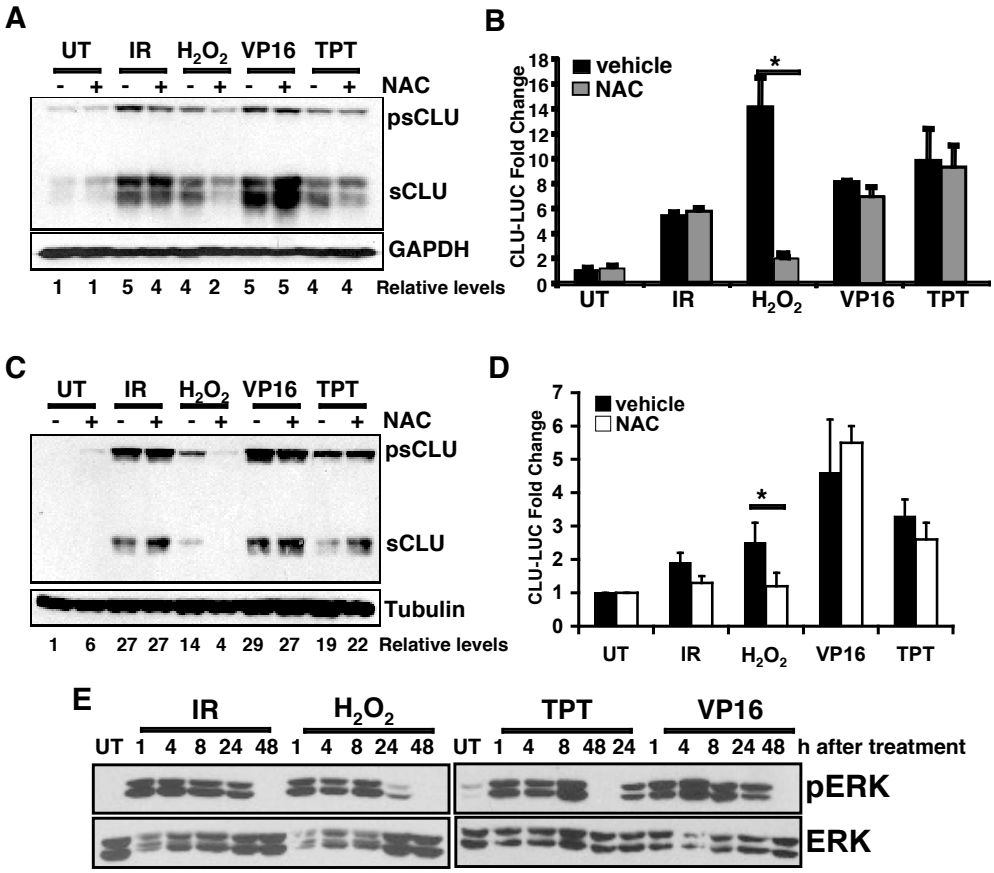

Figure 3.1 sCLU is induced after DNA damage. ***A, C.*** MCF-7 (A) or RKO7 (C) cells were mock-treated or exposed overnight with 5 mM NAC and then exposed to various DNA damaging agents (IR, 5 Gy; H_2O_2, 50 M for 5 h; VP16, 10 M for 5 h; TPT, 2.2 M for 5 h). Controls received DMSO (UT). Whole cell extracts were harvested at 72 h for western analyses. ***B, D.*** MCF-7 (B) or RKO7 (D) cells were transiently transfected with CLU-LUC (clusterin promoter fused to luciferase) and RSV-β-gal. Cells were pre-treated overnight with 5 mM NAC or vehicle and exposed to DNA damaging agents as explained in *A and C*. Cells were harvested 48 h after treatment and luciferase activities were normalized to β-gal expression. *p values $\leq$ 0.05; H_2O_2, NAC versus vehicle. ***E.*** MCF-7 cells were treated with various damaging agents as above. Controls received DMSO (UT). Whole cell extracts were harvested at the indicated times for western analyses of phosphorylated ERK (pERK) and total ERK. Note 24 h and 48h are reversed for TPT treatment.

expression (Figures 3.1 A and C) and CLU promoter activities (Figures 3.1 B and D) in MCF-7 and RKO7 cells. NAC also protected against H_2O_2-induced DNA damage visualized by comet assays and spared the lethal effects of H_2O_2 monitored by colony forming assays in both RKO7 and HCT116 cells (Shankar, BS; data not shown). In addition, NAC offered significant protection against H_2O_2-induced phosphorylation of serine 1981 on ATM ($pATM^{S1981}$) and serine 139 on H2AX (γ-H2AX) (Shankar, BS; data not shown). In contrast, NAC pre-treatment could not block DNA damage due to γ-radiation, topoisomerase I or IIα poisons. These data strongly suggested that DNA damage, in general, can provide a significant signal for induction of sCLU expression.

sCLU is induced by DSB

To examine whether DNA SSBs or DSBs were required for induction of CLU expression, we exposed cells to the DNA polymerase α inhibitor, aphidicolin (Aph), prior to treatment with DNA damaging agents. Topotecan (TPT) is a topoisomerase-I poison, therefore, DSBs are only formed when cells replicate. TPT causes SSBs that are converted to DSBs during DNA synthesis (D'Arpa, Beardmore et al. 1990). In MCF-7 cells, Aph abrogated TPT-induced sCLU protein expression and promoter activity (Figures 3.2 A and B). As expected, TPT induced phosphorylation of ATM and H2AX in S-phase cells (Figure 3.2 C). Treatment of MCF-7 cells with Aph abrogated TPT activation of ATM, and ATM-

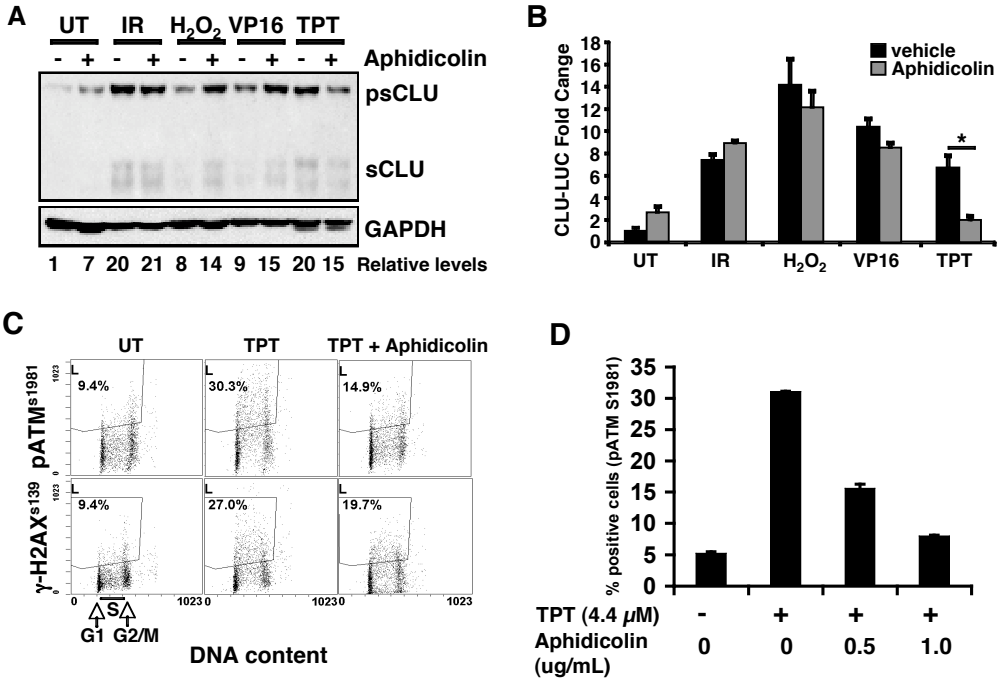

Figure 3.2 Double strand breaks are sufficient for sCLU induction *A.* MCF-7 cells were pretreated for 1 h with aphidicolin (100 ng/ml) or vehicle control prior to exposure to various DNA damaging agents, or DMSO (UT). Whole cell extracts were harvested at 72 h for western analyses. ***B.*** MCF-7 cells were transiently transfected with CLU-LUC and RSV-β-gal and pretreated with Aph (100 ng/ml) or vehicle control, then treated with various DNA damaging agents or DMSO (UT). Cells were assessed for luciferase activities and β-gal expression 48 h after treatment. Luciferase activities were normalized by β-gal expression. *$p \leq 0.05$, TPT, vehicle versus aphidicolin. ***C.*** Flow cytometric assessments of $pATM^{S1981}$ and γ-H2AX in MCF-7 cells treated with 4.4 μM TPT with or without aphidicolin (1 μg/ml) for 2 h. ***D.*** Graphical representation of data in *C*. Percent cells positive for $pATM^{S1981}$ in MCF-7 cells following TPT and aphidicolin treatment.

mediated downstream γ-H2AX (Figures 3.2 C and D). Since TPT-induced sCLU expression was abrogated by Aph treatment and Aph abrogated TPT-induced ATM phosphorylation, we concluded that ATM was a candidate for transmitting signals from DNA damage (most probably DSBs) to sCLU expression. In contrast, Aph did not affect sCLU expression, CLU promoter activity, or ATM phosphorylation by agents that induce other forms of DNA damage.

DNA damage-induced IGF-1 is upstream of sCLU expression

To investigate the role of IGF-1R signaling in DNA damage-induced sCLU expression, we exposed cells to AG1024, a specific IGF-1R tyrosine kinase inhibitor. AG1014 inhibited basal sCLU expression and sCLU induction in response to all DNA damaging agents tested, suggesting a common IGF-1/IGF-1R signaling pathway for the induction of this expression axis (Figure 3.3 A). Next, we examined whether IGF-1 ligand gene expression was induced after DNA damage. RKO7 cells treated with DNA damaging agents (IR, H_2O_2, TPT, or VP16) had higher levels (~5-fold) of IGF-1 ligand in the media (Figure 3.3 B) and higher IGF-1 mRNA (Figure 3.3 C) than untreated cells. Additionally, IGF-1 ligand expression was induced with the same kinetics and dose-response characteristics as CLU promoter activity and sCLU protein expression in RKO and MCF-7 cells after IR exposure (Figure 3.3 D). Collectively, the data in figures 3.1 to 3.3

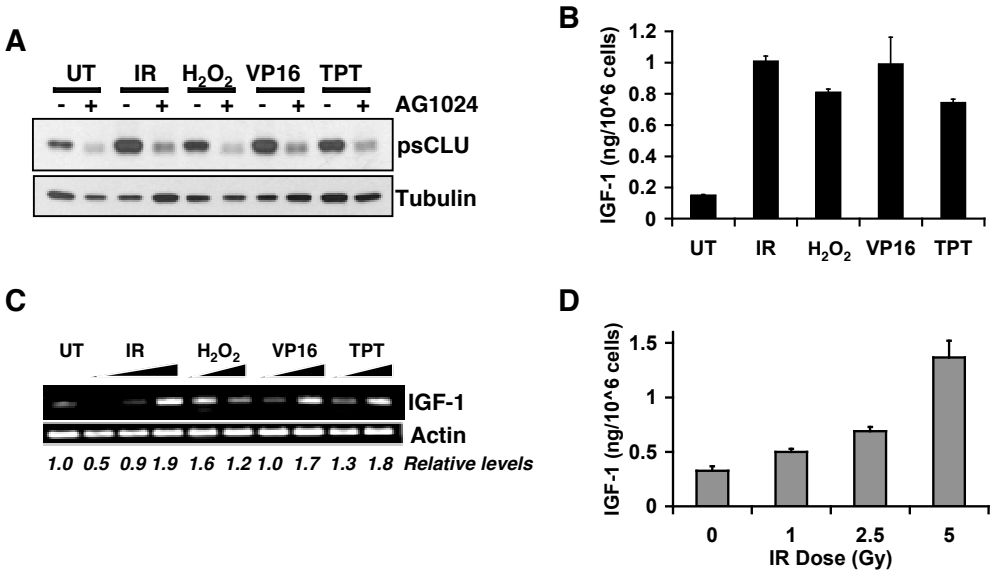

Figure 3.3 IGF-1 ligand expression is induced by DNA damage and required for sCLU induction ***A.*** MCF-7 cells were serum starved, and pre-treated with AG1024 (4 μM, overnight) or DMSO control before exposure to DNA damaging agents, or DMSO (UT). Whole cell extracts were prepared at 72 h for western analyses. ***B.*** MCF-7 cells were treated with different DNA damaging agents and media was collected 48 h later for determination of IGF-1 ligand levels by ELISA. The amount of IGF-1 in media was normalized to the number of cells on the plate. ***C.*** mRNA was isolated from RKO7 cells 48 h after treatment with DNA damaging agents (IR, 1, 2.5 5 Gy; H_2O_2, 50 and 100 μM; VP16, 5 and 10 μM; TPT, 2 and 4 μM) and processed for semi-quantitative RT-PCR as described in experimental procedures. ***D.*** RKO7 cells were harvested 48 h after treatment with an IR dose response and assessed for the secretion of IGF-1 ligand by ELISA.

strongly suggested that ATM induction in response to various DNA damaging agents up-regulated a common IGF-1-sCLU expression axis that has been linked to pro-survival responses, and is initiated by a transient genomic instability caused by DNA damage to the genome.

The IGF-1-sCLU expression axis is upregulated in genomically unstable cells

Since DSBs were minimally required for IGF-1-sCLU expression and genomically unstable cells commonly exhibit constitutive lesions, we examined whether cells with endogenous genomic instability would have constitutive elevation of the IGF-1-sCLU expression axis. MEFs deficient in H2AX, a mammalian histone 2A variant that undergoes rapid phosphorylation of its carboxy-terminal by ATM and other PIKK kinases to form γ-H2AX over large chromatin domains surrounding DSBs (Burma, Chen et al. 2001), were examined. Cells deficient in H2AX have a defect in localization of DNA repair factors BRCA1 and 53BP1, and have defective homologous recombination and persistent DSBs that contribute to their genomically unstable phenotype (Bassing, Chua et al. 2002; Celeste, Petersen et al. 2002). Indeed, dramatically increased levels of sCLU protein and CLU promoter activity were observed in H2AX-/- cells compared to their genetically matched wild-type MEF counterparts (Figures 3.4 A and B).

Since IGF-1 signaling was downstream of exogenous damage, but required for sCLU expression (Figure 3.3), we analyzed H2AX-/- cells for alterations in IGF-1 ligand expression and IGF-1R signaling. H2AX-/- cells showed significantly higher IGF-1 promoter activity than corresponding wild-type MEFs (Figure 3.4 C) using a -1630 to +322 base pair segment of the IGF-1 promoter fused to luciferase (IGF-1-LUC) (Mittanck, Kim et al. 1997). Higher levels of intracellular IGF-1 ligand were also noted in H2AX-/- versus H2AX+/+ MEFs (Figure 3.4 D).

Higher basal expression of IGF-1 ligand in H2AX-/- cells were consistent with downstream activation of IGF-1 signaling. Constitutive elevated basal phosphorylation of IGF-1R, AKT, and ERK was observed in H2AX-/- MEFs compared to H2AX+/+ MEFs (Figures 3.4 E and F). Additionally, IGF-1-induced phosphorylation of IGF-1R and downstream target Akt was higher in H2AX-/- MEFs (Figure 3.4 E), indicating that the entire IGF-1/IGF-1R/sCLU pathway was up-regulated in these cells (Figure 3.4). In addition, increased ERK phosphorylation in H2AX-/- MEFs suggested that the Src/MAPK/ERK signaling pathway was constitutively active due to elevated IGF-1/IGF-1R signaling. Consistently, AG1024 inhibited basal sCLU protein expression in both H2AX-/- and H2AX+/+ cells (Figure 3.4 G). Knocking down IGF-1R expression using siRNA resulted in dramatic decreases in sCLU protein expression (data not

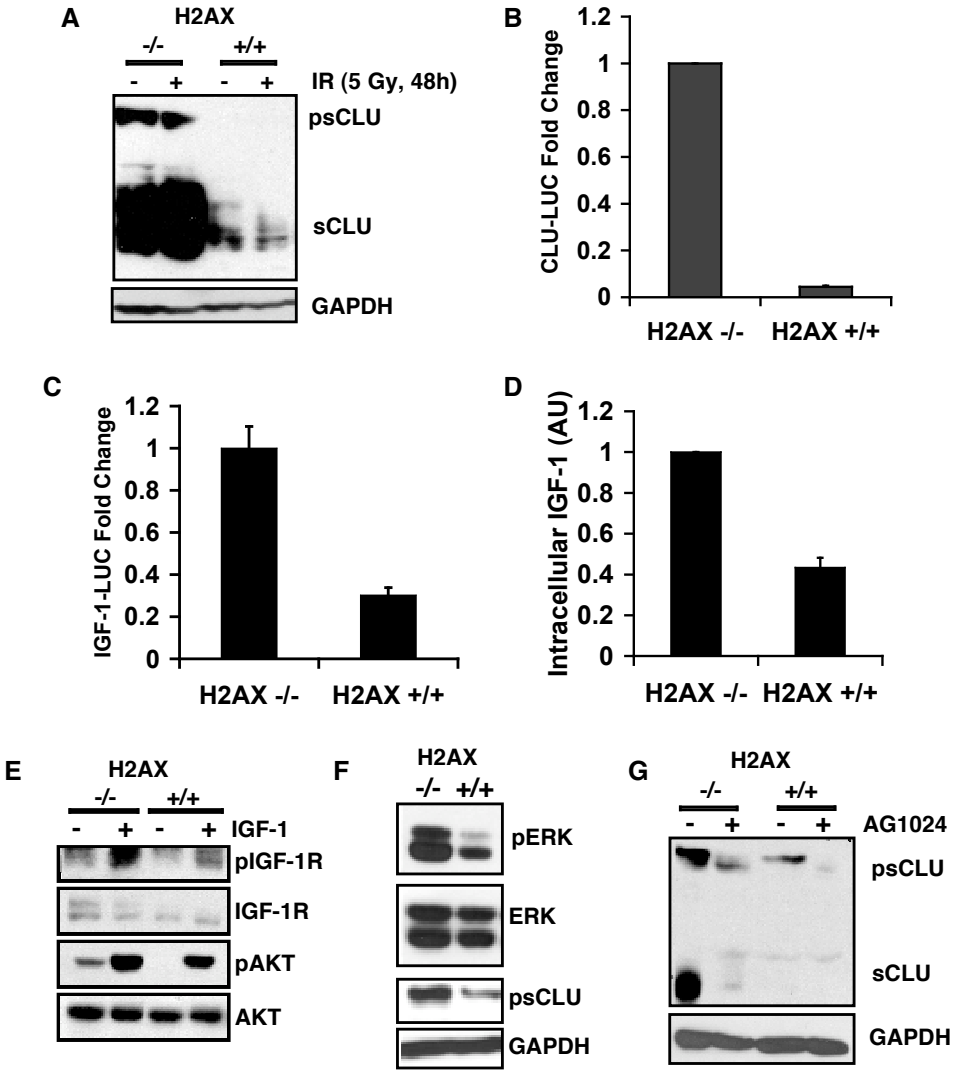

Figure 3.4 H2AX deficient cells have heightened IGF-1-sCLU expression. ***A***. MEFs generated from H2AX deficient (-/-) or wild-type (+/+) mice were treated with 5 Gy and cells were harvested 48 h later for western analyses. ***B***. H2AX-/- or H2AX+/+ MEFs were transiently transfected with CLU-LUC and RSV-β-gal and analyzed for luciferase activity and β-gal expression. ***C.*** H2AX-/- and H2AX+/+ MEFs were transiently transfected with the IGF-1 promoter (IGF-1-LUC, -1630 to +322 bp of the IGF-1 promoter fused to luciferase) or RSV-β-gal. Luciferase readings were normalized with β-gal expression. ***D.*** H2AX-/- and H2AX+/+ MEFs were analyzed for IGF-1 expression by flow cytometry labeling. A.U. is arbitrary units.

Figure 3.4 Continued

E. H2AX-/- and H2AX+/+ MEFs were treated with IGF-1 for 1 h and cells were analyzed for IGF-1 signaling by western analyses. Blots were probed with antibodies specific to phosphorylation of IGF-1R and Akt (pIGF-1R and pAkt) ***F.*** H2AX-/- and H2AX+/+ MEFs were analyzed for their basal expression of phosphorylated Erk (pErk) by western analyses. ***G.*** H2AX-/- and H2AX+/+ MEFs were treated with 2 μM AG1024 or vehicle control and collected 48 h later for western analyses.Type to enter text

shown). These data strongly suggested that increased sCLU expression in genomically unstable cells were regulated by the same IGF-1/IGF-1R/SRC/ MAPK/ERK signaling pathway noted in response to DNA damage induced by various cytotoxic agents (Criswell, Beman et al. 2005).

We then examined sCLU expression in a variety of other genetically matched genomically unstable cells. As in H2AX-/- MEFs, higher basal expression of sCLU was noted in other genomically unstable cell lines. sCLU expression was constitutively elevated in MDC1-/- MEFs compared to MDC1+/+ MEFs (Figure 3.5A). MDC1 is a DNA repair adaptor protein, linking H2AX and ATM by its BRCT and FHA domain, respectively, and acts as an enhancer in DNA damage signaling, promoting accumulation of ATM and H2AX phosphorylation at sites of DNA breaks, and localization of cell-cycle checkpoint factors (Lou, Minter-Dykhouse et al. 2006). MDC1-/- MEFs have defects in intra-S phase checkpoint regulation after DNA damage and ATM recruitment to DNA breaks, leading to genomic instability. sCLU expression was also elevated in fifth generation MEFs defective for the RNA component of mouse telomerase, mTR (Figure 3.5 B). mTR-/- MEFs are genomically unstable due a failure in these cells to extend telomere ends, ultimately revealing DSBs causing chromosomal rearrangements and end-to-end fusions (Hao and Greider 2004). As with H2AX-/- MEFs, sCLU basal expression was significantly decreased after AG1024 treatment in both

MDC1-/- and mTR-/- MEFs (Figures 3.5 C and D), indicating that IGF-1/IGF-1R signaling was constitutively active in these genomically unstable cells. Genomically unstable NBS1-/- MEFs (Zhu, Petersen et al. 2001), and hMLH1-deficient HCT116 cells unable to perform DNA MMR (Koi, Umar et al. 1994), have increased sCLU expression compared to their genetically matched counterparts (Figures 3.5 E and F). Overall, all genomically unstable cells examined had higher sCLU expression, indicating that the IGF-1-sCLU pathway is constitutively up-regulated in genomically unstable cells.

We next examined whether sCLU expression would be induced in genetically deficient cells in response to IR. sCLU expression was not induced in wild-type or deficient MEFs exposed to 5 Gy that were harvested at 48 h (Figures 3.4 A, 3.5 A and B), however, induction of sCLU was observed in wild-type MEFs 72 h after 5 and 10 Gy exposures. In contrast, MDC1-/- MEFs showed minimal induction (data not shown), strongly suggesting that the constitutively elevated basal level of IGF-1/IGF-1R/CLU signaling in MDC1 -/- MEFs left little capacity for further induction.

sCLU induction following DNA damage is mediated by ATM

In order to determine the upstream signal from DNA to IGF-1-sCLU expression, we examined activation of ATM and indirect activation of ATR in the

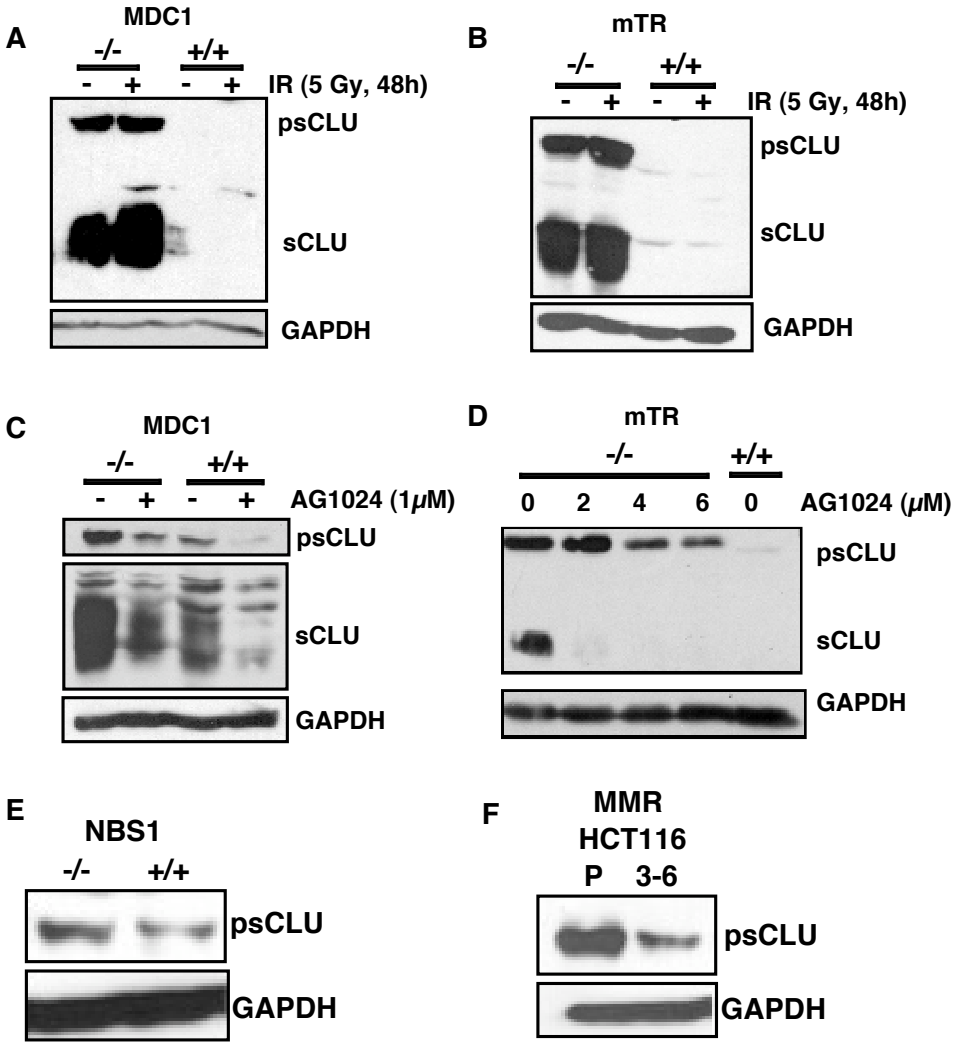

Figure 3.5 sCLU expression is constitutively expressed in genomically unstable cells. ***A, B*** MDC1 (A) or mTR (B) wild type (+/+) or deficient (-/-) MEFs were treated with 5 Gy and harvested 48 h later for sCLU expression by western analyses. ***C.*** MDC1 -/- and MDC1+/+ MEFs were treated with 1 μM AG1024 and collected 48 h later for sCLU expression by western blot analyses. ***D.*** mTR -/- MEFs were treated with 2-6 μM AG1024 and collected 48 h later for sCLU expression by western blot analyses. ***E.*** NBS1 wild-type (+/+) and deficient (-/-) MEFs were analyzed for basal sCLU expression by immunoblotting. ***F.*** HCT116 parental (P) or chromosome 3 expressing (3-6) human colon cancer cells were analyzed for basal sCLU expression by immunoblotting.

genomically unstable cell lines. Wild-type MEFs showed increased phosphorylation of ATM and H2AX following exposure to IR (Figures 3.6 A-C). As expected, both H2AX-/- and MDC1-/- MEFs did not show an increase in γ-H2AX staining (Figures 3.6 A and B, white bars), however both cells showed increased basal and IR-induced levels of $pATM^{S1981}$ (Figures 3.6 A and B, dark bars). mTR-/- MEFs displayed increased basal phosphorylation of ATM and H2AX that was induced after IR (Figure 3.6 C). To examine ATR activity indirectly, we monitored phosphorylation of Chk1 on serines 317 and 345 in the genomically unstable cells; ATR phosphorylates S317 and S345 after DNA damage (Zhao and Piwnica-Worms 2001). There was very little difference in S345 Chk1 phosphorylation ($pChk1^{S345}$), however phosphorylation of S317 on Chk1 ($pChk1^{S317}$) was higher in unstable MEFs compared to their wild-type counterparts (Figure 3.6 D). As a control for the affinity of the anti-$pChk1^{S317}$ antibody, mTR+/+ MEFs were exposed to UV which predominately activates ATR, and phosphorylation of Chk1 was confirmed (Figure 3.6 E). These data suggested that both ATM and ATR activity were elevated in these genomically unstable cells.

Since we noted elevated activation of ATM and ATR in both H2AX-/- and MDC1-/-, as well as mTR-/- and MMR-deficient cells (data not shown, and (Wagner, Li et al. 2008)), we investigated whether ATM or ATR was required for IGF-1/IGF-1R/sCLU signaling. MCF-7 cells were exposed to AAI (an ATM and

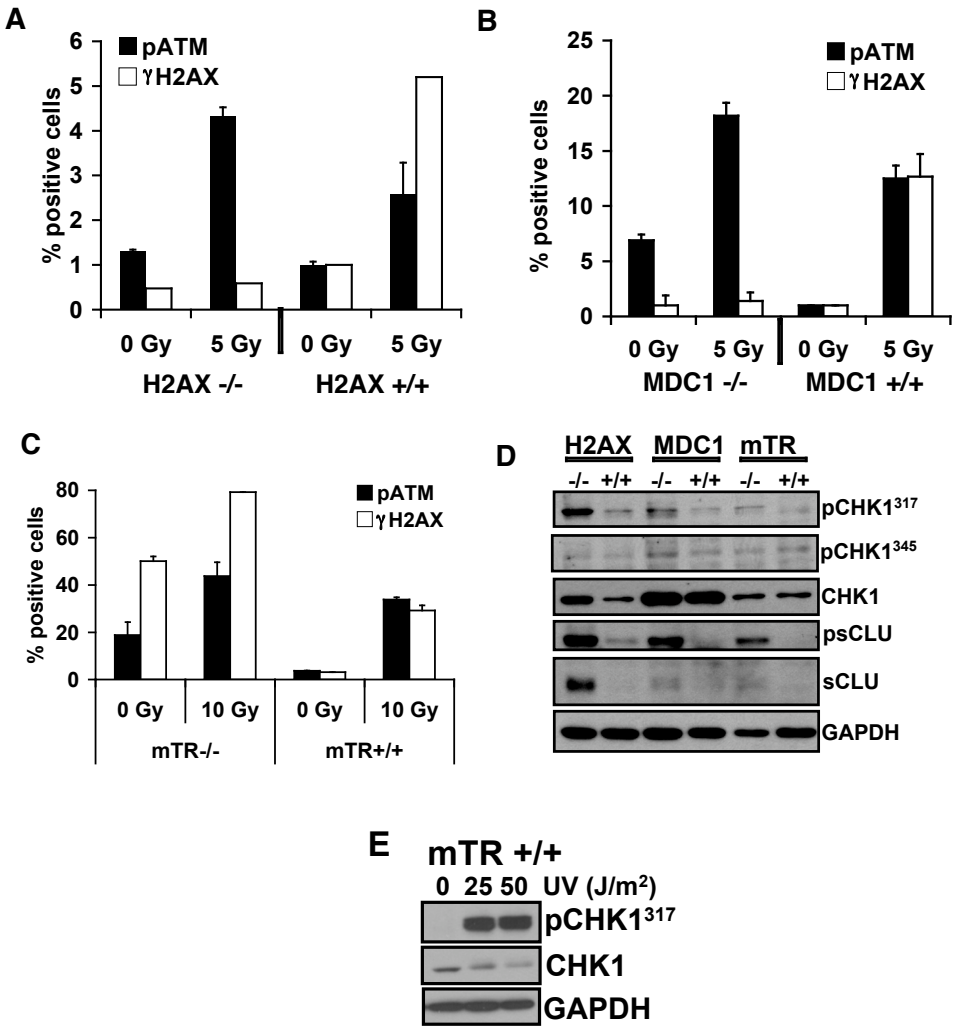

Figure 3.6 ATM and ATR signaling are constitutively activated in genomically unstable cells *A, B, C.* Percentage of cells positive for pATM^{S1981} and γ-H2AX in H2AX. MDC1, or mTR wild-type (+/+) and deficient (-/-) MEFs 1h after 5 Gy. ***D.*** H2AX, MDC1, and mTR wild-type (+/+) and deficient (-/-) MEFs were harvested for analysis of Chk1 phosphorylation by western blotting. Blots were probed with specific antibodies to phoshorylated Chk1 on S317 and S345, sCLU and GAPDH for loading. ***E.*** mTR wild-type cells were treated with 25 or 50 J/m^2 UV and analyzed for pChk1^{S317} by western blotting.

ATR kinase inhibitor (Bentle, Reinicke et al. 2006)), and IR-induced auto-phosphorylation of ATM (pATM^{S1981}) and γ-H2AX was abrogated in human cells (Figure 3.7A). Additionally, AAI was able to inhibit ATM autophosphorylation in MEFs after IR exposure (Figure 3.7A). AAI blocked sCLU protein expression in both genetically unstable cell lines (Figures 3.7 B, C) and induction of sCLU in MCF-7 cells following IR and TPT exposure (Figure 3.8 A). Collectively, these data suggested that either ATM or ATR was the DNA damage sensor kinase upstream of IGF-1/sCLU expression, since blocking ATM and ATR down-regulated its expression.

To determine if ATM or ATR was the major upstream factor for sCLU induction, we directly examined the role of ATM in genetically defined SV40 immortalized, ATM-deficient AT fibroblasts (ATM-/-). Even though ATM deficient cells are genomically unstable, the basal expression of sCLU was equal to or even lower in AT cells compared to genetically matched ATM reconstituted AT fibroblasts (Figures 3.8 B, D). Furthermore, sCLU protein expression was not induced following IR treatment in ATM-/- fibroblasts. In contrast, ATM+ fibroblasts induced sCLU with similar dynamics as noted in other wild-type cells (Figure 3.8 B). In primary AT fibroblasts (AT2052 cells), sCLU expression was not affected by IR exposure (Figure 3.8 C). In contrast, both ATM-/- and ATM+ fibroblasts induced sCLU and phosphorylation of IGF-1R and Akt in response to

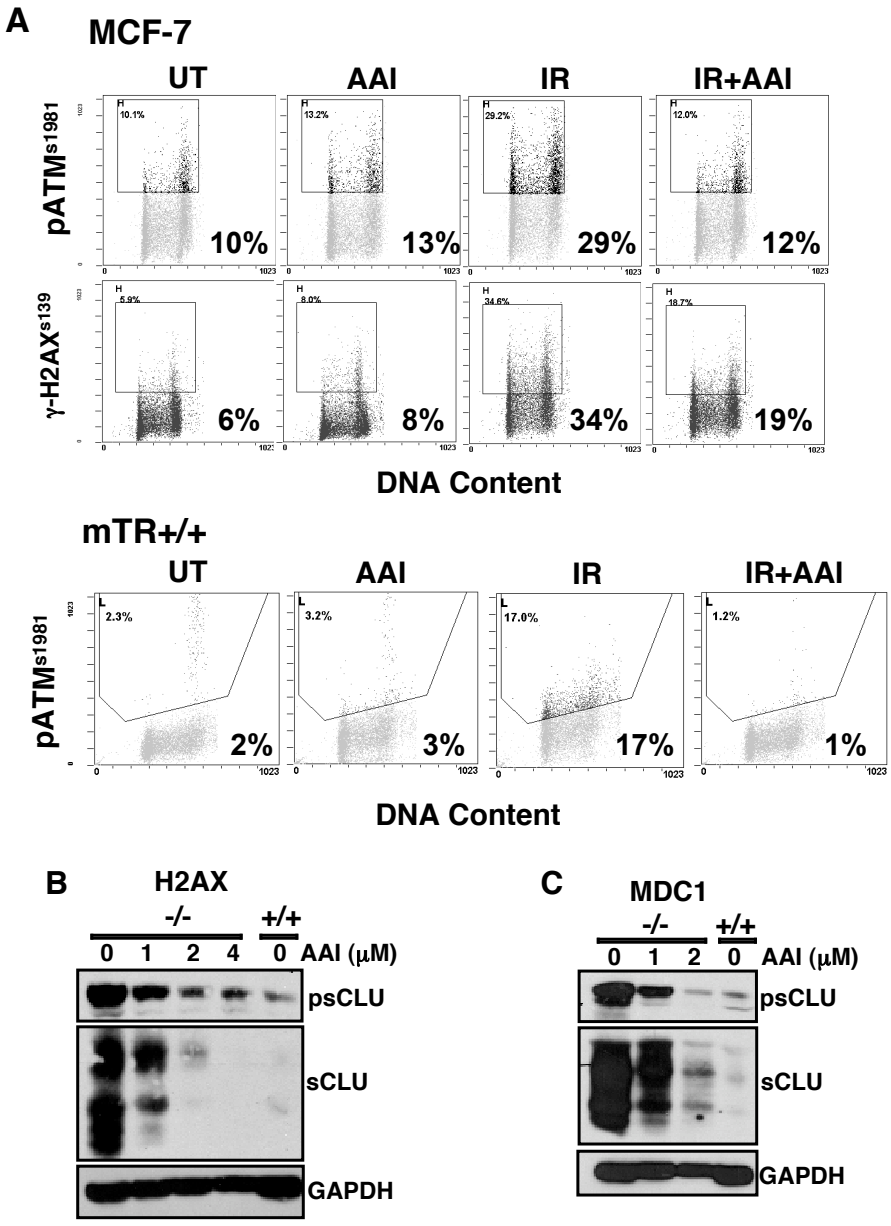

Figure 3.7 ATM signaling is required for sCLU up-regulation ***A.*** MCF-7 or mTR +/+ cells were pre-treated with ATM and ATR inhibitor (AAI) for 1 hour before irradiation. Cells were stained for $pATM^{S1981}$ and γ-H2AX 1 hour after 10 Gy exposure. Positive cells are boxed, and percent staining is indicated in the lower right of each dot plot. ***B.*** H2AX-/- cells were exposed to 1-4 μM AAI for 48 h for analysis of clusterin expression. ***C.*** MDC1-/- MEFs were treated with 1-2 μM AAI for 48 h and sCLU expression was examined by immunoblotting.

IGF-1 exposure (Figure 3.8 D, and data not shown), indicating that the IGF-1 to sCLU signaling axis was intact in the AT cells. Consistent with CLU induction being controlled by the IGF-1/IGF-1R pathway, we noted dramatically lowered basal pIGF-1R, as well as pAKT, in ATM-/- cells (Shankar, BS; data not shown), consistent with previous observations (Peretz, Jensen et al. 2001). These data strongly suggested the importance of ATM in up-regulation of the IGF-1/sCLU expression axis.

We then indirectly explored the role of ATR in IGF-1-sCLU expression by exposing AT fibroblasts or ATM-corrected AT fibroblasts with various doses of UV irradiation. ATR is the major DNA damage sensor activated after UV. Induction of sCLU was observed only in ATM+ cells, indicating that activation of ATR was not sufficient to induce sCLU in AT cells (Figure 3.8 E). Mdm2 accumulation was also noted in both ATM-/- and ATM+ fibroblasts after UV exposure that was independent of p53 (Figure 3.8 E). To confirm that AT cells can activate ATR signaling, pChk1^{S317} was examined 1 h and 48 h after UV exposure. Both ATM-/- and ATM+ fibroblasts phosphorylated Chk1 on S317 after UV (Figure 3.8 E and F), strongly suggesting that ATM was the DNA damage sensor kinase that signaled induction of IGF-1-sCLU expression in genomically unstable cells, and after DNA damage.

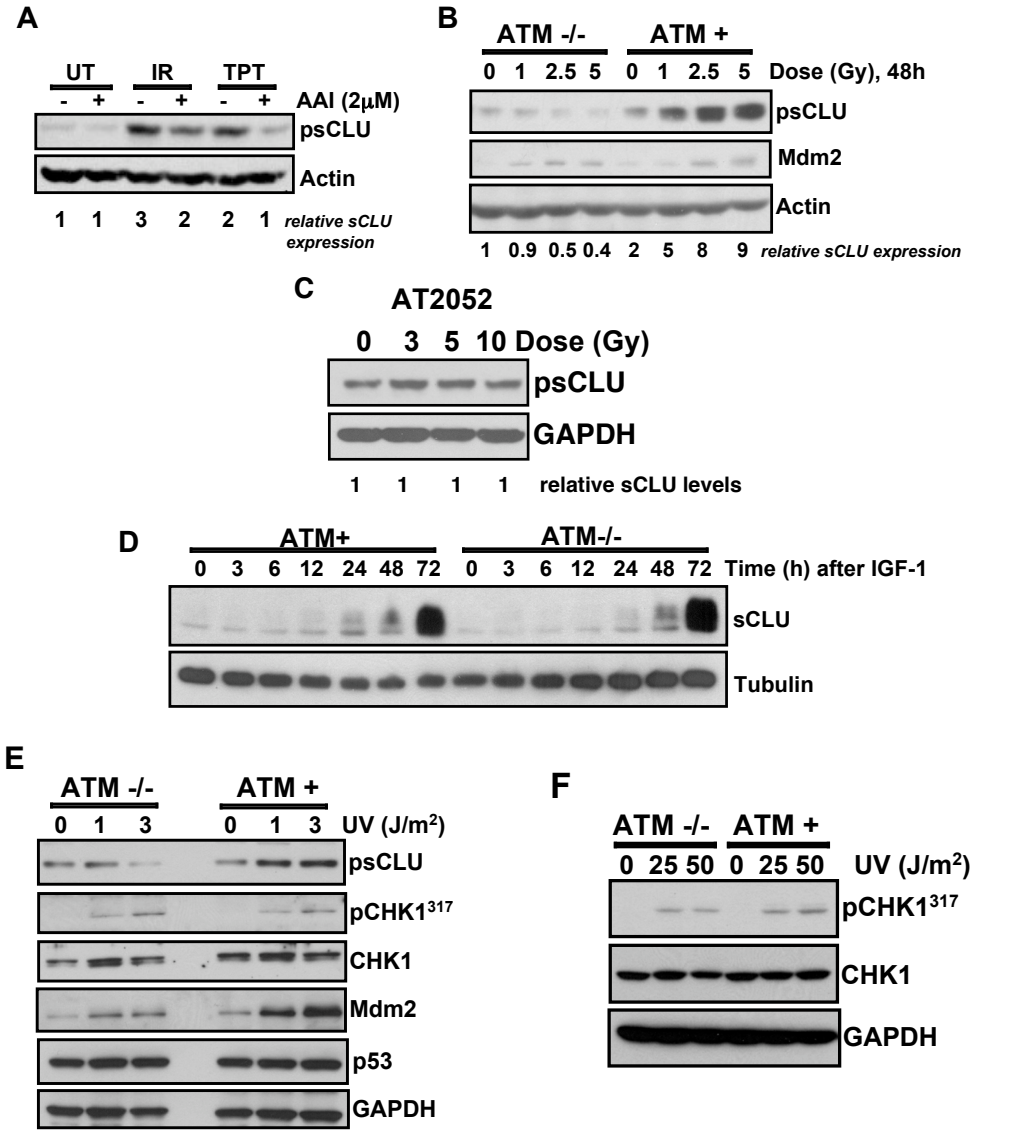

Figure 3.8 sCLU induction after DNA damage is mediated by ATM. ***A.*** MCF-7 cells were pretreated with AAI (2 μM, 1 h) before treatment with IR or TPT. Whole cell lysates were taken 72 h after IR or TPT, and prepared for western analyses of sCLU expression. ***B.*** Immortalized AT cells (ATM-/-) and AT cells reconstituted for ATM (ATM +/+) were treated with 0-5 Gy and analyzed 48 h later for sCLU, and Mdm2 expression by immunoblotting. ***C.*** Primary human AT deficient fibroblasts, AT2052, were treated with 0-10 Gy and analyzed for sCLU expression 72 h later. ***D.*** ATM+ and ATM-/- fibroblasts were treated with IGF-1 (10 ng/ml, 3-72 h) and whole cell extracts analyzed by western blotting.

Figure 3.8 Continued

E. ATM-/- and ATM+ cells were exposed to 1 or 3 J/m^2 UV or mock treated, and harvested for western analysis 48 h later. Blots were probed with antibodies to sCLU, p53, pCHK1^{S317}, total Chk1, Mdm2, and GAPDH for loading. ***F.*** ATM-/- and ATM+ cells were left untreated, or treated with 25 or 50 J/m^2 UV. Whole cell extracts were collected one hour later, and analyzed for pChk1^{S317}, total Chk1, and GAPDH by immunoblotting.

Discussion

Genomic instability is a hallmark of cancer (Lengauer, Kinzler et al. 1998) and can trigger changes that induce a switch from normal to uncontrolled growth. Pathways required for the survival of genomically unstable cells are important for cancer promotion and subsequent progression. Our results reveal, for the first time, that ATM regulates the pro-survival IGF-1-sCLU expression axis up-regulated in response to DNA damage, from either endogenous or exogenous sources. All cytotoxic agents that induced DSBs led to induction of IGF-1-sCLU expression. Importantly, aphidicolin (a DNA polymerase α inhibitor that prevents DNA synthesis) only inhibited TPT-induced sCLU expression, suggesting that formation of DSBs was sufficient to induce IGF-1-sCLU expression; TPT causes DNA SSBs that are converted to DSBs during replication. Additionally, stress-induced sCLU expression was a result of IGF-1/IGF-1R signaling, since treatment with AG1024 blocked sCLU induction, after all cytotoxic agents tested. These data complement our prior studies, where sCLU induction after IR was promoted by IGF-1/IGF-1R/ Src/MAPK signaling that activated Egr-1 to bind and transactivate the CLU promoter (Criswell, Beman et al. 2005), strongly suggesting a common IGF-1/ IGF-1R induction pathway of sCLU after any condition that causes DNA damage. In normal cells, transient up-regulation of the IGF-1-sCLU pathway after DNA damage is beneficial, since this pathway gives a survival advantage to the damaged

cells, however, when this pathway is uncontrolled, such as in genomic instability, constitutive up-regulation of IGF-1-sCLU can promoter cancer.

Our current data strongly suggest that ATM is the DNA damage sensor involved in mediating IGF-1-sCLU expression, however, the mechanism by which ATM stimulates or leads to activation of the IGF-1 promoter is currently unknown (Figure 3.9). Up-regulation of sCLU closely parallels activation of ATM kinase in genomically unstable cells or after DNA damage, and ATM was required for up-regulation of sCLU. MEFs deficient in MDC1, H2AX, and mTR display increased amounts of genomic instability, ATM S1981 phosphorylation, and constitutively elevated basal sCLU expression. Genomically unstable NBS1- and MMR-deficient cells also show increased basal expression of sCLU. Administration of the ATM and ATR inhibitor, AAI, significantly reduced basal expression of sCLU in genomically unstable cells and blocked IR induction of sCLU. Furthermore, Chk1 phosphorylation on S317 was noted in all genomically unstable cells examined. Even though S317 is a major site of phosphorylation by ATR, ATM can also phosphorylate this site within Chk1 (Gatei, Sloper et al. 2003). Immortalized AT fibroblasts did not induce sCLU after IR or UV exposure, while ATM reconstituted AT fibroblasts responded strongly to these cytotoxic agents to induce sCLU expression. Although ATR is the major DNA damage sensor kinase activated after UV, ATM is also activated after UV (Stiff, Walker et al. 2006). These data strongly

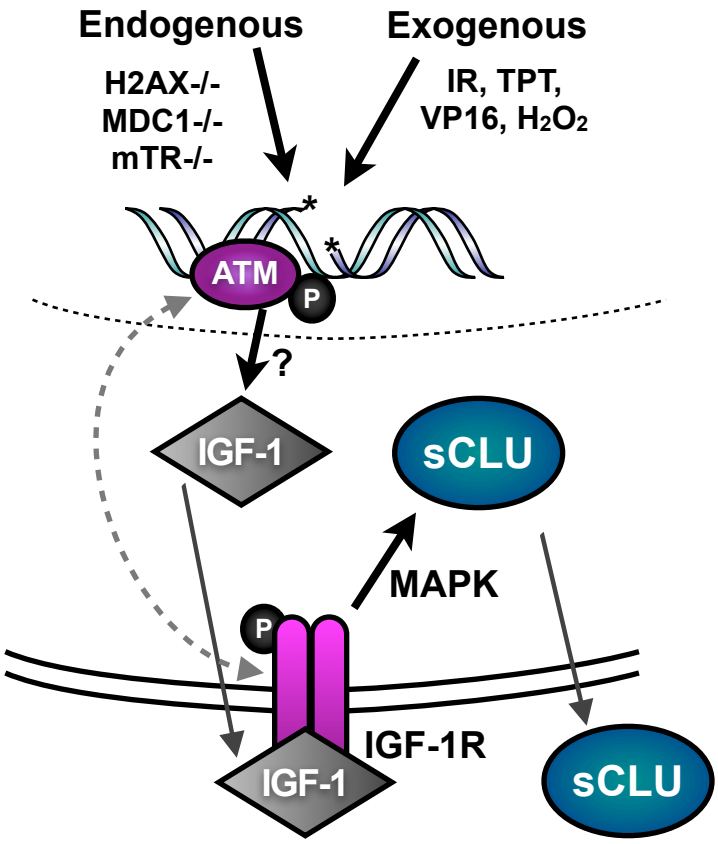

Figure 3.9 Model depicting ATM-dependent induction of sCLU by endogenous and exogenous DNA damage Model depicting induction of sCLU gene expression by DNA damage through activation of ATM. DNA damage caused by endogenous or exogenous means is detected by ATM. Activation of ATM (auto-phosphorylation of S1981), leads to production of IGF-1 ligand by an undefined mechanism. IGF-1 ligand is then secreted from the cell (dotted arrow), activating IGF-1R. Activation of IGF-1R stimulates MAPK/ERK/Egr-1 and eventually sCLU. sCLU is then released from the cell (dotted arrow), where it can have a pro-survival effect. The double-headed dashed gray arrow indicates the known dependency between ATM and IGF-1R.

indicate that that even though ATR signaling is concomitantly activated with ATM, ATM is the major DNA damage sensor kinase upstream of IGF-1-sCLU expression after DNA damage.

Although ATM is clearly required for sCLU up-regulation in response to genotoxic stress, we did observe basal levels of sCLU in AT cells. We propose two possibilities for this finding: i) Basal expression of sCLU is regulated in an ATM-independent manner; ii) Redundancy at the substrate level between ATM and the other PIKK family members, for example ATR and DNA-PKcs, may allow for these kinases to maintain a basal sCLU expression, but are not able to compensate for ATM after DNA-damage in induction of sCLU; or iii) Exogenous IGF-1 in the cell culture media. Further studies using ATR- or DNA-PKcs-deficient cells are ongoing to elucidate their roles in regulating basal sCLU expression and CLU promoter activity.

In previous studies, sCLU was shown as a late induced gene, with peak expression noted at 72-96 h after IR exposure (Criswell, Beman et al. 2005). Consistent with this, IGF-1-mediated induction of sCLU is delayed, coinciding with sCLU induction, starting at 24 hours (Figure 3.8D).

Loss of ATM leads to decreased expression of IGF-1R (Peretz, Jensen et al. 2001), and that loss of IGF-1R leads to decreased activity of ATM (Macaulay, Salisbury et al. 2001). Also, activation of IGF-1R signaling leads to

phosphorlyation of tyrosine and threonine residues on ATM (Suzuki, Kusakai et al. 2004), and we have noted an increase in autophosphorylation of ATM at S1981 after IGF-1 ligand exposure (described in Chapter V). These data indicate a positive feedback loop between ATM and IGF-1R that leads to robust and prolonged sCLU induction. However, this doesn't explain how activated ATM can lead to induction of IGF-1 ligand expression that would be required to initiate the positive feedback pathway. We hypothesize that ATM can activate a transcription factor complex that binds and transactivates the IGF-1 promoter. Amplified in breast cancer 1, AIB1, is a candidate transcription factor that is known to positively regulate IGF-1 expression in cancer cells, however, it is not known whether ATM regulates AIB1 expression or activity. Additionally, ATM may activate a yet unknown transcription factor that regulates IGF-1 ligand expression. Either way, ATM appears to play a major role in regulating the IGF-1-sCLU pro-survival expression axis that is activated after transient genotoxic agent-induced genomic instability or during long-term genomic instability caused by deficiencies in DNA repair factors such as, H2AX, MDC1, NBS1, and hMLH1. Understanding this pathway should allow its exploitation for cancer therapy, as well as for early detection of cancer and cancer prevention.

Besides IGF-1 promoting survival in cells with genomic instability, IGF-1 may also have important implications in cell metabolism. There is a link between

IGF-1 and glucose utilization, and this may promote the cell not only to stay alive, but alter its energy utilization from oxidative phosphorylation to glycolysis (Warburg Hypothesis (Warburg 1956)) during transformation. IGF-1 can increase glucose utilization in the brains of aged animals (Cheng, Reinhardt et al. 2000), and a downstream target of IGF-1, Akt, can stimulate aerob ic glycolysis in cancer cells (Elstrom, Bauer et al. 2004). This may suggest that genomically unstable cells may up-regulate IGF-1 to induce or maintain increased rates of glycolysis, allowing the survival of carcinogenic transformed cells in hostile microenvironments.

A vast majority of syndromes showing genomic instability have deficiencies or alterations in proteins intimately involved in DSB repair. These syndromes include *Ataxia Telangiectasia* (ATM), Nijmegen breakage syndrome (NBS1), AT-like disorder (Mre11), Werner's syndrome (WRN), Bloom's syndrome (BLM), Rothmund-Thompson syndrome (RTS), Fancoi anemia (FANC proteins), Xeroderma pigmentosa (XP proteins), and Cockayne's syndrome (CSA, CSB, XAB2). Individuals with these syndromes often have a pre-disposition to cancer (Duker 2002). Our data strongly suggest the IGF-1-sCLU expression pathway will be up-regulated in all cases of genomic instability, except when ATM is non-functional, protecting genomically unstable cells from cell death, and possibly leading to cancer. As a result of instability, cancer cells exhibit sustained markers

of genomic instability including mutations, alterations in chromosome number, chromosomal translocations, and microsatellite instability. These genetic alterations promote, over time, tumor formation, such as amplifications of oncogenes or deletions of tumor suppressors. Indeed, elevated sCLU expression has been reported as an early marker in genomically unstable adenomatous colon cancer (Stoler, Chen et al. 1999; Shih, Zhou et al. 2001; Chen, Halberg et al. 2003), and we expect that this increase in IGF-1-sCLU expression maintains survival of early genomically unstable cells, and promotes progression of these cancers as well.

Conventional markers of genomic instability consist of alterations in DNA sequence or changes in chromosome number, not readily detected unless DNA is directly analyzed. Thus, a secreted marker of genomic instability may promote early detection of cancer or initiated cells. Additionally, since sCLU is found in all bodily fluids, it is possible that sCLU could be used as a marker of genomically unstable cells in a whole organism. sCLU was up-regulated in the serum of individuals with endometrial adenocarcinoma (Abdul-Rahman, Lim et al. 2007), suggesting that tumor tissue can contribute to blood serum levels of sCLU.

The data presented in this study support the use of sCLU and IGF-1R signaling inhibitors for cancer therapy. IGF-1R antibodies are currently in phase I

and II clinical trials both alone and in combination with standard therapies for a variety of human cancers (Miller and Yee 2005). Also, blocking sCLU expression alone (using siRNA or OGX-011) greatly enhances various cancer therapies. Given the dramatic up-regulation of IGF-1 and sCLU in genomically unstable cells that can be blocked with IGF-1R inhibitors (Figure 3.4 and 3.5), and the sensitization of cells to chemotherapy when IGF-1 signaling or sCLU expression is inhibited (Mitsiades, Mitsiades et al. 2004; Gleave and Miyake 2005), we expect that trials combining inhibition of IGF-1 or sCLU with chemotherapies will be more successful than IGF-1 or sCLU inhibition alone. Collectively, our data demonstrate that the IGF-1-sCLU expression axis could be used as a potential biomarker of genomic instability and may provide potential targets for anti-cancer and/or chemo-preventative therapies.

CHAPTER IV: p53 suppresses induction of IGF-1 ligand by ionizing radiation

Abstract

IGF-1 is a potent growth factor that is up-regulated in cancer and enhances cancer risk. Here, the regulation of p53 on IGF-1 induction after IR is described. In p53 null, or knockdown cells, IGF-1 basal expression is higher and inducible by IR. In contrast, matched wild-type p53 expressing cells have lower basal expression of IGF-1 and minimal increases in IGF-1 after IR. Suppression of the IGF-1 promoter is mediated by interaction of p53 with the NF-Y transcription factor complex. p53 binds to NF-YA, and NF-YA-p53 complex binds to, and transcriptionally suppresses, the IGF-1 promoter. Transfection of DNA-binding domain mutants of p53 do not suppress IGF-1 promoter activity compared to wild-type p53. Treatment of wild-type p53 cells with Nutlin-3, a p53-Mdm2 inhibitor, caused dramatic decreases in sCLU protein expression, while having no effect on p53 knockout or knockdown cells. Nutlin-3 exposure also resulted in radiosensitization of HCT116 wild-type p53 cells. These findings explain the observed suppression of sCLU by p53, since IGF-1 signaling is upstream of sCLU, and supports the use of IGF-1/IGF-1R inhibitors for cancer therapy.

Introduction

Downstream signaling initiated after insulin-like growth factor-1 (IGF-1) stimulation is well studied, but the regulation and secretion of the IGF-1 ligand by cancer cells, with or without cell stress, is not known. In the body, IGF-1 is produced by endocrine regulation of growth hormone (GH), which stimulates secretion of IGF-1 from the liver. However, mice with a liver-specific deletion of IGF-1 still have 30% circulating ligand levels. These mice have normal growth and development, indicating the importance for IGF-1 production from tissues besides the liver (Yakar, Liu et al. 1999). Furthermore, IGF-1 availability in the body is regulated by IGF-1 binding proteins (i.e., IGFBP1-6). IGFBPs prolong the half-life of IGF-1 in the blood, allowing its delivery throughout the body, while also modulating the availability of IGF-1 to its receptor (IGF-1R) and mediating its proliferative effects (Jones and Clemmons 1995).

Since IGF-1 is a growth factor, it can be predicted that tumors increase IGF-1 secretion and signaling to promote growth. Consistently, many different types of cancer have increased IGF-1 ligand expression (Minuto, Del Monte et al. 1986; Tricoli, Rall et al. 1986; Hankinson, Willett et al. 1998; Harman, Metter et al. 2000). Loss of IGF-1 in liver-specific deletion mice, resulted in a reduction of breast cancer growth and metastasis in two different models (Yakar, Pennisi et al. 2005), suggesting the importance of IGF-1 in cancer initiation and progression.

IGF-1 stimulated proliferation and migration of intestinal fibroblasts (Simmons, Pucilowska et al. 1999), explaining its role in tumor metastasis. Moreover, IGF-1 signaling can contribute to treatment resistance for a variety of cancers (Grimberg 2003). IGF-1R is also upregulated in cancer (Pollak, Perdue et al. 1987; Hellawell, Turner et al. 2002; Law, Habibi et al. 2008). Currently, there are several phase I and II clinical trials using IGF-1R antibodies alone, or in combination with standard chemotherapy (Rowinsky, Youssoufian et al. 2007; Lacy, Alsina et al. 2008; Descamps, Gomez-Bougie et al. 2009). There are also several IGF-1R chemical inhibitors in clinical development (Haluska, Carboni et al. 2006; Mulvihill, Ji et al. 2008; Zimmermann, Wittman et al. 2008). Additionally, strategies to target IGF-1 ligand expression with specific antibodies (Goya, Miyamoto et al. 2004) or growth-hormone antagonists (Divisova, Kuiatse et al. 2006) are in development. To date, stress-induced regulation of IGF-1 ligand by tumor suppressors (e.g., p53) have not been described.

Like IGF-1, sCLU is another pro-survival factor that is stress-inducible. sCLU protein expression is induced after various agents, including exposure to chemotherapeutic agents (Miyake, Nelson et al. 2000) and IR (Boothman, Meyers et al. 1993). Basal sCLU expression is constitutively elevated in many human cancers, including breast, prostate, lung, colorectal and others (Steinberg, Oyasu et al. 1997; Redondo, Villar et al. 2000; Chen, Halberg et al. 2003; Miyake, Hara et

al. 2003; July, Beraldi et al. 2004). Furthermore, overexpression of sCLU renders cancer cells resistant to doxorubicin, cisplatin, and taxol (Miyake, Chi et al. 2000; Miyake, Hara et al. 2003), and siRNA-mediated knockdown of sCLU leads to sensitization to paclitaxel and IR (Criswell, Beman et al. 2005; So, Sinnemann et al. 2005). As a result of these data, antisense to sCLU, OGX-011, is in phase II clinical trials in combination with chemotherapeutic agents for a variety of cancers (So, Sinnemann et al. 2005; Chi, Siu et al. 2008).

Our lab demonstrated that after IR, sCLU was induced from 5-to 40-fold in MCF-7 cells 24-72 h post-treatment, and this induction process was mediated through IGF-1R/MAPK signaling. This signaling culminated in the transactivation of the human CLU promoter by activation of the Egr-1 transcription factor (Criswell, Beman et al. 2005). The upstream activation of the IGF1/MAPK signaling pathway was subsequently shown to involve the activation of *Ataxia telangiectasia mutated* (ATM) kinase (Chapter III). Additionally, our lab demonstrated that sCLU mRNA and protein expression were suppressed by p53, but the exact mechanism of suppression remained undefined (Criswell, Klokov et al. 2003).

The suppression of IGF-1 promoter transactivation was found to be mediated by a p53-NF-Y complex that binds a -438 bp consensus site within the IGF-1 promoter. Expression of wild-type p53 suppressed basal IGF-1 expression

and induction after IR. In contrast, DNA binding domain mutants of p53 lacked the ability to suppress the IGF-1 promoter. Nutlin-3, a potent inhibitor of p53-Mdm2 interaction decreased basal and IR-induced sCLU expression, and sensitized wild-type p53 cancer cells to IR. These data reveal new signaling and mechanistic data that can be exploited for radio-sensitization of normal versus tumor cells to radiotherapy.

Materials and Methods

Cell lines PC-3 and PC-3 cells expressing wild-type p53 were a generous gift from Drs. G. Stark and M. Jackson (Case Western Reserve University, Cleveland OH). HCT116 cells were obtained from ATCC. HCT116:3-6 cells were a kind gift from Dr. R. Boland (Yan, Schupp et al. 2001). HCT116:3-6 cells and RKO7 (RKO cells stably expressing MLH1) were knocked down for p53 using an shp53 SUPER lentiviral vector (Li, Morales et al. 2008). HCT116 p53-/- and HCT116 p21-/- cells were kindly provided by Dr. B. Vogelstein (Waldman, Kinzler et al. 1995; Bunz, Dutriaux et al. 1998). Human bronchial epithelial cells, immortalized by viral transduction of Cdk4 and hTERT (HBEC 3kt) and stably infected with small hairpin p53 (shp53) or the R273H p53 mutant, were generously provided by Dr. J. Minna (Sato, Vaughan et al. 2006). PC-3, RKO, and HCT116 cell lines were maintained in 5% FBS containg DMEM (Hyclone) in a 10% CO_2, 90% air

incubator as described (Criswell, Klokov et al. 2003). HBECs were maintained in KSFM defined media (Gibco) as described (Sato, Vaughan et al. 2006).

Plasmids The CLU-LUC promoter (-4250 to +1 base pairs of the human CLU promoter fused to luciferase) was cloned by us (Criswell, Beman et al. 2005). The IGF-1-LUC promoter (-1630 to +322 base pairs of the human IGF-1 promoter fused to luciferase) was obtained from Dr. P. Rotwein (Mittanck, Kim et al. 1997). The p21-promoter-luciferase (p21-LUC) was obtained from Dr. B. Vogelstein (el-Deiry, Tokino et al. 1993). The flag-tagged CMV-p53 cDNA was created by subcloning p53 cDNA into PCDNA3.1-N-term-Flag construct.

Site Directed Mutagenesis Site-directed mutagenesis of p53 cDNA and the IGF-1 promoter was performed using PCR-based mutagenesis. Briefly, complimentary primers containing mutations were synthesized (IDT), and care was taken not to introduce new transcription factor binding sites in the IGF-1 promoter. Forward primers used in PCR reactions with full-length plasmid DNAs were (backward primers were reverse complement):

R175H: 5'-GTTGTGAGGCACTGCCCCCACCATGAG-3'

R248Q: 5'-CGGCATGAACCAGAGCGGCATCCTCAC-3'

R248W: 5'-CGGCATGAACTGGAGGCCCATCCTCAC-3'

R273C: 5'-CAGCTTTGAGGTGTGTGTTTGTGCCTG-3'

R273H: 5'-CAGCTTTGAGGTGCATGTTGTGCCTG-3'

S15A: 5’ -GAGCCCCCTCTGGCTCAGGAAACATTTTCA-3’

S15D: 5’-GAGCCCCCTCTGGATCAGGAAACATTTTCA-3’

S15E: 5’-GAGCCCCCTCTGGAACAGGAAACATTTTCA-3’

S20A: 5’-CAGGAAACATTTGCAGACCTATGGAAACTACTTC-3’

S20E: 5’-CAGGAAACATTTGAAGACCTATGGAAACTACTTC-3’

IGF-1 NFY mut: 5’- GCCCTAAAGGGATACATCCAATGCTGCCTG CCCCTCC - 3'

After PCR, products were digested with DpnI, and then transformed into MaxEfficiency DH5α competent *E. coli* (Invitrogen). Plasmids were isolated and sequenced for correct mutation.

Luciferase Assays Cells (5 x 10^4) were plated in 12-well dishes and left overnight. The next day, cells were transiently transfected in triplicate using Fugene 6 (Roche) with indicated promoter-luciferase constructs, each with an appropriate transfection control: SV40-RL (renilla luciferase) or SV40-β-Gal (β-Galactosidase). After transfection (24 h), cells were treated with either AG1024/ DMSO and/or mock or IR treated and luciferase activities were monitored 48-72 h after treatment. For p53 co-transfections, IGF-1-LUC or CLU-LUC was transfected with 5-50 ng of p53 (wild-type or mutant flag-tagged-cDNA), which was not shown to alter cell cycle or cell death responses. Luciferase activities were measured using the Dual Luciferase Assay Reagent (Promega), or Luciferase

Assay Reagent (Promega) and Galacto-Star Reagent (Applied Biosystems). Data were graphed as mean +/- standard deviation (SD). Statistical significance was calculated using paired Student's t-tests.

RT-PCR Total RNA was harvested from approximately 1 x 10^6 cells using RNAeasy kit (Qiagen). mRNA was converted to cDNA using the cDNA Archive Kit (Applied Biosystems) and IGF-1 and actin cDNA were amplified using specific primers. Amplification was monitored by agarose gel electrophoresis. Experiments were performed at least three times. Primers are as follows:

IGF-1 forward: 5'-AACACCATCCATTTGGGAAA-3'

IGF-1 reverse: 5'-TGACATATTGCCCCCATTTT-3'

β-actin forward: 5'-GGACTTCGAGCAAGAGATGG-3'

β-actin reverse: 5'-AGCACTGTGTTGGCGTACAG-3'

ELISA Cells were plated in 6-well plates or 10-cm dishes and media was collected and stored at -80°C. IGF-1 in the media was measured using sandwich ELISA. The capture antibody, MAB291, and the detection antibody, BAF291, were obtained from R&D. The capture antibody was incubated overnight in 96-well plates, and then plates were blocked with PBS containing 5% sucrose and 5% Tween-20. 100 µL of media was added to each well overnight. Next, the biotinylated detection antibody was placed in the wells, followed by streptavidin HRP. The concentration of IGF-1 was determined by comparing to a standard

curve, and then normalized to cell number. All samples were run in triplicate, and data graphed are the mean +/- SD. Statistical significance was calculated using a paired Student's t-test.

Western Blotting Cells (2.5-5 x10^5) were plated in 10-cm dishes and treated or not with IR, AG1024 (EMD) or Nutlin-3 (Sigma) and harvested in RIPA buffer (0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris). Cell lysates were probed with Mdm2 Ab-2, GAPDH, p21/WAF1 from EMDBiosciences and sCLU B-5, p53 DO-1, NF-YA were from Santa Cruz. Flag-M2 antibody was from Sigma. All experiments were performed at least three times.

Chromatin Immunoprecipitation (ChIP) Briefly, cells were fixed in 1% formaldehyde, and then quenched with 1.25 mM Glycine. Cells were harvested in lysis buffer (50 mM Tris pH 8, 2 mM EDTA, 150 mM NaCl, 1% Triton-100, 0.1% DOC, 0.1% SDS) and sonicated to shear chromatin (Zhou and Chiang 2002). Sonicated chromatin was incubated overnight with antibodies (p53, NF-YA, NF-YB, NF-YC (Santa Cruz)), and protein A/G beads were added the next day. Beads were washed in lysis buffer, high salt buffer (Lysis buffer with 500mM NaCl), LiCl salt buffer (50 mM Tris pH 8, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% DOC), and two times with Tris-EDTA pH 8. Chromatin was eluted from the beads, cross-links reversed, and the DNA was purified. Experiments were

performed at least three times. PCR was performed using the following primers to amplify the IGF-1 and p21-promoters:

IGF-1 Forward: 5'-TCTATTTCAGTTGGGTTTTACAGCT-3'

IGF-1 Reverse: 5'-CTCACTAGTGCTTCTGAAGTACAAAG-3'

p21 Forward: 5'-CGACTCTTGTCCCCCAGGCT-3'

p21 Reverse: 5'-GGTCTCCTGTCTCCTACCAT-3'

Colony Formation Assay HCT116, HCT116 p53-/-, and HCT116 p21-/- cells were plated so approximately one hundred individual cells would be plated in 60-mm dishes before treatments. The next day, cells were pretreated or not with 4 μM Nutlin-3 one hour before IR. Cells were allowed to grow for 12 days, with media replacement every 3-4 days. After 12 days, colonies were stained and fixed with a methanol crystal violet solution. Colonies that contained more than 100 cells were counted as positive. Experiments were performed in triplicate and data graphed are the mean +/- standard deviation. Statistical significance was calculated using a paired Student's t-test.

Flow Cytometry To analyze the cell cycle, cells were harvested by trypsin, washed, and immediately incubated in cold 100% ethanol overnight. The following day cells were washed with PBS, then incubated with PI staining solution (50 μg/mL propidium iodide, 2% FBS, 100 μg/mL RNaseA in PBS) for 2 hours to overnight,

and then analyzed by flow cytometry for cell cycle. Experiments were performed at least three times.

For cell viability experiments, HCT116 p53-/- cells were co-transfected with equal amounts of CMV-GFP and wild-type p53, mutant p53, or vector cDNA using Fugene 6. After transfection (24 h), GFP transfected cells were treated with H_2O_2 (1.2 mM, 1 hour) or staurosporine (10 μM, 1 hour) as controls. Both floating and attached cells were harvested for flow cytometry analysis. Cells were washed in PBS, then incubated with PI (50 μg/mL) in PBS for 30 minutes, then analyzed by flow cytometry and gated for GFP and PI staining.

Results

p53 protein expression negatively regulates IGF-1

The IGF-1 promoter fused to luciferase (IGF-1-LUC) was transfected into various cell lines with varying p53 expression. In all cells examined, expression of wild-type (WT) p53 led to lower IGF-1 promoter activity compared to null, knockdown, or mutant p53 expressing cell lines. p53 null PC3 prostate cancer cells had higher IGF-1-LUC activity than PC3 cells stably expressing WT p53 (Figure 4.1 A). Scrambled-control, WT p53 expressing RKO7 colon cancer cells (RKO7 SCR), had lower IGF-1-LUC activity compared to cells stably expressing small hairpin knockdown of p53 (RKO7 shp53). WT p53 HCT116 colon cancer cells had lower IGF-1-LUC activity compared to p53 knock out cells (HCT116 p53-/-). Comparably, HCT116:3-6 cells stably knocked down for p53 (HCT116:3-6 shp53) had higher IGF-1-LUC activity compared to the parental wild-type p53 cells (Figure 4.1A). Human bronchial epithelial cells (HBEC, wild-type p53) stably infected with shp53 or R273H mutant p53 (mp53) were transfected with IGF-1-LUC. HBEC cells transfected with shp53 had an approximately 2-fold increase in IGF-1-LUC activity, consistent with small-hairpin knockdown of p53 in RKO7 and HCT116:3-6 cells. HBEC cells with R273H dominant negative p53 had an even higher IGF-1-LUC activity (Figure 4.1 A). These data indicated that

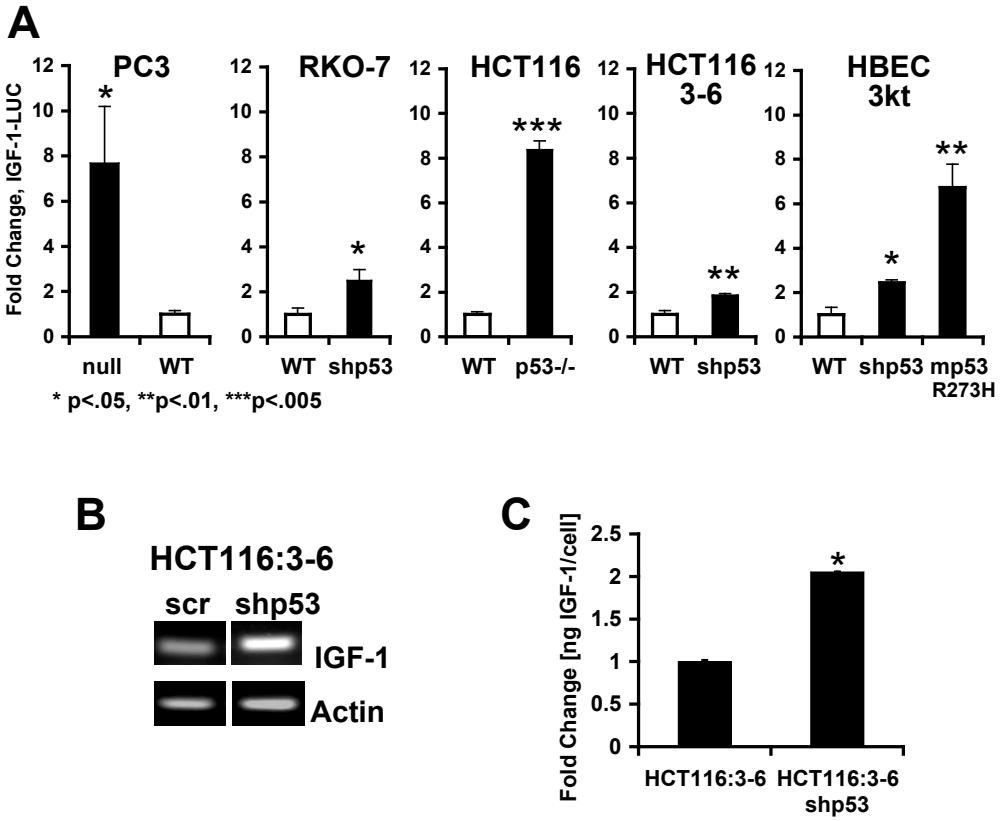

Figure 4.1 p53 suppresses IGF-1 ligand. ***A*,** PC3, PC-3+p53, RKO7 SCR, RKO7 shp53, HCT116, HCT16 p53-/-, HCT116:3- 6, HCT116:3-6 shp53 and human bronchial epithelial cells (HBEC WT, shp53, and mutant p53 (mp53)) were transiently transfected with IGF-1-LUC and RSV-β-Gal and monitored for luciferase expression 72 hours after transfection. ***B,*** RNA was collected from HCT116:3-6 and HCT16:3-6 shp53 cells and subjected to reverse-transcription PCR to amplify IGF-1 and Actin mRNA. ***C,*** Media was collected from HCT116:3-6 and HCT116:3-6 shp53 cells and analyzed for IGF-1 concentration by ELISA.

expression of WT p53 led to decreased expression of the IGF-1 promoter.

Next, basal IGF-1 mRNA and protein expression in scrambled control HCT116:3-6 (SCR) and HCT116:3-6 shp53 cells was examined. Both IGF-1 mRNA and IGF-1 ligand concentration was higher in HCT116:3-6 shp53 cells (Figure 4.1 B and C). These data indicated that IGF-1 expression was negatively regulated by p53, and suggested that IGF-1 expression may be regulated by a dose-dependent suppression by p53 since p53 knockout or null cells have ~8 fold increase in IGF-1 expression, compared to a ~2 fold in increase in p53 knockdown cells.

p53 suppresses IGF-1 induction after IR

IGF-1 was induced after IR exposure (Chapter III) and p53 suppressed sCLU induction after IR (Criswell, Klokov et al. 2003), however, it was unknown whether p53 suppressed IGF-1 induction after IR. RKO7 SCR and RKO7 shp53 cells were transiently transfected with IGF-1-LUC, and analyzed luciferase activity after IR. IGF-1-LUC activity was induced in a dose dependent manner after IR exposure in p53 knock down cells (Figure 4.2 A), coinciding with release of IGF-1 into the cell culture media (Figure 4.2 C). Additionally, IGF-1-LUC activity corresponds with downstream CLU promoter activity (CLU-LUC) and protein expression (Figures 4.2 B, D).

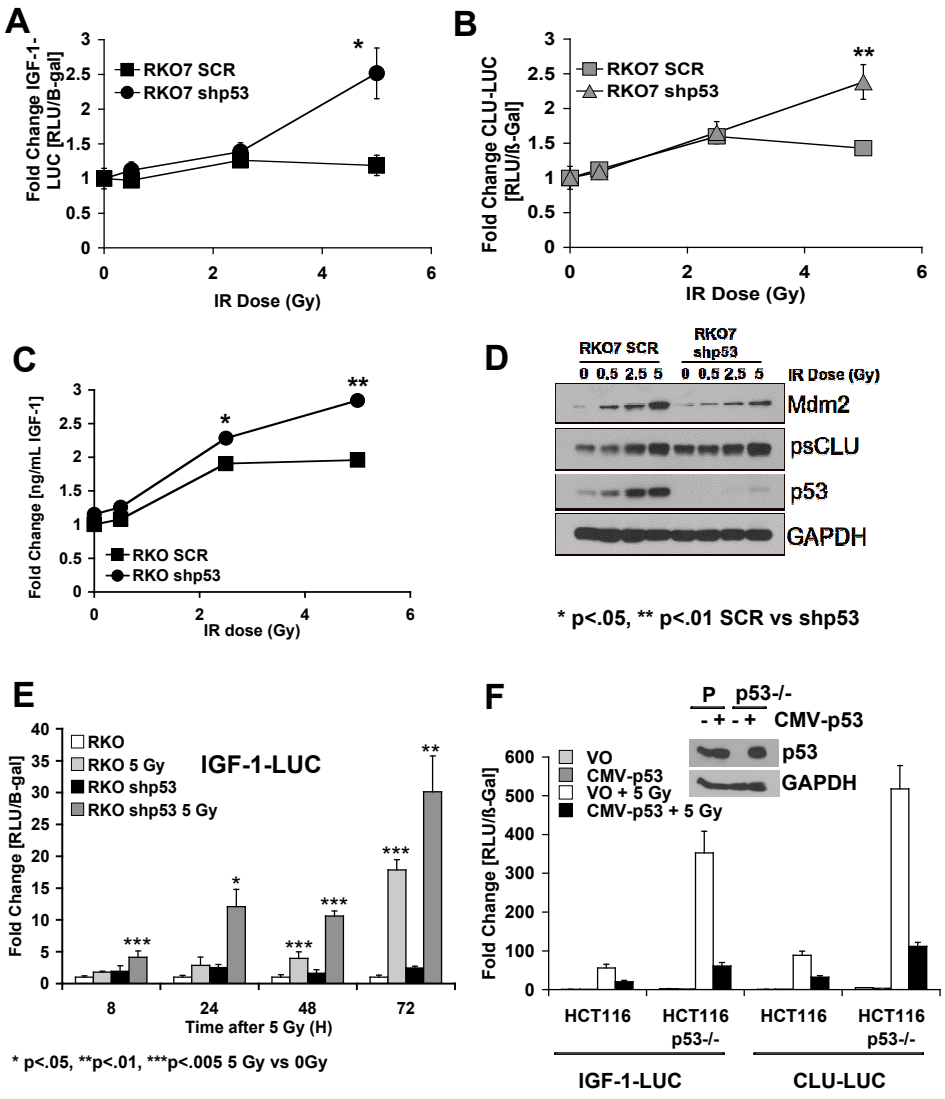

Figure 4.2 IGF-1 is induced by IR in p53 knockdown cells. ***A,B,*** RKO7 SCR and RKO7 shp53 cells were transiently transfected with IGF-1-LUC (*A*) or CLU-LUC (*B*) and RSV-β-Gal as a transfection control. Cells were irradiated 24 hours after transfection and analyzed for luciferase activity 72 h later. Activity is normalized with 0 Gy for each cell line. */**: RKO7 SCR versus RKO7 shp53. ***C,*** RKO7 SCR and RKO7 shp53 cells were irradiated and the media was collected at 72 h after IR and subjected to ELISA analysis. Data are normalized to unirradiated RKO7 SCR cells. */**: RKO7 SCR versus RKO7 shp53.

Figure 4.2 Continued

D, RKO7 SCR and RKO7 shp53 cells were irradiated at 0-5 Gy, harvested 72 h after IR, and subjected to SDS-PAGE. Blots were probed for Mdm2, sCLU, p53 and GAPDH for loading. . ***E,*** RKO7 SCR and RKO7 shp53 cells were transiently transfected with IGF-1-LUC and RSV-β-Gal and then exposed to 5 Gy 24 h later. Luciferase activity was monitored 8-72 h after IR exposure. Data are normalized to untreated RKO7 SCR cells. */**/***: 0 Gy vs 5 Gy ***F,*** HCT116 and HCT116 p53-/- cells were co-trasfected with wild-type p53 expression plasmid (CMV-p53, 5 ng) and IGF-1-LUC or CLU-LUC. Twenty four hours later cells were either mock or IR treated. Luciferase activity was measured 48 h after IR and samples were normalized to mock treated, VO transfected HCT116 cells. Inset shows immunoblotting of lysates to confirm p53 expression.

Next, induction of IGF-1 promoter activity was examined at specific intervals after IR. Beginning at 8 h, induction of IGF-1-LUC was observed in RKO7 shp53 cells, and continued until 72 h after IR exposure (Figures 4.2 E), similar to sCLU (Criswell, Klokov et al. 2003). Induction of IGF-1 promoter activities was noted in RKO7 SCR cells, however the extent of induction was greater when p53 was knocked down. HCT116 and HCT116 p53-/- cells were transiently transfected with flag-tagged WT p53 cDNA (CMV-p53) and the IGF-1-LUC or CLU-LUC plasmid. Transient transfection of WT p53 lowered both basal and IR induced activity of the IGF-1 and CLU promoters (Figure 4.2 F). Consistent with the RKO7 cell system, induction of IGF-1 and CLU promoter activity after IR was dependent on p53. Both IGF-1 and CLU basal and IR-induced promoter activity was lower in HCT116 cells and HCT116 p53-/- transfected with WT p53 (Figure 4.2 F). While induction of IGF-1 and CLU was observed in HCT116 cells, the fold induction was much higher in the HCT116 p53-/- cells. These data indicated that endogenous and exogenous expression of WT p53 suppressed both IGF-1 and downstream CLU induction after IR.

DNA binding domain mutants abrogate p53 suppression of IGF-1 and sCLU

Next, the function of Ser 15 and 20 phosphorylation, the major phosphorylation sites on p53 after IR, was analyzed for suppression IGF-1 and

CLU. ATM and ATM activated proteins, such as Chk2, phosphorylate p53 at serines 15 and 20 after IR exposure, however, other factors can phosphorylate these sites after other insults, as well. Phosphorylation of serine 15 and 20 is thought to play a role in its stabilization after IR (through interaction with Mdm2) and enhance its transactivation, however, the importance of these sites has recently come into question (Shieh, Ikeda et al. 1997; Dumaz and Meek 1999; Thompson, Tovar et al. 2004). HCT116 p53-/- cells were transiently transfected with wild-type p53 (WT), or mutated p53 constructs containing serine 15 to alanine (S15A), aspartate (S15D), or glutamate (S15E) mutations. Mutation of serine 15 to alanine makes p53 non-phosphorylatable, and mutation of serine to aspartate or glutamate makes p53 a phospho-mimetic. In addition, serine 20 was mutated in the same manner, to alanine or glutamate. All the serine 15 and 20 mutants suppressed IGF-1 and CLU promoter activity similar to wild-type p53 (Figures 4.3 A, B). These data suggested that phosphorylated p53 does not increase the suppressive action of p53, however, did not discount the importance of these sites for stabilization of p53 after IR.

Finally, p53 mutations that are commonly observed in cancer were analyzed for their ability to suppress IGF-1 and sCLU. p53 cDNA was mutated to the top five p53 mutations in cancer (Hjortsberg, Rubio-Nevado et al. 2008), including, from the most common, R175H, R248Q, R273H, R248W, and R273C.

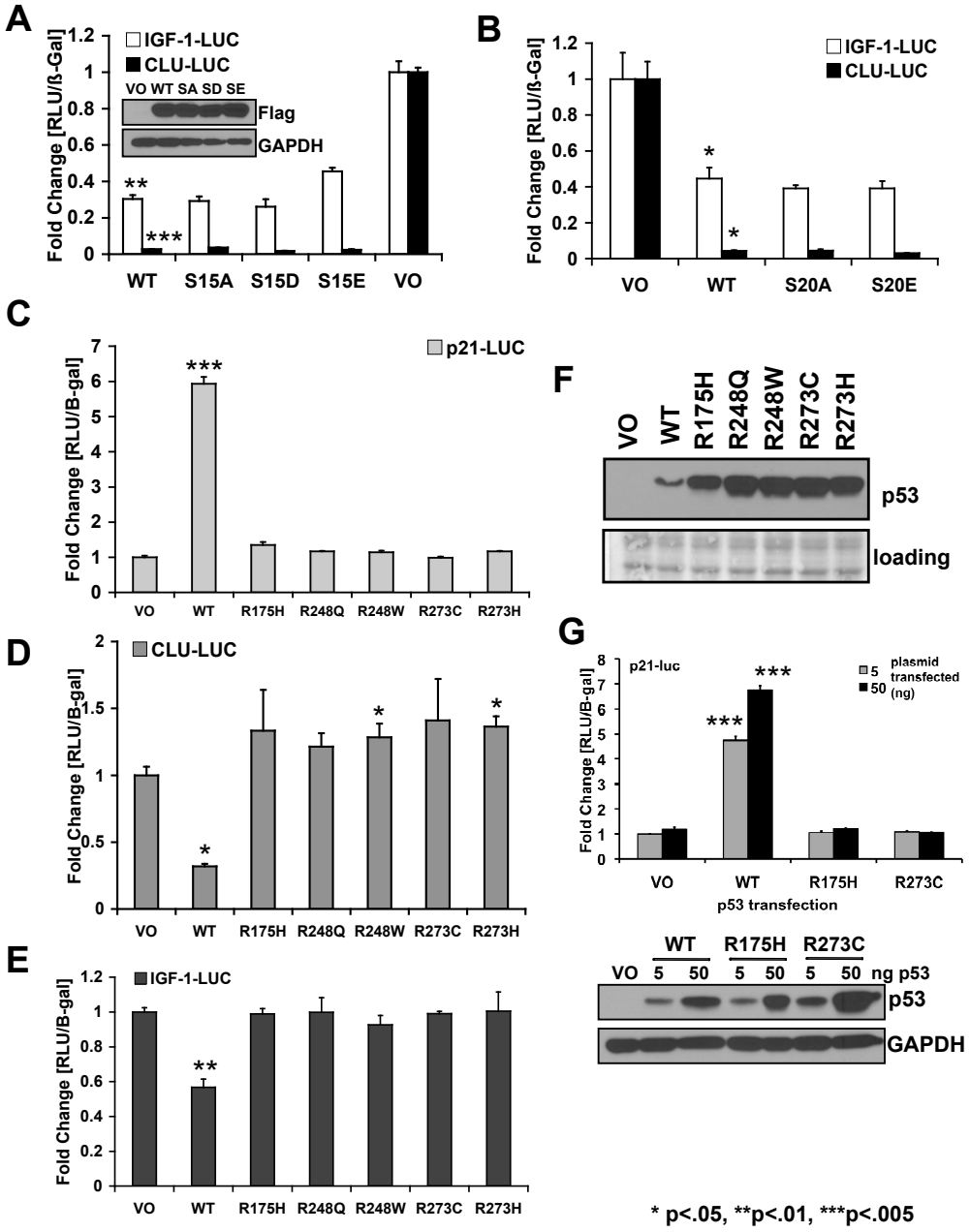

Figure 4.3 DNA binding domain mutants of p53 do not repress IGF-1 and CLU promoter activities

Figure 4.3 Continued

A, B, HCT116 p53-/- cells were co-trasfected with vector only (VO), wild-type p53 CMV expression plasmid (WT), serine 15 mutants (Serine 15 to Alanine (S15A), Aspartate (S15D), or Glutamate (S15E)), or serine 20 mutants (Serine 20 to Alanine (S20A) or Glutamate (S20E)), and IGF-1-LUC or CLU-LUC. Luciferase activity was measured 48 h after transfection and all samples were normalized to HCT116 p53-/- cells transfected with VO. */*/***: VO versus WT transfected. ***C, D, and E,*** HCT116 p53-/- cells were co-trasfected with VO, WT p53, or one of five mutant (MUT) contstructs (R175H, R248Q, R248W, R273C, R273H), and p21-LUC, CLU-LUC, or IGF-1-LUC. Luciferase activity was measured 24 h after transfection and all samples were normalized to VO. */**/***: VO versus WT or MUT transfected. ***F*,** Luciferase extracts from *C* were immunoblotted for p53 and expression. ***G,*** *Top,* HCT116 p53-/- cells were transfected with two different amounts of WT or MUT p53 cDNA and p21-LUC. Cells were harvested 24 h later for luciferase activity, and data are normalized to cells transfected with 5 ng of vector DNA. *Bottom,* lysates used for luciferase assay were run on SDS-PAGE gel and immunoblotted for p53 expression. ***: VO versus WT transfected.

These sites are located in the DNA binding domain of p53, and disrupt p53-DNA binding, and therefore, disrupt the transactivation capability of p53. As a control, WT and mutant p53 constructs were co-transfected with p21-promoter-luciferase (p21-LUC) in HCT116 p53-/- cells. As expected, the mutant p53 constructs did not induce p21-promoter activity compared to WT p53 (Figure 4.3C). Additionally, two different amounts of p53 cDNA were co-transfected into HCT116 p53 -/- cells, and transfection of an additional 45 ng of p53 only minimally increased the induction of p21-LUC (Figure 4.3 G, 5 ng versus 50 ng), indicating that the amount of p53 transfected in Figures 4.3 A-F was appropriate (25 ng). Next, the p53 constructs were co-transfected with IGF-1-LUC and CLU-LUC in HCT116 p53-/- cells. IGF-1 and CLU promoter activity were suppressed by WT p53 (Figures 4.3 D, E), however, the p53 mutants did not suppress IGF-1- or CLU-promoter activity. Expression of p53 was confirmed by western blotting (Figure 4.3 F). These results suggested that cancer cells containing DNA binding domain mutations in p53 will have higher expression of IGF-1 and sCLU protein expression.

To make sure that transient transfection of p53 was not inducing a cell death response resulting in lowered IGF-1 and sCLU expression, HCT116 p53-/- cells were co-transfected with GFP and 10 times the relative amount of p53 cDNA used in the transient luciferase assays and cell viability was measured. As

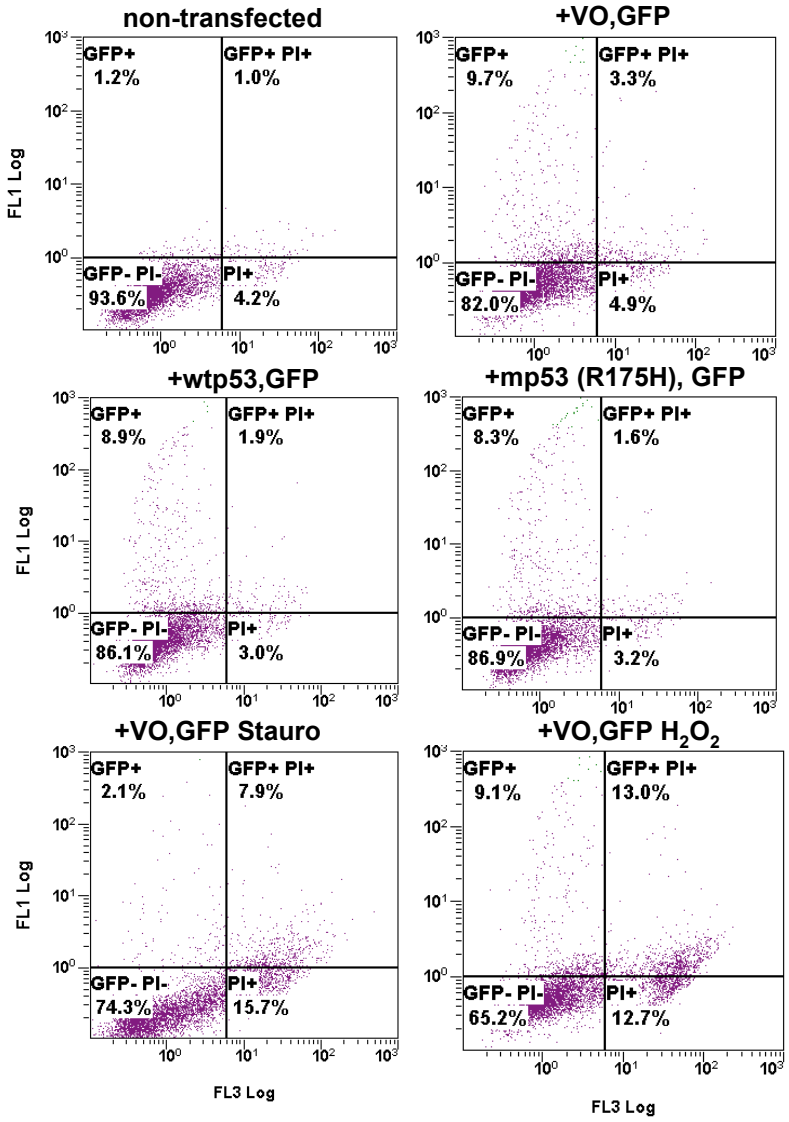

Figure 4.4 p53 transfection does not affect cell viability. HCT116 p53-/- cells were untransfected, or co-transfected with VO, WT p53, or R175H p53 (mp53) and CMV-GFP. As a positive control, GFP-transfected cells were treated with staurosporine (10 μM) or H_2O_2 (1.2 mM) for 1 h to induce cell death. Cells were harvested, immediately stained with PI, and run on flow cytometry.

expected, transfection of GFP resulted in increased detection of green fluoresnce by flow cytometry (Figure 4.4, y-axis). Propidium iodide (PI) was used as an indicator of cell viability; PI is membrane impermeable, and therefore, only stains DNA in non-viable cells. As a positive control, GFP-transfected cells treated with staurosporine or H_2O_2 took up PI (Figure 4.4, upper right quadrant), suggesting cells death. However, PI was not taken up by the VO, WT, or mutant p53 and GFP co-transfected cells (Figure 4.4), suggesting the cells were viable. These results indicated that suppression of IGF-1 and sCLU by p53 was not due to cell death.

IGF-1-R tyrosine kinase inhibitor AG1024 blocks sCLU induction after IR.

To dissect the IGF-1-sCLU pathway, RKO7 SCR and RKO7 shp53 cells were pretreated with AG1024, the IGF-1R tyrosine kinase inhibitor, and analyzed for IGF-1 and CLU promoter activity after IR. Pretreatment with AG1024 blocked CLU-LUC induction in RKO7 shp53 cells after IR, but not IGF-1-LUC activity, as expected (Figure 4.5).

NF-Y mediated transcriptional co-repression of p53.

NF-Y is trimeric transcription factor necessary for p53 to suppress certain genes, such as Cdc2 and Chk2 (Yun, Chae et al. 1999; Matsui, Katsuno et al. 2004). The NF-Y complex binds to DNA, and p53 binds to NF-Y, without p53-

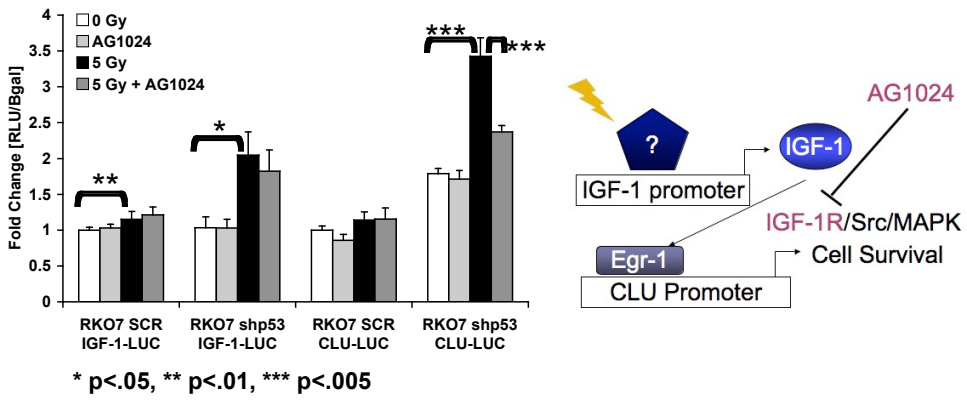

Figure 4.5 AG1024 blocks CLU induction, but not IGF-1 induction, after IR. *Left*, RKO7 SCR or RKO7 shp53 cells were transiently transfected with IGF-1-LUC or CLU-LUC and RSV-β-Gal. The next day cells were pre-treated with AG1024 for 1 h before irradiation at 5 Gy. Cells were harvested 48 h later for luciferase activity, and data normalized to RKO7 SCR cells. *Right*, Schematic indicating where AG1024 acts on the IGF-1-CLU pathway.

DNA contact (Imbriano, Gurtner et al. 2005). To determine the mechanism of p53 suppression of IGF-1 ligand, the IGF-1 promoter was examined for potential p53 regulatory elements and an NF-Y consensus site was found (Figure 4.6 A, B). To determine whether this site was required for p53 suppression, the NF-Y consensus site in the IGF-1 promoter was mutated using site-directed mutagenesis. Transfection of this NF-Y mutant promoter (MUT, Figure 4.6 A) led to an increase in IGF-1-LUC activity in HCT116:3-6 cells (Figure 4.6 C), suggesting that p53 suppression was lost when the NF-Y site was mutated. Next, chromatin immunoprecipitation (ChIP) analyses were performed to examine p53 and NF-YA

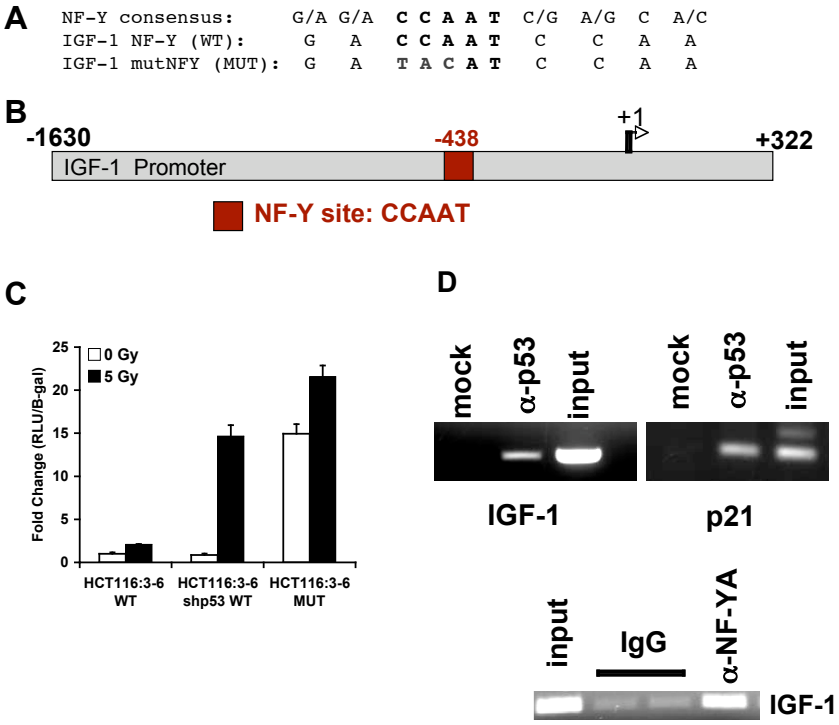

Figure 4.6 NF-Y mediates p53 suppression of the IGF-1 promoter. ***A*****,** Consensus sequence of NF-Y binding site compared to the IGF-1 promoter NF-Y site, and mutation made to the NF-Y binding site. ***B*****,** Schematic of the IGF-1 promoter with the NF-Y consensus site indicated. ***C*****,** HCT116:3-6 cells or HCT116:3-6 shp53 cells were transfected with IGF-1-LUC (WT) or IGF-1-LUC mutNF-Y (MUT) and RSV-β-Gal. Cells were irradiated the next day and harvested for luciferase activity 48 h after IR. Cells are normalized to luciferase activity of untreated HCT116:3-6 cells transfected with IGF-1-LUC (WT). ***D,E,*** HCT116 cells were harvested for ChIP and p53 and NF-YA were immunoprecipitated using specific antibodies. Primers specific to the IGF-1 promoter and the p21 promoter were used to analyze p53 binding.

binding to the endogenous IGF-1 promoter. Endogenous p53 bound to the IGF-1 promoter, and the p21 promoter as expected (Figure 4.6 D). Also, NF-YA bound to the IGF-1 promoter in the basal state (Figure 4.6 E). These data indicated that p53 and NF-Y could bind to the IGF-1 promoter, and that an NF-Y consensus site was necessary for p53 suppression of IGF-1.

Nutlin-3 treatment leads to decreased sCLU expression

Nutlin-3 is a small molecule inhibitor of the p53-Mdm2 interaction (Vassilev, Vu et al. 2004). Exposure of HCT116 and RKO7 SCR cells to Nutlin-3 resulted in stabilization of p53, and a decrease in sCLU expression (Figures 4.7 A, B). Induction of p53 gene targets Mdm2 and p21 were also observed. As expected, HCT116 p53-/- and RKO7 shp53 cells did not show a decrease in sCLU expression after Nutlin-3 exposure, or induction of Mdm2 or p21 (Figure 4.7 A, B), indicating that repression of sCLU by Nutlin-3 was p53 dependent. These data suggested that there was a 'p53 dose' dependent regulation of sCLU, regulated by the level of p53 in the cell, where more stabilized p53 results in less sCLU expression.

Since Nutlin-3 can lower sCLU basal expression, Nutlin-3 was examiend for the ability to suppresses IR-mediated induction of sCLU expression. HCT116 cells were pre-treated for 1 h with Nutlin-3 prior to IR exposure, and again,

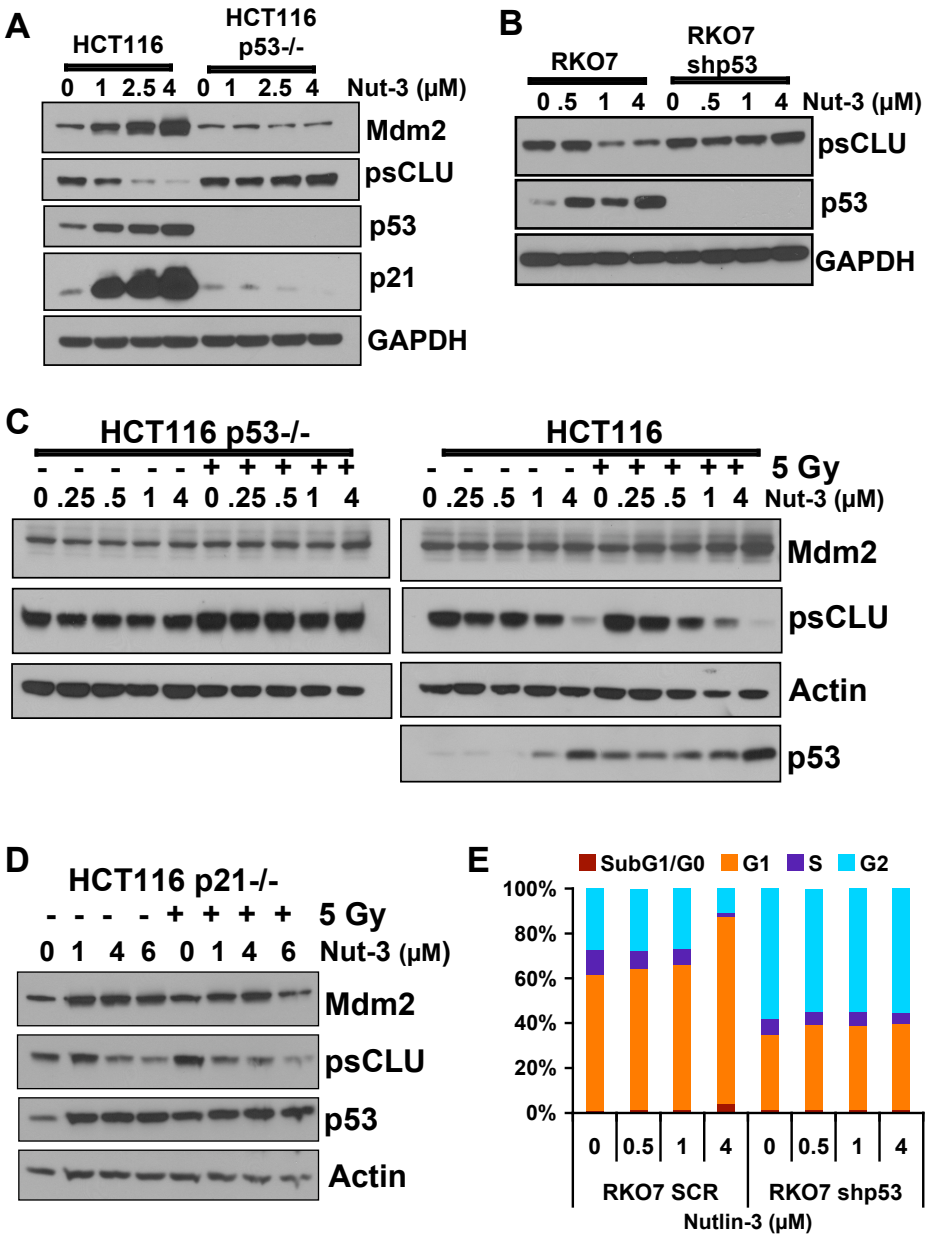

Figure 4.7 Nutlin-3 suppresses basal and IR induced sCLU protein expression.

Figure 4.7 Continued

***A*,** HCT116 or HCT116 p53-/- cells were treated with increasing doses of Nutlin-3 for 72 h. Cells were harvested for immunoblotting. Blots were probed with Mdm2, sCLU, p53, p21 and GAPDH for loading. ***B*,** RKO7 SCR and RKO7 shp53 cells were treated with Nutlin-3 for 72 h. Whole cell lysates were analyzed by immunoblotting. Blots were probed with sCLU, p53, and GAPDH for loading. ***C*,** HCT116 or HCT116 p53-/- cells were pre-treated with nutlin-3 for 1 h before irradiation. Cells were exposed to Nutlin-3 until cells were harvested 72 h after IR. Cell lysates were analyzed by immunoblotting and analyzed for Mdm2, sCLU, p53, and Actin as a loading control. ***D*,** HCT116 p21-/- were treated and analyzed as in *B*. ***E*,** RKO7 SCR or RKO7 shp53 cells were treated with 0-4 μM Nutlin-3 for 48 h, stained with PI, then analyzed for cell cycle.

Nutlin-3 alone decreased sCLU expression (Figure 4.7 C). Suppression of sCLU was even greater in IR-exposed cells, probably due to both Nutlin-3 and IR stabilizing p53 (Figure 4.7 C). As in figure 4.6 A, HCT116 p53-/- cells were not affected by Nutlin-3 treatment. Consistently, HCT116 cells somatically knocked out for p21 (HCT116 p21-/-), but WT for p53 expression, showed a decrease in sCLU expression with Nutlin-3 treatment, both with and without IR (Figure 4.7 D). However, HCT116 p21-/- cells seemed to be less sensitive to Nutlin-3 than HCT116 cells, since a greater concentration of Nutlin-3 (6 μM) was required for equivalent sCLU suppression (Figure 4.7 C versus D).

Since Nutlin-3 induced 10 to 30% apoptosis in HCT116 and RKO cells, albeit at much higher doses (Tovar, Rosinski et al. 2006), the affect of Nutlin-3 on cell cycle response for HCT116 and RKO7 SCR cells was examined. In both wild-type p53 cell lines, Nutlin-3 exposure induced substantial G1 arrest and complete loss of S-phase, but did not induce apoptosis (Figure 4.7 E, data not shown). The cell cycle profiles of HCT116 p53-/- and RKO7 shp53 cells remained unchanged with 0-4 μM Nutlin-3 treatment (Figure 4.7 E, data not shown). These data suggested that the decrease in sCLU by Nutlin-3 treatment was not due to an increase in apoptosis.

Nutlin-3 is a radiosensitizer

Since Nutlin-3 decreased sCLU expression, sCLU is a pro-survival factor, and knockdown of sCLU sensitized cells to IR, HCT116 cells were examined for IR-sensitivity with and without Nutlin-3. HCT116 cells were pretreated with Nutlin-3 immediately before exposure to IR, and Nutlin-3 IR-sensitized WT p53 expressing HCT116 cells (Figure 4.8 A). HCT116 p53-/- cells were not IR-sensitized by Nutlin-3, and the survival curve for p53-/- Nutlin-3 treated cells (black squares) overlaid all the untreated cells (open shapes). The HCT116 p21-/- cells were not as sensitized as the parental cell line even though they expressed wild-type p53, indicating that p21 may play a role in the IR sensitivity of Nutlin-3 treated cells.

Next, the time-dependent suppression of sCLU after Nutlin-3 and IR treatment was examined. HCT116 cells were pretreated or not with Nutlin-3 immediately before ionizing radiation exposure. sCLU expression remained unchanged from 1 to 8 hours after IR or IR and Nutlin-3 treatments. In contrast, both p21 and Mdm2 expression was elevated starting at 8 hours after Nutlin-3 and IR exposure, and stabilization remained elevated 96 h later (Figure 4.8 B). sCLU was dramatically induced 24-96 hours after IR, however Nutlin-3 completely blocked sCLU induction after IR, and at late times, sCLU expression was repressed below basal levels. These data indicated the importance of p53

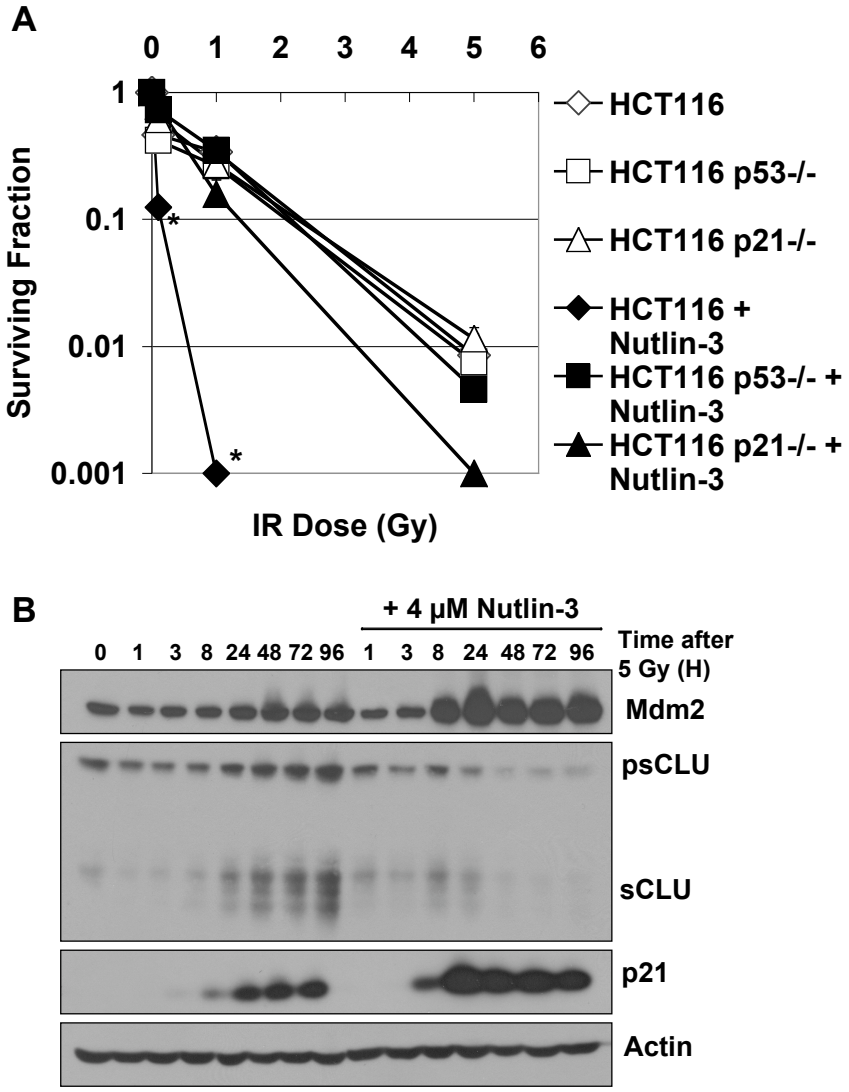

Figure 4.8 Nutlin-3 radio-sensitizes cells. ***A,*** HCT116, HCT116 p53-/-, or HCT116 p21-/- cells were treated with Nutlin-3 and then irradiated immediately after (10 min). Tweleve days later, cells were analyzed for the ability to form colonies. ***B*****,** HCT116 cells were pre-treated with Nutlin-3 or not for 1 hour and then irradiated with 5 Gy. Cells were harvested at the indicated times and processed for immunoblotting. Blots were probed with Mdm2, sCLU, p21, and Actin as a loading control.

regulation on the IGF-1-sCLU expression axis.

Discussion

In this study, IGF-1 gene transcription was suppressed by p53, occurring through NF-Y mediated promoter binding and repression (Figure 4.9). This model explains our observation of p53 suppression of sCLU, and why a p53 mediated repression site was not found in the CLU promoter (Criswell, Klokov et al. 2003). Several studies show an interaction between p53 and the IGF-1 signaling pathway, but this is the first report to show that p53 suppresses the IGF-1 ligand promoter, resulting in decreased IGF-1 mRNA and protein expression. Werner et al., showed a very similar effect of p53 on the IGF-1R promoter, with wild-type p53 suppressing the IGF-1R promoter, and mutant p53 activating the IGF-1R promoter (Werner, Karnieli et al. 1996). In this case, p53 interacted with the TATA-binding protein to suppress transcription. This group also showed that IGF-1 ligand induced Mdm2-dependent degradation of p53 by MAPK in response to DNA damage (Heron-Milhavet and LeRoith 2002), setting up a possible positive feedback mechanism. This is in addition to the canonical IGF-1R-PI3K-Akt pathway that induces Mdm2-dependent degradation of p53. Also, Mdm2 promoted ubiquinitation and degradation of IGF-1R in p53 knockdown cells (Girnita, Girnita et al. 2003). These activating pathways and feedback loops

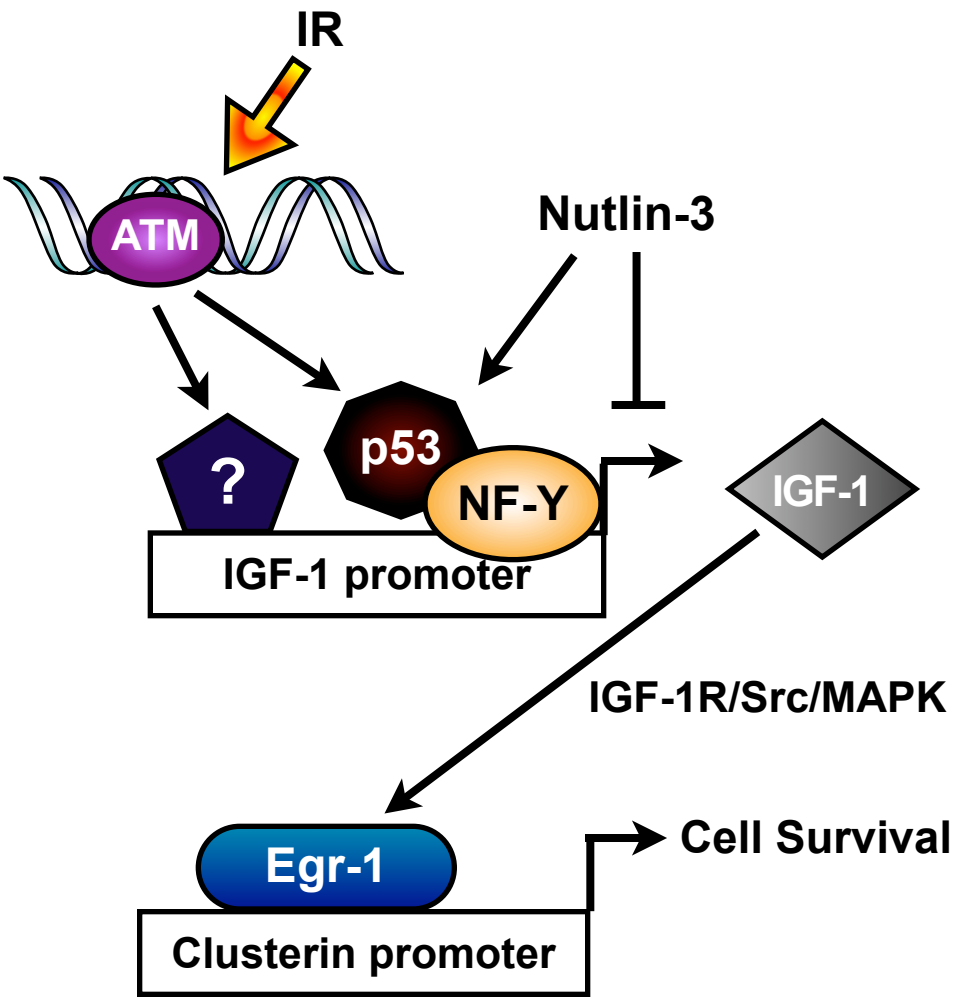

Figure 4.9 Model of the IGF-1-sCLU pathway. DNA damage is recognized by ATM, which then signals to the IGF-1 promoter. p53 negatively suppresses IGF-1 via NF-Y binding to the promoter, and this, along with any positively regulated transcription factors, creates a balance between the positive and negative factors to alter IGF-1 gene expression. IGF-1 released from the cell can then activate IGF-1R and promote activation of the MAPK pathway. Activated ERKs are translocated to the nucleus where Egr-1 (early growth response 1) is activated and binds to the CLU promoter. Nutlin-3 stabilizes p53 and blocks IGF-1 mediated signaling to sCLU.

propose a very confusing and highly regulated signaling event. Our finding that p53 suppresses IGF-1 ligand adds another mechanism of regulation onto this important signaling pathway.

This study shows p53 mutations commonly observed in cancer do not suppress IGF-1/sCLU signaling like wild-type p53. Therefore, we hypothesize that tumors with mutant p53 will have higher IGF-1 and sCLU compared to tumors with wild-type p53. We propose that tissue microarray studies, when examined for IGF-1 ligand or sCLU, will show up-regulation of these factors, and the amount of up-regulation will be dependent on p53 status, whether p53 expression is high or low, or mutant.

Mutation of the p53 DNA binding domain abrogated suppression of IGF-1-sCLU signaling, however, in our model, p53 does not directly bind to DNA, therefore, should not affect suppression of IGF-1. Consistently, Cyclin B2 and Cdc2, which are both suppressed by NF-Y/p53, are not suppressed by mutant p53 (Di Agostino, Strano et al. 2006). The Di Agostino study also revealed that when mutant p53 bound to NF-Y, p300 was recruited, whereas binding of WT p53 and NF-Y to promoters recruited HDACs. Also, the transactivation domain of p53 was dispensable for NF-Y-mediated promoter transactivation (Di Agostino, Strano et al. 2006). These findings provide a potential mechanism for the lack of IGF-1 promoter suppression by p53 DNA binding domain mutations.

In this study, we show IGF-1 ligand induction after IR is at the promoter level, which complements our and other's studies showing ionizing radiation activates IGF-1R phosphorylation (Criswell, Beman et al. 2005; Cosaceanu, Budiu et al. 2007). The majority of IGF-1 promoter activation and ligand secretion does not occur until 24-48 hours after IR exposure, explaining why other studies did not see an increase in IGF-1 ligand when measured within 8 h of IR exposure (Cosaceanu, Budiu et al. 2007). Alternatively, we observed an uptake of IGF-1 from the media immediately after IR (10 min), that returns to normal culture media levels by 24 hours, and increases beyond normal culture levels by 48 hours (unpublished observations), that would not be observed when using serum-free medium. This also explains the biphasic activation of IGF-1R/MAPK signaling observed in MCF-7 cells after IR (Criswell, Beman et al. 2005). The initial uptake of IGF-1 from the media could initiate a positive feedback loop where IGF-1 signaling leads to sCLU protein expression and degradation of p53 (Leri, Liu et al. 1999), resulting in additional synthesis of IGF-1 and sCLU.

IGF-1 was shown to protect against apoptosis (van Golen, Castle et al. 2000) and inhibition of IGF-1R signaling by chemical inhibitors or knockdown promotes radio-sensitization (Wen, Deutsch et al. 2001). These data indicate the importance of initial activation of IGF-1/IGF-1R signaling after IR exposure, and then, over time, promoting cellular synthesis of IGF-1, as extracellular availability

decreases, to maintain constitutive IGF-1R signaling. IGF-1 can attenuate cisplatin-induced γ-H2AX formation and DNA damage observed by comet assay in lung cancer cells. This was thought to be due to an interaction between IRS-1 and ATM (Jeon, Kim et al. 2008). IRS-1 is a docking protein that binds IGF-1R and mediates PI3-K activation, but also translocates to the nucleus when stimulated with IGF-1 (Baserga 2000; Tu, Batta et al. 2002). Studies have shown that IRS-1 and Rad51 bind in the cytoplasm, that is disrupted upon activation with IGF-1, allowing Rad51 to translocate to the nucleus to participate in DNA repair (Trojanek, Ho et al. 2003). However, Rad51 and IRS-1 interaction was shown in the nucleus as well (Trojanek, Ho et al. 2006). These results suggest that IRS-1 may be playing a role in the induction of IGF-1 or sCLU after DNA damage, and may protect cells from IR-induced DNA damage.

Nutlin-3 suppressed IGF-1/sCLU and radiosensitized cells, only in cells expressing WT p53 (Figures 4.7 and 4.8). Therefore, in order for Nutlin-3 to be used as a cancer therapy, it must be only be used in wild-type p53 tumors. This limits its use, since p53 is mutated in over 50% of cancers. Still, Nutlin-3 prevented tumor growth of WT p53 cells in mice (Vassilev, Vu et al. 2004; Tovar, Rosinski et al. 2006). Nutlin-3 radiosensitized prostate and lung cancer cells (Cao, Shinohara et al. 2006; Lehmann, McCubrey et al. 2007), however, this is the first evidence that Nutlin-3 exposure sensitizes HCT116 colon cancer cells to IR. Also,

a potential role was uncovered for p21 in Nutlin-3 radio-sensitization of cancer cells. Overall, these studies indicate that Nutlin-3 may be a effective therapy for WT p53 expressing cancer cells, however, IGF-1 or IGF-1R inhibitors may be a more effective treatment strategy in p53 null or mutant cancer cells.

Besides playing a role in radioresistance, IGF-1 is also involved in cancer treatment resistance to commonly used chemotherapeutic drugs and has been associated with increased cancer risk (Grimberg 2003). This study supports the basis of clinical trials using IGF-1R antibodies in combination of chemotherapy, such as figitumumab (CP-751871) (Lacy, Alsina et al. 2008) and development of new IGF-1 ligand inhibitors for use in combinations with chemotherapeutic agents or IR. Currently, there are humanized antibodies against IGF-1 in pre-clinical development, however, our data support additional screens to identify new IGF-1 ligand synthesis inhibitors specific to tumor cells, and not the liver. In conclusion, we have identified a mechanism of IGF-1 regulation that is altered in tumor cells, and may play a role in resistance to therapy that could be overcome with IGF-1/ sCLU pathway inhibition.

CHAPTER V: Conclusions and Future Directions

Conclusions

The goal of this dissertation was to determine how sCLU protein expression was regulated after DNA damage. The major motivation behind determining the regulation of this pro-survival pathway was to uncover new targets for cancer therapy. However, signaling induced after IR exposure is also pertinent to exposures to IR, both controlled and uncontrolled. Our lab found that induction of sCLU by ionizing radiation involved IGF-1R to MAPK signaling, and we elucidated the transcription factor responsible for transactivation of the CLU promoter, Egr-1 (Criswell, Beman et al. 2005). However, we did not determine how IGF-1 signaling was being activated, the initiating signal from DNA damage, or whether other agents that cause DNA damage induced the same pathway. We also found p53 suppressed sCLU expression after IR, but did not determine the mechanism of suppression or whether IGF-1 signaling was involved. Even after these studies were concluded there were still questions remaining.

In Chapter III, we found that all DNA damaging agents tested (IR, topotecan, etoposide, hydrogen peroxide) induced sCLU through the IGF-1R/ MAPK pathway. Also, activation of IGF-1R after IR was due to production of IGF-1 ligand, and this induction was regulated at the promoter level. Consistently,

other cytotoxic agents induced IGF-1 promoter activity and ligand secretion into the media. Next, we discovered that genomically unstable cells, with elevated levels of endogenous DNA damage due to loss of DNA repair factors (MDC1, H2AX, NBS1, or hMLH1) or loss of telomere elongation (mTR), expressed higher levels of sCLU protein. Additionally, ATM signaling was activated after exposure to DNA damaging agents, and in the genomically unstable cells, measured by serine 1981 auto-phosphorylation. These genomically unstable cells also up-regulated basal ATR signaling, measured indirectly by Chk1 phosphorylation on serine 317. Exposure to an ATM and ATR inhibitor led to decreased sCLU expression in genomically unstable cells, and blocked both IR and TPT-induced sCLU expression. Additionally, ATM-deficient cells were unable to induce sCLU after IR, or UV - even though ATR signaling was activated. Thus ATM and not ATR is strongly indicated in IGF-1-sCLU expression. This study answered some of our initial questions, however, did not indicate the mechanism of suppression by p53.

In Chapter IV, we determined whether p53 would suppress IGF-1 signaling, since a p53 repression site was not identified in the CLU promoter. p53 is known to suppress IGF-1R promoter activity (Werner, Karnieli et al. 1996) and transactivate IGFBP3 (Buckbinder, Talbott et al. 1995), which would lead to down-regulation of sCLU expression (Criswell, Beman et al. 2005). However, we found

that p53 also suppressed the IGF-1 promoter and subsequent protein expression, adding another mechanism of p53 regulation on the IGF-1 signaling cascade. p53 suppressed IGF-1 induction after IR exposure in wild-type p53 cells and by transient overexpression of p53 in p53 null cells. Loss of p53 (knockout or knockdown) promoted IR-mediated induction of IGF-1, as well as increasing basal IGF-1-sCLU expression. Incidentally, mutation of the ATM phosphorylation sites on p53 (serines 15 and 20) did not alter repression of IGF-1, however, mutation of three different sites within the p53 DNA binding domain abrogated p53 mediated repression. An NF-Y site in the IGF-1 promoter was responsible for p53 suppression, and both p53 and NF-YA bound to the IGF-1 promoter. Finally, we observed that Nutlin-3, a p53-Mdm2 inhibitor, led to increased p53 expression, and down-regulation of sCLU protein levels. Additionally, Nutlin-3 radio-sensitized HCT116 cells in a p53 dependent manner. These data indicated that cancer cells with mutant or null p53 expression would have higher expression of IGF-1/sCLU, and cancer cells could be radio-sensitized by Nutlin-3 exposure.

In normal cells, up-regulation of sCLU protects cells from exogenous insults to promote cell survival (Wilson and Easterbrook-Smith 2000). However, cancer cells take advantage of this pro-survival pathway to prevent the death of cells fated for destruction, such as cells with genomic instability. Since IGF-1/ sCLU is overexpressed in different conditions that are directly linked with cancer

and chemotherapy resistance, blocking IGF-1 or sCLU may enhance tumor cell killing with standard chemotherapeutic agents.

Currently, there are 23 clinical trials using IGF-1R blocking antibodies, either alone or in combination with standard chemothearpies, to treat a wide variety of tumors. The IGF-1R antibodies include BIIB022, AMG-479, AVE1642, MK-0646, IMC-A12, R1507, and CP-751,871[1]. The trials are aimed to treat lymphomas, sarcomas, general solid tumors, and cancers of the lung, breast, colon, ovary, pancreas, liver, and adrenal cortex. Additionally, there is one phase I trial using an IGF-1/2 humanized monoclonal antibody, MEDI-573[2], aimed against solid tumors. Our data suggests that tumors treated with IGF-1R or IGF-1 antibodies would have lower sCLU expression. In addition, we expect that tumors treated with IGF-1/IGF-1R antibodies will be sensitized to DNA damaging agents. Currently, the trials with the IGF-1R antibodies are either alone, in combination with other antibodies, such as α-EGFR, or with standard chemotherapeutic regimens. We would expect that combinations of IGF-1/IGF-1R antibodies, with chemotherapy that causes DNA damage or leads to sCLU induction, would be more effective than combinations of antibodies or antibodies alone. For antibody combinations, the tumor cell must be 'addicted' to the inhibited signaling pathways

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[1] http://clinicaltrials.gov/ct2/results?term=IGF-1R+antibody (accessed 3/20/09)

[2] http://www.clinicaltrials.gov/ct2/show/NCT00816361?term=medi-573&rank=1 (accessed 3/20/09)

that are necessary for survival. For EGFR inhibitors, cancer cells can escape EGFR inhibition by corresponding mutation of another site on EGFR, rendering it resistant to the inhibitor. Also, cellular up-regulation of other growth factors, such as VEGF, has been observed in inhibitor resistant cancer cells (Viloria-Petit, Crombet et al. 2001; Shih, Gow et al. 2005). In contrast, by combining growth factor inhibitors with chemotherapeutic agents, the cytotoxic agents will kill the tumor cells, while the growth factor inhibitor will block the cells that have begun to repair damage. The data presented in this dissertation, combined with other published reports, indicate that inhibiting either IGF-1 signaling or sCLU expression would be sufficient for enhancing chemotherapy induced cell death.

Since knockdown of sCLU enhanced sensitivity to chemotherapeutic agents, antisense to sCLU was developed into a deliverable anti-cancer agent. The antisense molecule, OGX-011, is currently used in combination with conventional chemotherapy (Chi, Zoubeidi et al. 2008). Knockdown of sCLU, by itself, induced p53-enhanced apoptosis, since p53 WT, mutant, and null tumor cells induced apoptosis after sCLU knockdown, with highest cell killing observed in p53 WT cells (Trougakos, Lourda et al. 2009). We expect that a combination of OGX-011 with IGF-1 or IGF-1R blocking antibodies would not be successful, since blocking IGF-1/IGF-1R will lead to a decrease in sCLU expression, leaving no need for further sCLU reduction by anti-sense. Besides OGX-011, there is a new clusterin

targeted therapy in preliminary testing called CGEN-25008. CGEN-25008 is a peptide that binds to and inhibits sCLU, and may be a promising anti-sCLU therapy, but only after further testing[3]. In conclusion, inhibition of IGF-1, and thereby sCLU, should sensitize tumor cells that have up-regulated IGF-1 signaling or sCLU protein expression.

This data in this dissertation suggests that the observed up-regulation of IGF-1, IGF-1R, and/or sCLU in tumor cells is due to their inherent genomic instability or due to the stressful conditions in which tumors have to grow. Also, since p53 is mutated in ~50% of cancers, and IGF-1/sCLU is not suppressed by DNA binding domain mutants of p53, we expect tumors with mutations in p53 to have higher expression of IGF-1/sCLU. These studies suggest that Nutlin-3 may be a good chemotherapeutic agent for WT p53 cells, while IGF-1, IGF-1R, or sCLU inhibitors may be effective for null or mutant p53 cells. Either way, the two major studies outlined in this thesis reveal a greater understanding of the regulation of the ATM-IGF-1-sCLU pathway. However, like all effective science projects, completion of this project also reveals new questions that need to be addressed.

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[3] http://www.cgen.com/Content.aspx?Page=CGEN_25008, accessed 03/20/2009

Future Directions

ATM induction of IGF-1

One of the questions left by this work is the link between activation of ATM after DNA damage and induction of the IGF-1 promoter. There is a connection between ATM and p53, since ATM phosphorylates p53 after DNA damage. However, mutation of these sites in p53 did not affect the ability of p53 to suppress IGF-1 (Figure 4.3). Still, IGF-1 was induced after IR exposure in p53-/- cells (Figure 4.2), indicating the presence of a positive transcriptional activator that can induce independent of p53. Since ATM is a major regulator of the DNA damage response, and ATM is upstream of IGF-1, the positive transcription factor is likely activated by ATM signaling. There are several candidate factors that could be involvement in regulating the IGF-1 promoter after IR. The studies outlined below should allow determination the positive regulatory factor necessary for the induction of IGF-1 by cytotoxic agent exposure.

NF-Y, the transcription factor involved in p53 mediated repression of IGF-1, is activated after IR. After IR, NF-Y and PCAF bind on the von Willebrand factor promoter to induce transcription, while NF-Y and HDAC1 association decreases (Peng, Stewart et al. 2007). We hypothesize that p53 could bind NF-Y, preventing association with PCAF. Consistently, when p53 is absent, NF-Y would

be free to bind PCAF and transactivate *IGF-1*. However, it is unknown whether the change in NF-Y association from HDAC1 to PCAF after IR is dependent on ATM, however it has not been ruled out.

AIB1 is another transcription factor that regulates the IGF-1 promoter that could act in opposition with NF-Y-p53, as a positive regulator of IGF-1. Amplified in breast cancer 1, AIB1, is a steroid receptor coactivator, but has been suggested to act as a coactivator for other transcription factors (Gnanapragasam, Leung et al. 2001). AIB1 expression (also known as steroid receptor coactivator-3, SRC-3) is directly correlated with IGF-1 expression. Loss of AIB1 results in decreased IGF-1 expression, and overexpression of AIB1 leads to increased IGF-1 (Wang, Rose et al. 2000; Torres-Arzayus, Font de Mora et al. 2004). Also, AIB1 binds the IGF-1 promoter and is necessary for transcriptional regulation of IGF-1 (Yan, Yu et al. 2006). AIB1 mRNA was induced following IR exposure (Jen and Cheung 2003) and AIB1 can be phosphorylated by c-Abl, and this phosphorylation is necessary for its function (Oh, Lahusen et al. 2008). This links AIB1 to ATM, since ATM can activate c-Abl after IR (Baskaran, Wood et al. 1997). These studies indicate that AIB1 could be acting in a transactivation complex to induce IGF-1 after IR, acting as a 'balance' to the suppressive effect of NF-Y/p53, to regulate sCLU expression in WT p53 cells.

If NF-Y or AIB1 does not regulate IGF-1 induction after IR, it is possible that any of the ATM effectors could regulate IGF-1 induction after IR. This would be more complicated because of the number of ATM effectors, but involvement could still be determined by knocking out the effectors singly with siRNA before IR exposure, and then examining IGF-1 promoter activity. Once an effector is identified, appropriate hypotheses can be drawn from the known targets or functions of the effector. If this method does not identify an effector, then the transcription factor must be directly regulated by ATM.

Once we identify a potential transcription factor required for *IGF-1* gene regulation, we can investigate whether it is involved in IGF-1 and sCLU regulation using similar strategies used to determine p53 regulation of the IGF-1 promoter. The factor can be overexpressed and knocked down, with and without IR exposure and analyzed for IGF-1 promoter activities. If known dominant negative or constitutively active forms exist, these can also be used in the transient promoter assays. Finally, binding of these factors to IGF-1 promoter can be confirmed using ChIP to examine the endogenous promoter. DNA pulldown assays can be used to introduce mutations into the consensus binding site of the IGF-1 promoter, and then loss of transcription factor binding can be determined.

Basal expression of sCLU

We still do not know how basal expression of sCLU is regulated. By comparing the ATM-/- and ATM+ cells, we can conclude that deficiency in ATM does not completely abrogate sCLU basal expression. Therefore, there must be some other factor(s) that regulate(s) sCLU basal expression. Treatment with AG1024, the IGF-1R tyrosine kinase inhibitor, decreased basal sCLU expression in many different cell lines, indicating that expression may be due to IGF-1 in the cell culture media. Also, the basal expression of sCLU observed in cell lines could be due to the inherent 'stress' of tissue culture. sCLU is a sensitive measure of stress, and poor culturing conditions, such as confluence or acidified medium, or even extended periods in low-serum medium, can induce sCLU. We also hypothesize that the high oxygen conditions of conventional incubators can also elevate the basal expression of sCLU. Consequently, when cells are switched from a 95% air oxygen incubator to a low-oxygen incubator (2%) sCLU expression decreases, indicating that the high oxygen environment and increased amounts of ROS cause stress, and lead to sCLU expression (Luo et al. unpublished observations). To fully understand how sCLU basal expression is regulated, experiments should be performed in the least stressful environment possible, and this may include reduced oxygen conditions. These experiments should also be

performed in the ATM-/- cell line, which would remove the stress-induced IGF-1-sCLU signaling.

Role of IGF-1 in IR protection

Since IGF-1 has been linked to cancer treatment resistance (Dunn, Hardman et al. 1997), high IGF-1 expression from tumor cells, or the tumor cell microenvironment, may alter the cell's ability to respond to DNA damaging agents. IGF-1 has already been shown to decrease DNA damage after cisplatin treatment (Jeon, Kim et al. 2008) and activate DNA damage repair after UV exposure (Heron-Milhavet, Karas et al. 2001). Preliminary data indicated that phosphorylation of DNA repair factors ATM (serine 1981), H2AX (serine 135), and 53BP1 (serine 25) after IR exposure, could be detected by flow cytometry (Figure 5.1). Therefore, we examined the effect of IGF-1 on untreated cells, by exogenously applying IGF-1 to HCT116 cells in low serum medium. We found that HCT116 cells treated with IGF-1 for 24 hours induced phosphorylation of ATM and 53BP1 without corresponding DNA damage (no increase in γ-H2AX, Figure 5.2 A). This data indicated that IGF-1 may 'prime' cells for DNA damage.

Since IR first induces DNA damage, we used the comet assay to measure DNA damage with and without IGF-1 pretreatment. HCT116 cells pretreated with IGF-1 had a small decrease in DNA damage after both IR and H_2O_2 exposures

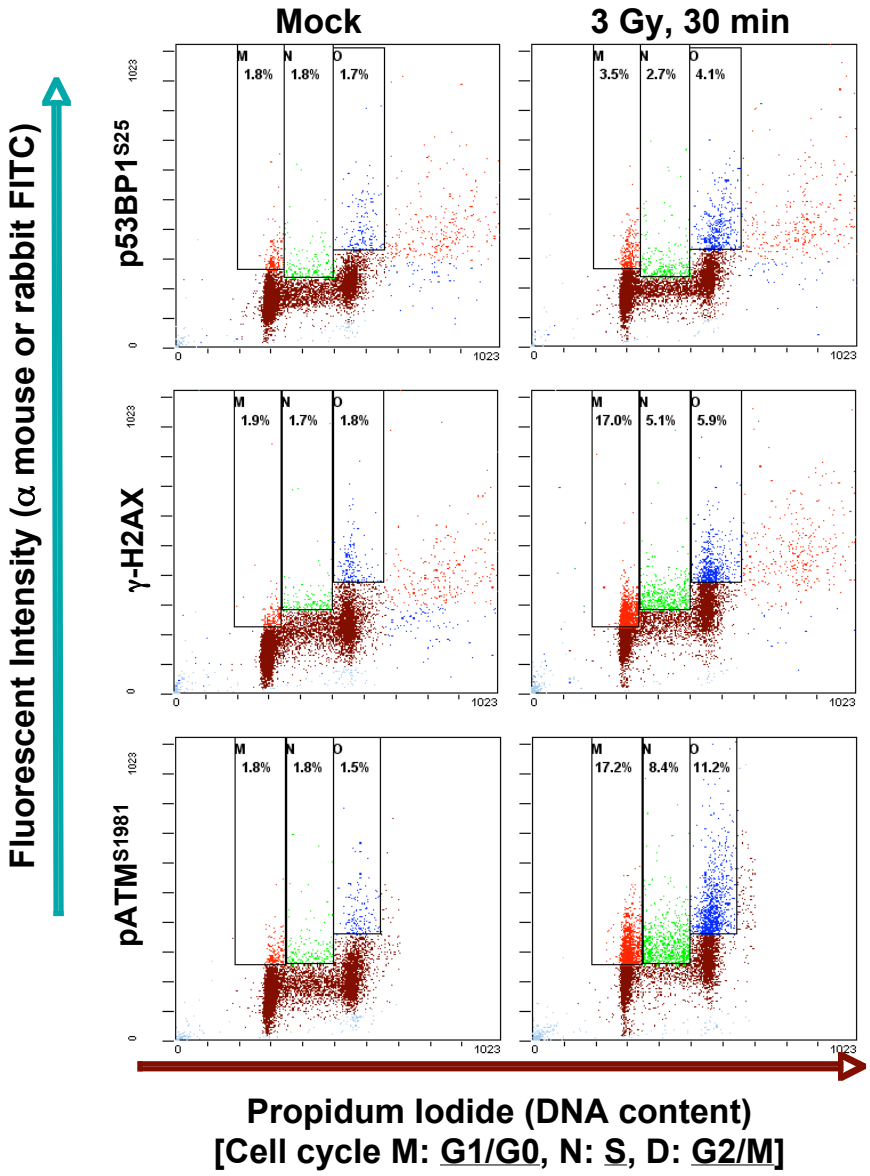

Figure 5.1 Phosphorylation of DNA damage repair factors after IR HCT116 cells were treated with 3 Gy and then harvested 30 minutes later. Cells were fixed in formaldehyde and permeabilized in ethanol. Cells were stained with antibodies to p53BP1^{S25}, pATM^{S1981}, or γ−H2AX and propidium iodide to detect DNA content. Boxes indicate FITC positive cells in each stage of the cell cycle (red is G0/G1 phase (M), green is S phase (N), and blue is G2/M phase (D)).

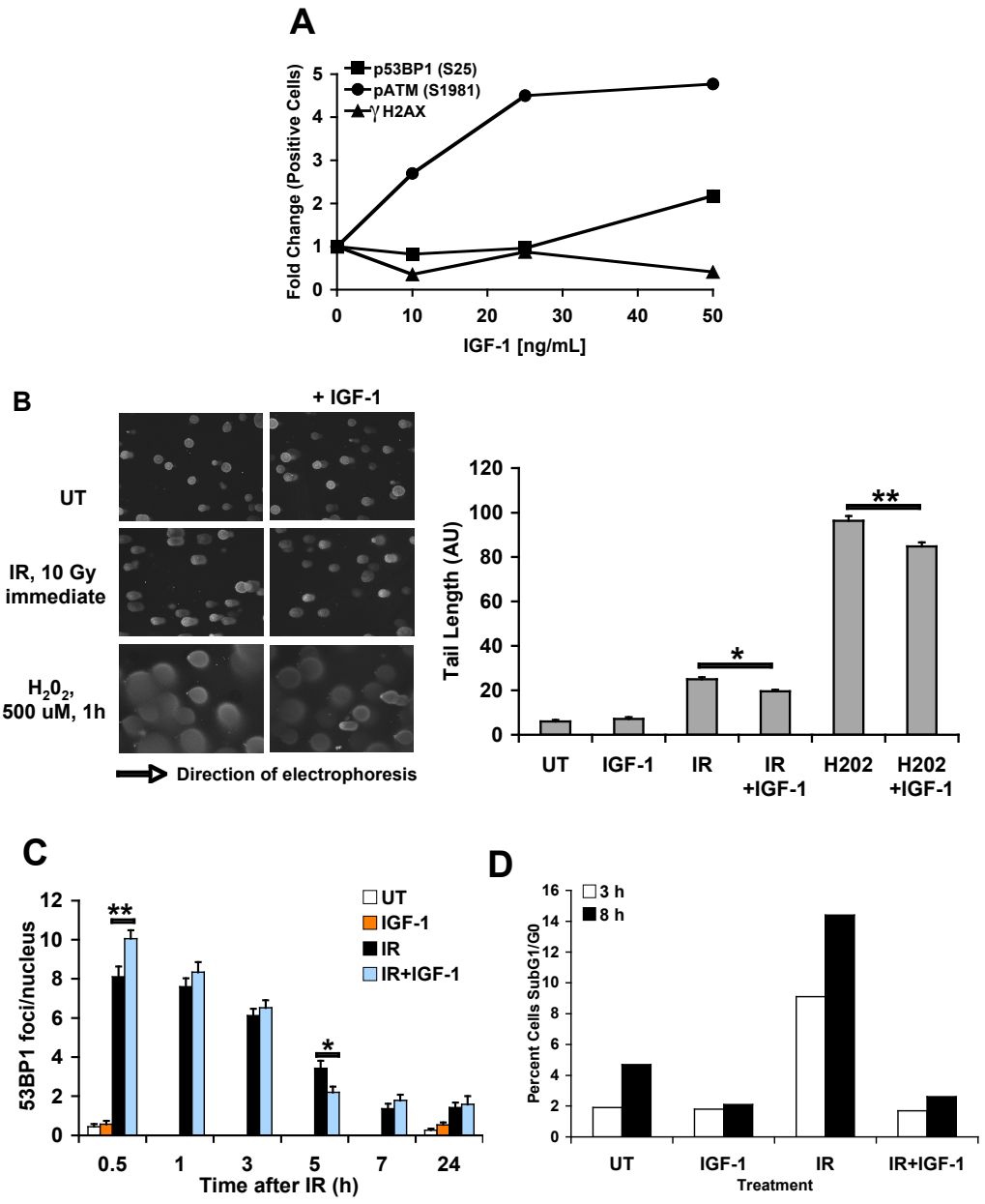

Figure 5.2 IGF-1 treatment enhances repair of IR-induced DNA breaks *A*, HCT116 cells were treated with varying doses of IGF-1 overnight, and phosphorylation of ATM, H2AX, and 53BP1 was monitored using flow cytometry analyses.

Figure 5.2 Continued

B*,** HCT116 cells were pretreated overnight with 25 ng/mL IGF-1 (or vehicle), and irradiated the following day with 10 Gy or treated with H_2O_2 (500 μM, 1 h). DNA damage was analyzed using single cell gel electrophoresis for the formation of comet tails. Tail length was measured using Image J software, AU = arbitrary units. ***C, HCT116 cells were grown on glass coverslips, serum starved overnight and then pre-treated with 25 ng/mL IGF-1 in 0.5% FBS medium for 24 h before IR exposure. Cells were fixed at the indicated times, then stained with 53BP1 antibody and foci were counted. ***D***, HCT116 cells were treated with 25 ng/mL IGF-1 (or vehicle) overnight, and were irradiated the next day (3 Gy). Cells were harvested three and 8 h later for cell cycle analysis. Cells in SubG1/G0 are shown, indicating cell death.

(Figure 5.2 B). After DNA damage, DNA repair factors localize to DNA breaks and localization can be observed as foci by fluorescence microscopy. Therefore, we analyzed the number of 53BP1 foci per nucleus in HCT116 cells exposed to IR. Cells that had been treated with IGF-1 for 24 hours before IR exposure had an average of 2 more foci per nucleus compared to IR-alone treated cells 30 minutes after IR (Figure 5.2 C). These data indicated that decreased DNA damage observed in IGF-1 treated cells was due to increased DNA repair.

Finally, we examined the effect of IGF-1 on IR-induced cell death. HCT116 cells were pretreated with IGF-1 for 24 hours before IR exposure, and then harvested for cell cycle analysis using PI staining. We noticed a dramatic decrease in $SubG_1/G_0$ in cells pretreated with IGF-1 (Figure 5.2 D). These data indicated that cells exposed to IGF-1 during IR have less DNA damage, possibly due to increased DNA repair capacity, leading to a decrease in cell death.

These preliminary experiments, although statistically significant, may not show biological significance. However, it does hint that IGF-1 may play a small role in protecting cells from DNA damage. These small differences could just be due to the methods employed, and it is possible these differences could be improved by overexpression of IGF-1 or sCLU, for example. By providing exogenous IGF-1, we may be taking advantage of a system the cell already has in place to 'prime' the cells to deal with DNA damage by promoting 'pre-activation'

of DNA repair factors and activation of pro-survival signaling allowing cells to stay alive. This would prevent death by apoptosis, or metabolically active death also known as stress-induced premature senescence (SIPS) (Di Leonardo, Linke et al. 1994). These studies implicate the importance of the IGF-1-sCLU expression axis and its regulation in cancer cell survival.

Mechanism of IGF-1 protection

Since we showed that IGF-1 pre-treatment can alter the amount of DNA damage, it suggests that IGF-1 may be activating DNA repair. IRS-1 has been shown to be involved in DNA repair and is also up-regulated in cancers. IRS-1 is a docking protein that promotes association of effectors activated by IGF-1/IGF-1R and insulin signaling. Like IGF-1 and IGF-1R, IRS-1 expression is overexpressed in human tumors (Chang, Li et al. 2002). Overexpression of IRS-1 promotes cell transformation, and knockdown of IRS-1 reverses transformation (D'Ambrosio, Keller et al. 1995). Transgenic expression of IRS-1 in the mouse mammary induces tumor development and metastasis (Dearth, Cui et al. 2006). IRS-1 translocates to the nucleus after IGF-1 stimulation, and has been shown to bind to UBF1, Rad51, β-catenin, PP2A, and histone H1, and is thought to act as a transcriptional co-factor (Tu, Batta et al. 2002; Drakas, Prisco et al. 2005; Urbanska, Pannizzo et al. 2008; Wu, Chen et al. 2008). In addition, IGF-1 has

been shown to stimulate homologous recombination by regulating the IRS-1-Rad51 cytoplasmic complex (Trojanek, Ho et al. 2003). Also, binding of IRS-1 and ATM was dependent on DNA damage (Jeon, Kim et al. 2008). Overall, these studies indicate a role for IRS-1 in regulating DNA repair after IR. Future studies to investigate the role of IRS-1 in the ATM to sCLU pathway, may link the enhanced repair of HCT116 cells after IR with IGF-1 pretreatment.

IGF-1 phosphorylation of ATM and 53BP1

We showed that IGF-1 stimulated auto-phosphorylation of ATM^{S1981}, and phosphorylation of 53BP1 on S25, however, the mechanism of this phosphorylation was not explored. It was previously shown that IGF-1 exposure led to Tyr and Thr phoshorylation on ATM (Suzuki, Kusakai et al. 2004). However, due to the difference in localization of IGF-1R and ATM proteins, direct phosphorylation of ATM by IGF-1R seems unlikely. In a study from the same group, ATM was phosphorylated by ARK5, a novel AMPK family member, when ARK5 was phosphorylated and activated by glucose-stimulated Akt signaling (Suzuki, Kusakai et al. 2003). This group also shows the presence of Akt and AMPK consensus phosphorylation sites in ATM protein. Besides ARK5, IGF-1 also activates nuclear translocation of IRS-1 (Tu, Batta et al. 2002), as mentioned above. Since IRS-1 is a docking protein and binds to ATM (Jeon, Kim et al. 2008),

we hypothesize that a complex between ATM, IRS-1, and a protein kinase, such as PI-3K or AKT (Backer, Myers et al. 1992), could lead to phosphorylation and activation of ATM. Then, these activated phosphorylations would induce phosphorylation of ATM at S1981 by auto-activation or by other kinases, such as ATR (Stiff, Walker et al. 2006). The phosphorylation of ATM after IGF-1 ligand exposure may be key to the perceived protection of cells from IR exposure. Understanding how ATM is phosphorylated may reveal a novel signaling cascade that could be targeted for anti-tumor therapies.

Tumor Microenvironment

Another aspect of tumor biology, the microenvironment, could also play a role in this pathway. Both IGF-1 and sCLU are secreted by cells. Therefore, both tumor cells and surrounding stroma could produce these pro-survival factors. Also, sCLU is up-regulated during cellular senescence and in aged individuals (Trougakos and Gonos 2006), indicating that normal cells have a potential to secrete IGF-1/sCLU during normal aging and stress-induced premature senescence, enhancing the survival of initiated cells that would otherwise undergo cell death. This suggests that studies concerning the regulation of IGF-1 and sCLU should be carried out in normal and tumor cells, since both types of cells can

contribute to the microenvironment and affect cell growth, and the ability of cells to respond to stress or therapeutic agents.

Nutlin-3 as a radiosensitizer

Nutlin-3 is a potent inhibitor of the Mdm2-p53 interaction, leading to p53 stabilization. Nutlin-3 has been proposed for use as an anti-cancer agent, and has been used in an animal model to decrease tumor growth (Vassilev, Vu et al. 2004; Tovar, Rosinski et al. 2006). Since Nutlin-3 led to a dramatic decreases in sCLU expression, cell cycle arrest, and radio-sensitized cells based on their p53 status, we hypothesize that IGF-1 and sCLU will play a role in the cell growth arrest and/or radio-sensitization by Nutlin-3. In order to examine this, sCLU and/or IGF-1 cDNA would be transfected into wild-type p53 cells, and then treated with Nutlin-3 and IR. Stable transfection of sCLU or IGF-1 cDNA would bypass the p53 regulation since they would both be under the control of a CMV-promoter, which is not dependent on p53 expression like their own promoters. First, we would confirm that IGF-1 and sCLU expression was not altered by Nutlin-3 exposure, then cells examined for cell cycle arrest and radio-sensitization. In addition, Nutlin-3 induces apoptosis at doses greater than 10 μM (4 μM maximum dose was used in this study), and although unexplored in this dissertation, IGF-1 or sCLU may affect the ability of Nutlin-3 to induce apoptosis. Completion of these

experiments would uncover the role of IGF-1 and sCLU in the response of cells to Nutlin-3.

Other mutations or post-translational modifications of p53

Most of the mutations of p53 in cancer are in the DNA binding domain, however, there are mutations in other domains as well. This leaves a gradient of p53 activity dependent on the mutation. The studies detailed in this dissertation only examine the top five most common mutations of p53 in cancer. Even though we observed the same effect with all five mutants, mutation of other p53 sites may have a different effect. Creation of other common mutations of p53, including those outside of the DNA binding domain, could be easily examined for their ability to suppress IGF-1 and sCLU.

There are also extensive post-translational modifications of p53, and many of these modulate p53's function. For example, one of the most important post-translational modifications is ubiquination, since it targets p53 to the proteasome for degradation (Honda, Tanaka et al. 1997). In addition, p53 is acetylated by p300 and PCAF after UV or IR exposure, and acetylation of these sites can enhance p53 binding to consensus DNA sequences (Barlev, Liu et al. 2001). Alternatively, histone deacetylases, such as HDAC1, remove acetylation groups from p53, decreasing its ability to bind DNA (Luo, Su et al. 2000). These post-translational

modifications may alter the ability of p53 to bind to NF-Y after radiation, and enhance suppression. To examine this, site-directed mutagenesis of p53 cDNA can be performed to change acetylated lysine residues to glutamate, which mimics acetylation, or to alanine, to prevent acetylation. These constructs can then be transfected in comparison to WT p53, and suppression of IGF-1 and sCLU promoters examined by luciferase assays, and p53-NF-Y binding to the IGF-1 promoter examined by ChIP and/or DNA pulldowns experiments. Understanding the role of different p53 mutations my be important to determine whether or not anti-IGF-1 or sCLU therapies should be used.

p53 and NF-Y promoter binding after IR

This dissertation only investigated the binding of NF-YA and p53 to the IGF-1 promoter in the basal state. It is expected that binding of these factors may change after IR exposure. For example, p53 is stabilized after DNA damage, leading to increased expression. Therefore, in ChIP or DNA pulldown assays, we anticipate p53 binding to the IGF-1 promoter will be enhanced after IR exposure. We may also gain additional information by transfecting the mutant p53 constructs and examining mutant p53 and NF-Y binding to the IGF-1 promoter before and after IR exposure. The conclusion of these studies will greatly enhance the

mechanistic understanding of p53 regulation of sCLU, and may also determine whether NF-Y is involved in the induction of IGF-1 in p53 null cells.

sCLU mediated inhibition of IGF-1 signaling

Recently, it was proposed that sCLU bound to IGF-1 to block IGF-1R/Akt signaling (Jo, Jia et al. 2008). They showed that sCLU was secreted from cancer cells where it bound to IGF-1 and prevented binding to IGF-1R, thereby inhibiting IGF-1 signaling. Additionally, cells with constitutively activated IGF-1R signaling were resistant to sCLU-mediated inhibition. This study suggests that there may be a negative feedback loop between IGF-1 and sCLU. So far, we have not observed a decrease in sCLU in cell lines after IR exposure (up to 96 h) however it could be a possible mechanism for eventual decrease in sCLU back to basal expression.

Summary

Overall, the studies performed in this dissertation, greatly enhances our knowledge of IGF-1 and sCLU protein regulation. These studies revealed two previously unknown factors required for IGF-1-sCLU expression and clarified other aspects of this complicated signaling pathway. This study also revealed that mutant p53 does not suppress IGF-1-sCLU expression, which was previously unknown.

Since both IGF-1 and sCLU are pro-survival factors that protect cancer cells from endogenous and exogenous insults, they are good targets for cancer chemotherapy, especially when used in combination with IR or cytotoxic agents. However, determining how this pathway is regulated may also uncover new targets, or explain how current drug therapies kill cancer cells. Also, this pathway may also important for determining total dose received after unexpected IR exposures, where levels of IGF-1 and sCLU could be measured up to days after exposure, due to the delayed and extended up-regulation of sCLU. In conclusion, these studies show that IGF-1-sCLU is important pro-survival, pro-cancer signaling axis that is highly regulated by both positive and negative factors and feedback mechanisms.

Materials and Methods

Comet Assay Cells ($1x10^5$) were irradiated (10 Gy) or treated with H_2O_2 (500μM, 1 hour), and trypsinized to obtain single cells. 25 μL of the single cell suspension was combined with 250 μL of 1% low melting point agarose in PBS, which was then added to comet slides (Trevigen). After agarose solidified, cells were immersed in lysis solution (2.5% SDS, 34 mM N-Lauroyl-Sarcosine, 25 mM EDTA, pH 9.5) and then immersed in alkaline solution (300 mM NaOH, 1mM

EDTA). Cells were then washed in TBE buffer, and then subject to electrophoresis at 2.5 v/cm for 5 minutes. After electrophoresis, slides were dried and stained with SYBR Green, and comet tails imaged by fluorescence microscopy. Comet tail lengths for a least 100 cells were measured using image J software, length is in arbritary units (AU). Data graphed are the mean +/- SEM. Statistical significance was calculated using a paired Student's t-test.

Flow Cytometry After IR or IGF-1 exposure, cells were trypsinized, counted, and then spun down. For antibody and PI staining, the cells were resuspended in PBS containing 1% formaldehyde and placed on ice for 30 minutes, then washed twice in PBS, resuspended in ice cold 100% ethanol, and stored at 4°C overnight. The next day, 1 x 10^5 cells were stained with 0.25 μg of primary antibodies (α-phospho S25-53BP1 (Bethyl), α-phospho-S1981-ATM (Rockland), or α-γ-H2AX (Upstate), washed with wash buffer (PBS with 0.5% BSA and 0.05% sodium azide) and stained with 0.25 μg of fluorescent secondary antibodies (Invitrogen). The cells were washed again and stained with propidum iodide staining solution (50 μg/mL propidium iodide, 2% FBS, 100 μg/mL RNaseA in PBS) for 2 hours to overnight, and then analyzed by flow cytometry for cell cycle and antibody staining.

Fluorescence Microscopy HCT116 cells were grown on sterilized glass coverslips, and treated with or without IGF-1 for 24 hours in low serum medium (0.5%) before IR. Cells were fixed at indicated times in 70% Methanol and 30% Acetone

at -20°C for at least 20 minutes. Cells were washed in PBS, blocked with 5% FBS in PBS for one hour, then incubated with primary and fluorescent secondary antibodies. Nuclei were stained with Hoechst 33258. Foci per nucleus were counted in at least 100 cells using a fluorescence microscope. Data are graphed as the mean +/- SEM. Statistical significance was calculated using a paired Student's t-test.

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