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Radiation Sensitization of Breast Cancer Cells by Vitamin D Through the Promotion of Autophagic Cell Death

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RADIATION SENSITIZATION OF BREAST CANCER CELLS BY VITAMIN D

THROUGH THE PROMOTION OF AUTOPHAGIC CELL DEATH

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

1,25 -D3 1,25 dihydroxyvitamin D3

CaMKK- Ca^{2+}/calmodulin-dependent kinase kinase

DAPI- 4′,6-diamidino-2-phenylindole

eif2α -eukaryotic initiation factor (eif2α

AMPK- 5' adenosine monophosphate-activated protein kinase

AO- Acridine orange

AVOs- Acidic vacuolar organelles

CQ- Chloroquine

ER- Endoplasmic Reticulum

FACS- Fluorescence-activated cell sorting

GRP78- glucose-regulated protein of 78 kDa

PERK- protein kinase-like endoplasmic reticulum kinase

IR- Ionizing radiation

mTOR- Mammalian Target of Rapamycin

PI- Propidium iodide

TUNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling

TEM- Transmission electron microscopy

BAF- Bafilomycin A1

SS- Serum starvation

ULK1- unc-51-like kinase 1

List of Contributions

All of the experiments performed in this dissertation were performed by Eden Nicole Wilson.ZR-75-1 cells in which ATG 5 and ATG 7 were genetically silenced were kindly provided by Dr. William Maltese.

Abstract

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Major Director: David A Gewirtz, Ph.D.

Professor, Pharmacology and Toxicology

Radiation therapy is a widely used tool in cancer therapy and is frequently offered as the first line of treatment for cancers of the breast. While radiotherapy is often initially effective in killing tumor cells or suppressing their growth, there are factors that confer tumor cell resistance to irradiation. Development of resistance may lead to disease recurrence despite the use of surgery, chemotherapy and radiation therapy. A primary goal of the studies in Dr. Gewirtz's laboratory is to develop strategies to overcome resistance to radiation (and chemotherapy) in breast cancer, with the ultimate goal of preventing or attenuating disease recurrence. One of these approaches involves combining the active form of vitamin D, 1,25-di hydroxy vitamin D3 or its analogs with radiotherapy.

Our proposed studies were designed to build upon and extend previous work from this laboratory focused on determining the nature of cell death when vitamin D3 is combined with ionizing radiation in breast tumor cells. Studies were extended to the wild type p53, estrogen receptor positive, ZR-75-1 breast cancer cell line. We were able to validate that vitamin D3 does in fact, sensitize ZR-75-1 breast cancer cells to radiation therapy and substantiate that autophagy is the mode of sensitization by vitamin D3. Interestingly, our experimental system demonstrated that autophagy can actually have dual roles. Specifically, inhibition of autophagy both enhanced sensitivity to radiation and attenuated radiation sensitization by 1,25D3. Moreover, this experimental model proved to be a useful tool in trying to distinguish the factors involved in cytoprotective and cytotoxic autophagy, as we were able to demonstrate a potential role of 5' adenosine monophosphate-activated protein kinase in the sensitization of breast tumor cells to radiation by vitamin D3 as well as cytotoxic autophagy.

Chapter 1

Introduction

Cancer

Cancer is the leading cause of death worldwide and the second leading cause of death in the United States (Lyn, 2012). Deaths are projected to continue rising, with an estimated 13.1 million deaths in 2030 (Boehringer Ingelheim, 2012). Cancer is defined as the uncontrolled growth of cells and can develop in almost every tissue and organ in the body (American Cancer Society, 2011b). Benign tumors do not have the capacity to invade other tissues, in contrast to malignant tumors that have these properties (Kleinsmith et al., 2005). Cancerous cells stop responding to normal growth regulatory factors as a result of multiple mutations in genes involved in cell growth (National Cancer Institute, 2000). Mutations can occur in oncogenes which promote growth, tumor

suppressor genes that inhibit growth, genes involved in programmed cell death and DNA repair genes (Chow, 2010). One of the most widespread mutations to occur in cancer cells is in p53, a tumor suppressor gene and "the guardian of the genome" that is involved in apoptosis, maintaining genomic stability and regulating angiogenesis (Ames et al., 1995; Bai and Zhu, 2006).

The most common cancer diagnosed in men is lung cancer followed by prostate cancer, whereas breast cancer is the most common cancer found in women (Centers for Disease Control and Prevention, The, 2011). It is estimated that 1 in 8 women will develop breast cancer in her lifetime and of this population 1 in 36 women will die from the disease (U.S. breast cancer statistics.2012). There are two primary forms of breast cancer, ductal and lobular. Lobular carcinomas originate in lobules of the breast that produce milk whereas ductal carcinomas originate in the tubes that move milk from the breast to the nipple; ductal carcinomas are more prevalent (Mayo Clinic, 2012). Breast Cancers are broken down into three categories: Hormone receptor positive (estrogen or progesterone receptor), HER2 positive, and triple negative (Breast Cancer: Know your type.2011 . Approximately 75% of all breast cancers are estrogen receptor (ER) positive and about 65% of these cancers are also progesterone positive (Wax, 2010). Breast cancer, which is hormone-receptor positive is most likely to respond to hormonal therapies. Approximately 20% to 25% of breast cancers, are her2/neu positive (overproduction of HER2/neu) and, unlike breast cancers which are hormone receptor positive, these breast cancers tend to be much more aggressive and fast-growing (Pohlmann et al., 2009; Pruthi, 2010). It is estimated that between 10% and 17% of breast cancer cells are triple negative, meaning that these cancers lack the estrogen and

progesterone receptors and do not overexpress HER2 (Wax, 2010). These cancers are often very aggressive and prone to metastasis as well as disease recurrence (How triple-negative breast cancer behaves and looks.2012).

Cancer Treatments

Cancer treatments are dependent upon the type of cancer and stage of the disease. Cancer treatments include **surgical removal**, chemotherapy, hormone antagonist, gene therapy and radiation therapy. The decision to conduct surgery depends on the location and size of the tumor. For instance, a breast cancer patient may receive a lumpectomy or a mastectomy to remove cancerous tissue. Mastectomy refers to the surgical removal of the entire breast whereas a lumpectomy refers to removal of only a portion of the breast that has cancerous tissue (Mastectomy vs. lumpectomy.2012). For many tumors, cancer surgery is the best treatment if the tumor is localized and has not metastasized (Cancer surgery: Physically removing cancer.2011). However, in some cases surgery is not the best option as some cancerous tissue is not completely accessible.

Chemotherapy is the use of anti-neoplastic agents to treat cancer (Fayed, 2009). Most chemotherapies are designed to target cells that divide rapidly and act by promoting cytotoxicty which leads to cell death (Chemotherapy and you: Support for people with Cancer.2011). Chemotherapies are divided in to five classes, which include: alkylating agents, anti-metabolites, plant alkanoids, topoisomerase inhibitors and cytotoxic antibiotics. Moreover, chemotherapy can eliminate cancer cells that have metastasized to other parts of the body and therefore is a systemic approach to cancer treatment as it treats the primary site as well secondary sites (American Cancer Society, 2011c).

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Unfortunately, chemotherapy can target normal cells that are actively dividing and can lead to many adverse side effects.

Hormone therapy is another systemic approach to treat cancer. It is an adjuvant treatment and often used to help reduce recurrence after surgery, but it can also be used to treat cancer that has spread or recurred (American Cancer Society, 2012). Hormone therapies generally involve interfering with the actions of hormones that promote growth and consequently hormone therapy is effective in cancers such as the breast and prostate. A commonly used drug to treat breast cancer is Tamoxifen. Estrogen receptor positive breast cancers require estrogen to proliferate and Tamoxifen, an estrogen receptor antagonist, prevents their proliferation (American Cancer Society, 2012). Another class of hormone therapy is aromatase inhibitors. Aromatase inhibitors prevent the synthesis of estrogen in the breast tumor tissue, thereby inhibiting the growth of breast cancer cells (American Cancer Society, 2012). However, treatment is ineffective against ER negative tumors.

Radiation therapy is one of the most widely used tools in cancer therapy and is a fundamental component in the treatment of breast cancer. Radiation therapy uses high-energy radiation to shrink tumors and kill cancer cells (How radiation works.2012; Radiation therapy.2012). X-rays, gamma rays, and charged particles are types of radiation used for cancer treatment (National Cancer Institute, 2010b; National Cancer Institute, 2010b). Patients receive either external-beam radiation therapy or internal radiation therapy (brachytherapy) (American Cancer Society, 2009). External-beam radiation therapy uses an external source of radiation, which is directed at a particular

part of the body whereas brachytherapy is radiation delivered from radiation sources (radioactive materials) placed inside or on the body (National Cancer Institute, 2007).

Exposure to ionizing radiation leads to rapid generation of reactive oxygen species (ROS) and oxidative stress that produce indirect DNA damage (Chen and Nirodi, 2007). One of the most lethal radiation induced lesions in DNA is the double strand break, as these lesions are not as easily repaired by DNA damage repair mechanisms as single strand breaks (Spitz et al., 2004). Moreover, double strand breaks in the DNA can lead to growth arrest or cell death by the activation of several signaling pathways (Rothkamm and Lobrich, 2002). Other types of DNA damage caused by radiation include single strand breaks, DNA-protein cross-links and addition of DNA adducts (Davidson and Coh, 1988; Hall and Giaccia, 2006). DNA damage caused by ionizing radiation can be direct or indirect. Most often damage is indirect, which occurs through the ionization of atoms (Hall and Giaccia, 2006). Specifically, indirect DNA damage occurs via the ionization of water, which in turn leads to the formation of O2 and hydroxyl radicals (OH) (Fig 1.1) (Hall and Giaccia, 2006).

The amount of radiation used in photon radiation therapy is measured in gray (Gy), and varies depending on the type and stage of cancer being treated. For curative cases, the typical dose for a solid epithelial tumor ranges from 60 to 80 Gy, while lymphomas are treated with 20 to 40 Gy (National Cancer Institute, 2010b). Patients typically receive fractionated radiation doses of 2 Gy (National Cancer Institute, 2010b). As ionizing radiation is not specific for cancer cells, fractionating the radiation dose allows normal cells time to recover from treatment. Due to decreased repair mechanisms, tumor cells are generally less efficient in recovering from radiation

treatment (National Cancer Institute, 2010b). Another benefit of fractionated radiation is the ability to allow tumor cells that were in a relatively radio-resistant phase of the cell cycle during one treatment to cycle into a sensitive phase of the cycle before the next fraction is given (National Cancer Institute, 2010b). Likewise, tumor cells that are more radioresistant due to hypoxia may have time to reoxygenate between fractions, which can improve tumor cell killing (National Cancer Institute, 2010b). The typical fractionation schedule for adults is 1.8 to 2 Gy per day, five days a week (National Cancer Institute, 2010b).

As with many therapeutic approaches, there are limitations associated with radiation therapy. While ionizing radiation is often initially effective in killing tumor cells or suppressing their growth, there are factors that confer resistance to irradiation in breast tumor cells, such as refractoriness to apoptosis and increased DNA repair capacity (Dent et al., 2003). Development of resistance may lead to disease recurrence despite the use of surgery, chemotherapy and radiation therapy.
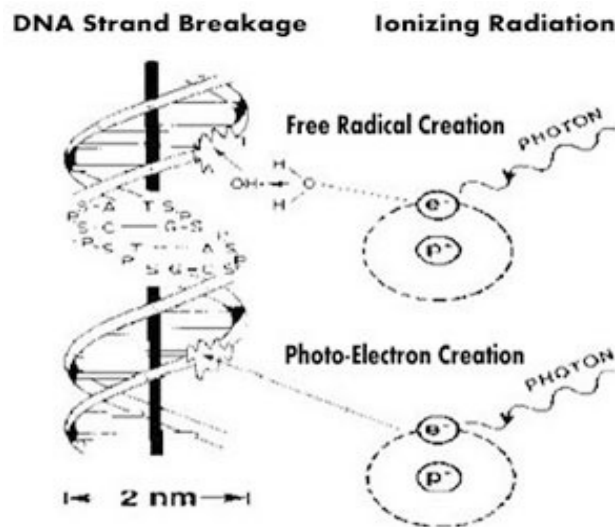
Figure 1.1 DNA Damage Induced by Ionizing Radiation (Goodall, 2004)

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Cellular Responses to Cancer Therapy

Apoptosis is a caspase dependent programmed cell death pathway (Widmann et al., 1998). Apoptosis is a normal physiological process that occurs during cellular development and serves to maintain cellular homeostasis and protect against abnormal cell division (Schwartzman and Cidlowski, 1993). It can also be activated following extreme cellular stress conditions such as DNA damage or hypoxia and is often thought to be the main response to cancer treatments (Hannun, 1997; Lowe and Lin, 2000). Apoptosis is characterized by morphological features such as cell shrinkage, membrane blebbing and nuclear fragmentation (Boe et al., 1991). There are two main apoptotic pathways, the extrinsic and intrinsic which respond to external and internal death signals respectively. Both pathways lead to transduction of intracellular signals that ultimately result in the activation of a complex cleavage cascade, converging on caspase-3 (an effector protein that initiates degradation) and subsequent destruction of the cell (Ashkenazi, 2008; Elmore, 2007). Other executioner caspases such as 6 and 7 are also involved in mediating apoptosis (Tong et al., 2002).

The extrinsic apoptotic pathway involves the activation of transmembrane death receptors (Fesik, 2005). These receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily, where the most well characterized receptor ligands include FasL, TNF-alpha, Apo3L, and Apo2L (Fesik, 2005). The corresponding receptors are FasR, TNFR1, DR3, and DR4/DR5, respectively (Fesik, 2005). The binding of the FasL to Fas receptors triggers multiple receptors on the cell surface to aggregate together. Formation of this aggregate results in the recruitment of Fas-associated death domain protein (FADD), an adaptor protein on the cytoplasmic side of the receptor (Fesik, 2005).

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FADD, in turn, recruits caspase-8, an initiator protein, to form the death-inducing signal complex (DISC) (Elmore, 2007; Fesik, 2005). Moreover, the activated caspase-8-DISC complex directly activates caspase-3 to initiate degradation of the cell (Fig 1.2) (Fesik, 2005).

Unlike the extrinsic pathway, the intrinsic apoptotic pathway is not receptor-mediated, but in turn responds to internal stimuli such as cellular stress, specifically mitochondrial stress caused by factors such as DNA damage and heat shock (Fulda and Debatin, 2006). After receiving a stress signal, the cytoplasmic pro-apoptotic proteins, BAX and BID, bind to the outer membrane of the mitochondria to signal the release of internal content (Ashkenazi, 2008; Fulda and Debatin, 2006). Another pro-apoptotic protein, BAK which resides within the mitochondria, is recruited for cytochrome c release (Fulda and Debatin, 2006). Following its release, cytochrome C forms a complex in the cytoplasm with adenosine triphosphate (ATP) and Apaf-1, which leads to activation of caspase-9 (Fulda and Debatin, 2006). The cytochrome c, ATP and Apaf-1 complex is termed the apoptosome. In a similar manner to the extrinsic pathway, the apoptosome activates caspase-3 (Fig 1.2) (Elmore, 2007). It is believed that many cancers arise from the dysfunction in the apoptotic pathway (Kasibhatla and Tseng, 2003).

Mitotic Catastrophe is cellular response due to abnormal cell division and believed to maintain genome stability by preventing division of cells with damaged DNA (Vitale et al., 2011). Mitotic catastrophe has been defined in morphological terms as a mechanism of cell death occurring during or after aberrant mitosis (Vitale et al., 2011). It is believed to occur as a result of aberrant chromosome segregation during early mitosis.

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Mitotic Catastrophe is also correlated with incomplete DNA synthesis (Castedo et al., 2004).
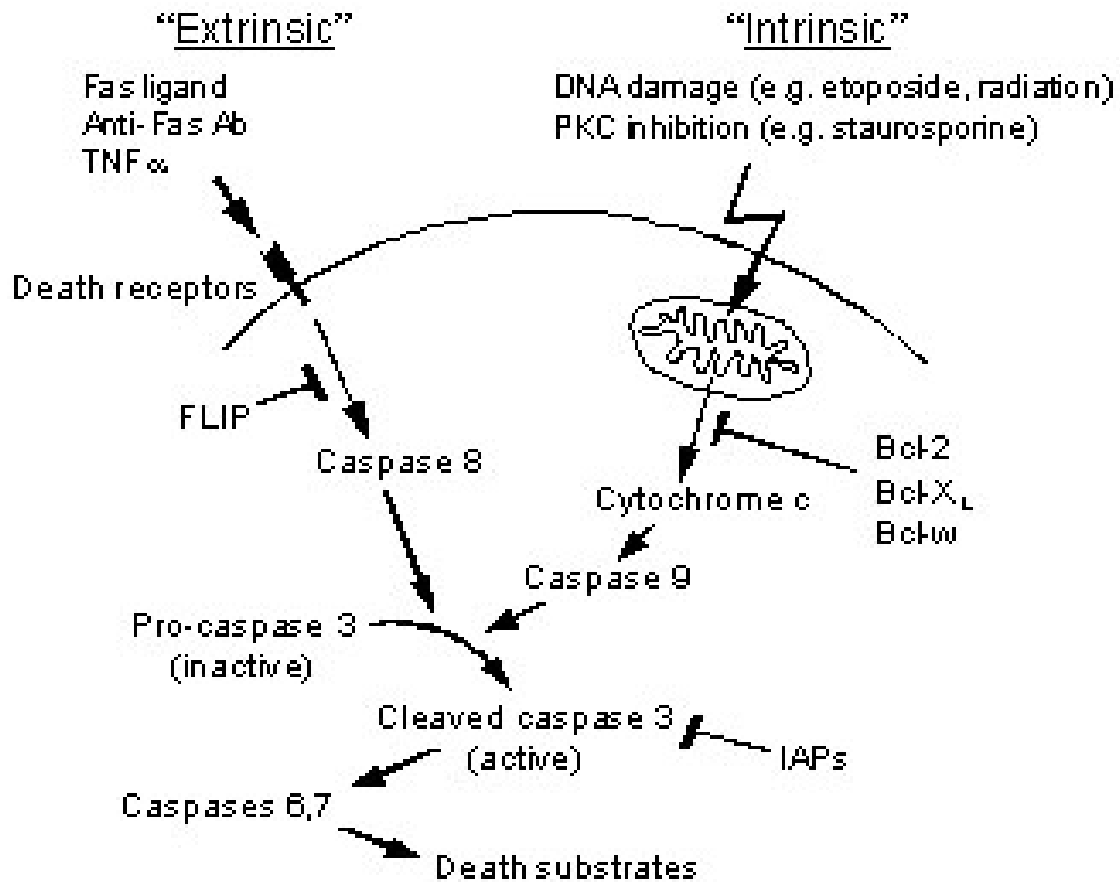
Figure 1.2 Intrinsic and Extrinsic Apoptosis Pathways (Method used by COX-2 enzymes in development of colon cancer described by UCSD medical researchers.2003)

In a similar fashion to apoptosis, cells undergoing mitotic catastrophe display chromosome condensation (Castedo et al., 2004; Vakifahmetoglu et al., 2008) . However, unlike apoptotic cells, cells undergoing mitotic catastrophe do not undergo shrinkage of the cytoplasm and nuclear fragmentation (Castedo et al., 2004). All the morphological and biochemical changes involved in mitotic catastrophe are not fully understood; however Castedo et al proposes that mitotic catastrophe is a result of the combination of deficient cell cycle checkpoints and cellular damage (Castedo et al., 2004). Cells undergoing mitotic catastrophe are characterized by an accumulation of multiple micronuclei and one of the most prominent morphological characteristics of mitotic catastrophe is the formation of giant cells with abnormally large nuclei (Castedo et al., 2004; Vakifahmetoglu et al., 2008; Vitale et al., 2011). There is evidence that cell cycle specific kinases such as cyclin B1-dependent kinase, aurora kinases, survivin and p53 play a part in regulation of mitotic catastrophe (Castedo et al., 2004). It is still unclear whether mitotic catastrophe results in caspase-dependent or caspase-independent cell death.

Senescence is irreversible growth arrest caused by the shortening of telomeres or cellular stress such as extensive DNA damage caused by radiation or DNA damaging agents (Ben-Porath and Weinberg, 2004). During senescence (believed to be a state of permanent growth arrest), cells are refractory to physiological proliferation stimuli but they remain viable and have some metabolic activity (Ben-Porath and Weinberg, 2004). Senescent cells also exhibit altered cell morphology and gene expression patterns (Dimri et al., 1995). Senescent cells are characterized by an enlarged flattened morphology and an increase of β-galactosidase activity (Dimri et al., 1995).

There are three forms of senescence: oncogene-induced, replicative and stress induced. Replicative senescence is a fundamental feature of somatic cells as it prevents unlimited cell division and is believed to be a tumor suppressive mechanism (Campisi, 2001). Human diploid fibroblasts were the cell type initially used to define the mechanisms underlying replicative lifespan (Dimri, 2005). Replicative senescence is initiated by telomere shortening and reports in the literature suggest a role of cell cycle regulators such as p53, p16 and p21 (Dimri, 2005). On the other hand, oncogene-induced senescence (OIS) is a sustained antiproliferative response as a result of oncogenic signaling which results from oncogene mutations, or the inactivation of tumor-suppressor genes (Braig and Schmitt, 2006). Oncogene induced senescence (OIS) was first observed following expression of an oncogenic form of *RAS* in normal human fibroblasts (Lleonart et al., 2009). Moreover, OIS is telomere independent and is believed to be a tumor suppressive mechanism to prevent the development of malignant tumors (Braig and Schmitt, 2006). Stress-induced senescence (SIS) can be induced by DNA damage, ionizing radiation such as X-rays or UV, and oxidative stress (Lleonart et al., 2009).

Necrotic cell death is often referred to as the premature death of cells and unlike other cellular responses to therapy, it is not believed to occur by an orderly set of signaling cascades (Golstein and Kroemer, 2007). A classical definition of necrosis is based on morphological features, which include plasma membrane rupture, dilation of cytoplasmic organelles and spillage of intracellular proteins that activates a damage response regulated by the immune system (Golstein and Kroemer, 2007). Induction of the inflammatory response and immune amplification is in sharp contrast to apoptotic cells that are silently removed by tissue macrophages (Golstein and Kroemer, 2007).

Moreover, morphological features of necrotic cells are similar to apoptosis but there is no involvement of caspases (Vandenabeele et al., 2010).

Recent reports suggest that necrosis might in fact have distinct molecular markers. Genetic studies have identified death receptor adaptors, including receptor interacting protein kinase 1 (RIPK1) and the tumor necrosis factor (TNF) receptor – associated factor (TRAF2), as essential regulators of death receptor–induced necrotic cell death (Galluzzi et al., 2009). Receptor-interacting serine threonine kinase (RIP-1) is also thought to be involved in this pathway. RIP-1 is reported to induce necrosis via mitochondrial dysfunction (Vandenabeele et al., 2010). Cells deficient in RIPK1 or TRAF2 have been shown to be protected from necrotic cell death when treated with Fas ligands (Festjens et al., 2007).

Autophagy is an evolutionary conserved process whereby cytoplasm and cellular organelles are degraded in lysosomes for amino acid and energy recycling (Chen and Karantza-Wadsworth, 2009) (Fig 1.3). Autophagy is activated by various cellular stressors such as nutrient starvation, energy depletion, DNA damage, interruption of growth factor signaling pathways, activation of mitogen-activated protein kinase signaling, inhibition of proteasomal degradation, the accumulation of intracellular calcium, and endoplasmic reticulum stress (Chen and Karantza-Wadsworth, 2009; Shintani and Klionsky, 2004). Upon activation, there is the formation of a double membrane structure around the cytoplasm and cytoplasmic organelles termed an autophagasome (Chen and Karantza-Wadsworth, 2009; Levine and Klionsky, 2004). Various autophagy proteins (ATGS) are required for initiation and elongation of this structure. One of the most crucial ATGS recruited to the autophagasomal membrane is

light chain protein 3 (LC3/ATG 8) which is needed for stabilization of this structure (Tanida et al., 2004). Once autophagy is activated, LC3 I is converted to LC3 II by E1-like enzyme, ATG-7. When the autophagasome fully matures it fuses with the lysosome and the contents are degraded by hydrolases (Chen and Karantza-Wadsworth, 2009; Kroemer and Jaattela, 2005; Shintani and Klionsky, 2004). The degraded contents are used for substrates for amino acids to allow for synthesis of new macromolecules (Codogno and Meijer, 2005; Geng and Klionsky, 2008) (Fig 1.3)

Two unique ubiquitin-like conjugation systems, Atg8-phosphatidylethanolamine (Atg8-PE) and Atg12-Atg5, are involved in the biogenesis of the autophagic membrane (Fig 1.4). These conjugation systems are widely conserved in various eukaryotes and have an essential role in autophagy (Levine and Klionsky, 2004; Mizushima et al., 1998). In the Atg12-Atg5 conjugation system, an irreversible isopeptide bond is formed between a C-terminalglycine residue of Atg12 and a central lysine residue of Atg5 (Kilonsky, 2011). Two additional proteins are required to form this conjugate; one is Atg7, which is an E1 ubiquitin activating enzyme and the other is Atg10, which functions like an E2 ubiquitin conjugating enzyme (Geng and Klionsky, 2008; Mizushima et al., 1998). As in the ubiquitin system, the E1-like enzyme Atg7 binds the C-terminal glycine of Atg12 through its active site to form an intermediate complex via a thioester bond (Mizushima et al., 1998). ATP hydrolysis results in Atg12 activation, and activated Atg12 is then transferred to the E2 like enzyme, Atg10 (Mizushima et al., 1998).Finally, Atg12 is covalently bonded to an internal lysine residue of Atg5 to form the final conjugate (Mizushima et al., 1998). The Atg12- Atg5 conjugate noncovalently binds another protein Atg16, which can mediate the formation of a higher multimeric structure of

Atg12, Atg5, and Atg16 (Mizushima et al., 1998). Experiments have demonstrated that the formation of the multimeric Atg12-Atg5-Atg16 complex is functionally important for autophagy (Geng and Klionsky, 2008; Xie and Klionsky, 2007).
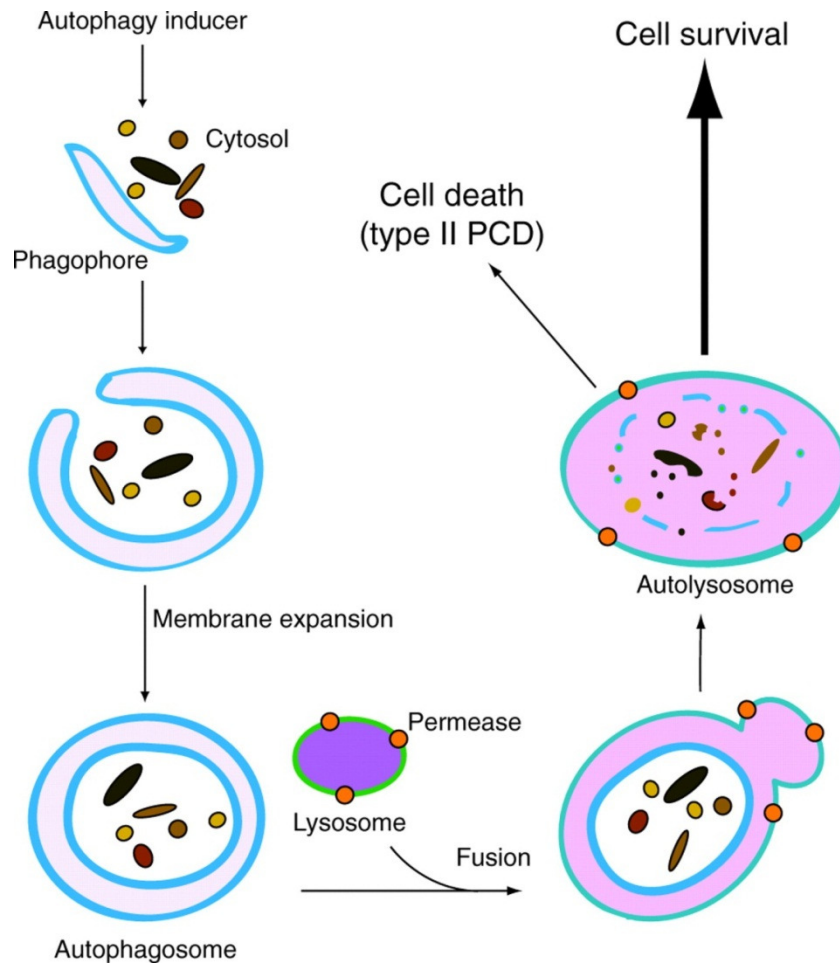
Figure 1.3 Autophagy: Autophagosome formation and degradation (Chen and Klionsky, 2011)

The other autophagy conjugation system involves Atg8 as a modifier of a lipid, phosphatidylethanolamine (Geng and Klionsky, 2008). Atg8 was originally characterized as a microtubule-associated protein required for autophagy. In the first step of Atg8 conjugation, Atg4 proteolytically removes a C-terminal arginine residue of Atg8, which exposes a glycine residue, making it accessible to the E1-like enzyme Atg7 (Geng and Klionsky, 2008). Atg7 activates Atg8, which is then transferred to another E2-like enzyme, Atg3, and eventually conjugated to phosphatidylethanolamine (PE) (Geng and Klionsky, 2008).

Autophagy is thought to be initially activated as a survival mechanism (Fung et al., 2008; Ito et al., 2005; Lum et al., 2005; Lum et al., 2005; Shintani and Klionsky, 2004; Shintani and Klionsky, 2004; Wu et al., 2008). However, data demonstrates that autophagy can become a cellular suicide pathway when apoptosis is defective and following extreme stress conditions (Debnath et al., 2005; Gorka et al., 2005; Maiuri et al., 2007; Mathew et al., 2007; Oh et al., 2008). Anticancer therapies such as drug treatment and gamma-irradiation have been shown to induce autophagy(Paglin et al., 2001a) and the autophgay induced can either contribute to cell death or represent a mechanism of resistance to these treatments(Apel et al., 2008; Gewirtz et al., 2009) .

There is some controversy in the literature as to whether autophagy is actually a cell death mechanism or rather just a precursor to other forms of cell death (Kroemer and Levine, 2008; Levine and Yuan, 2005; Shen and Codogno, 2011). There are, however, certain characteristics that define autophagic cell death. These characteristics include: i) cell death occuring without the involvement of apoptosis and necrosis; (ii) increased autophagic flux, rather than an increase of autophagic markers; and (iii) suppression of

autophagy via both pharmacological inhibitors and genetic approaches is able to rescue or

prevent cell death (Shen and Codogno, 2011).
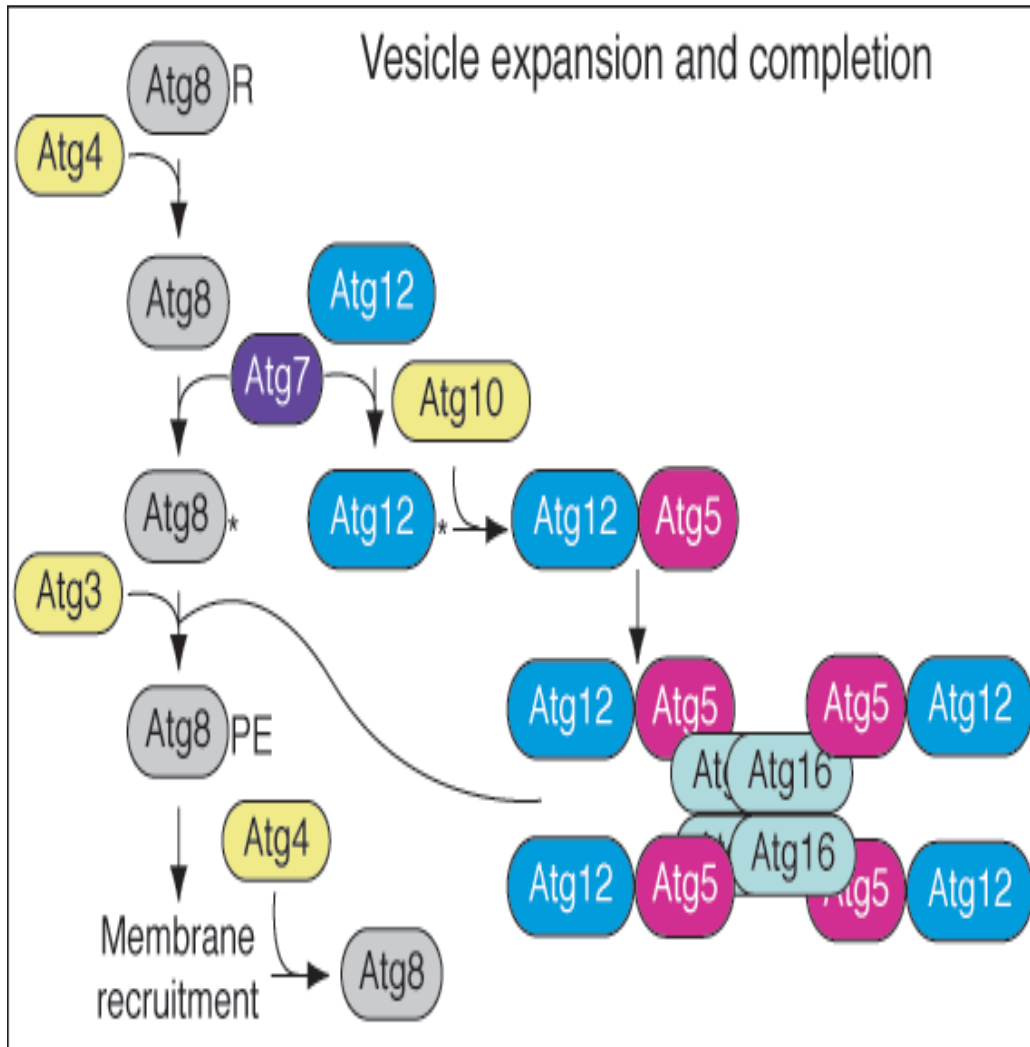
Figure 1.4 Autophagy Conjugation Systems (Kilonsky, 2011)

Autophagy Signaling (AMPK/mTOR, ER Stress)

Autophagy is a multi-step process that appears to be regulated by various signaling pathways (Codogno and Meijer, 2005). Suppression of mammalian target of rapamycin (**mTOR**) signaling has been implicated as one of the primary pathways associated with promotion of autophagy (Botti et al., 2006; Codogno and Meijer, 2005). mTOR is a sereine/thereonine kinase that belongs to the phosphatidylinositol kinase-related kinase (PIKK) family (Jung et al., 2010). mTOR is responsible for regulating cell growth, proliferation, cytoskeletal organization, transcription, protein synthesis, ribosomal biogenesis and cellular survival (Dowling et al., 2010; Jung et al., 2010).

mTOR is present in two distinct protein complexes commonly referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 ((Fig 1.6) (Dowling et al., 2010). mTORC1 signaling is frequently deregulated in cancer (Pantuck et al., 2007). Loss or inactivation of effectors upstream of mTOR, which antagonize activation of mTORC1, such as p53, LKB1, PTEN, and TSC1/2, can promote tumorigenesis via increased signaling through mTORC1 (Guertin and Sabatini, 2007). Moreover, increased levels and/or phosphorylation of downstream targets of mTORC1 have been reported in various human malignancies in which they correlate with tumor aggressiveness and poor prognosis (Hirashima et al., 2010; Pantuck et al., 2007). The activity of mTOR is inhibited by such stressors as nutrient starvation (amino acids and glucose), and energy depletion (Jung et al., 2010). Both stressors are known as crucial activators of autophagy induction in eukaryotes (Jung et al., 2010). Among the numerous components involved in the regulation of autophagy and growth, TOR (target of rapamycin) is a key component

that coordinately regulates the balance between growth and autophagy in response to cellular physiological conditions and environmental stress (Jung et al., 2010).
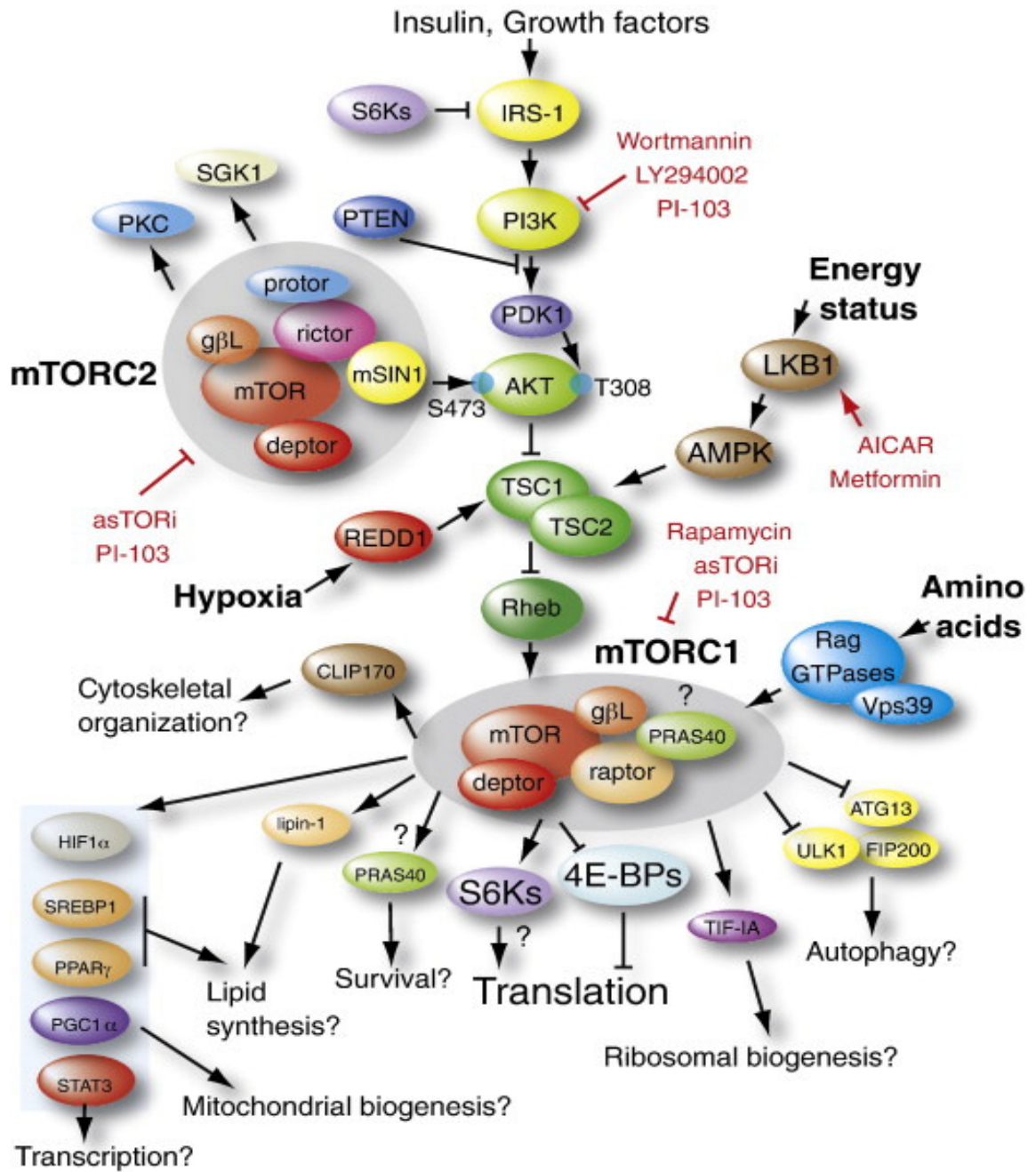
Figure 1.5 mTOR Signaling (Dowling et al., 2010)

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mTOR is also closely linked with **5′-adenosine monophosphate-activated protein kinase (AMPK)** as it represents an important regulator of mTOR activity (Chen and Klionsky, 2011; Motoshima et al., 2006). AMPK has emerged as an important regulator of cellular energy homeostasis that coordinates metabolic pathways in order to balance nutrient supply with energy demand in mammalian cells (Motoshima et al., 2006). AMPK is activated in response to an increase in the AMP:ATP ratio during hypoxia, starvation, or glucose deprivation (Hardie, 2011). The heterotrimeric AMPK contains a catalytic α-subunit, encoded by two genes α_1 and α_2 (Kodiha et al., 2007; Viollet et al., 2010).The regulatory β- and γ-subunits are encoded by two and three genes, respectively (Kodiha et al., 2007).

Activation of AMPK includes the phosphorylation of Threonine 172 of the α-subunit, which can be mediated by one of three upstream regulatory kinases LKB1, Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK) and TAK1 (Chen and Klionsky, 2011; Kim et al., 2011b; Luo et al., 2005). Numerous studies have determined an involvement of AMPK activation in autophagy induction (Hardie, 2011; Mihaylova and Shaw, 2011; Roach, 2011). It is believed that AMPK promotes autophagy by inhibiting mTOR activity through its phosphorylation of mTOR inhibitor, TSC2 (Chen and Klionsky, 2011; Hardie, 2011). Furthermore, the LKB1–AMPK–TSC1/2 and CaMKK–AMPK–TSC1/2 signaling pathways have been shown to play a role in mTORC1 inhibition under oxidative stress (Chen and Klionsky, 2011). It has also been proposed that AMPK can induce autophagy independent of mTOR by direct activation of autophagy inducer ULK1. Hoyer-Hansen et al demonstrated that calcium-mobilizing agents such as vitamin D can trigger an increase in intracellular calcium levels, which can

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ultimately lead to autophagy induction through the AMPK-mTOR pathway (Jung et al., 2010; Kim et al., 2011b; Roach, 2011). Inhibition of CaMKKβ using pharmacological inhibitor ST609 or knockdown using siRNA has been shown to attenuate autophagic cell death induced by vitamin D in MCF-7 breast cancer cells (Hoyer-Hansen et al., 2007). In addition, inhibition of intracellular calcium by BAPTA-AM and AMPK inhibition by pharmacological inhibitor compound C also inhibits autophagy induced by vitamin D in MCF-7 cells, suggesting a crucial role of this signaling pathway in autophagic cell death in breast cancer cells (Fig 1.6).
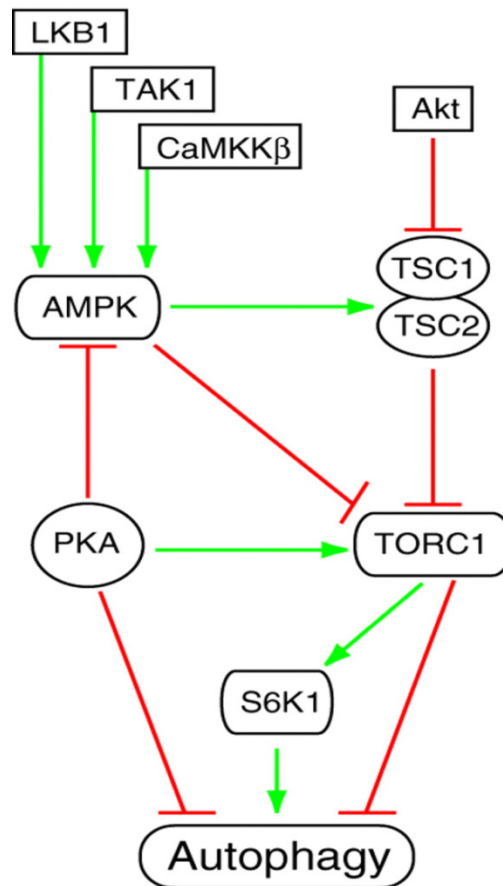
Figure 1.6 AMPK Signaling (Chen and Klionsky, 2011)

The **endoplasmic reticulum** serves two major functions in the cell. It facilitates the proper folding of newly synthesized proteins destined for secretion, cell surface or intracellular organelles, and it provides the cell with a Ca^{2+} reservoir (Hoyer-Hansen and Jaattela, 2007a). The endoplasmic reticulum (ER) is the compartment in which protein folding occurs prior to transport to the extracellular surface or various intracellular organelles (Hoyer-Hansen and Jaattela, 2007a). ER stress occurs in various physiological and pathological conditions where the capacity of the ER to fold proteins becomes saturated. For example, ER stress can become activated in response to the expression of folding incompetent or aggregation prone proteins, Ca^{2+} flux across the ER membrane, glucose starvation (reduced protein glycosylation), hypoxia (reduced formation of disulfide bonds) or defective protein secretion or degradation (Hoyer-Hansen and Jaattela, 2007a; Verfaillie et al., 2010). Furthermore, activation leads to the induction of a complex intracellular signaling cascade, termed the unfolded protein response (UPR), to remove misfolded and damaged proteins (Ogata et al., 2006). It is been shown that there is a direct link between the UPR and autophagy (Hoyer-Hansen and Jaattela, 2007a; Ogata et al., 2006). When the UPR becomes overwhelmed, autophagy is activated as a compensatory mechanism to remove the damaged proteins (Hoyer-Hansen and Jaattela, 2007a; Verfaillie et al., 2010).

While the UPR is usually a prosurvival response, under certain moderate and intermittent degrees of ER stress, chronic stress can lead to autophagic cell death (Verfaillie et al., 2010). Although autophagy has been associated with ER stress, the detailed molecular mechanisms behind activation have not been fully elucidated. However, there is increasing evidence that suggest that induction/activation of PERK,

eIF2α, IRE1, JNK, and XPB1 (Fig 1.7) are involved in ER stress mediated autophagy signaling (Hoyer-Hansen and Jaattela, 2007b; Verfaillie et al., 2010) In fact PERK-eif2a has been shown to mediate the conversion of LC3I to LC3II (Kouroku et al., 2007).
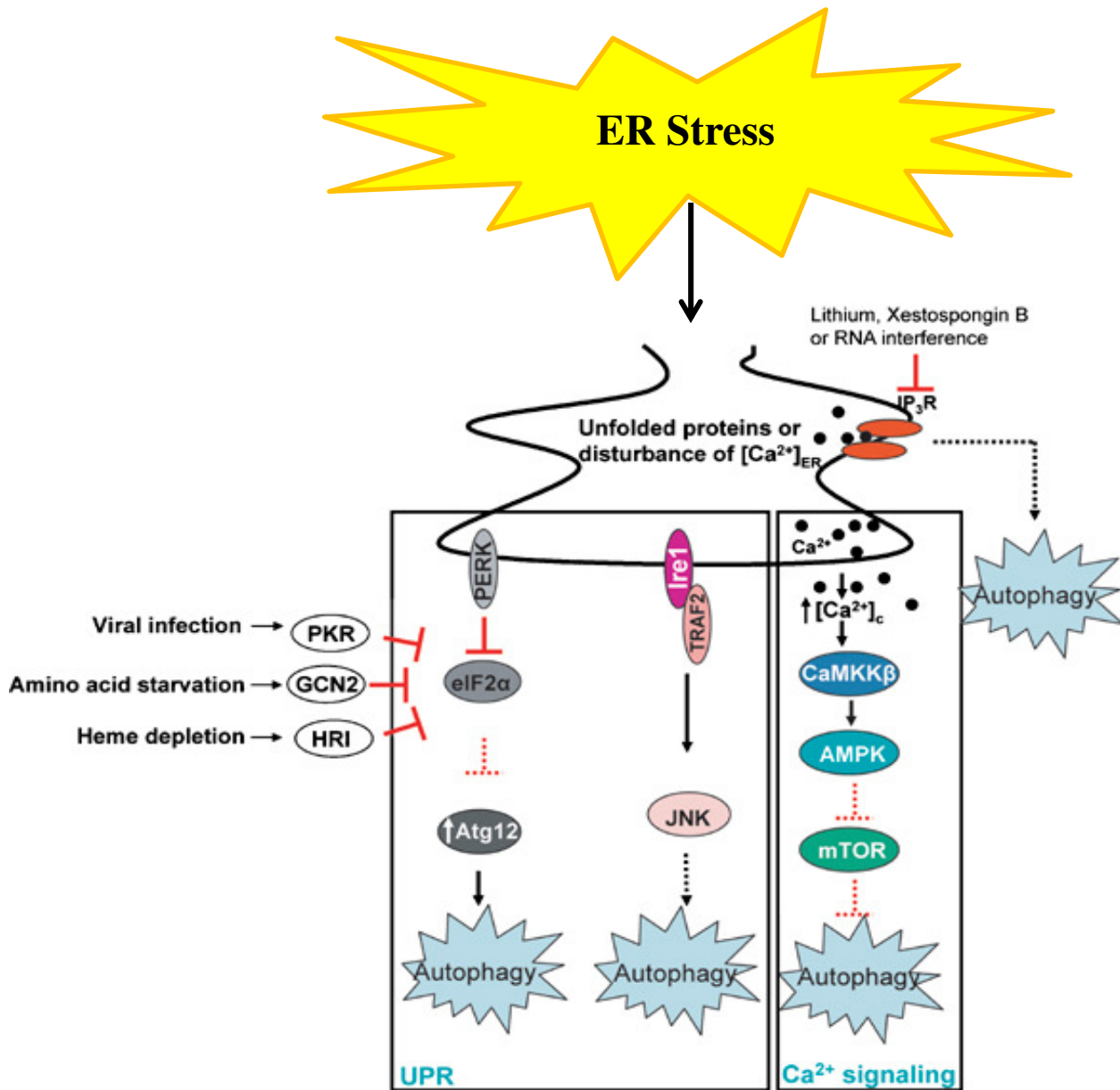
Figure 1.7 ER Stress Signaling (Hoyer-Hansen and Jaattela, 2007a)

Vitamin D

Vitamin D is a part of a group consisting of fat-soluble secosteroids (National Cancer Institute, 2010a) (Fig 1.8). Vitamin D functions as a pro-hormone and unlike most vitamins; it can be synthesized in the body (National Cancer Institute, 2010a). Vitamin D can be obtained from two sources, UV-B light from exposure to the sun and through dietary sources such as fatty fish, cod liver oil and milk (Cui and Rohan, 2006). Moreover, vitamin D is not a true vitamin because it does not require dietary supplements when sun exposure is adequate (National Cancer Institute, 2010a).

Vitamin D is synthesized from cholesterol, specifically 7-dehydrocholestorol (Cui and Rohan, 2006; Ma et al., 2010; National Cancer Institute, 2010a). Synthesis is triggered when ultraviolet rays from sunlight (UV-B) strike the skin and convert 7-dehydrocholestorol into Cholecalciferol (pre- vitamin $D_{3)}$, (Cui and Rohan, 2006). Pre-Vitamin D3 is inert and must be activated by 2 hydroxylation steps, first in the liver by 25-hydroxylase (CYP24B1) where it becomes 25-hydroxyvitamin D and second in the kidney by 1-alpha-hydroxylase (CYP24A1) (Fig 1.9) (Cui and Rohan, 2006). Pre-Vitamin D3 is transported in the blood stream on vitamin D binding proteins. The active form of vitamin D is termed calcitriol (1-alpha-dihydroxyvitamin D 1,25D3)(Cui and Rohan, 2006). Deactivation occurs when 1,25D3 is hydroxylatd by 24-hydroxylase, which yields 1-alpha-24,25 hydroxyvitamin D (Cui and Rohan, 2006).

Vitamin D's effects are mediated through its binding with the vitamin D receptor (VDR) (Cui and Rohan, 2006). The VDR can be found in both the cytoplasm and the nucleus, but in many cells it is predominately in the nucleus (Fleet et al., 2012). Once

activated by calcitriol (1,25D3), the active form of vitamin D, it forms a hetereodimer with the retinoid-X receptor (RXR) and translocates to the nucleus where it can bind to vitamin D response elements in DNA to affect gene transcription (Cui and Rohan, 2006).

The major biologic function of vitamin D is to maintain normal blood levels of calcium and phosphorus (American Cancer Society, 2010; Cui and Rohan, 2006). Vitamin D aids in the absorption of calcium in the bone, which enables bone growth, remodeling and mineralization (American Cancer Society, 2010; Cui and Rohan, 2006). Activation of 1,25D3 is triggered when calcium levels are low (Cui and Rohan, 2006). The parathyroid gland releases the parathyroid hormone, which in turn increases the synthesis of 1-alpha hydroylase and consequently results in an increase of active vitamin D (Silver et al., 1999).
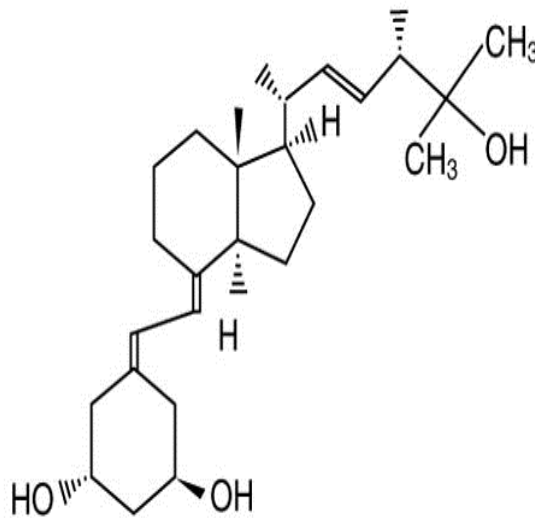
Figure 1.8 Vitamin D structure

Apart from the established role of vitamin D3 in regulation of calcium homeostasis, numerous studies have shown that the metabolically active form of vitamin D3, 1,25-di hydroxy vitamin D3, is actually involved in numerous biological processes. It has been demonstrated that Vitamin D can modulate cell growth, neuromuscular and immune function, reduce inflammation as well effect the tumor microenvironmnt (Deeb et al., 2007; National Cancer Institute, 2010a). Many genes encoding proteins that regulate cell proliferation, differentiation, and apoptosis are modulated in part by vitamin D (Trabert et al., 2007). Numerous types of cells have also been shown to express the vitamin D receptor and possess 1α-hydroxylase allowing for extrarenal activation of vitamin D on site (Buras et al., 1994; Chiang and Chen, 2009; Chung et al., 2009; Fleet et al., 2012).

Vitamin D has been shown to be involved in cancer prevention and treatment (Ma et al., 2010; National Cancer Institute, 2010a). The first results came from epidemiologic studies known as geographic correlation studies. In these studies, an inverse relationship was found between sunlight exposure levels in a given geographic area and the rates of incidence and death for certain cancers in that area (Fleet et al., 2012; National Cancer Institute, 2010a). Individuals living in southern latitudes were found to have lower rates of incidence and death for these cancers than those living at northern latitudes. Because sunlight/UV exposure is necessary for the production of vitamin D_3, researchers hypothesized that variations in vitamin D levels accounted for the observed relationships (National Cancer Institute, 2010a).Furthermore, women deficient in Vitamin D have been shown to have more advanced breast cancer that is prone to metastasis than women who have sufficient levels (National Cancer Institute, 2010a). Laboratory studies also

suggested a possible cancer-protective role for vitamin D using cell culture models (National Cancer Institute, 2010a). In fact, it is now known that a number of cancer cells express the vitamin D receptor and activation of the VDR can lead to differentiation, growth arrest, inhibition of metastasis and angiogenesis as well as cell death in a number of malignancies (Chiang and Chen, 2009; Fleet et al., 2012; Ma et al., 2010).
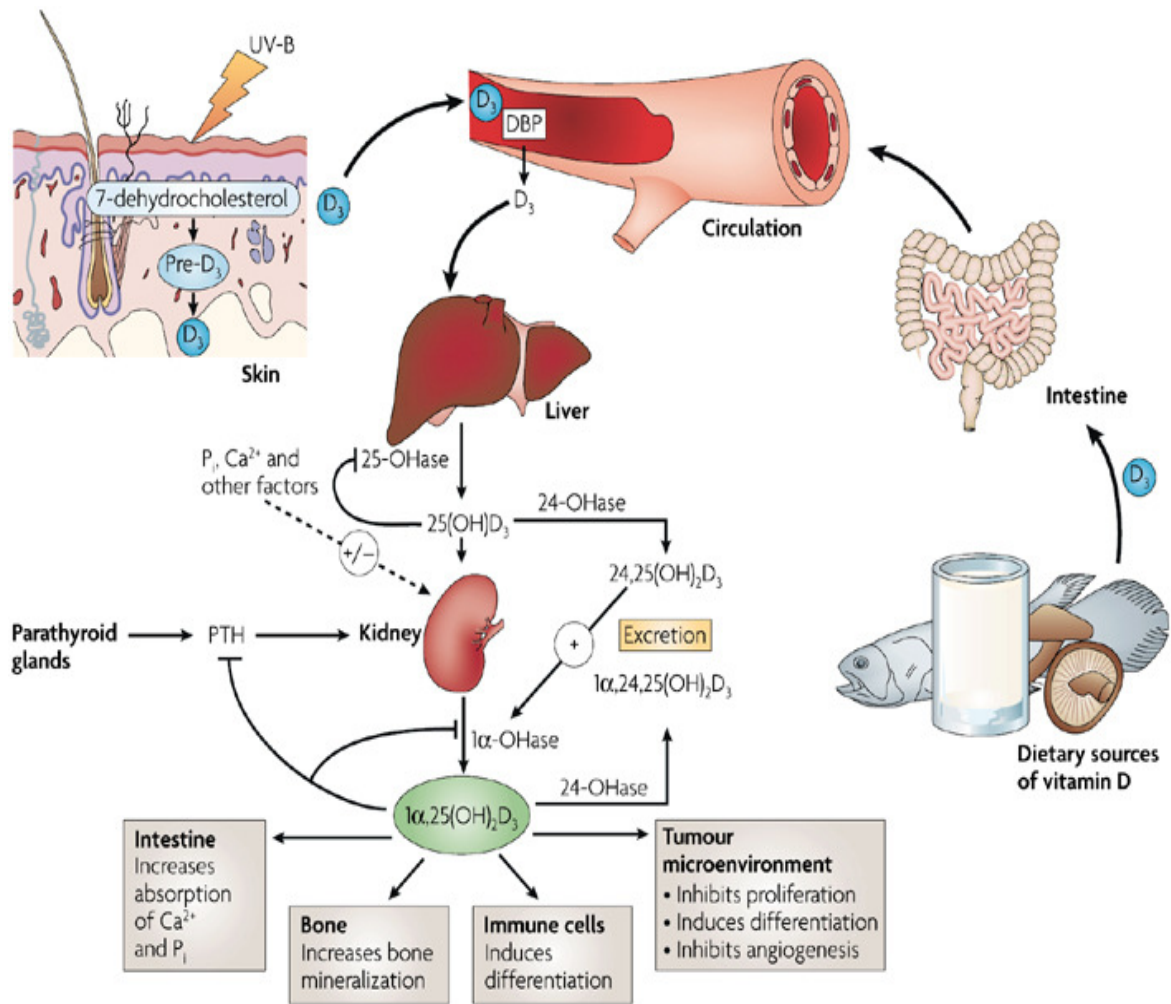
Figure 1.9 Vitamin D Activation (Deeb et al., 2007)

Genes involved in growth that are known to be regulated by vitamin D include p21, p27 and cyclin dependent kinases (Deeb et al., 2007; Ingraham et al., 2008; Wu and Sun, 2011). One interesting research finding showed that vitamin D is a partner in the antioxidant defense system of cells, helping to clear them of free radicals and thereby protecting them from DNA damage that can lead to mutations (Fleet et al., 2012). Moreover, studies have shown that mutations of the VDR can render prostate and breast cancer cells more aggressive and harder to treat (Deeb et al., 2007; Fleet et al., 2012). An additional mechanism by which vitamin D may influence cancer cell growth and viability is through modulation of growth factor signaling i.e. Insulin-like growth factor signaling (Fleet et al., 2012).

It is believed that vitamin D's anti-proliferative effects are due to modulating cell signaling pathways leading to casapase dependent and independent cell death such as apoptosis and autophagy (Deeb et al., 2007; Fleet et al., 2012). For instance, Hoyer-Hansen et al reported that vitamin D analog (100nM), EB1089, can induce cytotoxic autophagy in MCF-7 cells following a 5 day treatment (Hoyer-Hansen et al., 2005; Welsh, 2007). Studies by Welsh et al have demonstrated biochemical and morphological features of apoptosis in breast cancer in response to vitamin D (Welsh, 2007). In addition to vitamin D having anti-proliferative effects on its own, it has been shown that vitamin D can actually enhance the effects of other cancer therapies such as Adriamycin, Taxol and 5- Fluorouracil (Ma et al., 2010).

Previous Studies

A primary goal of research in our laboratory has been to develop strategies using natural compounds to enhance the response to radiation therapy by devising ways to

prevent proliferative recovery. We believe proliferative recovery is one basis for disease recurrence. Once disease recurs, it often very difficult to treat (American Cancer Society, 2011a). One strategy employed to enhance radiation response included using the active form of vitamin D, Calcitriol (1,25D3) as well as analogs of vitamin D to sensitize cells to radiation (Demasters et al., 2006; Gewirtz et al., 2009; Sundaram and Gewirtz, 1999). Previous work in our laboratory has demonstrated that the active form of vitamin D and vitamin D analogs can enhance radiation therapy in MCF-7 breast cancer cells (Demasters et al., 2006; Gewirtz et al., 2009; Sundaram and Gewirtz, 1999). Pre-treatment with 100nM EB1089 or 100nM 1,25D3 for 72 hours significantly reduced cell viability in cells receiving a fractionated dose of radiation (5x2gy) compared to cells that received radiation treatment alone (Demasters et al., 2006). Vitamin D and its analog appeared to interfere with proliferative recovery as the recovery was completely attenuated with vitamin D pre-treatment. Studies evaluating the cellular responses mediating vitamin D radiosensitization revealed that apoptosis and mitotic catastrophe were not responsible and promotion of autophagy was likely the mode of sensitization (Demasters et al., 2006). This hypothesis was based on the fact that there was a significant increase in autophagy in 1,25D3+IR treatment compared to cells that received 1,25D3 and IR treatment alone (Demasters et al., 2006).

Hypotheses and Specific Aims

Our proposed studies were designed to build upon and extend previous work from this laboratory focused on determining the nature of cell death when vitamin D is combined with ionizing radiation in the MCF-7 breast cancer cell line. We sought to extend these studies to other breast cancer cell lines believed to be highly resistant to

radiotherapy and to elucidate the autophagic signaling mechanisms by which 1,25-di hydroxy vitamin D3 sensitizes breast cancer cells to ionizing radiation therapy. We hypothesized that:

- Drug- and radiation-resistant breast cancer cell lines will be sensitized to radiation therapy following treatment with 1,25-di hydroxyl vitamin D3

- Autophagy is responsible for radiosensitization of breast tumor cells by 1,25-di hydroxy vitamin D3 and pharmacological and genetic inhibition of autophagy will block 1,25-di hydroxy vitamin D3 mediated radiosensitization.

- The ER stress and mTOR pathways are involved in the autophagic signaling cascade and may be critical signaling elements in the radiosensitization of breast tumor cells by 1,25-di hydroxy vitamin D3

To test our hypotheses we developed the following specific aims: I) proving that 1,25D3 sensitizes drug and radiation resistant breast cancer cells to radiotherapy using ZR-75-1, HS587T cells and 231-BR breast cancer cells; II) Substantiating that autophagy is the mode of radiation sensitization in breast tumor cells treated with 1,25 dihydroxy vitamin D3 by ruling out other forms of cell death and using pharmacological and genetic approaches to inhibit autophagy. III) Determining the involvement/modulation of the ER stress and mTOR pathways in radiosensitization of breast cancer cells by 1,25 dihydroxy vitamin D3.

Chapter 2

Materials and Methods

Cell Lines

The p53 wild-type (WT) ZR-75-1, MCF-7 and HS587t human breast tumor cell lines were obtained from ATCC. 231- BR cells were a generous gift from Dr. Palemiri. The ZR-75-1 ATG5-/-and ATG7-/- cells were generated as indicated below.

RNA Interference

The siRNA sequence to target Atg5 was obtained from a previous publication (Crighton et al., 2006): The sequence of the siRNA was: GCAACTCTGGATGGGATTG. It was ordered as a hairpin oligonucleotide with the sense and antisense sequence separated by a loop sequence and restriction sites at the 5' and 3' ends to facilitate cloning into System

Bioscience's pSIH-H1-puro lentiviral shRNA vector (which uses the H1 promoter to drive expression). With the shRNA inserted into this vector, lentivirus was produced in HEK 293TN cells co-transfected with the followingvectors that encode the necessary packaging components: pPack-Rev, pPack-Gag and pPack-VSVg (purchased from Systems Biosciences as a mix). The virus shed into the medium was then used to infect the ZR75 cells. The latter were selected in medium with 1 ug/ml puromycin to enrich the infected cells. A 70% KD of Atg5 in these cells was observed following selection. shRNA control cells were generated in similar fashion except that the siRNA sequence used was against the irrelevant *Aequorea victoria* green fluorescent protein (GFP).

Mission shRNA lentiviral transduction particles for Atg 7 (Sigma NM_006395), were purchased as a set of 5 different shRNA viral particles. After infecting the ZR-75- target cells with each of the 5 different viral populations, each at 3 different MOI's, the cells were checked for Atg7 expression and the culture that displayed the greatest decrease in Atg7 expression, was selected. The Sigma particles that worked best were #TRCN0000007584 (at an MOI of 0.5), with shRNA directed against the following sequence in the 3'UTR of Atg7: GCCTGCTGAGGAGCTCTCCA. The transduced cells were selected with in medium with 1 ug/ml puromycin to obtain stable cell lines.

Cell Culture and Treatment

All ZR-75-1 derived cell lines were grown from frozen stocks in basal RPMI 1640 supplemented with 5% FCS, 5% BCS, 2 mmol/L l-glutamine, and penicillin/streptomycin (0.5 mL/100 mL medium). ZR-75-1/ATG5-/- and ATG-7-/- cells were maintained using (1µg/ml) puromycin (Sigma p8833) for resistance. All cells were maintained at 37°C

under a humidified, 5% CO_2 atmosphere. Cells were exposed to γ-IR using a ^{137}Cs irradiator. In our studies, cells were exposed to 100 nmol/L 1,25 Vitamin D_3 (Sigma D1530) alone or concurrently with radiation treatment. In the cases where the radiation doses were fractionated, four fractions of 2 Gy radiation were administered over two consecutive days (two fractions separated by 6 hours on days 1 and 2).

Cell Viability and Clonogenic Survival

Cell viability was determined by trypan blue exclusion at various time points after treatment. Cells were harvested using trypsin, stained with 0.4% trypan blue dye (Sigma T8154), and counted using phase contrast microscopy. For clonogenic survival studies, cells were plated in triplicate in six well tissue culture dishes at the appropriate density for each condition. (500 cells per treatment). After 14 days, the cells were fixed with 100% methanol, air-dryed and stained with 0.1% crystal violet (Sigma C3886). For computing the survival fraction, groups of 50 or more cells were counted as colonies. Data were normalized relative to untreated controls, which were taken as 100% survival.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay

for Apoptosis

The method of Gavrieli et al.(Gavrieli et al., 1992) was used as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and nonadherent cells. Cells were fixed and the fragmented DNA in cells undergoing apoptosis was detected using the In situ Cell Death Detection kit (Roche 11373242910, 03333566001), where strand breaks are end labeled with fluorescein-dUTP by the enzyme terminal transferase. Cells were then fixed to glass slides using DAPI-containing

Vectashield mounting medium (Sigma D9542). Pictures were taken using an Olympus inverted fluorescence microscope. All images presented are at the same magnification.

Western Blot Analysis

After the indicated treatments, cells were washed in PBS and lysed using 500-1000 μL M-PER mammalian protein extraction reagent (Thermo Scientific #78501) containing protease and phosphatase inhibitors for 5 minutes on a shaker. Protein concentrations were determined by the Lowry method and equal aliquots of protein (40 μg) were separated using 12% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and using Licor blocking buffer. Membranes were immunoblotted with respective antibodies and then incubated with respective Licor secondary antibodies. Proteins were visualized using the Licor imaging system. Primary antibodies used were anti-p62 (SQSTM1 – Santa Cruz sc-28359), anti-ATG5 (APG5 – Biosensis R-111-100), anti-ATG7 (APG7 – Santa Cruz sc-33211, anti-ß actin (Santa Cruz sc-47778) anti α-tubulin (Santa Cruz sc-5286), anti-caspase 3(Cell Signaling 9665), anti-parp (Cell signaling 46D11). All primary antibodies presented were used at a 1:1000 dilution. All primary antibodies presented were used at a 1:1000 dilution

Detection and Quantification of Autophagic Cells by staining with Acridine Orange

As a marker of autophagy, the volume of the cellular acidic compartment was visualized by acridine orange staining (Paglin et al., 2001a). Cells were seeded in six well tissue culture dishes and treated as described above for the cell viability study. 72 hrs hours following initial treatment, cells were incubated with medium containing 1 μg/mL acridine orange (Invitrogen A3568) for 15 minutes; the acridine orange was then

removed, cells were washed once with PBS, fresh media was added, and fluorescent micrographs were taken using an Olympus inverted fluorescence microscope. All images presented are at the same magnification. The number of cells with increased acidic vesicular organelles (AVO) was determined by flow cytometry. Cells were trypsinized, harvested and analyzed by BD FACSCanto II using BD FACSDiva software. A minimum of 10,000 cells within the gated region were analyzed

Detection of Autophagic Cells by staining with Monodansylcadaverine (MDC)

The autofluorescent agent monodansylcadaverine (Sigma Chemical) was used as a specific autophagolysosome marker (Zakeri et al., 2008). Cells were seeded in six well tissue culture dishes and treated as described above for the cell viability study. 72 hrs hours following initial treatment, cells were incubated with medium containing 1 μg/mL MDC for 15 minutes; the MDC was then removed, cells were washed once with PBS, fresh media was added, and fluorescent micrographs were taken using an Olympus inverted fluorescence microscope. Again, all images presented are at the same magnification.

Cell Cycle Analysis by Propidium Iodide Staining

For the cell cycle analysis, tumor cells treated as described above were trypsinized, fixed with 70% ethanol, and stained with propidium iodide 72 hours post treatment. Protocol was adapted from Z. Darzynkiewicz et al (Debacq-Chainiaux et al., 2009). A minimum of 20,000 cells/events was analyzed.

Transmission Electron Microscopy

TEM services, including sample fixation, embedding, ultra-microtomy and staining were provided by the VCU Department of Anatomy and Neurobiology Microscopy Facility. Sections were imaged via Jeol JEM-1230 transmission electron microscope (EM) equipped with a Gatan UltraScan 4000SP 4K x 4K CCD camera. The scale bar at the bottom of the micrograph indicates the magnification of each image.

FACS Analysis for Annexin V and Propidium Iodide Positive Cells to Monitor Apoptosis and Necrosis

For the FACS analysis, cells treated as described above were collected and labeled fluorescently for detection of apoptotic and necrotic cells by adding 500uL of binding buffer, 5uL of Annexin V-FITC and 5uL of propidium iodide to each sample. Samples were mixed gently and incubated at room temperature in the dark for 15 min. The number of cells with increased annexin/PI staining was determined by flow cytometry and analyzed by BD FACSCanto II using BD FACSDiva software. A minimum of 10,000 cells within the gated region was analyzed.

Cytochemical Detection of β-galactosidase Staining.

β-galactosidase-positive cells were detected by the method of Dimri *et al.*(Dimri et al., 1995) Briefly, the monolayers of cells were washed two times with PBS and then fixed with 2% formaldehyde 1 0.2% glutaraldehyde (prepared in PBS) for 5 min. The cells were then washed again two times with PBS. After the last wash, staining solution was added [1mg/ml 5-bromo-4-chloro-3-inolyl-b-D-galactoside(X-gal) in dimethyformamide (20 mg/ml stock), 40 mM citric acid/sodium phosphate, pH 6.0,5 mM potassium

ferrocyanide, 5 mM potassium ferricyanide, 150mM NaCl, 2 mM MgCl2] and the cells were incubated in a 37°C for 24 hr. After incubation the cells were washed two times with PBS and visualized using an Olympus inverted microscope.

FACS Analysis of β-galactosidase Activity

Cells were treated as described above and analyzed using fluorescent β-galactosidase activity marker C12FDG. The protocol was adapted from Debacq-Chainiaux et al (Debacq-Chainiaux et al., 2009).

RFP-LC3 Redistribution

The MCF7 RFP-LC3 construct was a generous gift from Dr. Keith Miskimins. ZR-75-1 cells were stably transfected with RFP-LC3 using the standard effectene (Qiagen) protocol. Cells were treated as described above and visualized using a Leica Confocal laser-scanning microscope. Cells were counterstained with DAPI to visualize. Five fields were counted for each treated condition to determine the average number of LC3 puncta per cell.

Sytox Green and Propidium Iodide to Assess Cell Viability

For the FACS analysis, cells treated as described above were collected and labeled fluorescently for evaluation of cell viability. Sytox green (Invitrogen) was diluted 1:1000 in PBS. For Propidium iodide staining, a 1:1000 dilution was also used. Samples were mixed gently and incubated at room temperature in the dark for 15 min. The number of cells with increased sytox green staining was determined by flow cytometry and analyzed by BD FACSCanto II using BD FACSDiva software. A minimum of 10,000 cells within the gated region was analyzed

Kinase activation by Flow Cytometry

After the indicated treatments, cells were washed with 1X PBS, fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 100% cold methanol for 30 minutes and incubated with blocking buffer (bovine serum albumin in PBS) for 10 minutes. Cells were then incubated with a 1:50 dilution of primary antibody for 1 hour. Cells were washed again with PBS and incubated with a fluorochrome-conjugated secondary antibody (1:100) for 30 minutes. Cells were washed with blocking buffer and resuspended in 400uL PBS. Fluorescent intensity was measured by FACS analysis. A minimum of 10,000 cells was analyzed for each sample. Primary antibodies used were anti- p-AMPK α1/α2THR 139 (Santa Cruz sc-33524), p-MTOR SER 2448 (Cell Signaling #2976), p-eif2a SER 52 (Santa Cruz sc-12412), p-PERK THR 981 (Santa Cruz sc-32577) anti- y-H2AX Ser139 (Millipore 05-636)

Assessment of Intracellular Calcium using Flou-3am

Cells were treated as described above, washed with PBS and incubated with intracellular calcium marker, Fluo3am (1:1000 in PBS). Fluorescent intensity was analyzed by FACS analysis.

Statistical Analysis

Statistical differences were determined using StatView statistical software. The data were expressed as the means ± SE (as standard error of the mean) Comparisons were made using a one-way ANOVA followed by Tukey Kramer post-hoc test. Ps ≤ 0.05 were taken as statistically significant.

Chapter 3

Sensitization to Radiation by 1,25D3

1,25 D3 has Modest Growth Inhibitory Effects on ZR-75-1 Breast Cancer Cells

Initial studies assessed the influence of treatment with 1,25D3 on cell viability to determine an effective concentration for use in our radiation sensitivity studies. We have proposed that 1,25D3 treatment alone must be capable of producing at least a modest impact on cell viability in order to observe sensitization. Previous studies in our laboratory demonstrated that 1,25D3 treatment slows the growth of MCF-7 cells at 100nM (Demasters et al., 2006). Thus, initial studies were conducted to assess the effects of 1,25D3 treatment in ZR-75-1 breast cancer cells. ZR-75-1 cells are similar to MCF-7 cells in that they are estrogen receptor positive and contain wild type p53 (Neve et al., 2006). However, unlike MCF-7 cells, they possess functional caspase-3 (Neve et al., 2006).

To assess sensitivity to radiation by 1,25D3 in ZR-75-1 breast cancer cells, we conducted a clonogenic survival assay using 10nM, 50nM and 100nM 1,25D3 (Fig 3.1) Cells were treated with 1,25D3 for 72 hours and allowed to form colonies for 2 weeks. Both 50nM and 100nM 1,25D3 resulted in a modest but significant decrease in clonogenicity. A dose of 100nM was chosen for future studies. To confirm results obtained in the clonogenic survival assay, a trypan blue exclusion assay was performed to determine effects of 1,25D3 on cell viability. ZR-75-1 cells were treated with 100nM 1,25D3 for 72 hours and cell viability was assessed at days 3 and 6 post treatment (Fig 3.2). Similar to the clonogenic survival data, exposure of ZR-75-1 cells to 1,25D3 alone had a modest impact on cell growth; at day 3 control cells had doubled approximately 2.5 times whereas cells treated with 1,25 D3 had doubled approximately 2 times. Temporary growth inhibition is likely due to 1,25 D3's regulation of proteins involved in cell cycle progression such as p21 (Ingraham et al., 2008; Wu et al., 1997).

1,25D3 Sensitizes ZR-75-1 Cells to Radiation Therapy

Our next set of studies was conducted to determine if treatment with 1,25D3 sensitizes ZR-75-1 cells to radiation. Cells were treated with 1,25D3 concurrently with a fractionated dose of radiation (4x2 Gy) and viable cell number was determined by exclusion of trypan blue at days 3 and 6 following treatment (Fig 3.3). In previous studies in the laboratory, cells were pre-treated with 1,25D3 prior to receiving radiation. This method, however, did not result in enhanced sensitivity to radiation by 1,25D3 in ZR-75-1 cells. Consequently, studies were conducted using concurrent 1,25D3 treatment as this treatment paradigm resulted in reliable and reproducible results. The combination of 1,25D3 with radiation resulted in an approximate 40% reduction in cell viability (actual

cell killing) that was followed by growth arrest in the residual surviving cell population; in contrast, radiation alone appeared to inhibit cell growth without producing an actual reduction in viable cell number (compared to control) indicating that these cells were likely undergoing exclusively growth arrest (Fig 3.3). To confirm these results, we sought to measure cell viability by flow cytometry using sytox green as described in the Materials and Methods section. Similar to trypan blue exclusion studies, at day 3 the combination of 1,25D3+IR produced a significant increase in non-viable cells, whereas radiation treatment alone did not reduce cell viability (Fig 3.4.) Staurosporine was used as a positive control for cell killing.

Radiosensitization of ZR-75-1 breast cancer cells by 1,25 D3 was further assessed by the clonogenic survival assay using both single dose (Fig 3.5) and fractioned dose radiation (Fig 3.6); when combined with 1,25 D3, there was a significant decrease in clonogenicity compared to radiation alone at all radiation doses. As in the previous studies, 1,25D3 treatment alone produced a minimal impact on clonogenicity, indicating that the sensitization to radiation is likely not simply a result of additive toxicity between 1,25D3 and radiation.
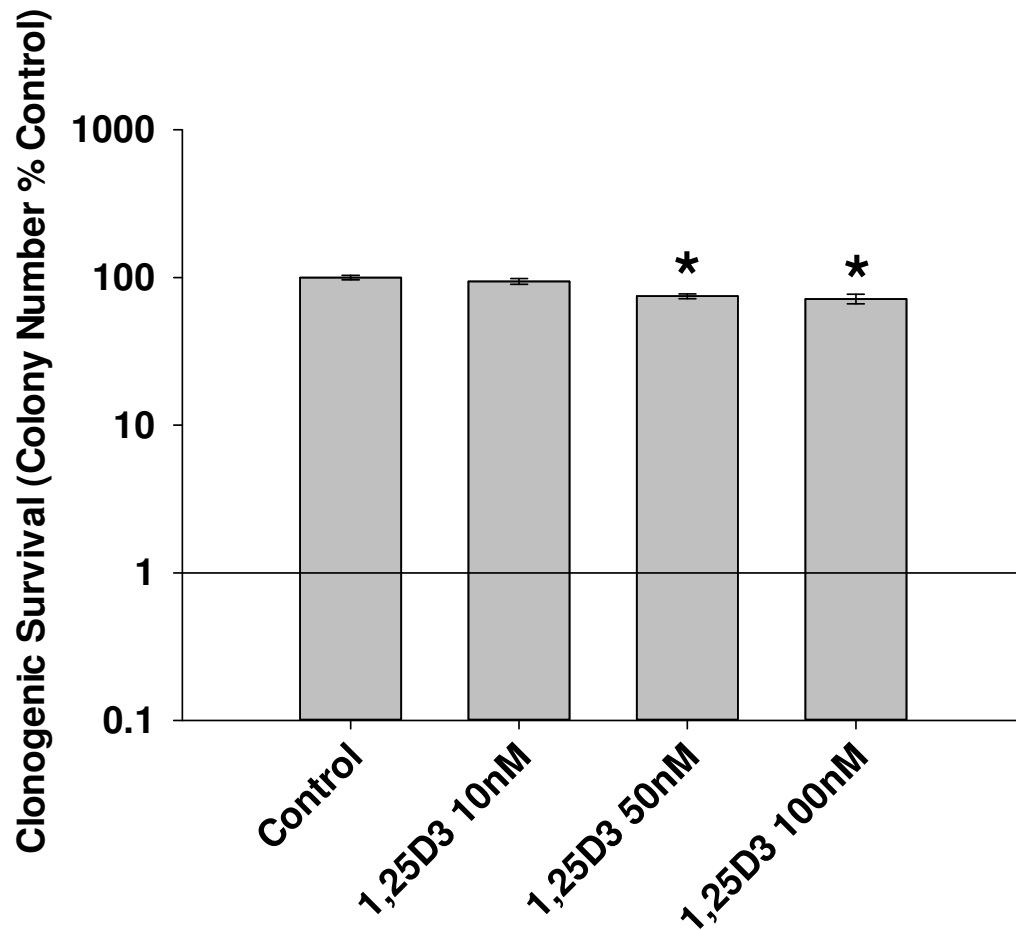
Figure 3-1 *<u>Modest Impact of 1,25D3 alone on Clonogenic Survival in ZR-75-1 Breast Cancer Cells</u>*. Cells were treated with various doses of 1,25D3 and clonogenic survival was assessed 14 days post treatment. Values shown are from a representative experiment with triplicate samples for each condition. * $p<0.05$ compared to control
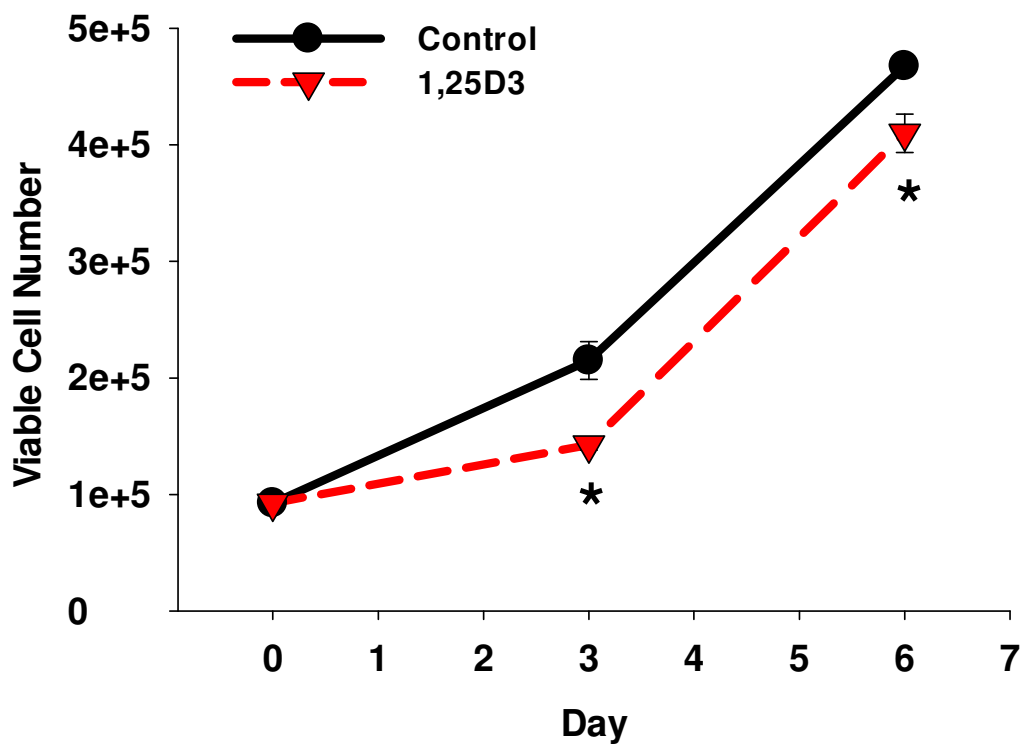
Figure 3-2 *Modest Impact of 1,25D3 Alone on Cell Viability.* ZR-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously. Viable cell number was determined by exclusion of trypan blue at days 3 and 6 post treatment. * $p < 0.05$ compared to control
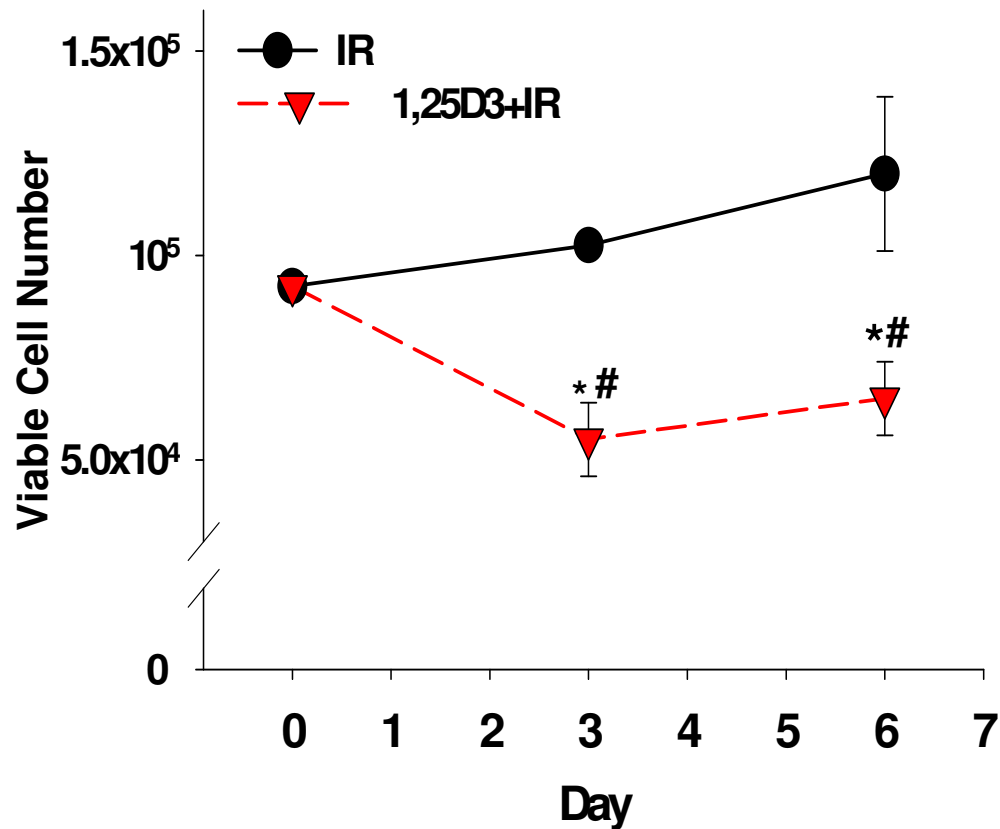
Figure 3-3 *Growth Arrest by IR and Cell Death by 1,25D3+IR.* ZR-75-1 cells received fractionated doses of 4x2 Gy (over a period of 2 days) or were treated concurrently with 1,25D3 and 4x2 Gy radiation.Viable cell number was determined by exclusion of trypan blue at days 3 and 6 post treatment. Values shown are from a representative experiment with triplicate samples for each condition. * $p < 0.05$ compared to control # $p < 0.05$ compared to IR alone
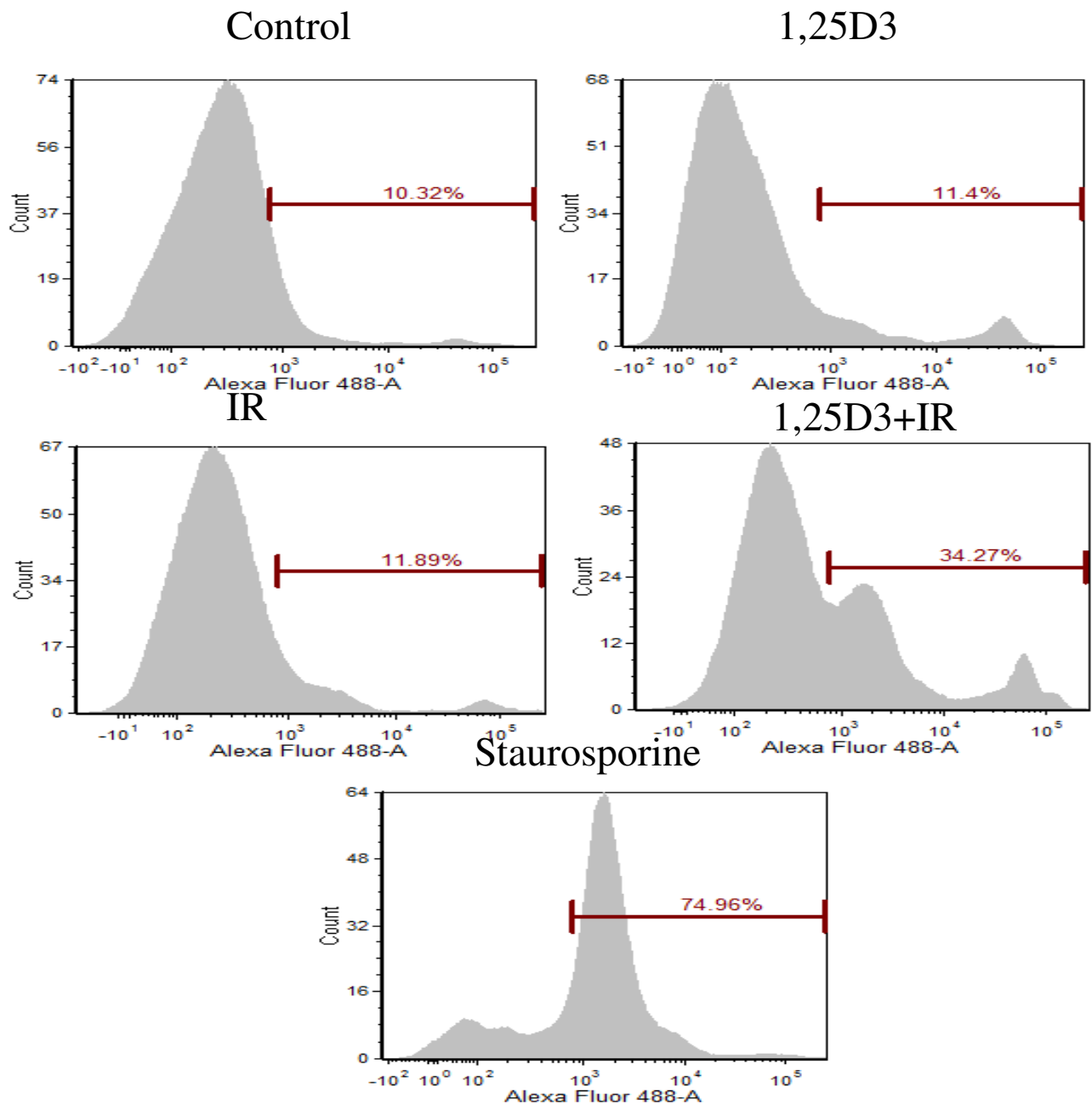
Figure 3-4 Increased Cell Death by 1,25D3+IR Treatment. ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, 4 Gy radiation or were treated concurrently with 1,25D3 and 4 Gy radiation. Viable cell number was determined by measuring intensity of Sytox Green at day 3 post treatment. Staurosporine was used as a positive control
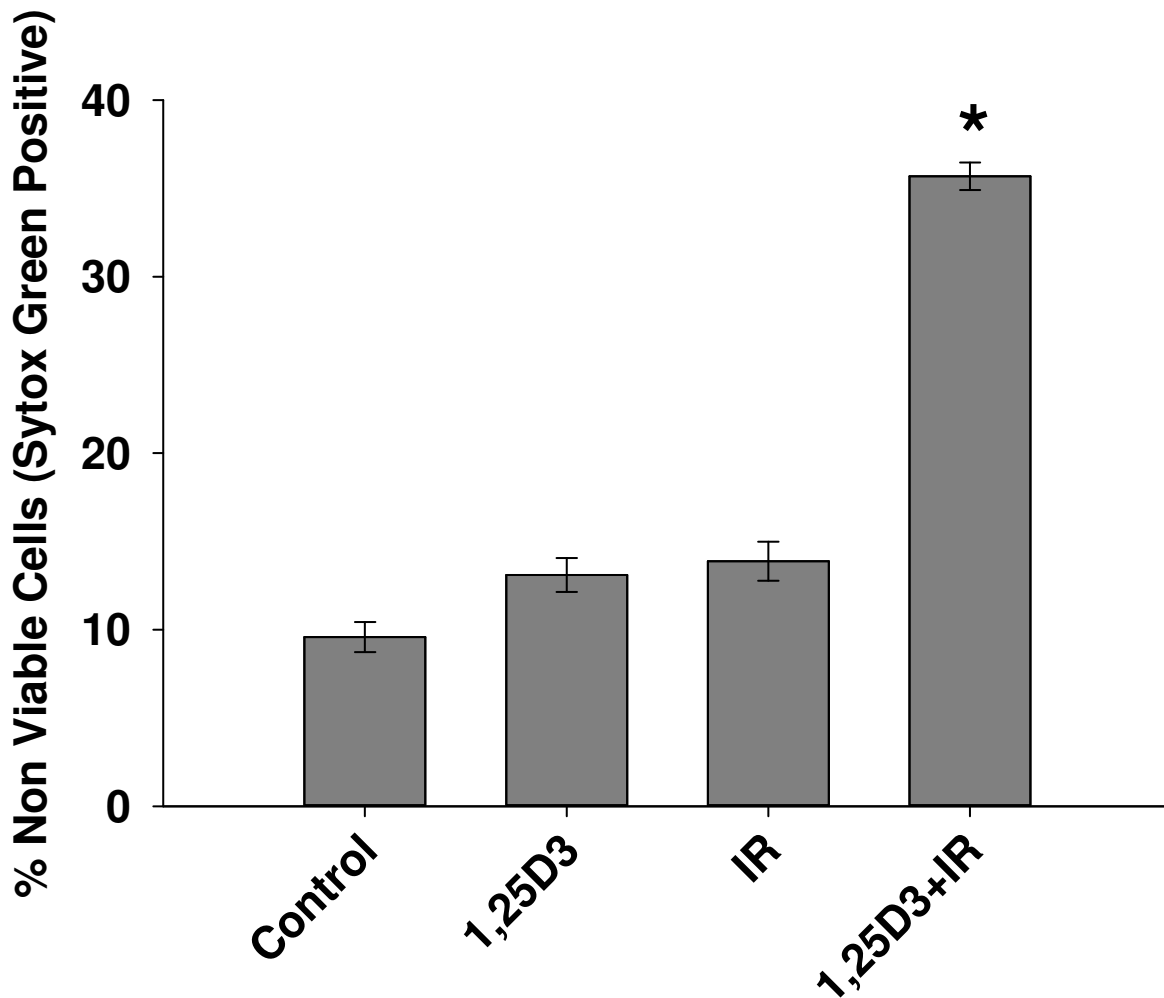
Figure 3-4 (Continued) <u>Increased Cell Death by 1,25D3+IR Treatment.</u> ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or were treated concurrently with 1,25D3 and 4 Gy radiation. Viable cell number was determined by measuring intensity of Sytox Green at day 3 post treatment. * $p < 0.05$ compared to control
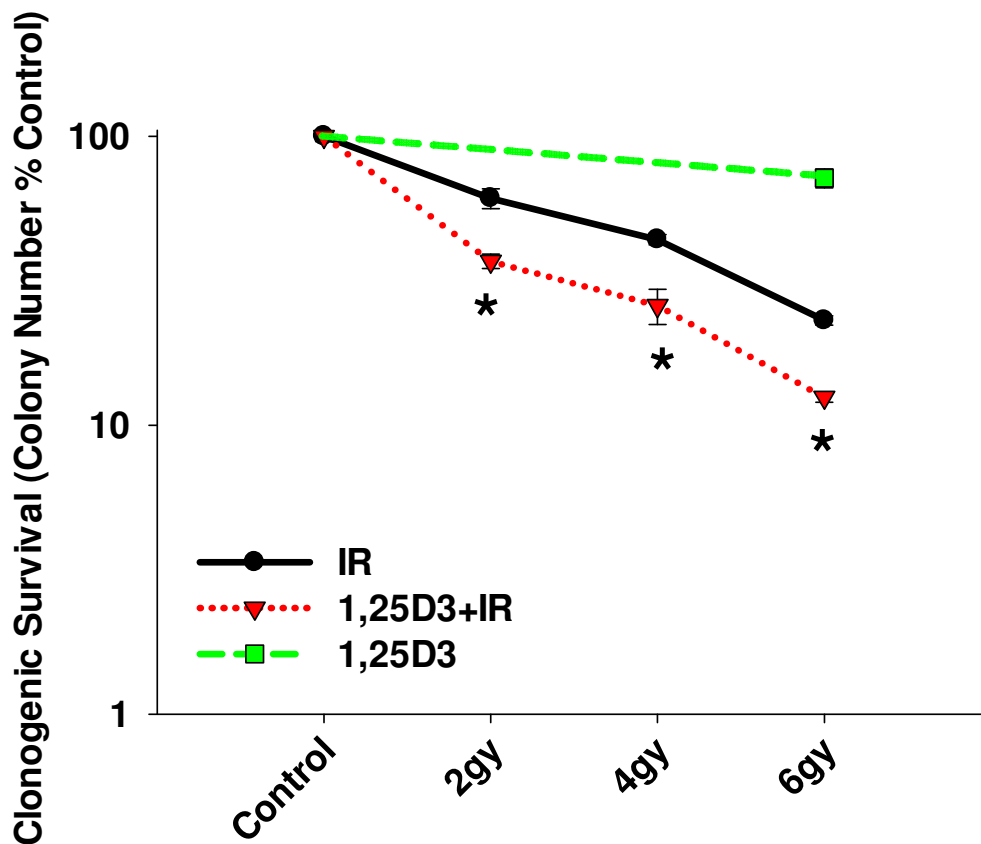
Figure 3-5 <u>*1,25D3 Increases Sensitivity to Radiation*</u> (single dose radiation) ZR-75-1 cells received 100nM 1,25D3 alone, various single doses of radiation or were treated concurrently with 1,25D3 and radiation. Clonogenic survival was assessed 14 days post treatment. Values shown are from a representative experiment with triplicate samples for each condition. * $p < 0.05$ compared to IR
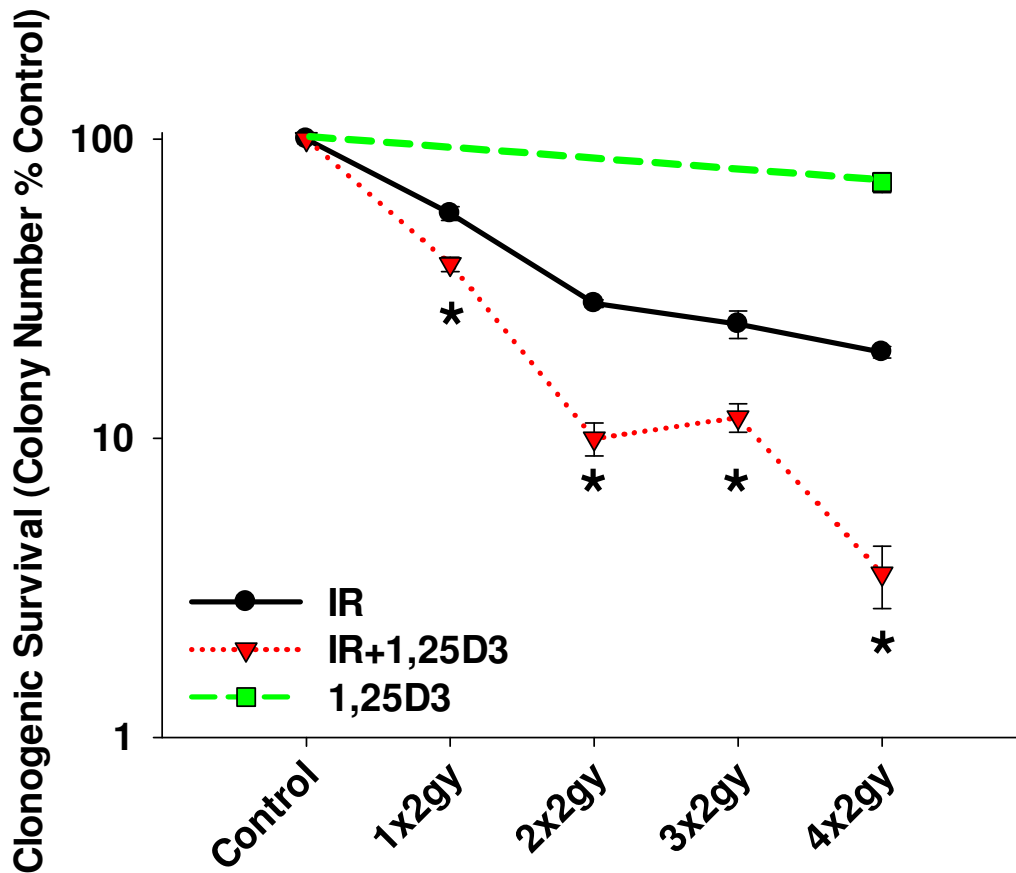
Figure 3-6 _1,25D3 Increases Sensitivity to Radiation_ (fractionated radiation) ZR-75-1 cells received 100nM 1,25D3 alone, various fractionated doses of radiation or were treated concurrently with 1,25D3 and radiation. Clonogenic survival was assessed 14 days post treatment. Values shown are from a representative experiment with triplicate samples for each condition. * p<0.05   compared to IR

Lack of Sensitization to Radiation by 1,25D3 in Cells Lacking Functional p53

To extend our radiosensitization studies to other breast cancer cells resistant to radiotherapy, we conducted studies in HS587t and 231-BR cells. HS587t breast cancer cells lack the estrogen receptor and are p53 mutant (Neve et al., 2006) . HS587t cells were treated with 1,25D3, fractionated radiation (4x2Gy) or 1,25D3+IR and cell viability was assessed at days 3 and 6 post treatment (Fig 3.7). Unlike the outcome of studies using ZR-75-1 cells, 1,25D3 treatment failed to sensitize HS578t cells to radiation therapy. DMSO, the solvent used to dissolve 1,25D3, was used as a control. In fact, while 1,25D3 had no effect on cell viability alone inclusion of 1,25D3 appears to protect the cells from radiation. However, the increase in cell viability with 1,25D3+IR treatment was not significant compared to IR treatment alone. To confirm the lack of radiosensitization by 1,25D3, we conducted a clonogenic survival assay; as was the case with the trypan blue exclusion data, 1,25D3 also failed to sensitize HS587t cells to radiation therapy (Figs 3.8-and 3.9).

We conducted the same studies in 231-BR breast cancer cells, a subclone of MDA-MB-231 human breast carcinoma cells that selectively metastasizes to brain (Gril et al., 2008). 231-BR cells originate from MDA-MB-231 breast cancer cells, which are triple negative, meaning they lack the estrogen receptor, functional p53 and the progesterone receptor. In addition, 231-BR cells overexpress the Epidermal Growth Factor Receptor (EGFR) (Gril et al., 2008). 231-BR cells were treated with 1,25D3 before IR or treated concurrently with 1,25D3+IR and cell viability was measured using trypan blue exclusion at days 3 and 6 post treatment (Fig 3.9). Neither pre-treatment nor

continuous exposure to 1,25D3 sensitized 231-BR cells to radiation. Thus, no further studies were conducted to assess radiosensitization.
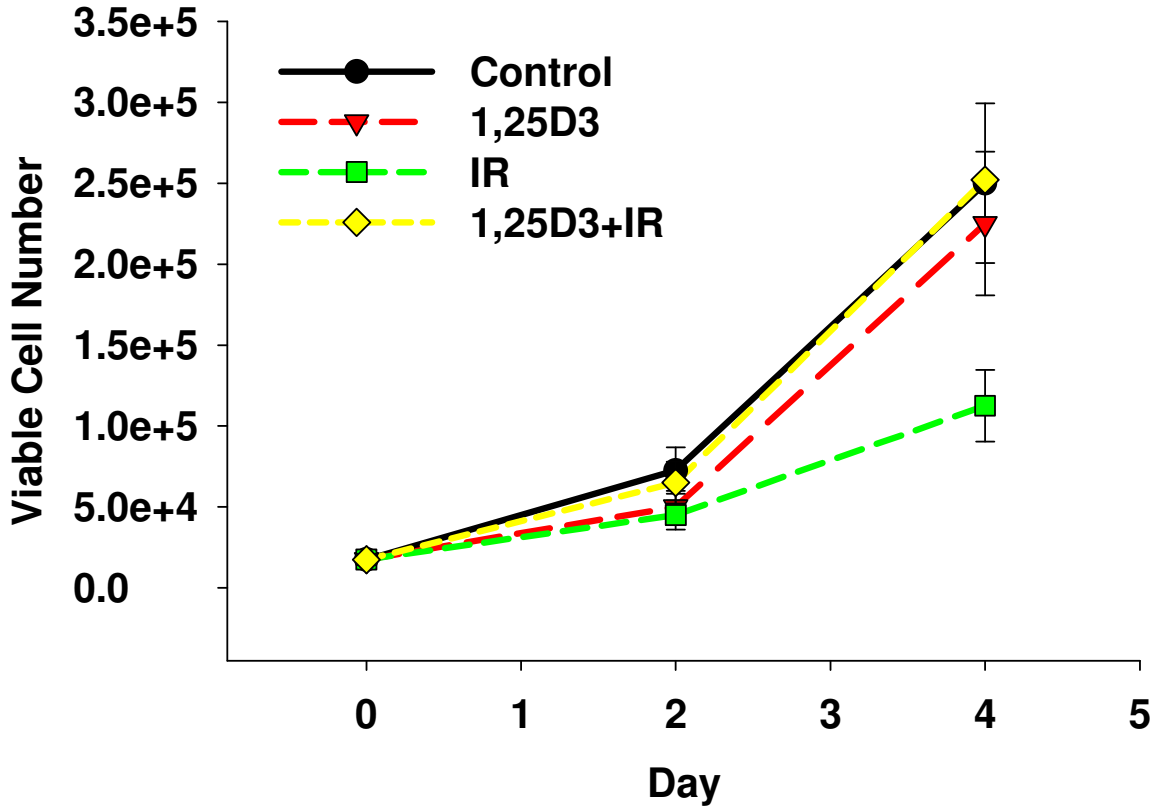
Figure 3.7 *Treatment with 1,25D3 does not Enhance Sensitivity to Radiation in HS587T Breast Cancer Cells*. Cells were treated with 1,25D3, a fractionated dose of radiation (4x2 Gy over a period of 2 days) or were pre-treated with 1,25D3 for 72 hours prior to receiving a dose of 4x2 Gy radiation. Viable cell number was determined by exclusion of trypan blue at days 2 and 4 following post treatment. Values shown are from a representative experiment with triplicate samples for each condition
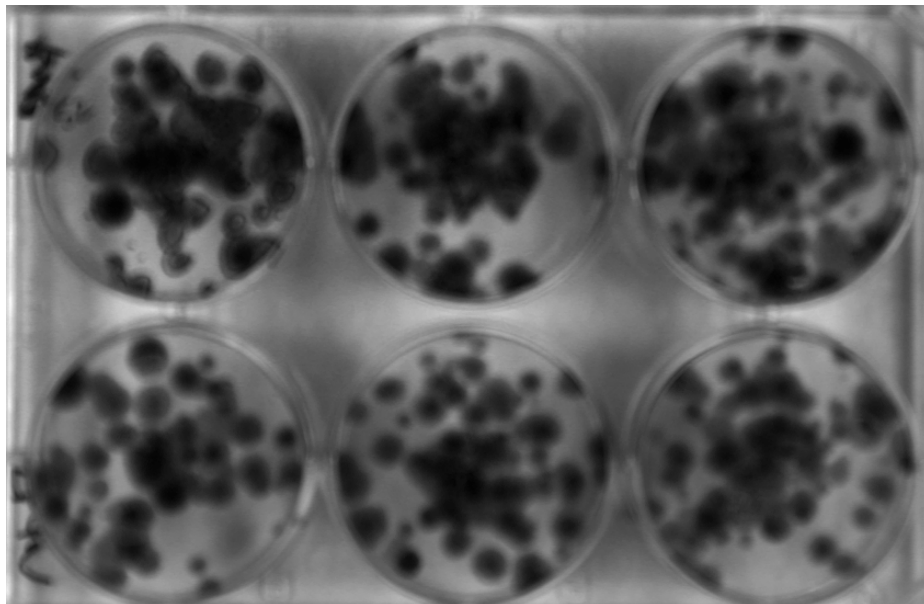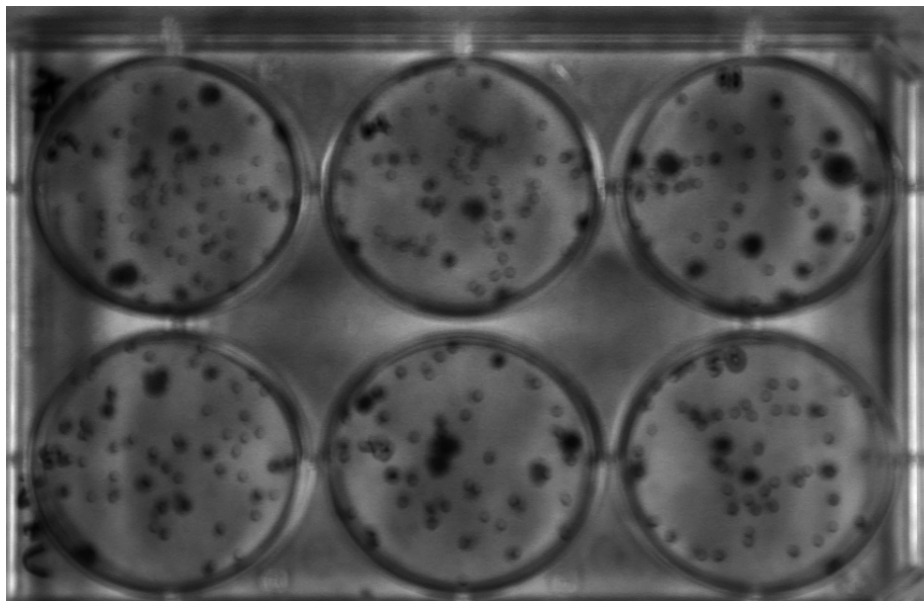
Figure 3-8 _**Lack of Sensitization to Radiation by 1,25D3 in HS587t Cells.**_ Cells were exposed to 100nM 1,25D3, fractionated doses of 4x2 Gy (over a period of 2 days) or were pre-treated with 1,25D3 for 72 hours prior to receiving a dose of 4x2 Gy radiation. Clonogenic survival was assessed 14 days post treatment. Values shown are from a representative experiment with triplicate samples for each condition.
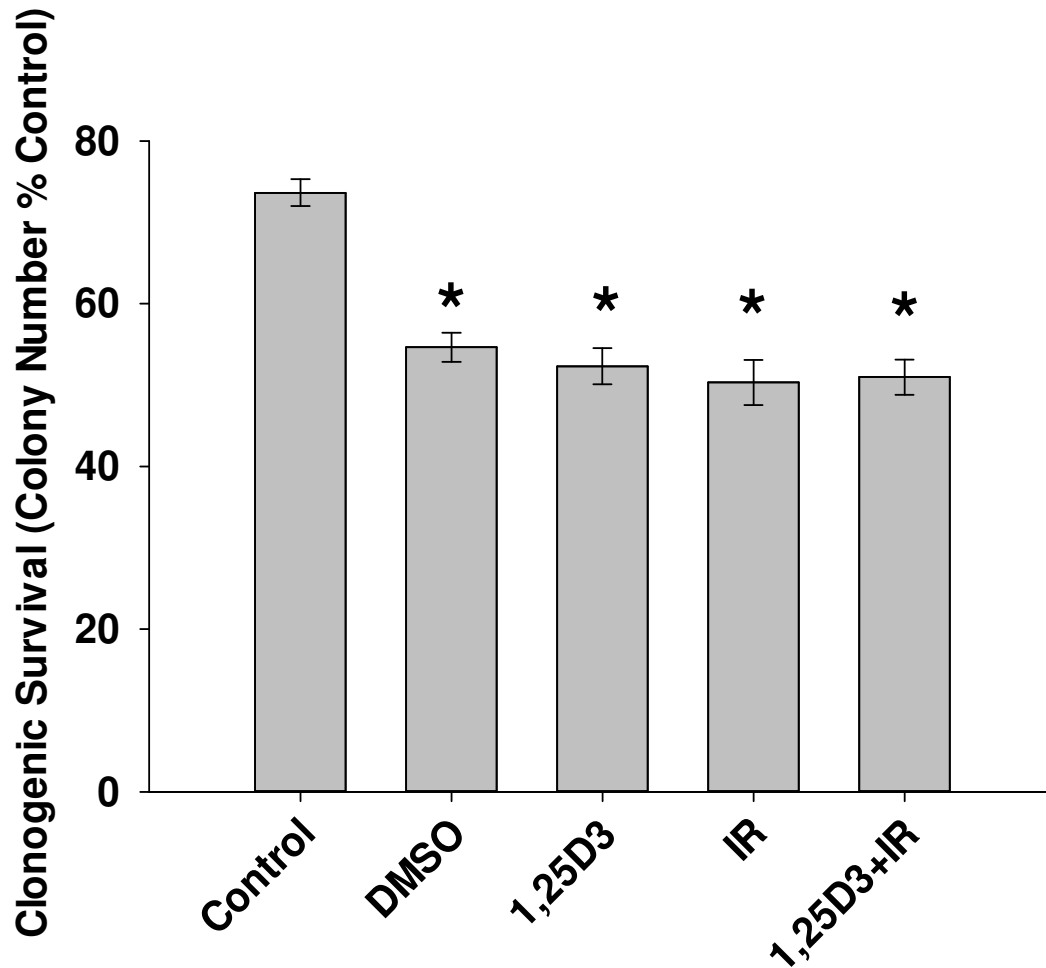
Figure 3-8 _**Lack of Sensitization to Radiation by 1,25D3 in HS587t cells (continued)**_ Cells were exposed to 100nM 1,25D3, fractionated doses of 4x2 Gy (over a period of 2 days) or were pre-treated with 1,25D3 for 72 hours prior to receiving a dose of 4x2 Gy radiation Clonogenic survival was assessed 14 days post treatment. Values shown are from a representative experiment with triplicate samples for each condition.   * $p < 0.05$ compared to control
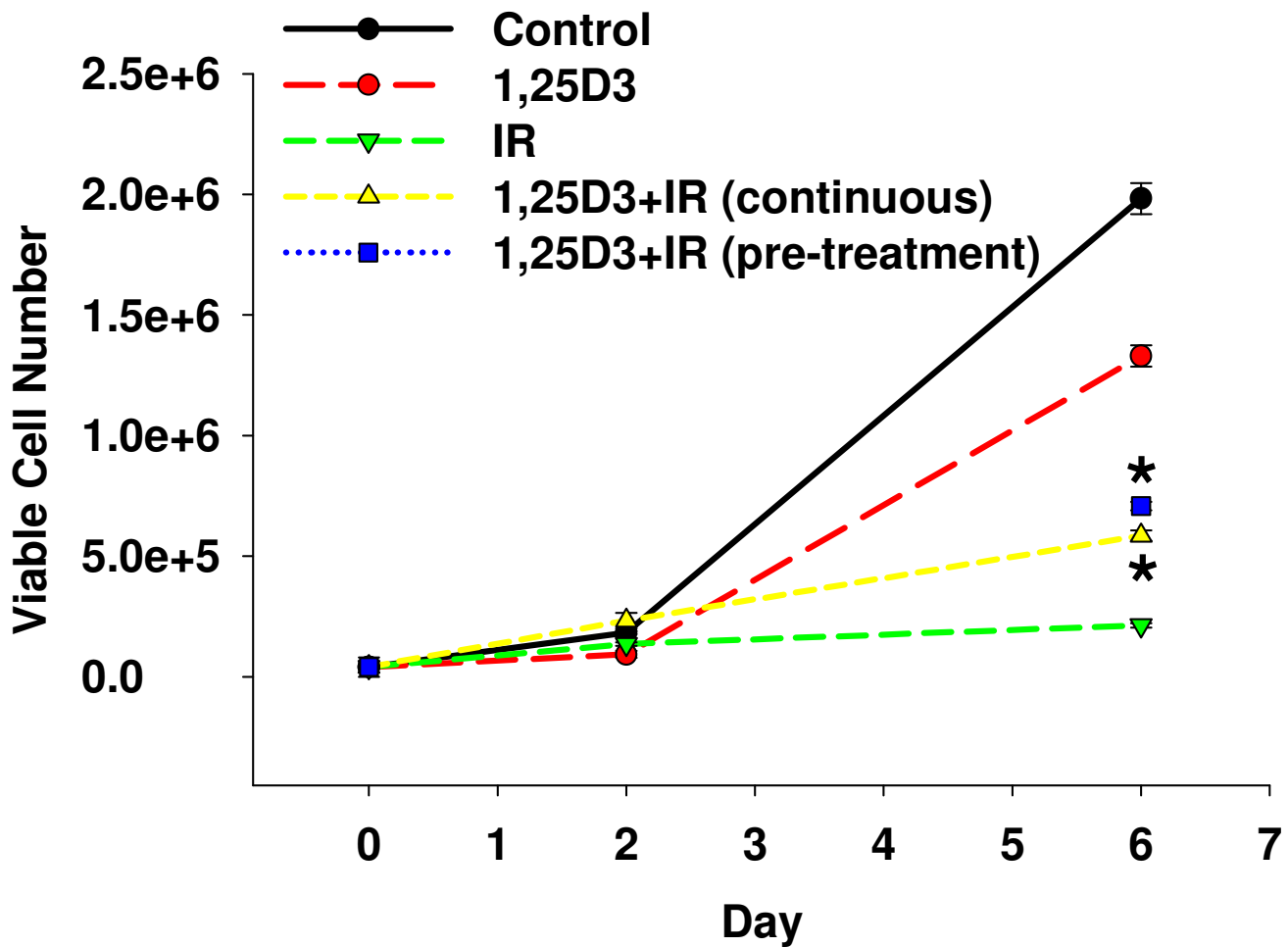
Figure 3.9 _Treatment with 1,25D3 does not Enhance Sensitivity to Radiation in 231-BR_ _Breast Cancer Cells._ Cells received 100nM 1,25D3, a 4 Gy dose of radiation or were pre-treated with 1,25D3 followed by 4 Gy radiation or concurrently treated with 1,25D3 and 4 Gy radiation. Viable cell number was determined by exclusion of trypan blue at days 3 and 6 following treatment. Values shown are from a representative experiment with triplicate samples for each condition.   * $p < 0.05$ compared to IR alone

Summary

1,25D3 alone had a modest impact on cell growth in ZR-75-1 breast cancer cells. However, 1,25D3 enhanced sensitivity to radiation as demonstrated by an increase in non-viable cells by trypan blue exclusion and sytox green assessment by flow cytometry. In addition, clonogenic survival assays showed a significant decrease in clonogenicity when 1,25D3 was combined with either single dose or fractionated radiation. These data are consistent with our previous studies in which a similar effect was observed with radiation and 1,25D3 in MCF-7 breast cancer cells (Demasters et al., 2006; Gewirtz et al., 2009; Sundaram and Gewirtz, 1999). In contrast, cells lacking p53 such as Hs587T and 231-BR breast cancer cells were not sensitized to radiation therapy when combined with 1,25D3. These findings are consistent with previous studies in our laboratory by Sundaram et al demonstrating that MDA-MB-231 and T47D breast cancer cells, both lacking functional p53, are not sensitized to radiation by 1,25D3 or vitamin D analog EB1089 (Sundaram and Gewirtz, 1999). Taken together, these studies suggest a clear requirement of functional p53 for sensitization to radiation by 1,25D3. Microarray data generated by Dr. Matthew Beckman support these conclusions.

Chapter 4

Substantiating that Autophagy is the Mode of Radiation Sensitization by Vitamin D

Minimal Induction of Apoptosis, Necrosis and Mitotic Catastrophe by Radiation and 1,25D3+IR

As mentioned in the Introduction, autophagic cell death can be characterized as occurring in the absence of apoptosis, necrosis or mitotic catastrophe. It should be noted that the absence of other cell death mechanisms alone, however, is not enough to prove that cells are undergoing autophagic cell death, but does provide some insight into how sensitization is and is not occurring. Therefore, initial studies were conducted to rule out

apoptosis (generally one of the primary modes of cell death) as the mode of radiationsensitization by 1,25D3 in ZR-75-1 breast cancer cells. We first assessed apoptosis by staining cells with DAPI and the TUNEL assay, which evaluate nuclear morphology and the presence of a specific form of DNA damage (cleavage) respectively. ZR-75-1 cells were treated with 1,25D3, radiation or the combination of 1,25D3+radiation and assessed at 72 hours post treatment (Fig 4.1) Stauroporine, a protein kinase C inhibitor and potent apoptosis inducer, was used as a positive control (Bertrand et al., 1994; Watson et al., 1988). Minimal apoptosis was detected as indicated by lack of nuclear shrinkage and fragmentation by DAPI staining as well as lack of positive green fluouescence by TUNEL, which would be indicative of DNA fragmentation and strand breaks. These characteristics were only observed with the positive control, Staurosporine.

Although TUNEL staining is relatively specific for apoptosis, we sought to confirm apoptosis or the lack thereof using Annexin V and propidium iodide (PI). Annexin V/PI staining can distinguish cells undergoing both early and late apoptosis. Cells undergoing apoptosis will display phosphatidylserine on their outer membrane. Annexin V recognizes and binds to phosphatidylserine and then fluoresces; fluorescent intensity can be measured using flow cytometry. Early apoptosis will only result in positive Annexin V staining whereas cells undergoing late apoptosis will have positive staining for both Annexin V and PI. In addition, this assay can detect cells undergoing necrosis. In contrast to apoptotic cells, necrotic cells only exhibit positive PI staining.

ZR-75-1 cells were treated with 1,25D3, radiation or 1,2D3+IR and Annexin V/PI staining was assessed 48 and 72 hours post treatment (Fig 4.2). Similar to the results of

TUNEL and DAPI staining, minimal apoptosis was evident based on minimal staining in quadrants Q2 and Q4, which are indicative of early and late apoptotic cells. There was also no evidence of necrosis by treatment with 1,25D3, IR or 1,25D3+IR, as indicated by lack of positive staining in quadrant Q1. Again Staurosporine, used as a positive control, resulted in an approximate 50% increase in apoptotic cells.

To further confirm the lack of apoptosis, we conducted western blot analysis for the cleavage of apoptotic markers caspase-3 and PARP (Boulares et al., 1999). There was no cleavage of caspase-3 or PARP in response to any of our treatments, again suggesting lack of apoptosis induction by 1,25D3, radiation or the combination of 1,25D3 + radiation (Fig 4.3). We also conducted cell cycle analysis to evaluate the population of cells in the sub-G1 phase, which would be indicative of cells undergoing apoptosis. Again there is no evidence of cells undergoing apoptosis as there was minimal accumulation of cells in this region (Fig 4.4).

Another possible mode of sensitization to radiation by 1,25D3 would be through mitotic catastrophe. There are not many assays to monitor mitotic catastrophe other than cell morphology. Cells undergoing mitotic catastrophe exhibit multinucleation and nuclear enlargement as a result of failed cellular division (Castedo et al., 2004; Vitale et al., 2011). Changes in morphology can be measured using Hoechst dye to stain the cell nucleus. One advantage of this assay is that it allows the cell membrane to stay intact, therefore allowing for the determination of cells that are multinucleated. At days 3 and 6 post treatment, there was little to no indication of extensive mitotic catastrophe for any of our treatment groups (Fig 4.5). To support this conclusion cell cycle analysis also

indicated that there was no mitotic catastrophe, due to the lack of accumulation of cells

with DNA content greater than 4n (Fig 4.4) (Kwong et al., 2009; Portugal et al., 2010).
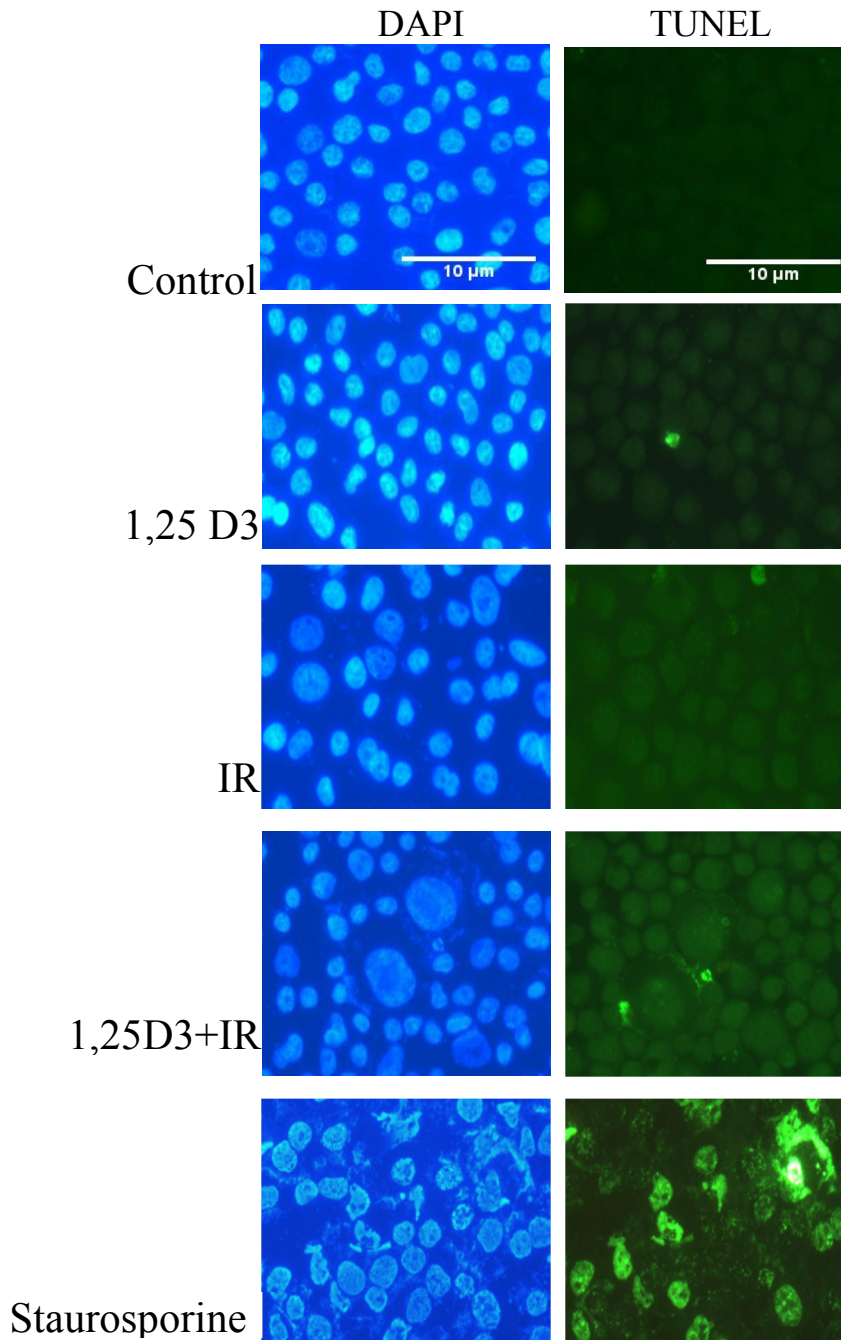
Figure 4.1 *__Minimal Apoptosis Induction as indicated by DAPI and TUNEL Staining__* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, fractionated doses of 4x2 Gy (over a period of 2 days) or were pre-treated with 1,25D3 followed by a dose of 4x2 Gy radiation. Images were taken 72 hours post treatment. Staurosporine was used as a positive control
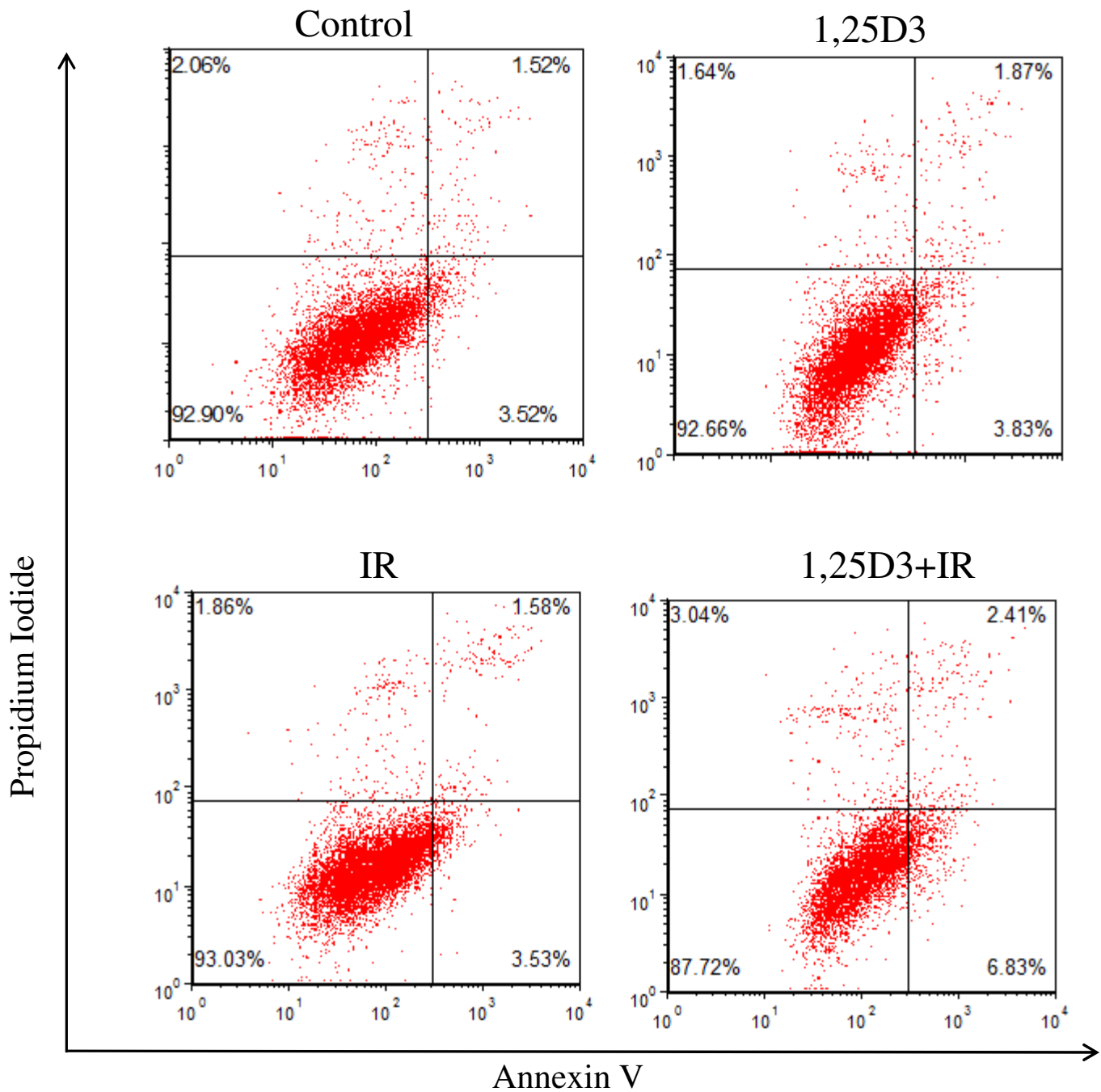
Figure 4.2 _**Minimal Apoptosis Induction as indicated by Annexin V/Propidium Iodide Staining (48 hours).**_ ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. Treated cells were stained with Annexin V/PI and fluorescence intensity was analyzed by flow at 48 hours post treatment. Staurosporine was used as a positive control

Control   1,25D3   IR

1,25D3+IR   Staurosporine

Propidium Iodide

Control: 4.6% | 0.4% | Q1 Q2 | 0.5% Q4 | Q3

1,25D3: 3.5% | 1.9% | Q1 Q2 | 3.1% Q4 | Q3

IR: 1.8% | 1.1% | Q1 Q2 | 1.8% Q4 | Q3

1,25D3+IR: 4.3% | 2.4% | Q1 Q2 | 3.3% Q4 | Q3

Staurosporine: 3.9% | 16.7% | Q1 Q2 | 25.7% Q4 | Q3

Q1 Necrosis | Q2 Late Apoptosis

Q3 Viable Cells | Q4 Early Apoptosis
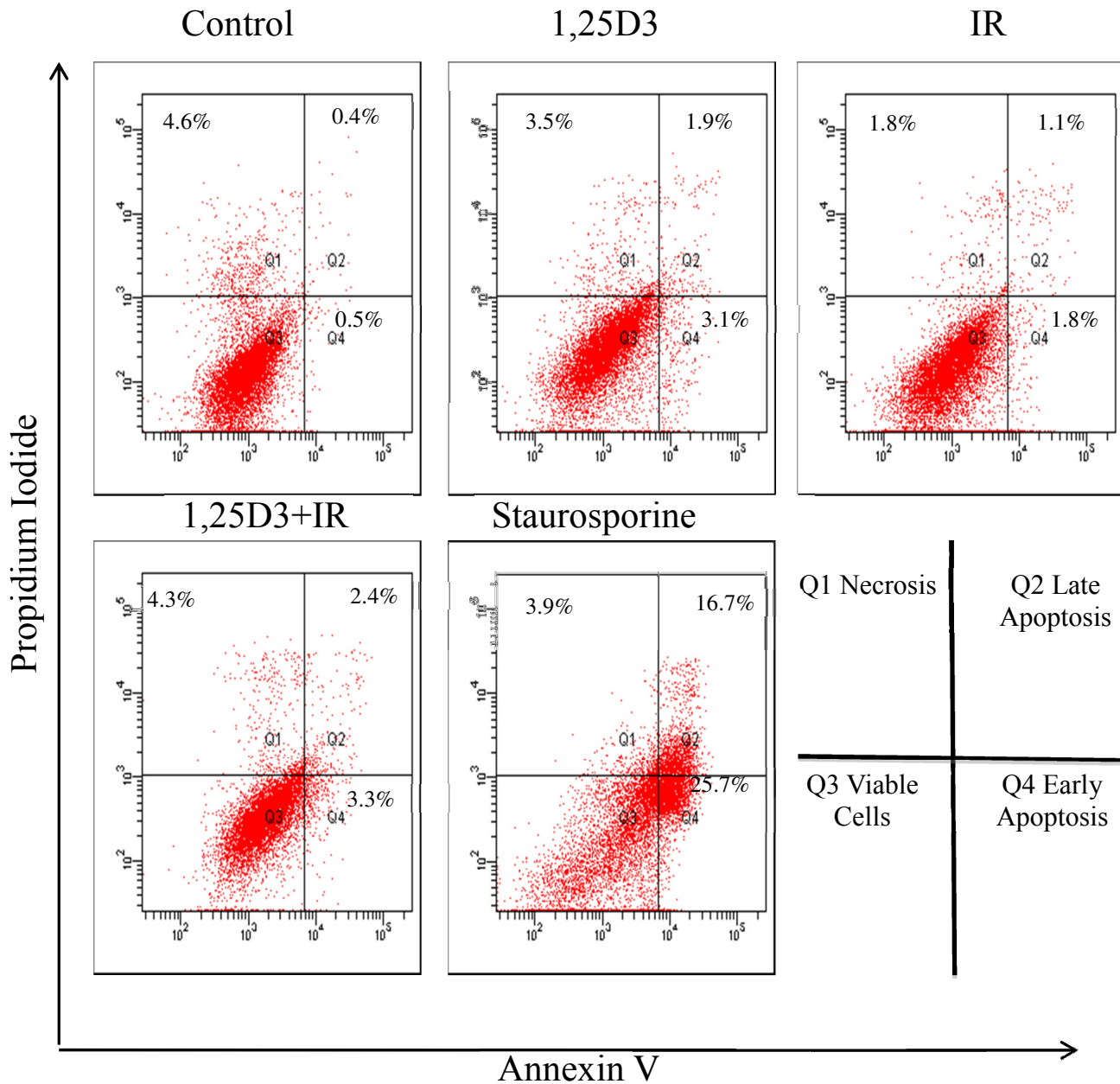
Annexin V

Figure 4.2 (Continued) *Minimal Apoptosis Induction as indicated by Annexin V/Propidium Iodide (72hours).* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. Treated cells were stained with Annexin V/PI and fluorescence intensity was analyzed by flow at 72 hours post treatment. Staurosporine was used as a positive control
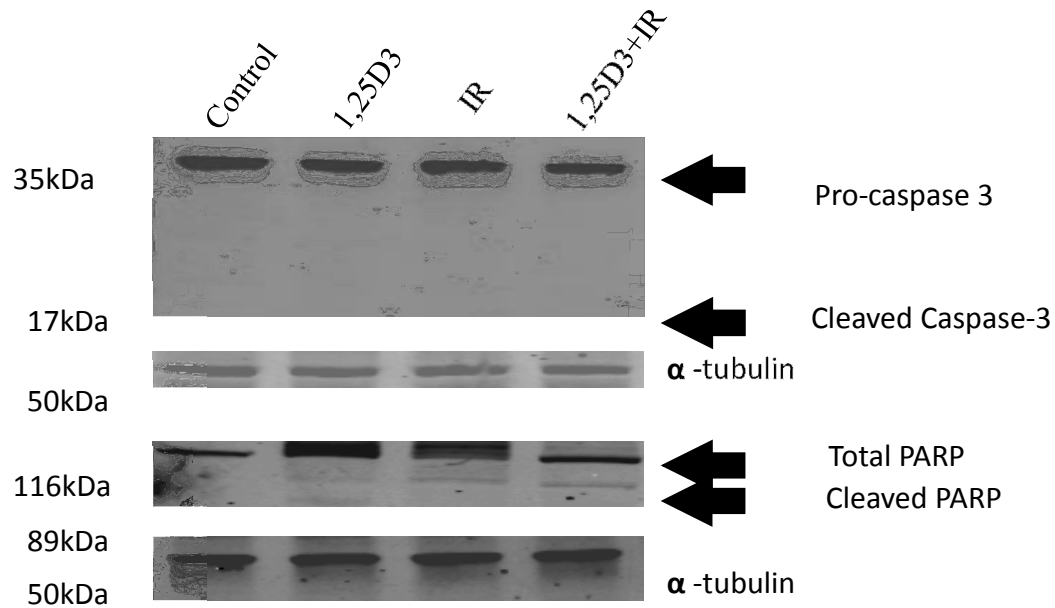
Figure 4.3 *<u>Western Blot Analysis Revealed Lack of Caspase-3 and PARP cleavage</u>* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, fractionated doses of 4x2 Gy (over a period of 2 days) or were treated concurrently with 1,25D3 and a dose of 4x2 Gy radiation. Cell lysates were taken 72 hours post treatment. **α** -Tubulin was used as loading control
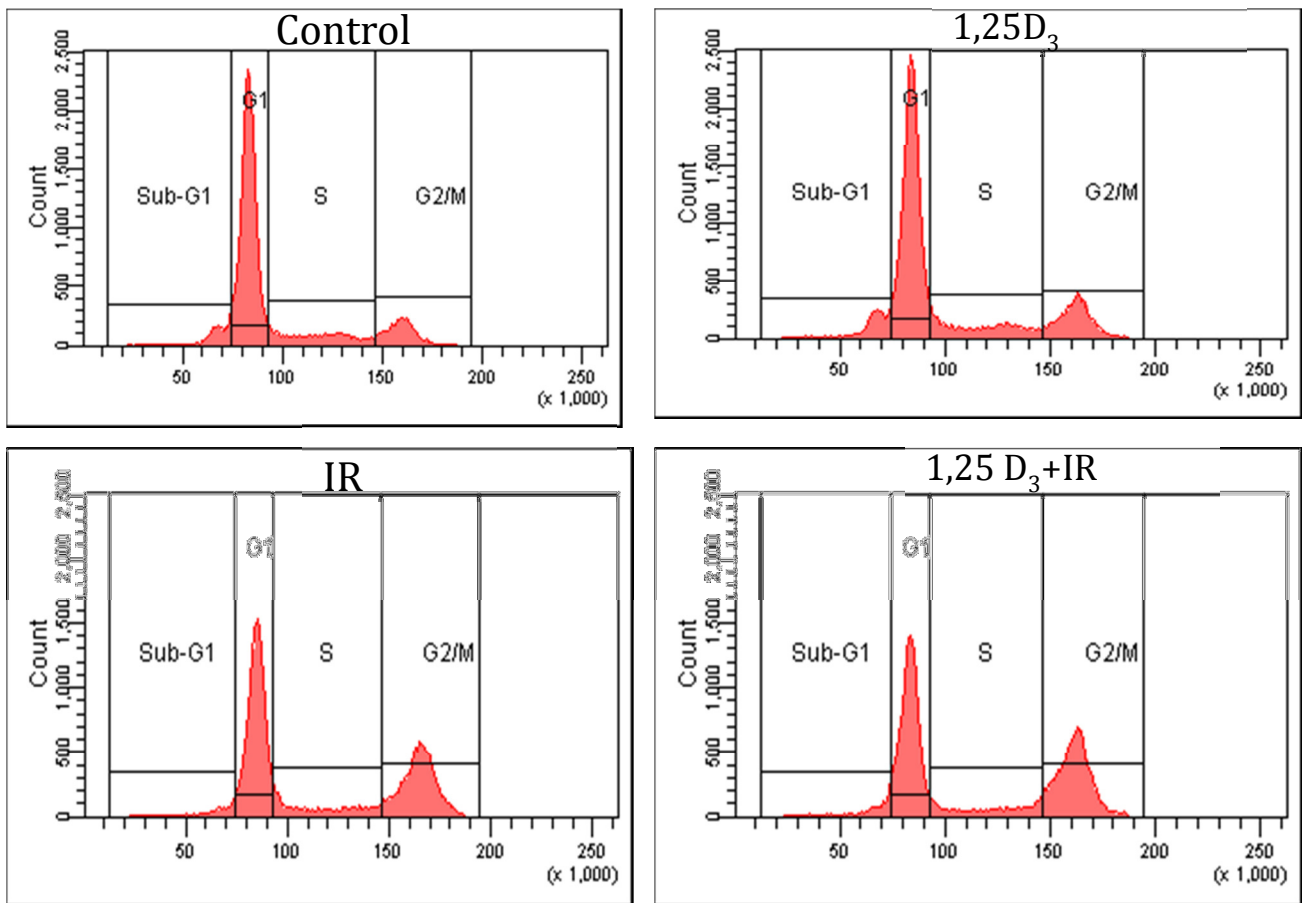
	G1	S phase	G2/M	Sub-G1
Control	65.8	14.3	12.5	7.5
1,25 D$_3$	60.2	14.5	16.7	8.6
IR	49.3	12.1	34	4.6
1,25 D$_3$+IR	45.1	11.1	38.1	5.8

Figure 4.4 *Cell Cycle Analysis by Flow Cytometry Indicates Minimal Accumulation of Cells in the Sub-G1 Phase* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, fractionated doses of 4x2 Gy (over a period of 2 days) or were treated concurrently with 1,25D3 and a dose of 4x2 Gy radiation. Cells were fixed, stained with PI and analyzed 72 hours post treatment. 20,000 cells were analyzed for each treatment.

Control   1,25D3 72 hrs   1,25D3 144 hrs

IR 72 hrs   IR 144 hrs

125D3+IR 72 hours   125D3+IR 144 hours
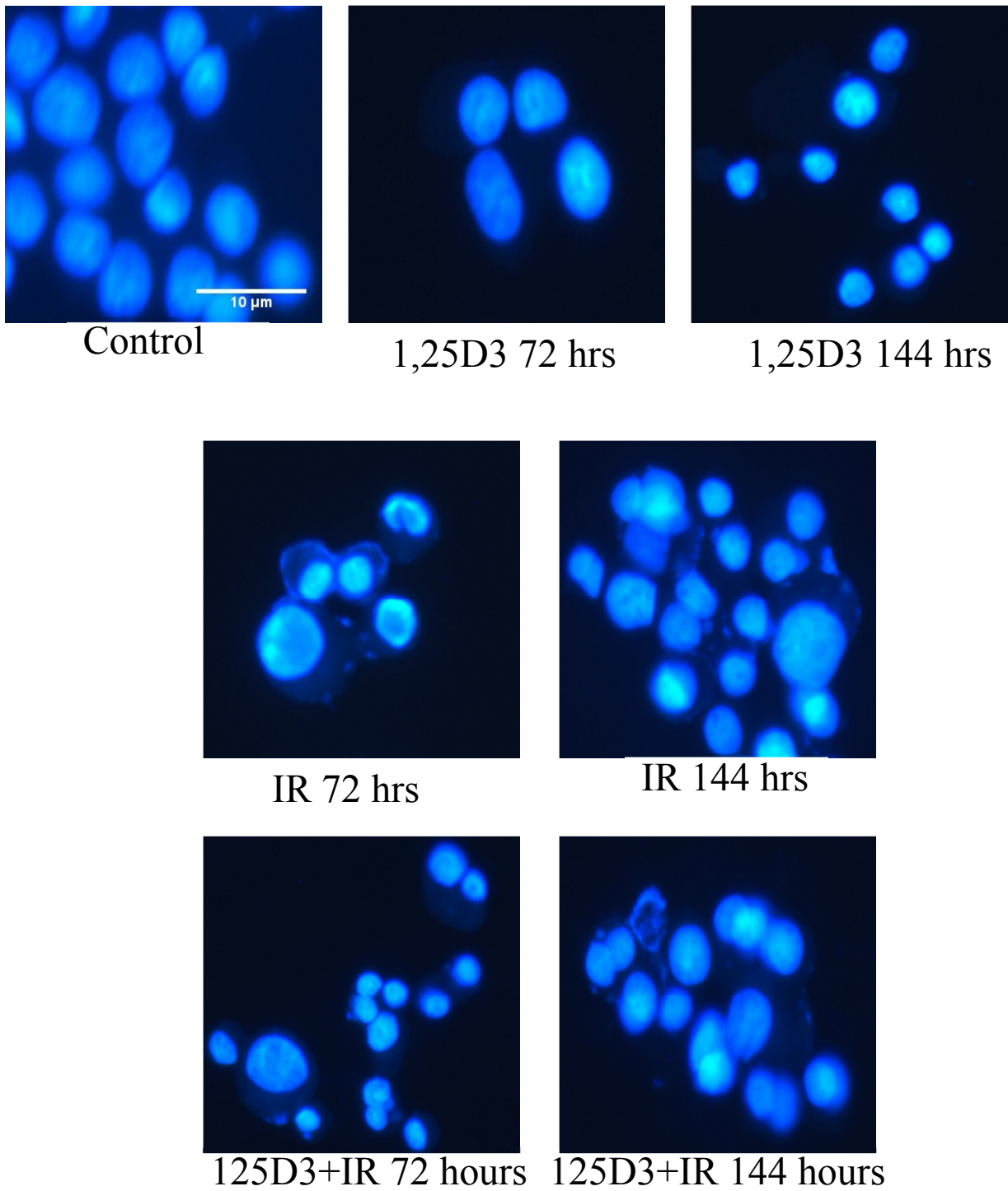
Figure 4.5 *Lack of Cells Undergoing Mitotic Catastrophe* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, fractionated doses of 4x2 Gy (over a period of 2 days) or were treated concurrently with 1,25D3 and 4x2 Gy radiation. Cells were stained with Hoechst dye and images were taken 72 hours post treatment

Autophagy Induction and Autophagic Flux by Radiation Alone and

1,25 D3+Radiation

Autophagy has been shown to play a role in cell death; positive characteristics of autophagic cell death include an increase in autophagic markers as well as autophagic flux (Pyo et al., 2005; Shen and Codogno, 2011). To investigate whether autophagy plays a role in radiation sensitization in the ZR-75-1 cells, cells were stained with acridine orange and the formation of punctate stained vesicles was monitored based on visual assessment using fluorescence microscopy as well as quantification using flow cytometry. Figure 4.6 presents representative acridine orange images at 48 and 72 hours post treatment. At 48 hours, only cells treated with 1,25D3+IR displayed increased punctate staining, where treatment with either IR or 1,25D3+IR resulted in increased punctate formation at 72 hours. Serum starvation was used a positive control. Images were also taken under natural white light to monitor vacuolization, which is also characteristic of cells undergoing autophagy (Fig 4.7). Red arrows indicate vacuolization.

To quantify the extent of autophagy induced following treatment, fluorescent intensity of acridine orange was monitored by flow cytometry. Figure 4.8 summarizes data collected from a timecourse experiment. At 72 hours post irradiation, both IR and 1,25D3+IR resulted in an approximate 5- fold increase in the percent of AVOs (acidic vacuolar organelles). However, time course data confirmed previous studies with visual assessment of acridine orange, which suggested that autophagy is initiated earlier in cells treated with 1,25D3+IR. The data further suggest that autophagy is sustained to a slightly higher extent in cel1s treated with 1,25D3+IR treatment compared to radiation treatment

alone as at day 5 there was a significant increase in the percent of positive AVO staining (Fig 4.8). Serum starvation using EBSS for 24 hours was used as a positive control.

The promotion of autophagy by both radiation alone and 1,25-D3 + radiation was confirmed by monodacylcadaverine (MDC) staining at 48 and 72 hours post treatment (Fig 4.9). Similar to acridine orange staining, there was no autophagy induction in control cells or cells treated with vitamin D for 48 or 72 hours. However, autophagy was induced at 48 hours and 72 hours post treatment with 1,25D3+IR compared to only at 72 hours with IR treatment alone, again suggesting earlier induction of autophagy in the combination treatment. Transmission electron microscopy further confirms autophagy induction at 72 hours post treatment with both IR and 1,25D3 treatment. Autophagosomes are indicated by the red arrows (Fig 4.10).

Another assay for monitoring autophagy induction is RFP-LC3 redistribution. Upon autophagy induction, RFP-LC3 displays punctate staining in the cytoplasm and can be visualized using confocal microscopy (Kirisako et al., 2000)**.** ZR-75-1 cells were stably transfected with a plasmid containing LC3 labeled with red fluorescent protein and treated with 1,23D3, IR or 1,25D3+IR. At 72 hours post treatment, cells were fixed and visualized. Confocal images and quantification of RFP-LC3 puncta for each treatment is presented in figure 4.11. Consistent with the acridine orange staining data, essentially the same number of LC3 puncta are detected per cell for IR and 1,25D3+IR treatment at 72 hours.

Since the presence of autophagic vesicles does not necessarily mean that the autophagosomes have fused with the lysosome and that the contents of the

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autophagosome have been degraded, autophagic flux was assessed by western blot analysis for p62 degradation (Larsen et al., 2010; Pankiv et al., 2007). p62 binds directly to LC-3 (Atg8), a critical protein involved in autophagosome formation to facilitate autophagic degradation (Larsen et al., 2010; Pankiv et al., 2007). Consistent with the acridine orange staining and electron microscopy data, there was no p62 degradation with 1,25D3 alone while p62 degradation is shown with serum starvation, which was used as a positive control. In the case of both radiation alone and 1,25D3 + radiation, p62 degradation appears to be virtually complete within 72 hours (Fig 4.12). We also monitored autophagic flux visually by assessing the colocalization of RFP-LC3 and fluorescent stained lysosomes (Fig 4.13). Images were taken at 24 hours and 72 hours post treatment. Autophagic flux is indicated by the presence of a yellow stain due to the colocalization of RFP-LC3 and lysotracker green. At 24 hours, only cells treated with 1,25D3+IR and serum starved cells displayed colocalization. By 72 hours, both IR and 1,25D3+IR treatment display colocalization. These data are consistent with the p62 western blot analysis, where autophagic flux appears to occur earlier in cells treated with 1,25D3+IR compared to IR treatment alone.

Control	1,25D$_3$	Serum Starvation

IR 48 hrs	IR 72 hrs

1,25D3+IR 48 hrs	1,25D3+IR 72 hrs
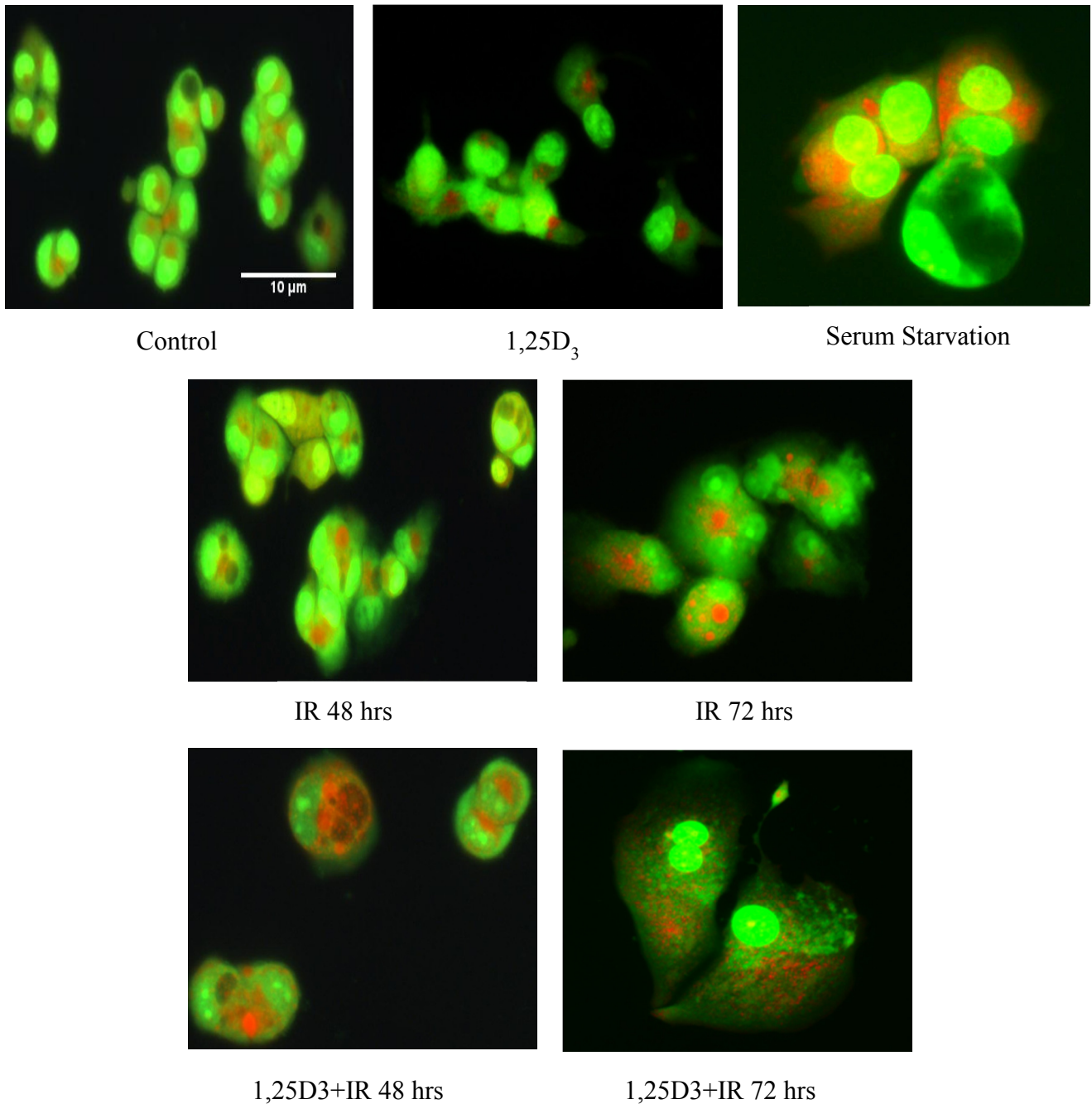
Figure 4.6 *__Analysis of Autophagy by Acridine Orange Staining__* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, fractionated doses of 4x2 Gy (over a period of 2 days) or were treated concurrently with 1,25D3 and 4x2 Gy radiation. Cells were stained with Acridine Orange and images were taken 48 hours and 72 hours post treatment. Serum Starvation was used as a positive control
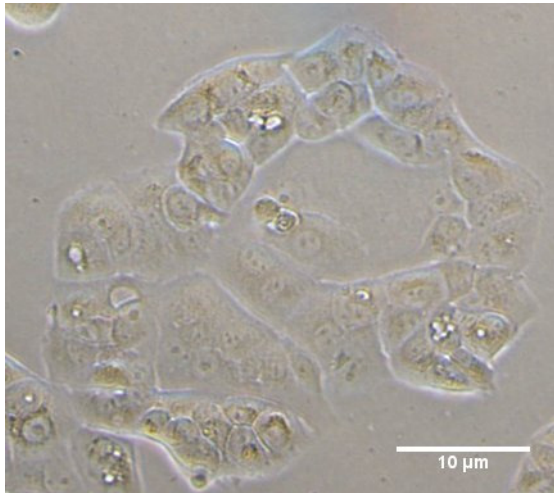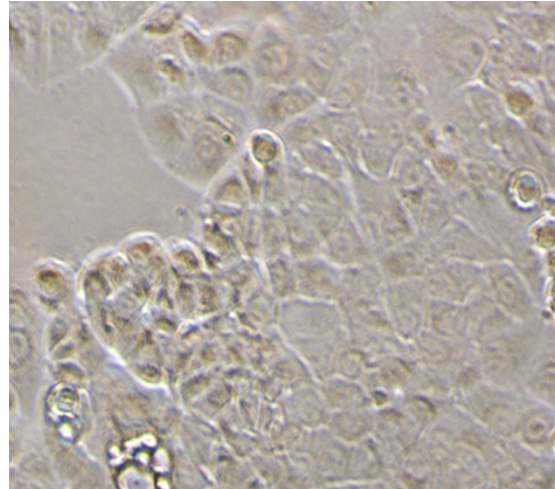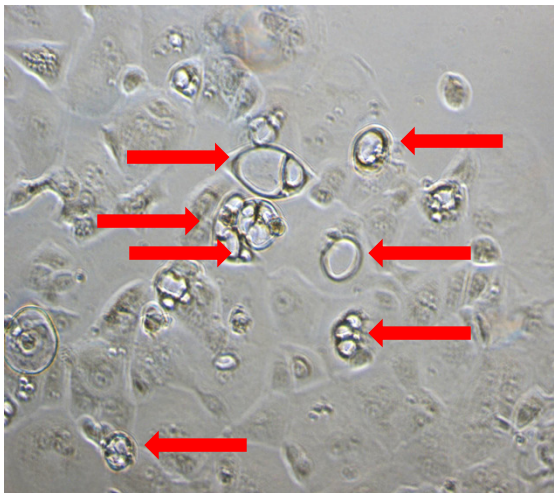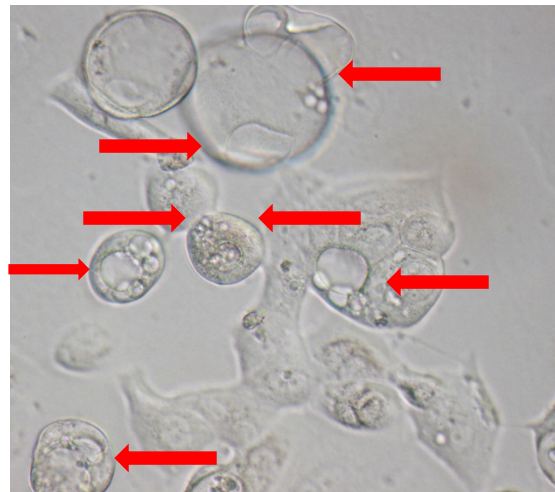
Figure 4.7 *Increased Vacuolization Following Treatment with IR and 1,25D3+IR* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, fractionated doses of 4x2 Gy (over a period of 2 days) or were treated concurrently with 1,25D3 and 4x2 Gy radiation. Cells were visualized using natural white light and images were taken 72 hours post treatment. Vacuoles are indicated by the red arrows
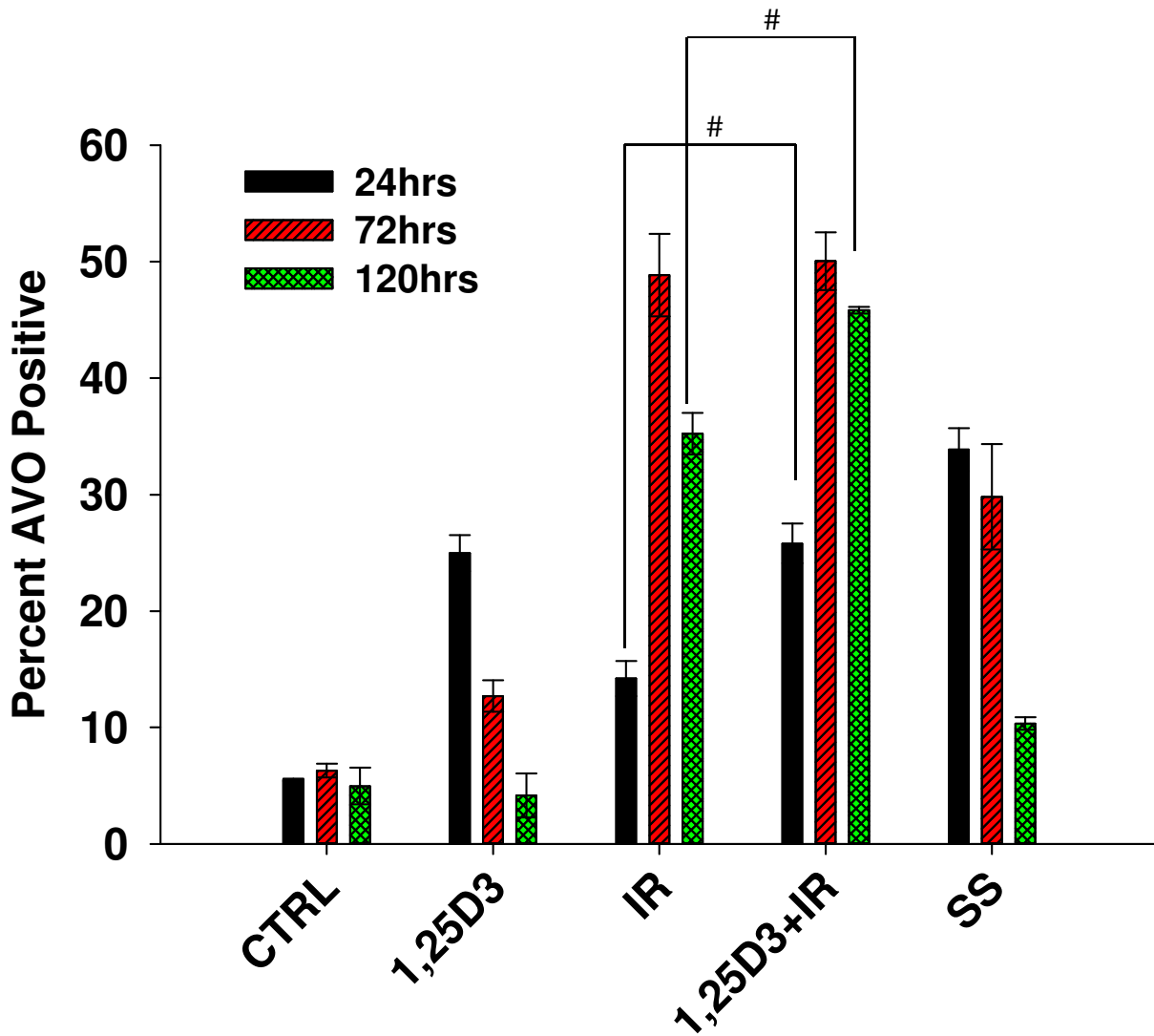
Figure 4.8 *Assessment of Acridine Orange Staining by Flow Cytometry.* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy or were treated concurrently with 1,25D3 and 4 Gy radiation. 10,000 cells were analyzed for each treatment by flow cytometry. Values shown are from a representative experiment with triplicate samples for each condition. Percentage of cells with positive acridine orange staining is represented as the mean ± SE # p< 0.05 compared to IR

Control   1,25D3 48 hrs   1,25D3 72 hrs

IR 48 hrs   IR 72 hrs

1,25D3+IR 48 hrs 1,25D3+IR 72 hrs
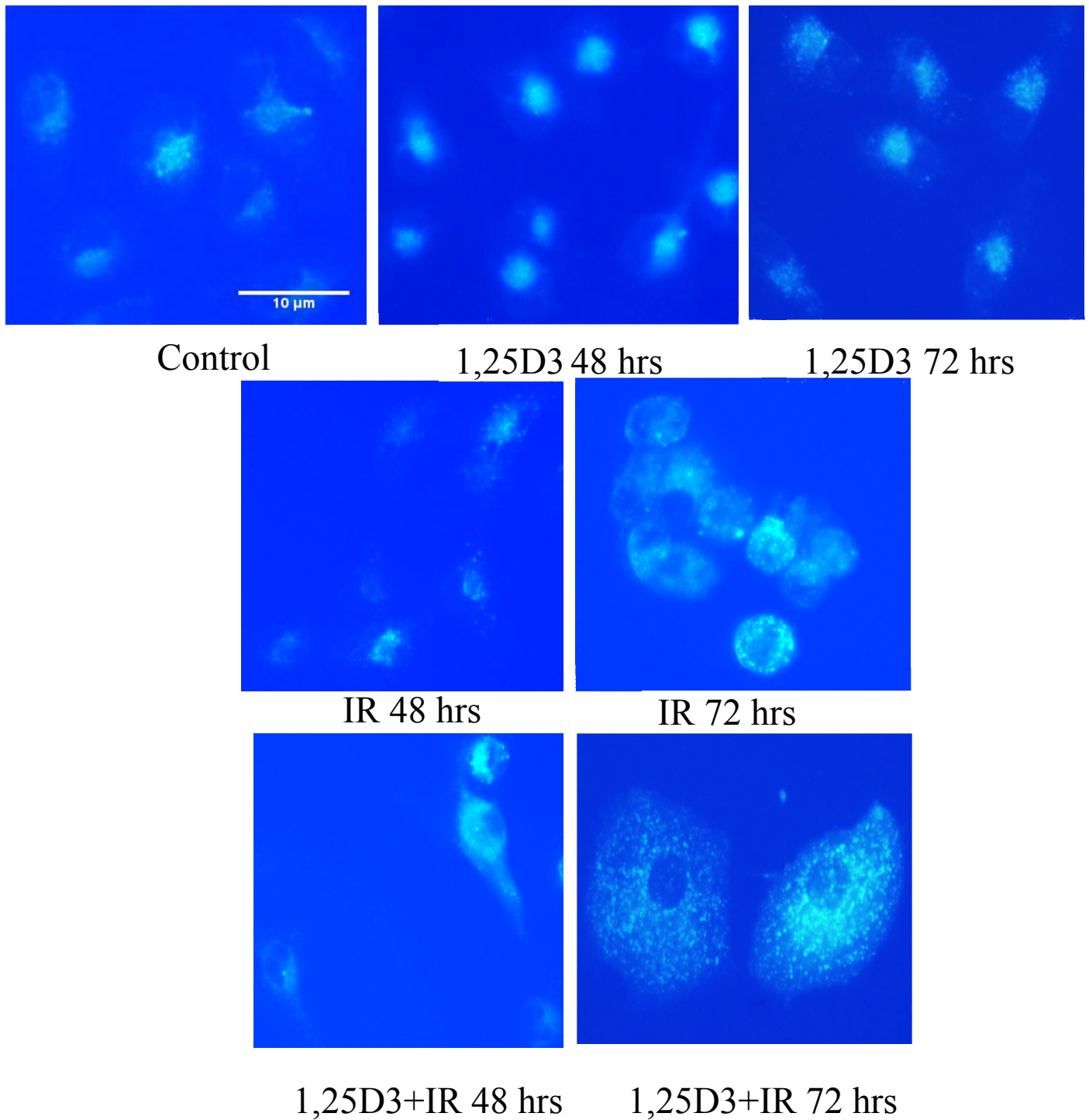
Figure 4.9 *Analysis of Autophagy by MDC staining* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, fractionated doses of 4x2 Gy (over a period of 2 days) or were treated concurrently with 1,25D3 and 4x2 Gy radiation. Cells were stained with MDC and images were taken 48 hours and 72 hours post treatment.
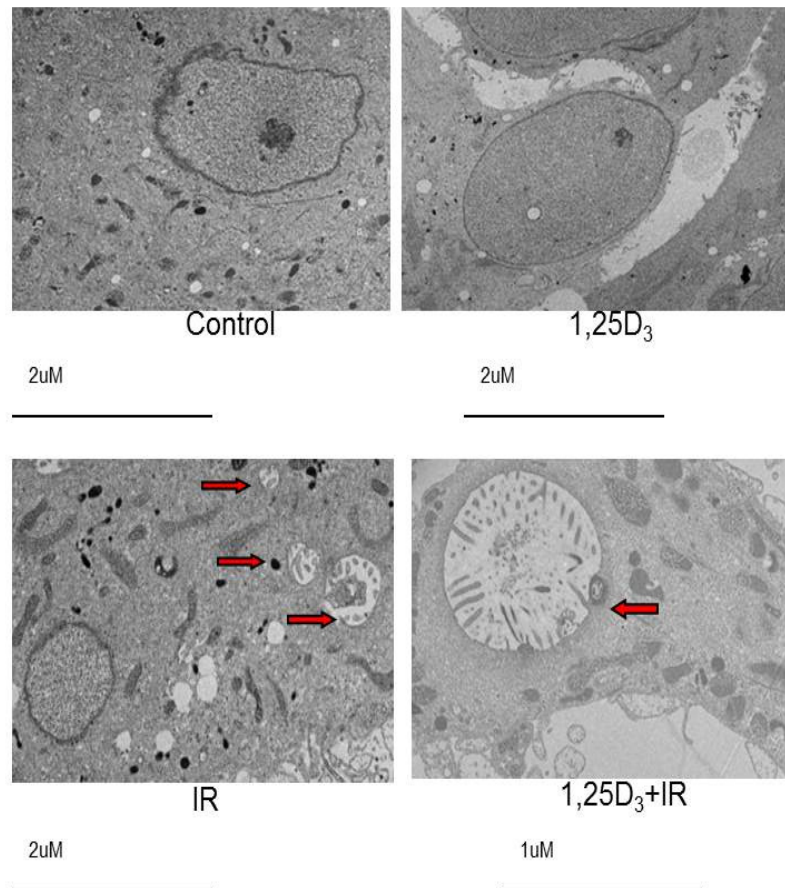
Figure 4.10 *Visualization of Autophagic Vacuole Formation by Transmission Electron Microscopy* ZR-75-1 cells were exposed to 100nM 1,25D3 continuously, fractionated doses of 4x2 Gy (over a period of 2 days) or treated concurrently with 1,25D3 and 4x2 Gy radiation. The presence of autophagic vacuoles was confirmed by electron microscopy at 72 hours post treatment. Autophagosomes are indicated by the red arrows
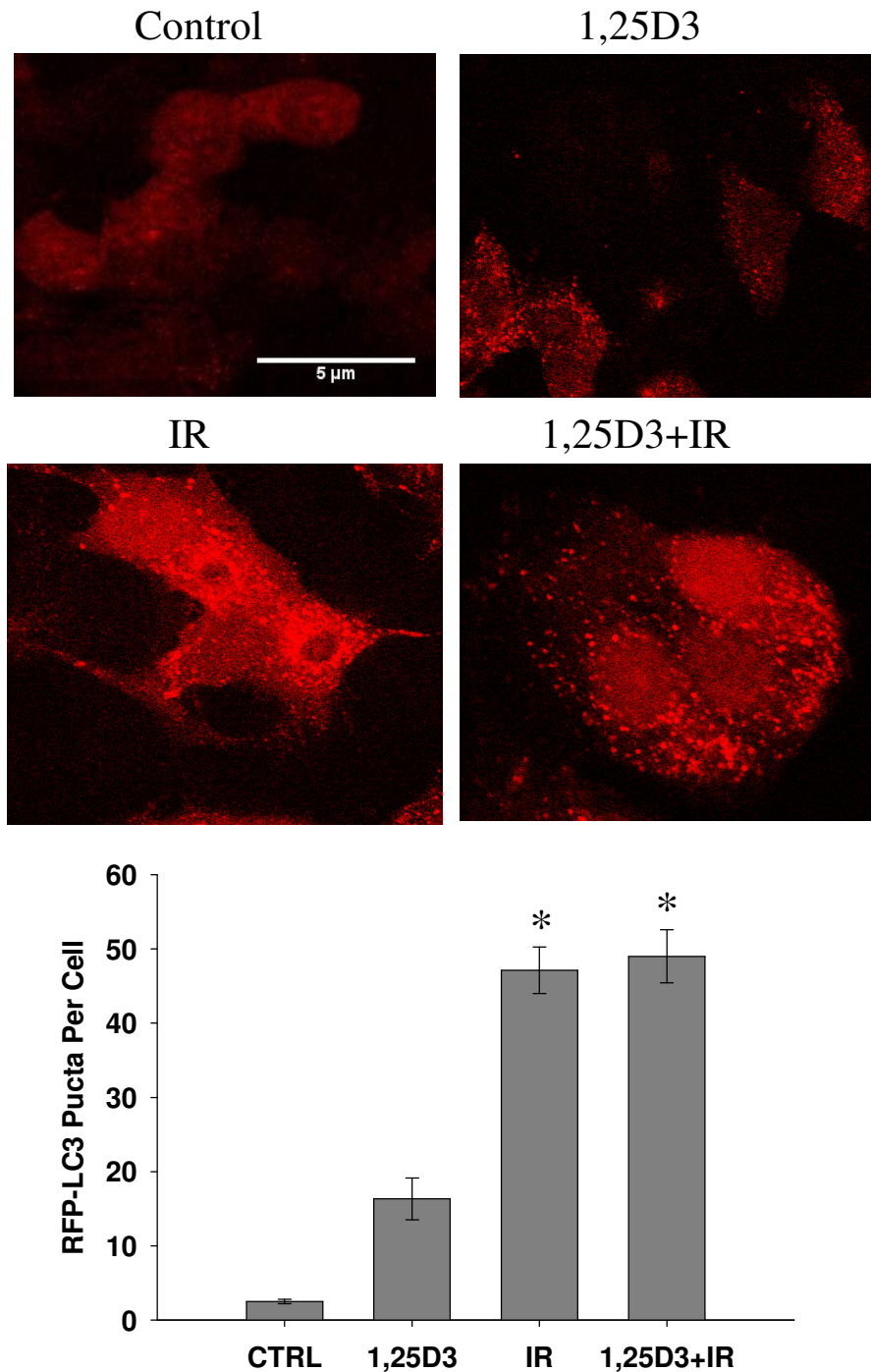
Figure 4.11 _Assessment of RFP-LC3 Redistribution._ ZR-75-1 cells were stably transfected with RFP-LC3 and exposed to 100nM 1,25D3 continuously, a dose of 4 Gy or were treated concurrently with 1,25D3 and 4 Gy radiation. The average number of puncta per cell was counted in five fields for each condition at 72 hours post treatment. Punctate formation is represented as the mean ± SE * $p < 0.05$ compared to control
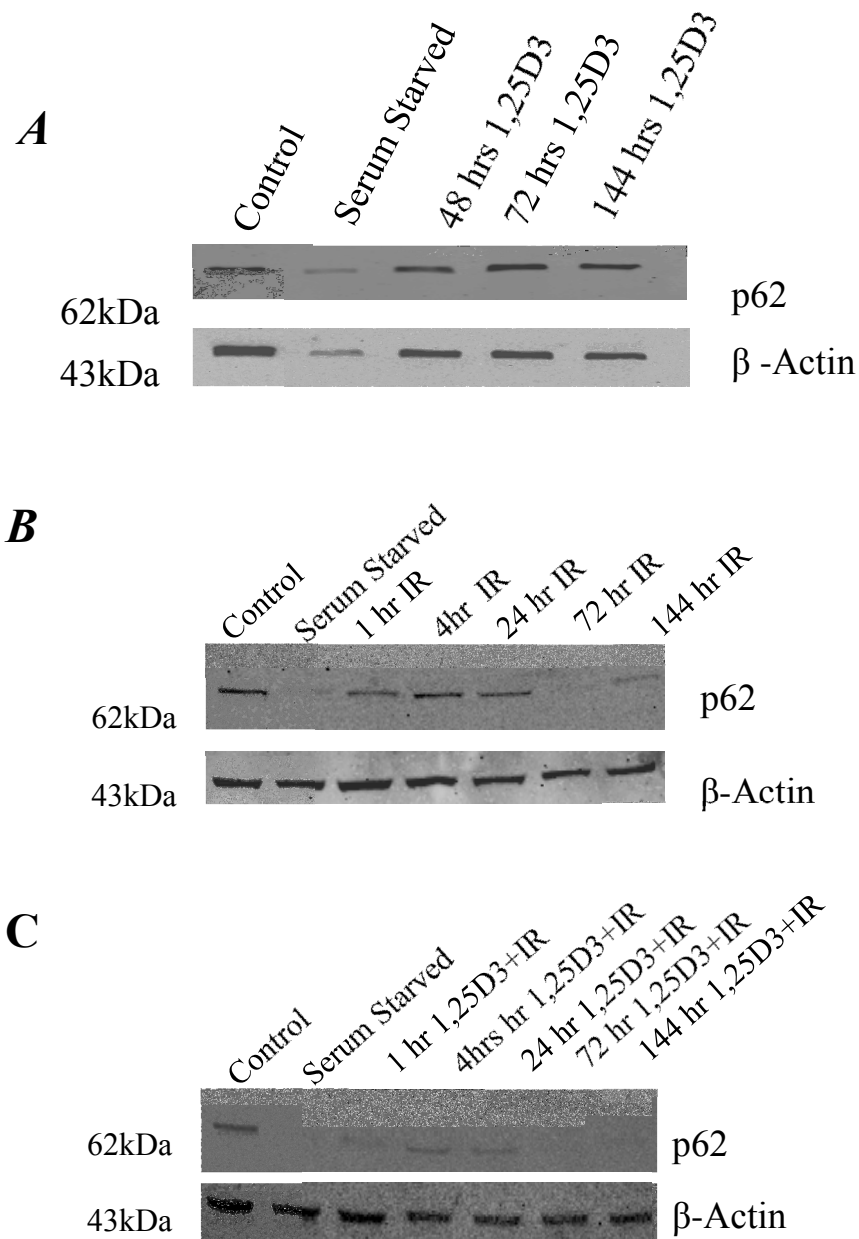
Figure 4.12 *Assessment of Autophagic flux by Western Blot Analysis of p62*
ZR-75-1 cells were exposed to 100nM 1,25D3 continuously (A) , a dose of 4
Gy (B) or were treated concurrently with 1,25D3 and 4 Gy radiation (C) .
Degradation of p62 was monitored at various time points post treatment.
Serum Starvation was used as a positive control.
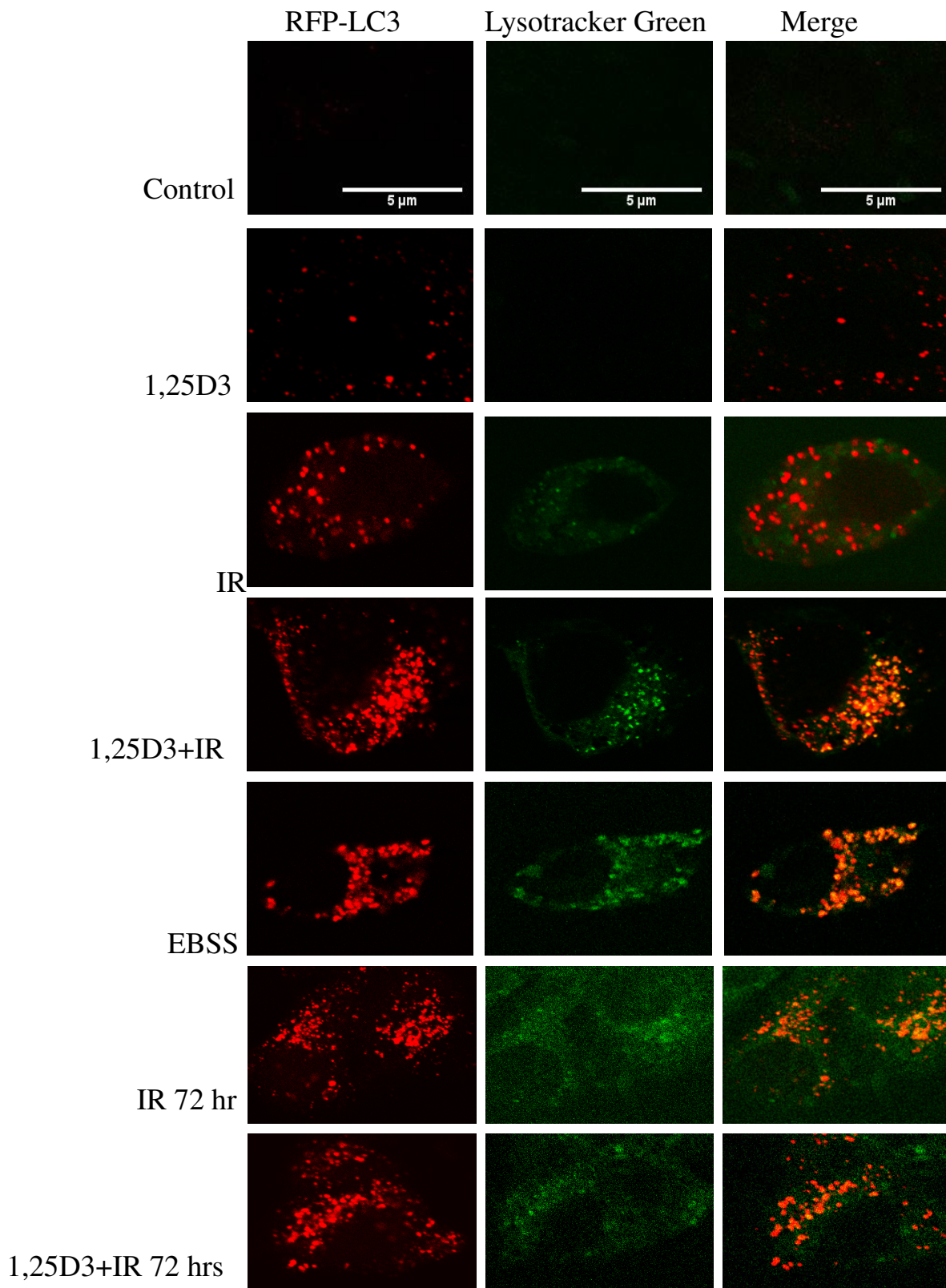
Figure 4.13 *Assessment of Autophagic Flux by Confocal Microscopy* ZR-75-1 cells stably transfected with RFP-LC3 were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy or were treated concurrently with 1,25D3 and 4 Gy radiation. At 24 hours and 72 hours post treatment cells were stained with Lysotracker Green and visualized by confocal microscopy. Serum starvation was used as a positive control for autophagic flux

Effects of Pharmacological Autophagy Inhibition on Cell Viability after IR and

1,25D3+IR

Confirmation of autophagic cell death requires evidence that suppression of autophagy via both pharmacological inhibitors and genetic approaches is able to rescue the cell by preventing cell death (Shen and Codogno, 2011). Thus, we initially employed a pharmacological approach to inhibit autophagy and assess effects on cell viability following treatment. In efforts to confirm that autophagy is the basis for sensitization by 1,25D3, studies were conducted using the pharmacological autophagy inhibitors Chloroquine and Bafilomycin A1. Bafilomycin A1 prevents the fusion of the autophagosome with the lysosome, while Chloroquine prevents the acidification of the lysosome (Carew et al., 2010; Eskelinen and Saftig, 2009; Shen and Codogno, 2011). We identified concentrations of Bafilomycin A1 and Chloroquine that would have little to no toxic effects, as indicated by a MTT assay (Fig 4.14) as well as by trypan blue exclusion (Fig 4.15).

It was important to first validate that the doses of Bafilomycin A1 and Chloroqine (200nM and 5uM respectively) did, in fact inhibit the autophagy induced by our treatments. Initial studies assessed the accumulation of autophagic vacuoles based on acridine orange staining. Due to the fact that Bafilomycin A1 and Choloroquine are both late stage autophagy inhibitors, cells undergoing autophagy will display an accumulation of autophagic vesicles resulting from impaired lysosomal activity. Indeed at 72 hours post treatment, there is an increased accumulation of autophagic vacuocles in cells treated with Bafilomycin A1 and Chloroquine in combination either with IR or 1,25D3+IR treatment (Fig 4.16). Accumulation was also observed at 48 hours post initial treatment

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with 1,25D3+IR following autophagy blockade, which was not observed with IR treatment alone. These data demonstrate the inhibitors are indeed working. 1,25D3 treatment alone did not display accumulation of autophagic vesicles (data not shown).

Inhibition of the autophagy induced by either radiation or 1,25D3 + radiation (specifically the formation of acidified autophagic vesicles) was confirmed through an assessment of acridine orange by flow cytometry using Bafilomycin A1 (Fig 4.17) There was a decrease in intensity of acridine orange staining which is consistent with Bafilomycin A1 inhibiting autophagy. As Bafilomycin A1 alters lysosomal pH, a decrease in intensity of acridine orange would be expected (Note light orange to yellowish staining in Fig 4.16).

Another assay to confirm autophagy blockade is to assess RFP-LC3 distribution. Similar accumulation of RFP-LC3 punctate was observed after IR and 1,25D3+treatment following blockade with Chloroquine at 72 hours post treatment (Fig 4.18). Quantification indicated that there is more accumulation of RFP-LC3 punctae in cells treated with 1,25D3+IR in the presence of the autophagy inhibitors, again suggesting increased autophagic flux by the combination treatment.

As these studies have established that both Bafilomycin A1 and Chloroquine inhibit autophagy, we proceeded to measure the effects of autophagy inhibition on radiation sensitivity by measuring cell viability using trypan blue exclusion. Treatment with radiation alone concurrently with Bafilomyin A1 or Chloroquine resulted in a significant enhancement of cytotoxicity as indicated by the decline in viable cell number (Fig 4.19A). These observations are consistent with the premise that autophagy induced

by radiation alone is cytoprotective, which has been demonstrated in our lab as well as by others (Paglin et al., 2001b; Wilson et al., 2011). In dramatic contrast, interference with autophagy in 1,25D3+IR treated cells results in reduced radiation sensitivity, which is the expected outcome if autophagy mediates the cytotoxicity of the 1,25 D3-radiation combination treatment (Fig 4.19B). In fact, the cell death induced by the combination treatment is attenuated and cell viability is restored to levels similar to what is observed with IR treatment alone (comparing the figures 4.19A and 4.19B).

We also conducted a clonogenic survival assay to assess proliferative capacity. As shown in figure 4.20, these studies verified that autophagy induced by 1,25D3+IR is cytotoxic as there is increased clonogenicity after 1,25D3+IR treatment when autophagy is blocked with Bafilomycin A1 and Chloroquine compared to 1,25D3+IR treatment alone (Fig 4.20). Unexpectedly, autophagy blockade with radiation treatment alone did not result in decreased clonogenicity, which was observed in trypan exclusion. This might be a consequence of a longer experimental window for this assay. Compared to other assays where experiments were analyzed at day 3 and 6 post treatment, this assay was analyzed at 14 days post treatment. It is plausible that the autophagy inhibiting effects of Bafilomycin A1 and Chloroquine have diminished by 14 days, which could explain why we fail to detect decreased sensitivity.

Up until this point, reports in the literature have argued for either cytoprotective or cytotoxic autophagy following treatment with chemotherapies. These findings provided the foundation for the existence of an "autophagic switch." It was the first time that **BOTH** cytoprotective and cytotoxic functions of autophagy were observed in the same experimental system. Moreover, this was a very exciting finding as we believe

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future studies assessing autophagy following treatment with radiation or 1,25D3+IR should shed light on how a cell determines whether autophagy will switch from a pro-survival mechanism to pro-death.

The next series of studies were designed to determine how autophagy inhibition sensitizes ZR-75-1 cells to radiation treatment. Thus, we monitored apoptosis induction following Chloroquine+IR treatment by assessing Annexin V/PI staining by flow cytometry (Fig 4.21). The data generated suggested that radiosensitization by chloroquine appears to be a result of the promotion of apoptosis. Approximately 20% of the cells were apoptotic. There was not a significant increase in the percent of necrotic cells. In addition we monitored mitotic catastrophe using Hoechst stain. Irradiated cells in which autophagy was blocked by Bafilomycin A1 or Chloroquine displayed binucleated cells and enormous nuclei both indicative of mitotic catastrophe (Fig 4.22). These characteristics were not observed with any other of our treatments.

Pharmacological inhibition studies were extended to MCF-7 breast cancer cells by measuring cell viability to validate the existence of cytotoxic autophagy as the mode of radiosensitization by 1,25D3. Previous studies in the laboratory assessing sensitization by 1,25D3 were performed by pre-treating MCF-7 cells with 1,25D3 followed by irradiation. Consequently, we wanted to verify that changing the treatment paradigm to concurrent exposure with IR resulted in similar radiosensitization. MCF-7 cells were treated with 1,25D3, IR or concurrently with 1,25D3 and IR, which is identical to the studies conducted in ZR-75-1 cells (Fig 4.23). Similar to previous studies, 1,25D3+IR treatment resulted in a significant decrease in viable cell number at day 6 post treatment. We next assessed the effects of pharmacological autophagy inhibition on viable cell number by

using a 5uM dose of Chloroquine. Chloroquine had a minimal impact on cell growth alone as indicated by trypan blue exclusion (Fig 4.23). However in a similar fashion to our ZR-75-1 studies, autophagy inhibition resulted in attenuation of 1,25D3 mediated radiosensitization (Fig 4.24). Inhibition of autophagy following treatment with IR resulted in a modest decrease in cell viability. This is important as it allows for detection of cytotoxic autophagy.
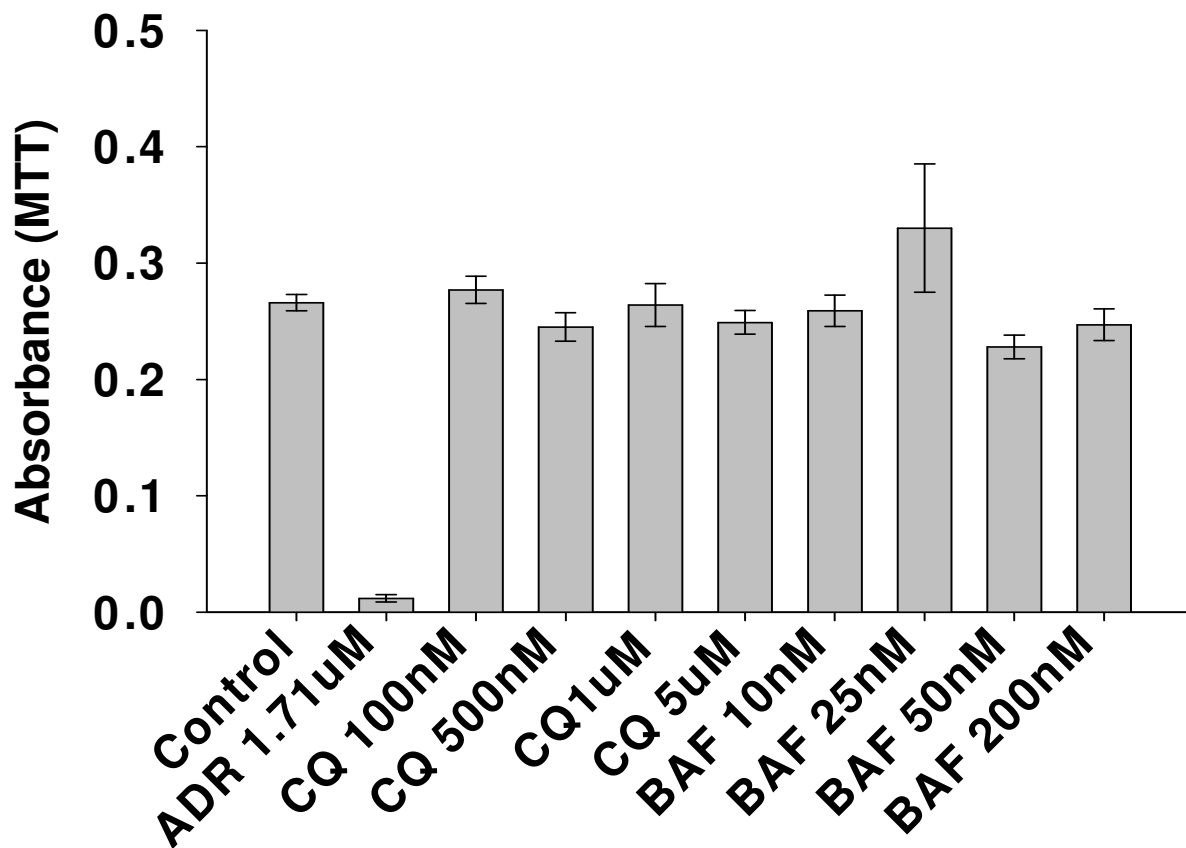
Figure 4.14 *Dose Response of Autophagy Inhibitors Chloroquine and Bafilomycin A1* ZR-75-1 cells were exposed to various does of Chloroquine and Bafilomycin A1 for 72 hours. Cell toxicity was assessed by MTT
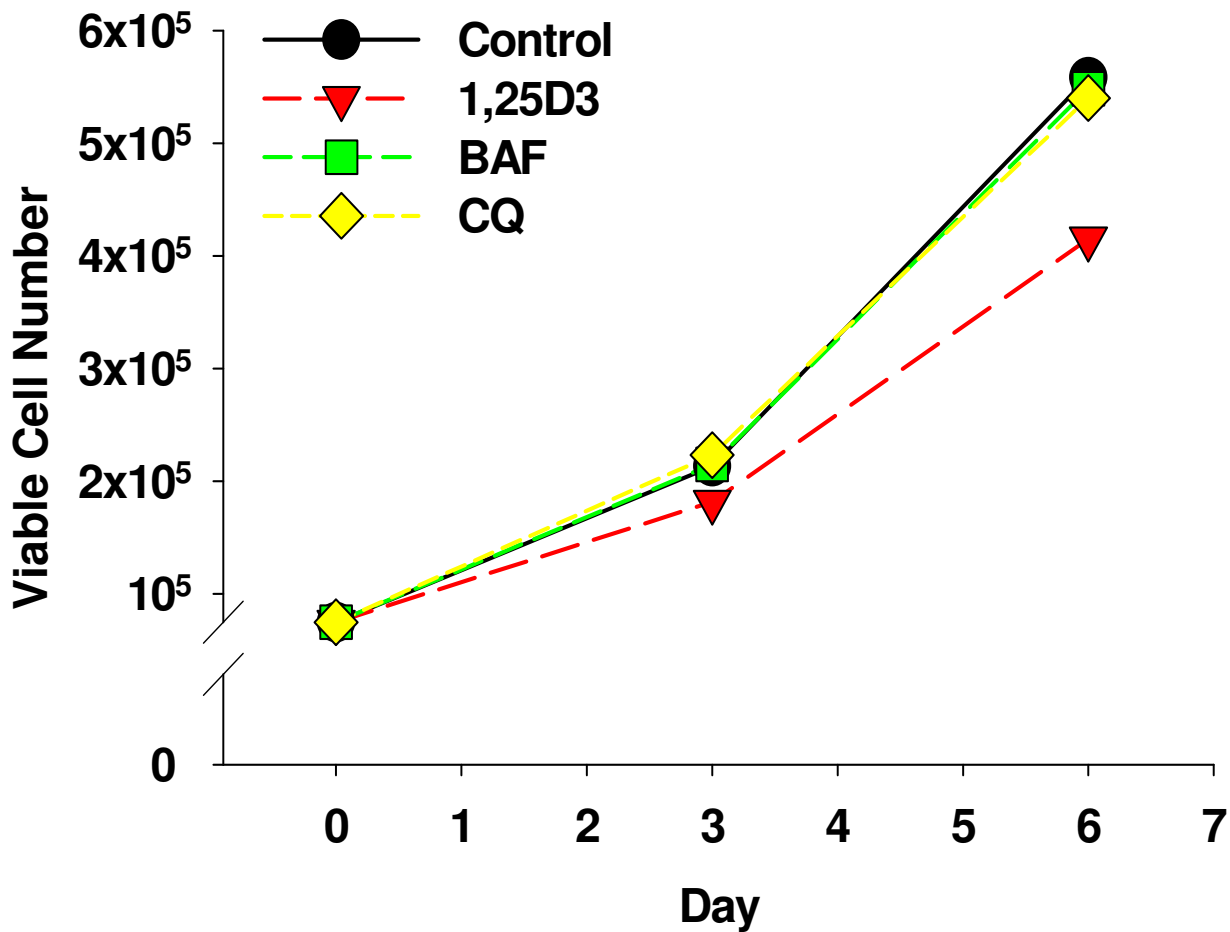
Figure 4.15 _**Minimal effect of Chloroquine and Bafilomyin A1 on Cell viability**_
ZR-75-1 cells were exposed to 100nM vitamin D, 5uM Chrloroquine or 200nM
Bafilomyin A1. Cell viability was assessed by trypan blue exclusion at 72 hours post
treatment
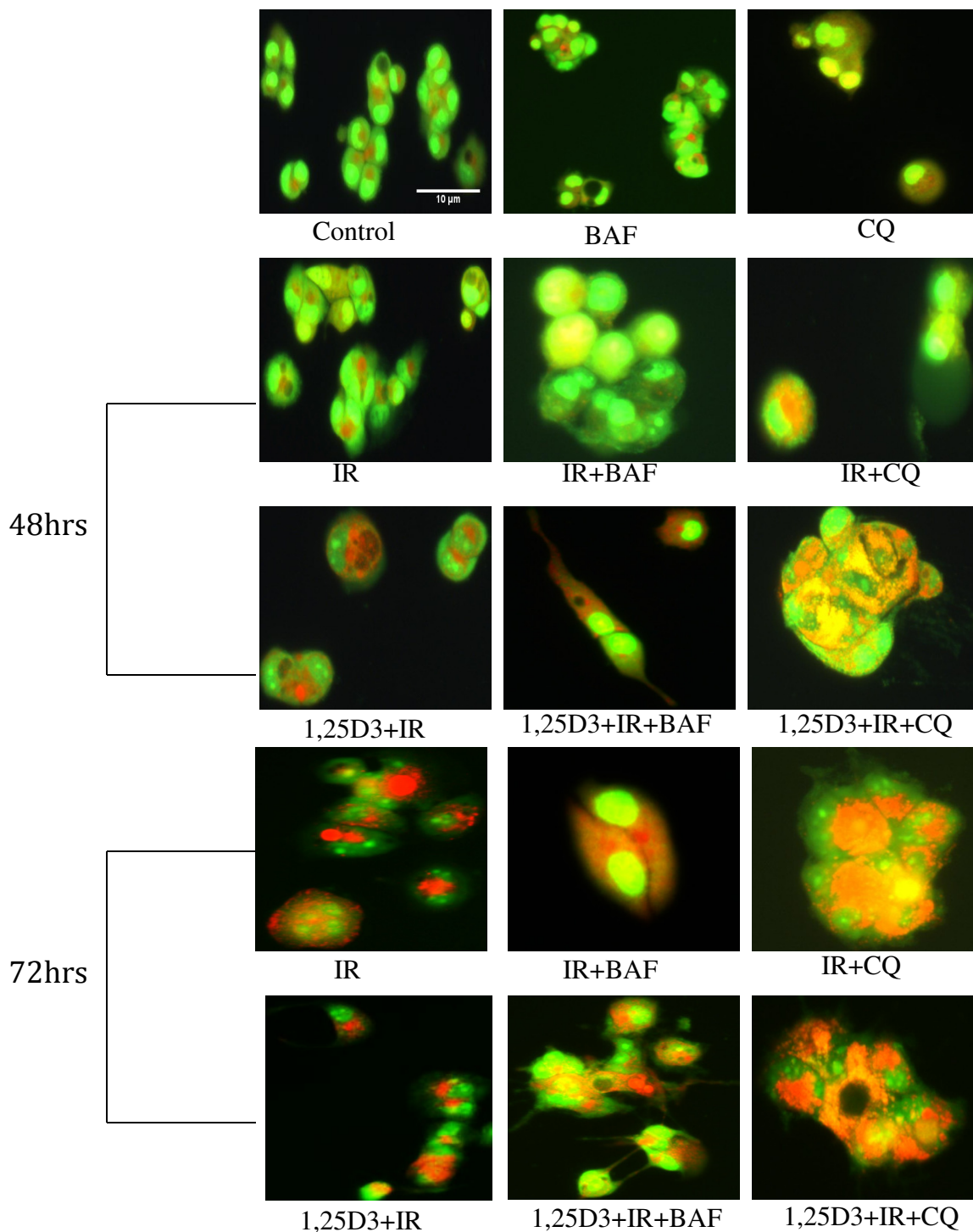
Figure 4.16 *Increase in Autophagic Vacuole formation in Acridine Orange Staining Following Autophagy Inhibition* ZR-75-1 cells were exposed to Bafilomycin A1 or Chloroquine alone, radiation in the absence or presence of either Bafilomycin A1 or Chloroquine or 1,25D3 concurrently with 4x2 Gy radiation in the absence or presence of either Bafilomycin A1 or Chloroquine. Cells were stained with Acridine Orange and images were taken 48 hours and 72 hours post treatment
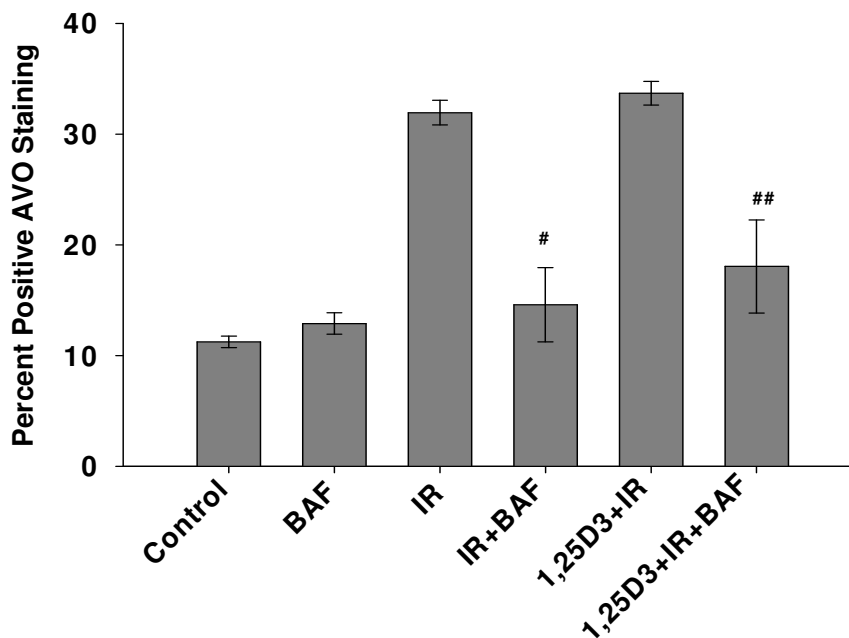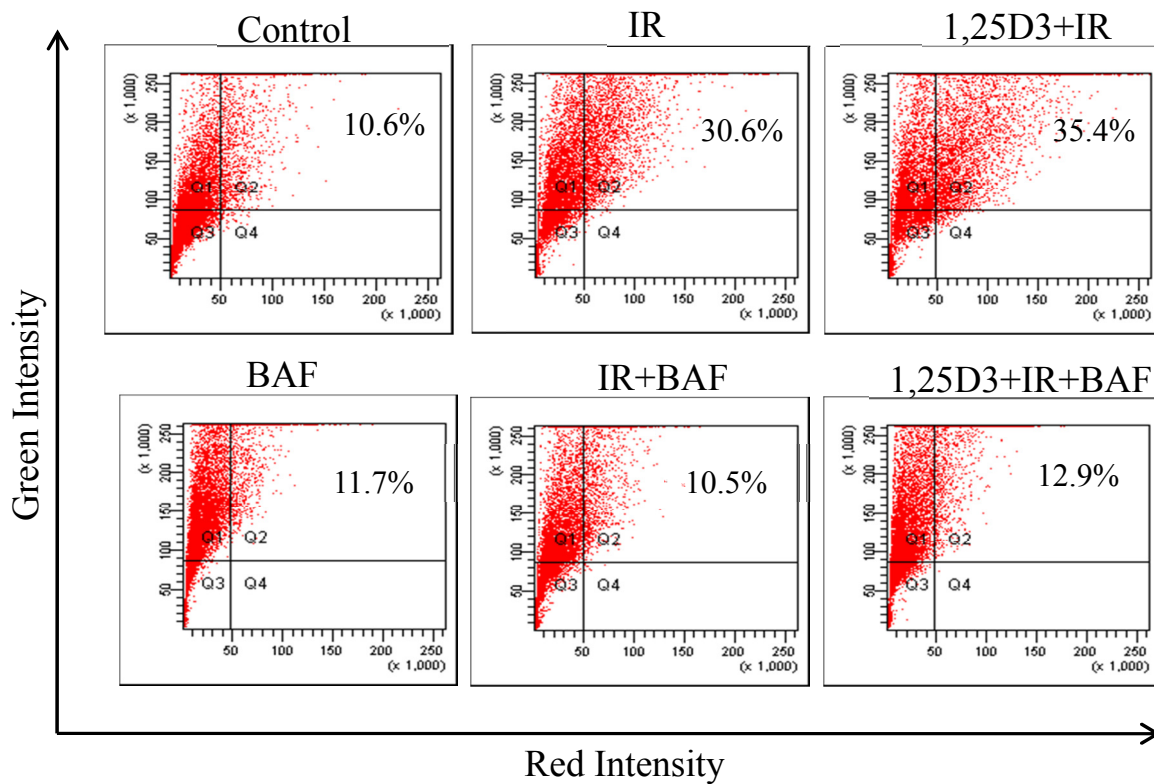
Figure 4.17 *Autophagy Inhibition by Bafilomycin A1* ZR-75-1 cells were exposed to 4x2 Gy radiation alone or concurrently with Bafilomycin A1 or 1,25D3 concurrently with 4x2 Gy radiation in the absence or presence of Bafilomycin A1. The percentage of cells with positive AVO staining was monitored using flow cytometry at 72 hours post treatment #p<0.05 compared to IR ##p<0.05 compared to 1,25D3+IR.
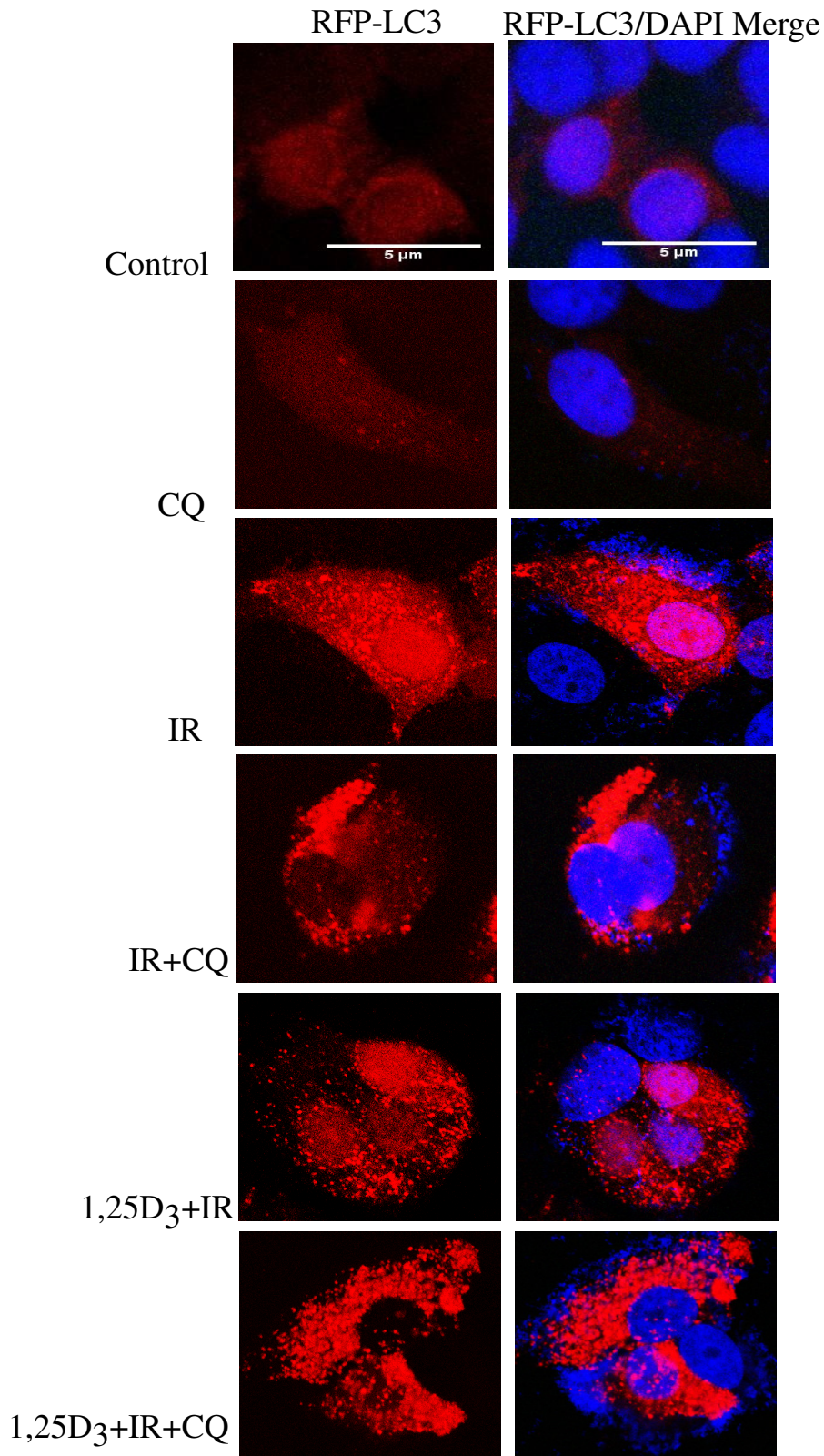
Figure 4.18 _Increase in RFP-LC3 Redistribution Following Autophagy Inhibition_ ZR-75-1 stably transfected with RFP-LC3 were treated with Chloroquine alone, a dose of 4 Gy or were treated concurrently with 1,25D3 and 4 Gy radiation in the absence or presence of Chloroquine. RFP-LC3 redistribution was monitored by Confocal Microscopy at 72 hours post treatment
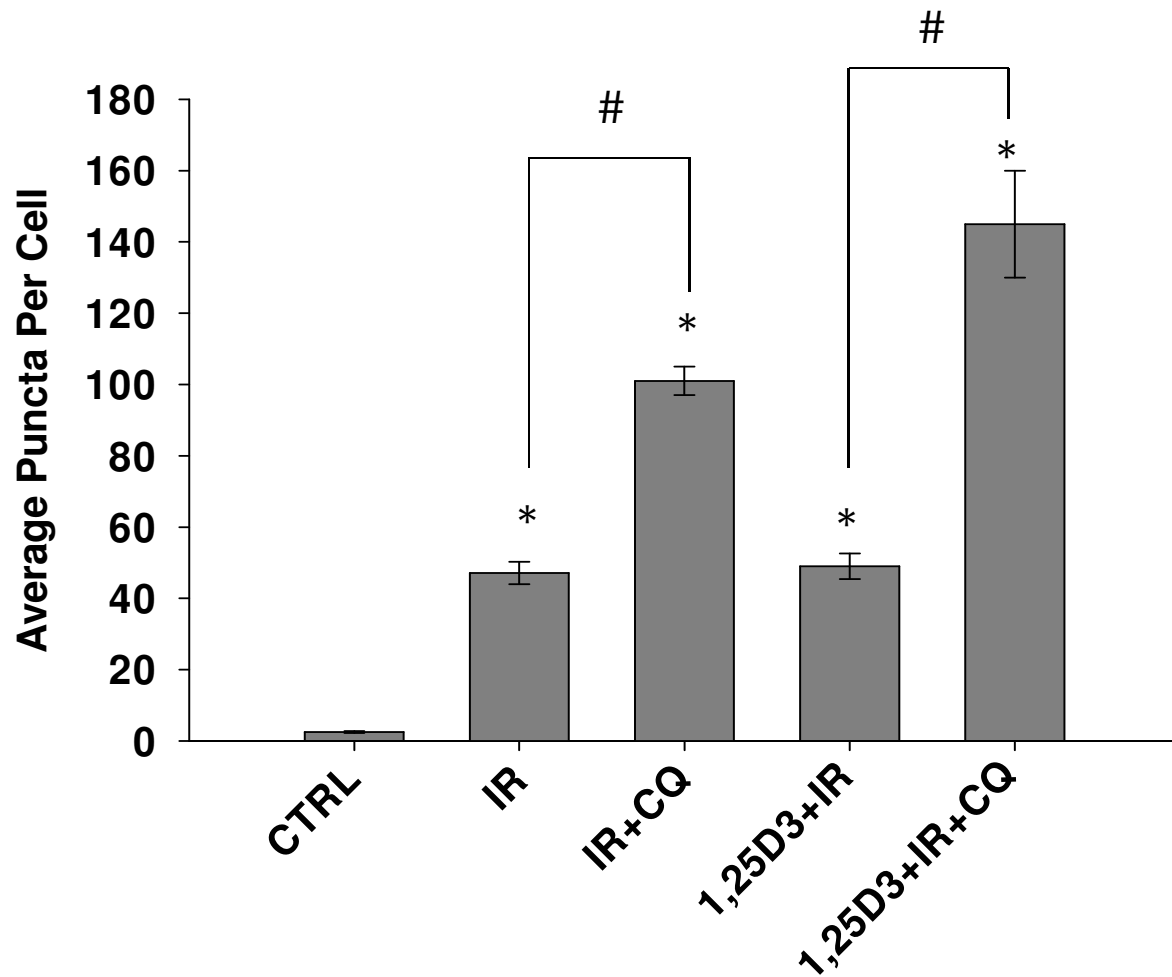
Figure 4.18 Continued *Increase in RFP-LC3 Redistribution Following Autophagy Inhibition* ZR-75-1 stably transfected with RFP-LC3 were treated with a dose of 4 Gy or were treated concurrently with 1,25D3 and 4 Gy radiation in the absence or presence of Chloroquine. RFP-LC3 redistribution was monitored by Confocal Microscopy at 72 hours post treatment p* <0.05 compared to control
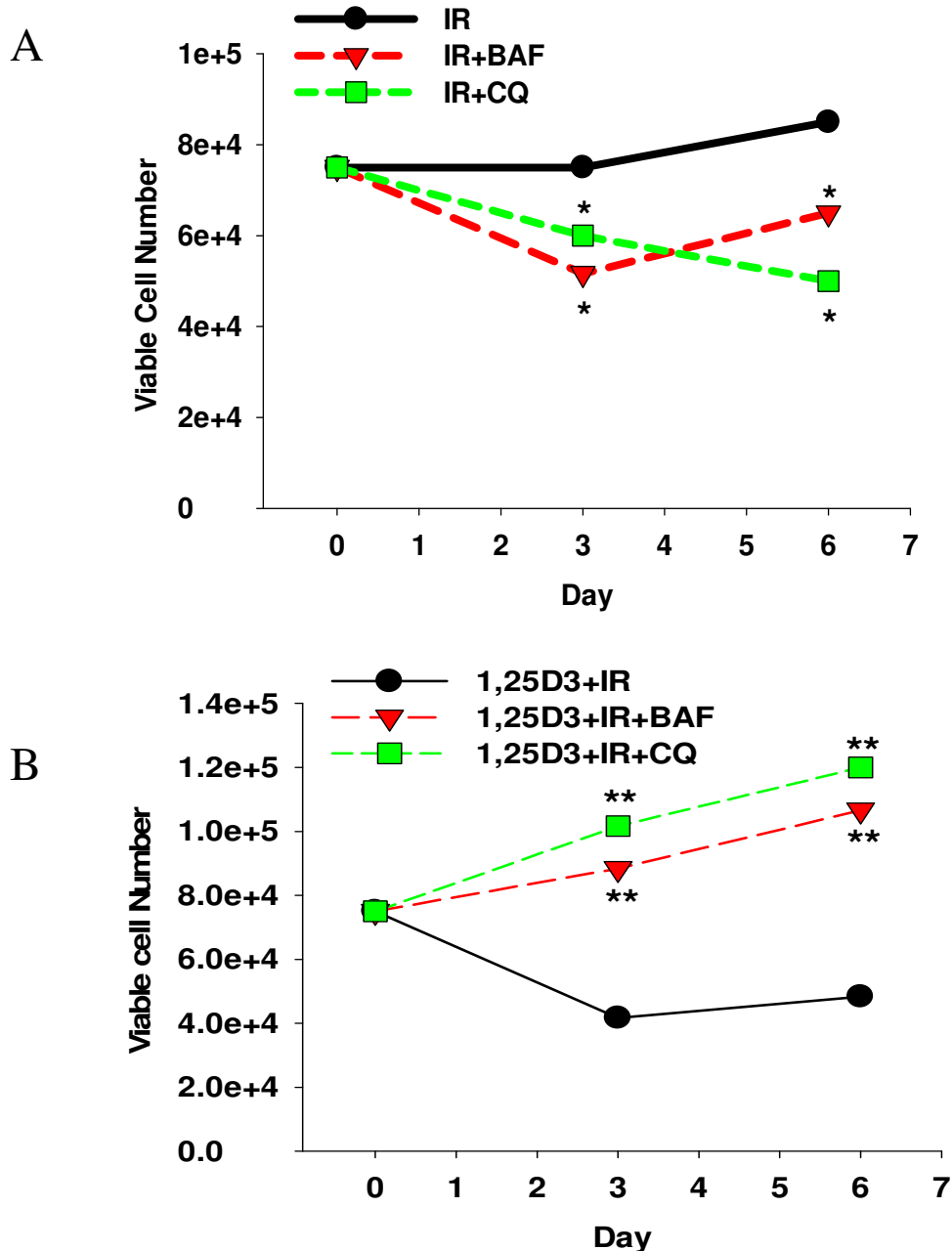
4.19 _Effects of Pharmacologic Autophagy Inhibition on Cell Viability by IR and 1,25D3+IR Treatment A_) ZR-75-1Cells were exposed to radiation in the absence or presence of either Bafilomycin A1 or Chloroquine and viable cell number was assessed by trypan blue exclusion at days 3 and 6 post treatment. B) ZR-75-1 cells were exposed to 1,25D3 concurrently with 4x2 Gy radiation in the absence or presence of either Bafilomycin A1 or Chloroquine and viable cell number was assessed by trypan blue exclusion at days 3 and 6 post treatment. Values shown are from a representative experiment with triplicate samples for each condition. *p<0.05 compared to IR **p<0.05 compared to 1,25D3+IR
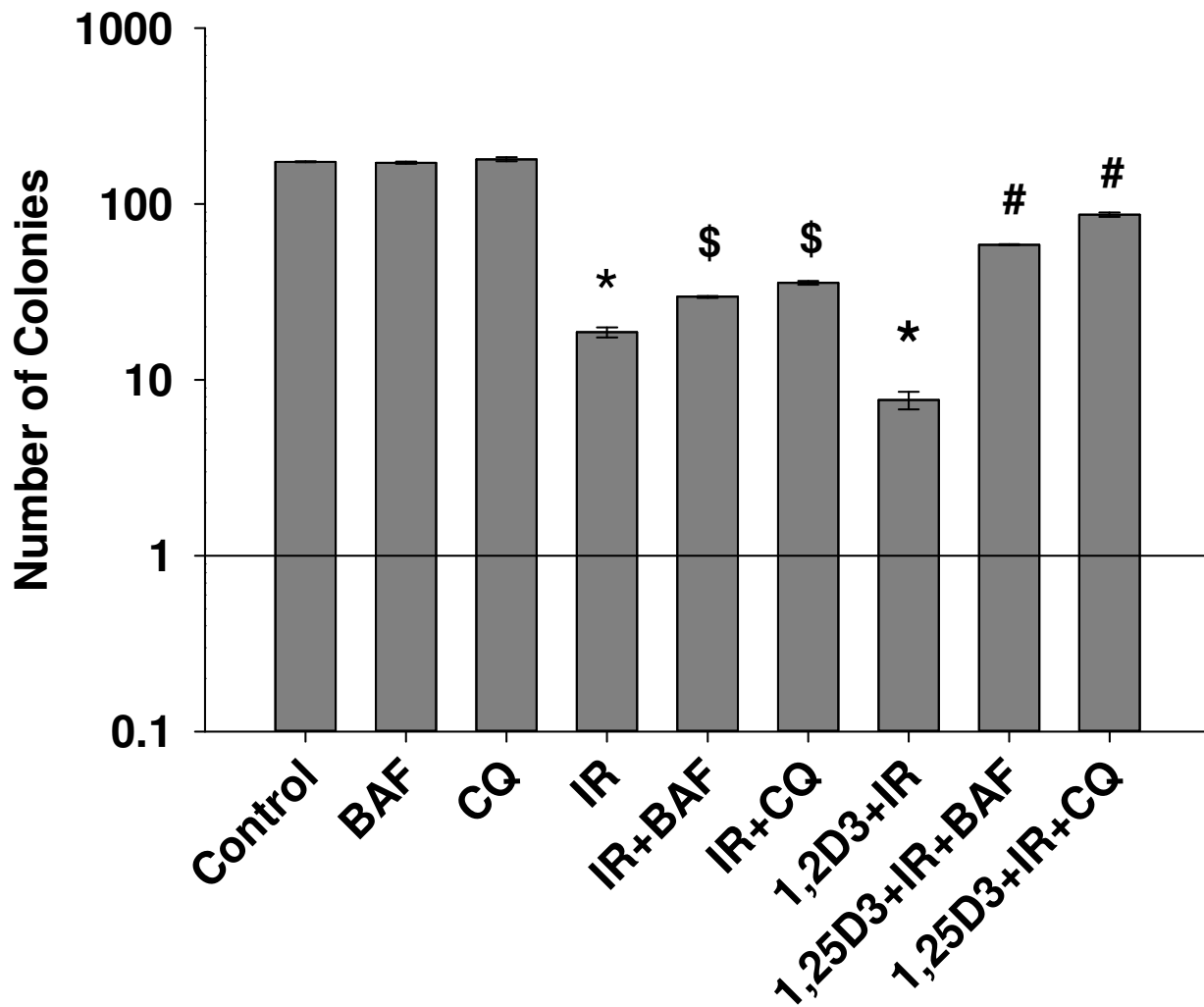
4.20 *Effects of Pharmacologic Autophagy Inhibition on Clonogenic Survival by IR and 1,25D3+IR Treatment* ZR-75-1 cells were exposed to Bafilomycin A1 or Chloroquine alone, radiation in the absence or presence of either Bafilomycin A1 or Chloroquine ,1,25D3 concurrently with 4x2 Gy radiation in the absence or presence of either Bafilomycin A1 or Chloroquine. Clonogenic survival was assessed at 14 days post treatment. *p<0.05 compared to control $p<0.05 compared to IR 1,25D3+IR # p<0.05 compared to1,25D3+IR
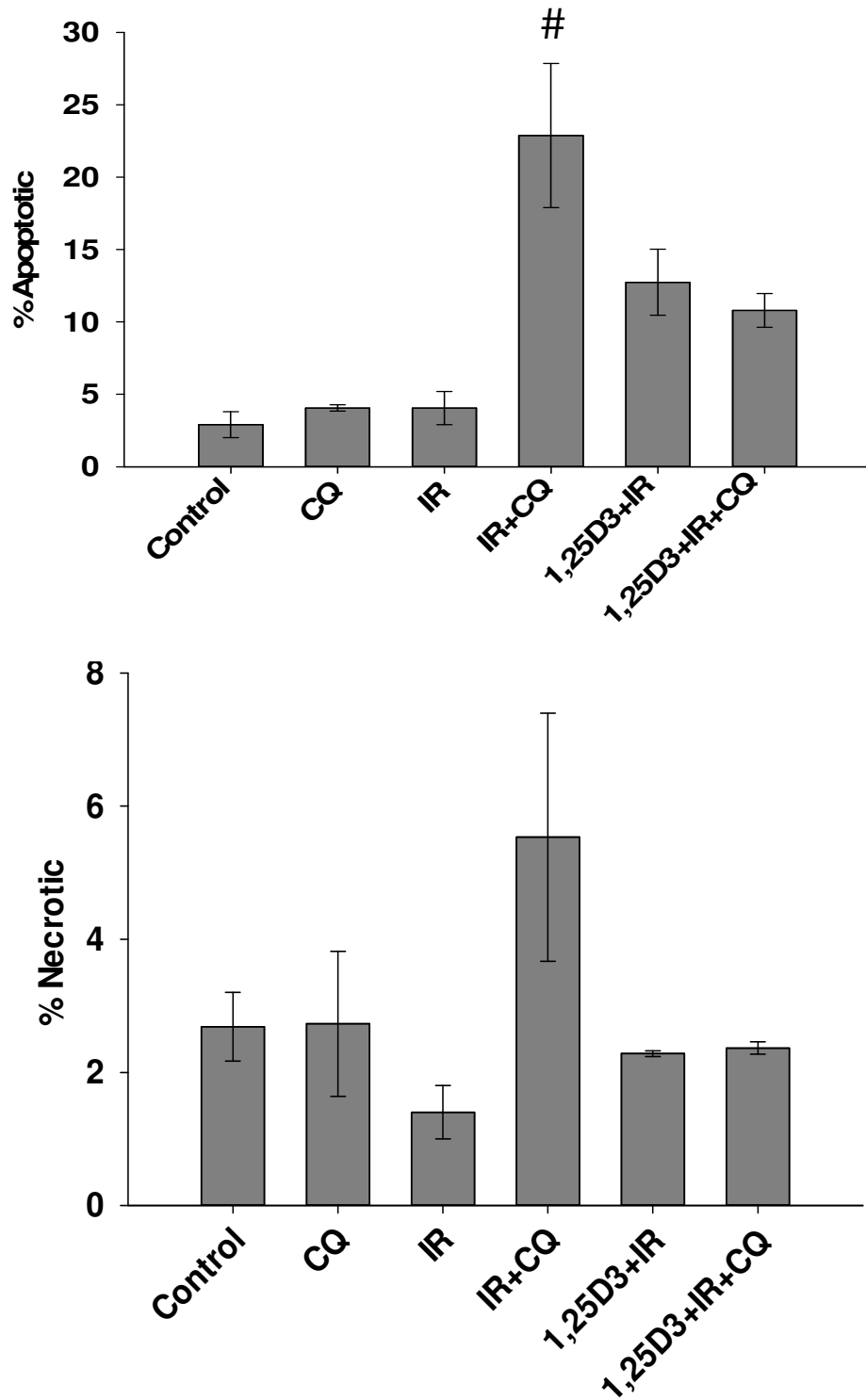
Figure 4.21 *Autophagy Inhibition Increases Extent of Apoptosis by IR.* ZR-75-1 cells were exposed to Chloroquine alone, radiation in the absence or presence of Chloroquine, or 1,25D3 concurrently with 4 Gy radiation in the absence or presence of Chloroquine. Annexin V/PI fluorescence intensity was analyzed by flow at 72 hours post treatment. # $p < 0.05$ compared to IR alone
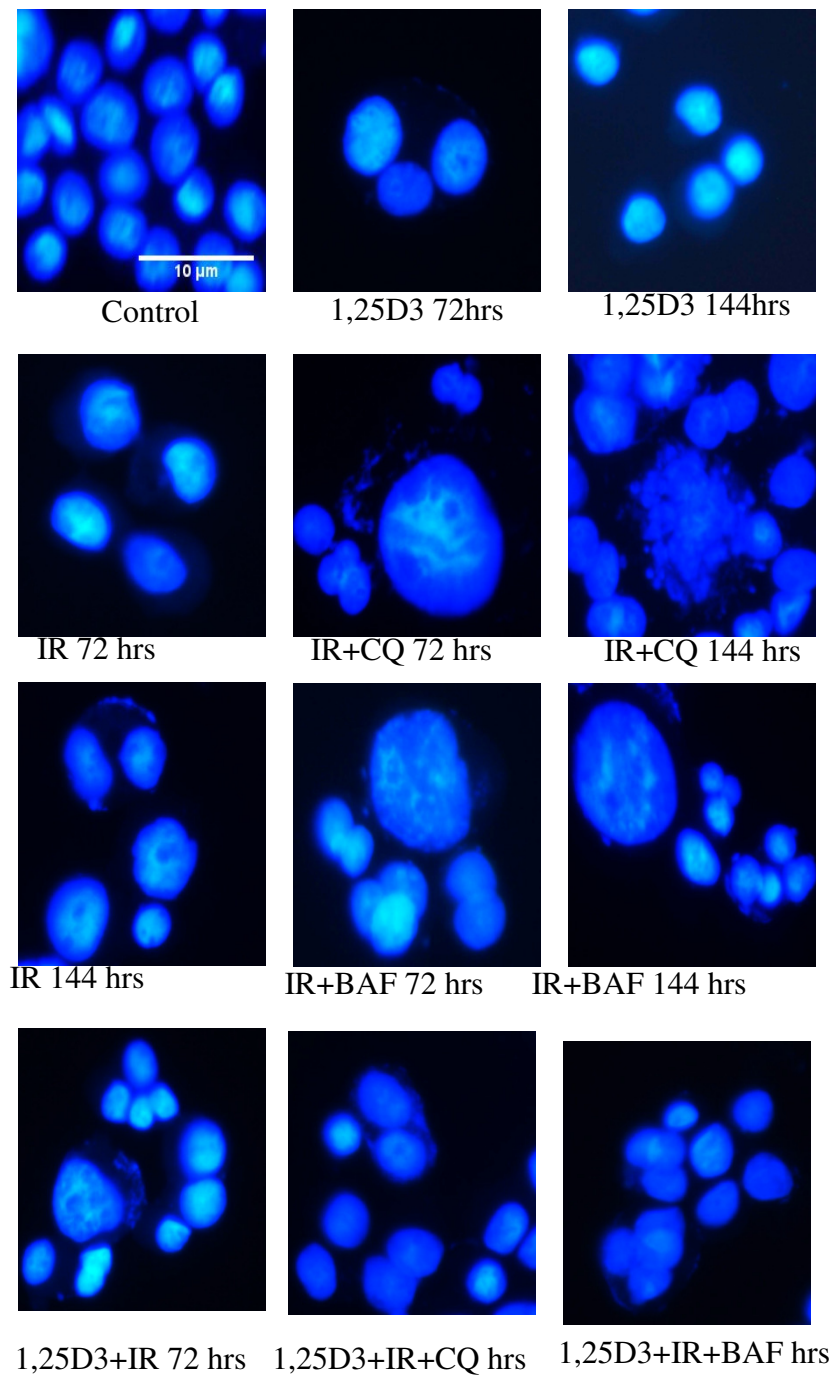
Figure 4.23 *Increased Mitotic Catastrophe following Autophagy Inhibition in Irradiated ZR-75-1Cells.* Cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation and stained with Hoechst dye. Images were taken 72 hours post treatment
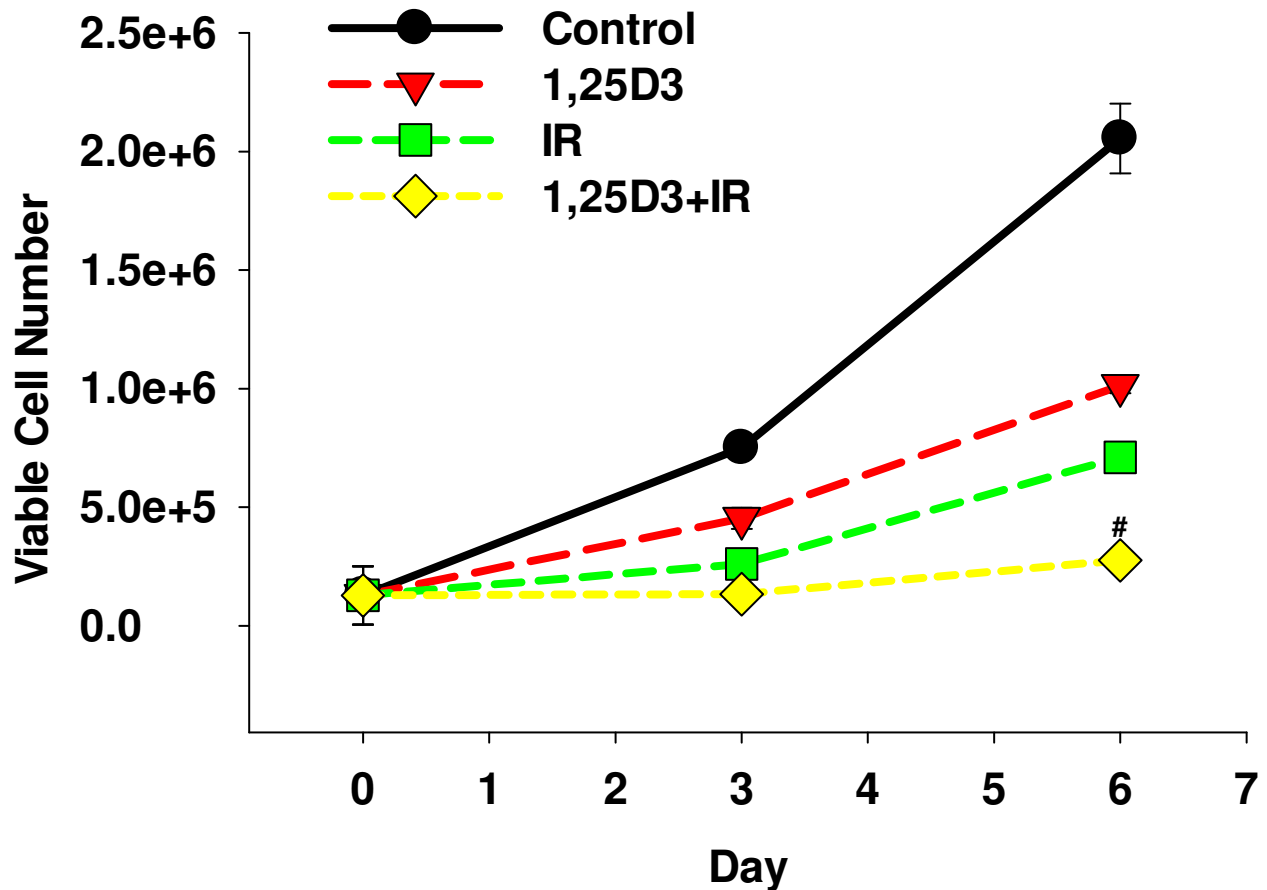
Figure 4-23 _Radiation Sensitization by 1,25D3 in MCF-7 Breast Cancer Cells._ Cells were treated with 1,25D3 alone, a dose of 4 Gy radiation or 4 Gy concurrently with 1,25D3.Viable cell number was determined by exclusion of trypan blue at days 3 and 6 following post initial treatment. Values shown are from a representative experiment with triplicate samples for each condition. # p<0.05 compared to IR alone

Expanded Figure
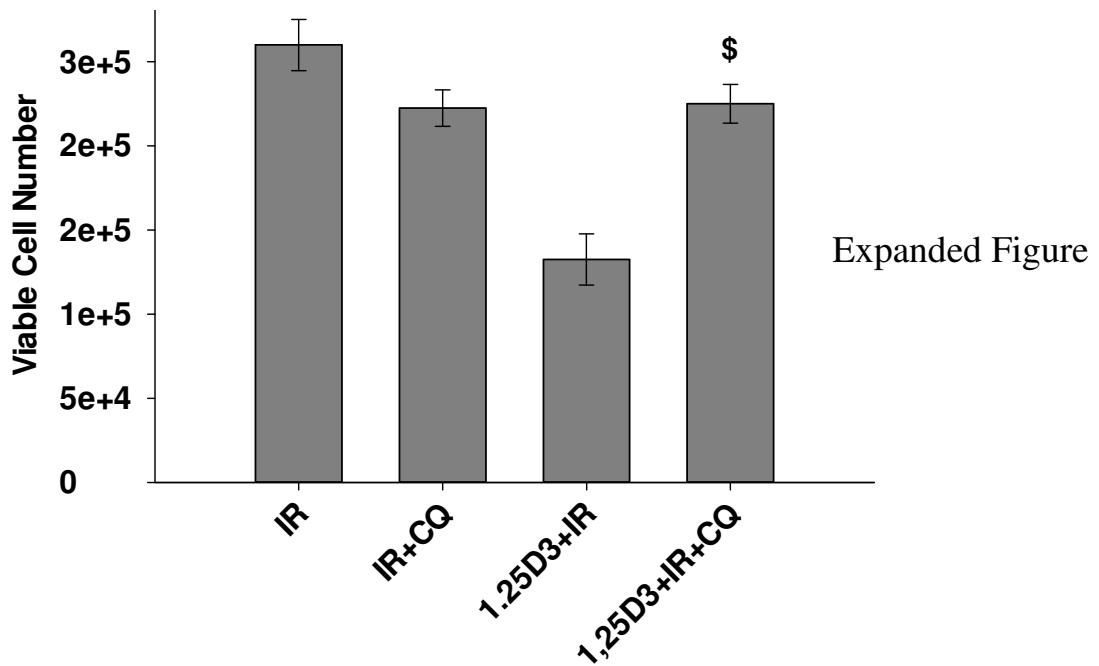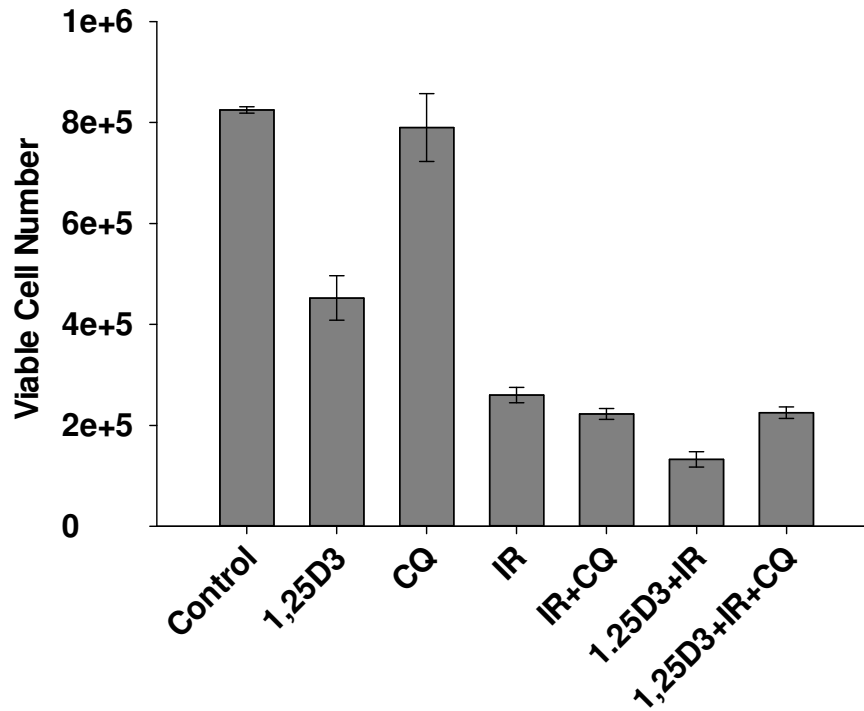
4.24 *Autophagy Inhibition Reverses Sensitization to Radiation by 1,25D3 in MCF-7 cells.*
Cells were exposed to 1,25D3, CQ, radiation in the absence or presence Chloroquine, or
1,25D3 concurrently with 4 Gy radiation in the absence or presence of Chloroquine. Viable
cell number was assessed by trypan blue exclusion at day 3 post treatment. Values shown
are from a representative experiment with triplicate samples for each condition. $p<0.05
compared to1,25D3+IR

Effects of Genetic Autophagy Inhibition on Cell Viability in IR and 1,25D3+IR treated cells

To confirm the results generated with pharmacological autophagy inhibition, additional studies were performed using ZR-75-1 cells in which expression of either ATG-5 or ATG-7 was stably suppressed by shRNA. ATG-5 combines with Atg12 in a multimeric protein complex that is required for autophagosome formation, whereas ATG-7 is an E1-like enzyme involved in activating both LC3II (Atg8) and Atg-5 (Klionsky, 2005). ATG-5 levels were reduced by approximately 77% and ATG-7 levels by approximately 60% as indicated by western blot analysis (Fig 4.25 A-B). Furthermore, to determine if our knockdown was sufficient to alter autophagic function, autophagic flux was monitored by p62 degradation in ATG-5 and ATG-7 knockdown cells (Fig 4.25C). Indeed, cells in which ATG-5 or ATG-7 had been knocked down displayed decreased autophagic flux compared (i.e. a reduction in degradation of p62) to vector control cells following serum starvation. In addition, we conducted acridine orange and transmission electron microscopy assays to assess autophagy induction in cells in which ATG-5 was knocked down. In cells where ATG 5 was knocked down, there was no increase in the amount of acridine orange staining induced by either IR or 1,25D3+IR (Fig 4.26A). Lack of autophagy was also observed in the EM images following treatment with IR or 1,25D3+IR (Fig 4.26B).

Studies were also conducted to confirm that 1,25D3, IR and 1,25D3+IR treatment produced the same relative effects in control cells expressing shRNA against GFP (shRNA control) as in the parental ZR-75-1 cells. A single dose of 4 Gy radiation was

used for these studies, as we found that this dose allowed us to more effectively distinguish the effects of radiation alone from the combination treatment in these genetically modified cells. Figure (4.27A) shows transient growth inhibition by 1,25D3 while Figure (4.27 B) indicates that indeed 1,25D3 sensitized shRNA control cells to IR treatment.

Our next series of studies were conducted to confirm that cells with knockdown of ATG-5 or ATG-7 retained sensitivity to 1,25D3, which was indeed observed (Fig 4.28). This result was the expected outcome, as 1,25D3 does not induce autophagy. Consequently, autophagy inhibition should not have an effect on 1,25D3's ability to moderately inhibit growth.

In ZR-75-1 cells where ATG-5 was partially silenced, sensitivity to radiation was increased compared to the shRNA control cells, again indicating that autophagy is a mode of cell protection for radiation alone (Fig 4.29A). In dramatic contrast, the impact of 1,25 D3 + radiation is blunted or attenuated when Atg-5 is suppressed, which supports the cytotoxic actions of autophagy for the 1,25-D3 + radiation combination treatment (Fig 4.29B). Studies conducted in ATG-7 suppressed cells demonstrated an essentially identical outcome as with the pharmacological inhibitors Chloroquine and Bafilomycin A1 as well as with suppression of Atg5. That is, sensitivity to radiation alone is increased (Fig 4.30A), while radiosensitization by 1,25D3 is markedly attenuated (Fig 4.30B).
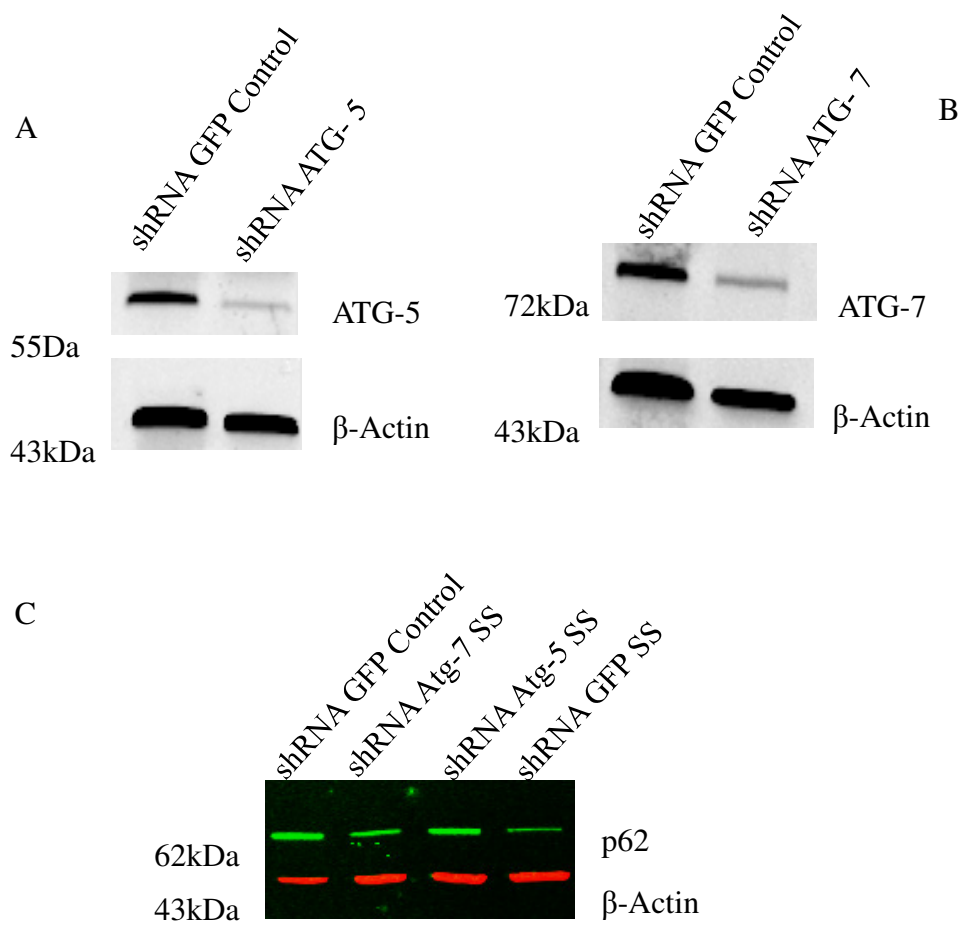
Figure 4.25 *__Knockdown of ATG-5 and ATG-7 Reduces Autophagic Flux Following__* *__Serum Starvation__* Knockdown of ATG-5 and ATG-7 in ZR-75-1 cells were verified by western blot analysis (A-B). Degradation of p62 was monitored at 24 hours following serum starvation (C).

A
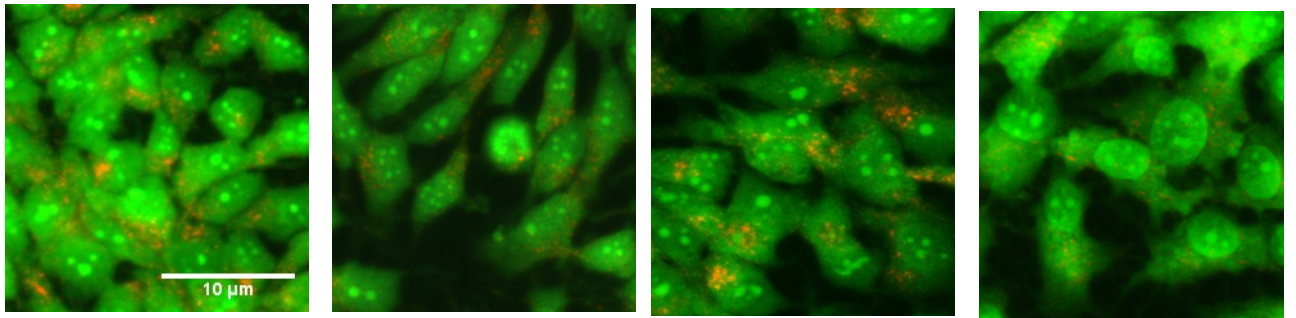
Control ATG- 5 KD   1,25D3 ATG- 5 KD   IR ATG-5 KD   1,25D$_3$+IR ATG-5 KD

B
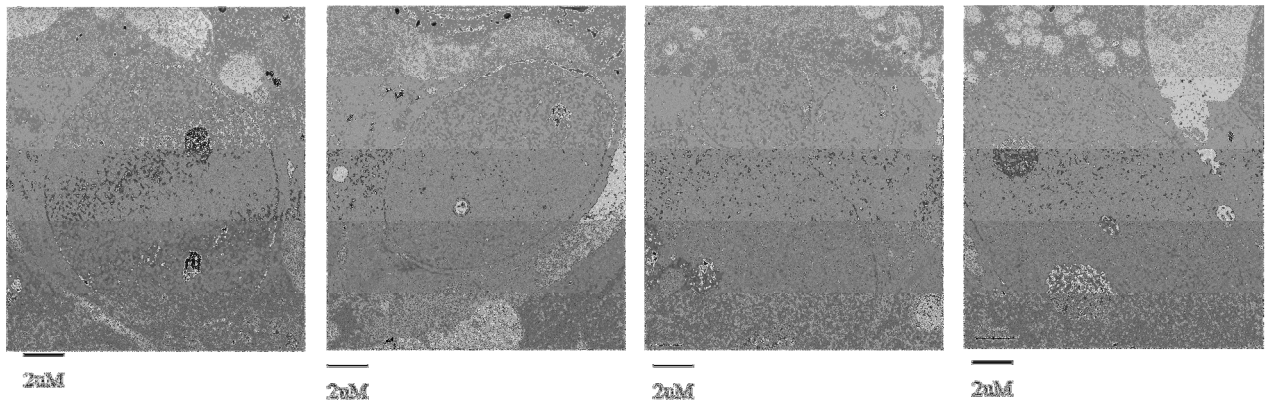
Control ATG- 5 KD   1,25D3 ATG- 5 KD   IR ATG-5 KD   1,25D3+IR ATG-5 KD

Figure 4.26 _**No Change in Autophagic Vacuole Formation with Suppressed ATG-5.**_ ZR-75-1 cells were exposed to 100nM 1,25D3, a dose of 4 Gy radiation or were treated concurrently with 1,25D3 and 4 Gy radiation. Cells were stained with Acridine Orange and images were taken 72 hours post treatment (A). The lack of presence of autophagic vacuoles was confirmed by electron microscopy at 72 hours (B).

A
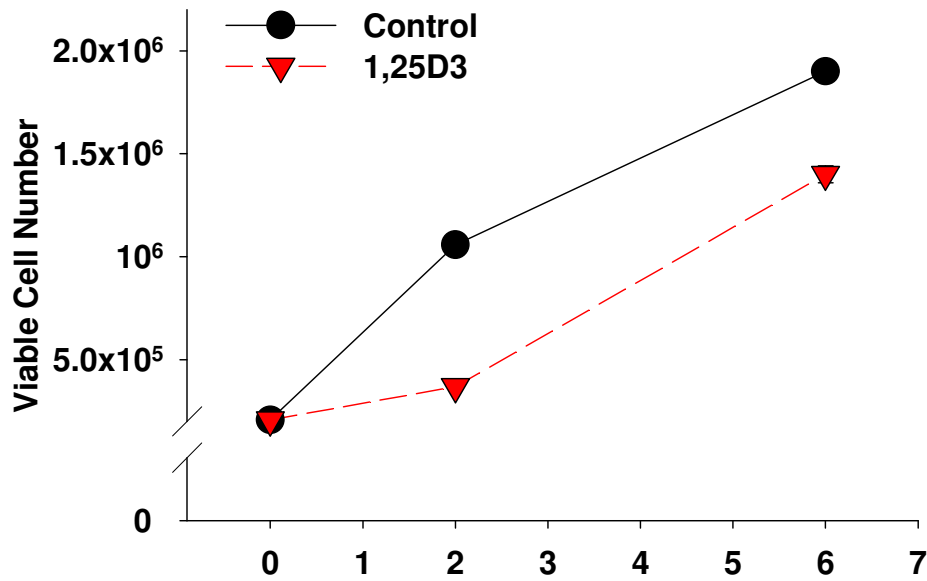
B
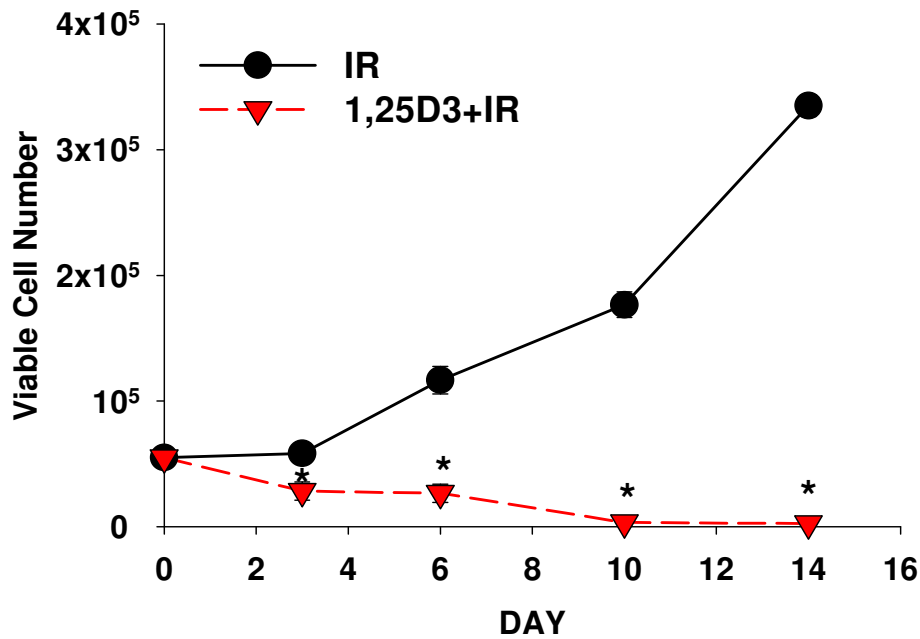
4.27 Confirmation of Sensitization to Radiation by 1,25D3 in ZR-75-1 shRNA Control Cells.
ZR-75-1 cells stably transfected with a lentivirus encoding shRNA against GFP (control) were
treated continuously with 1,25D3 (A), a single dose of 4 Gy or concurrently with 1,25D3 and IR
(B) Cell viability was assessed by trypan blue exclusion. * $p < 0.05$ compared to IR alone
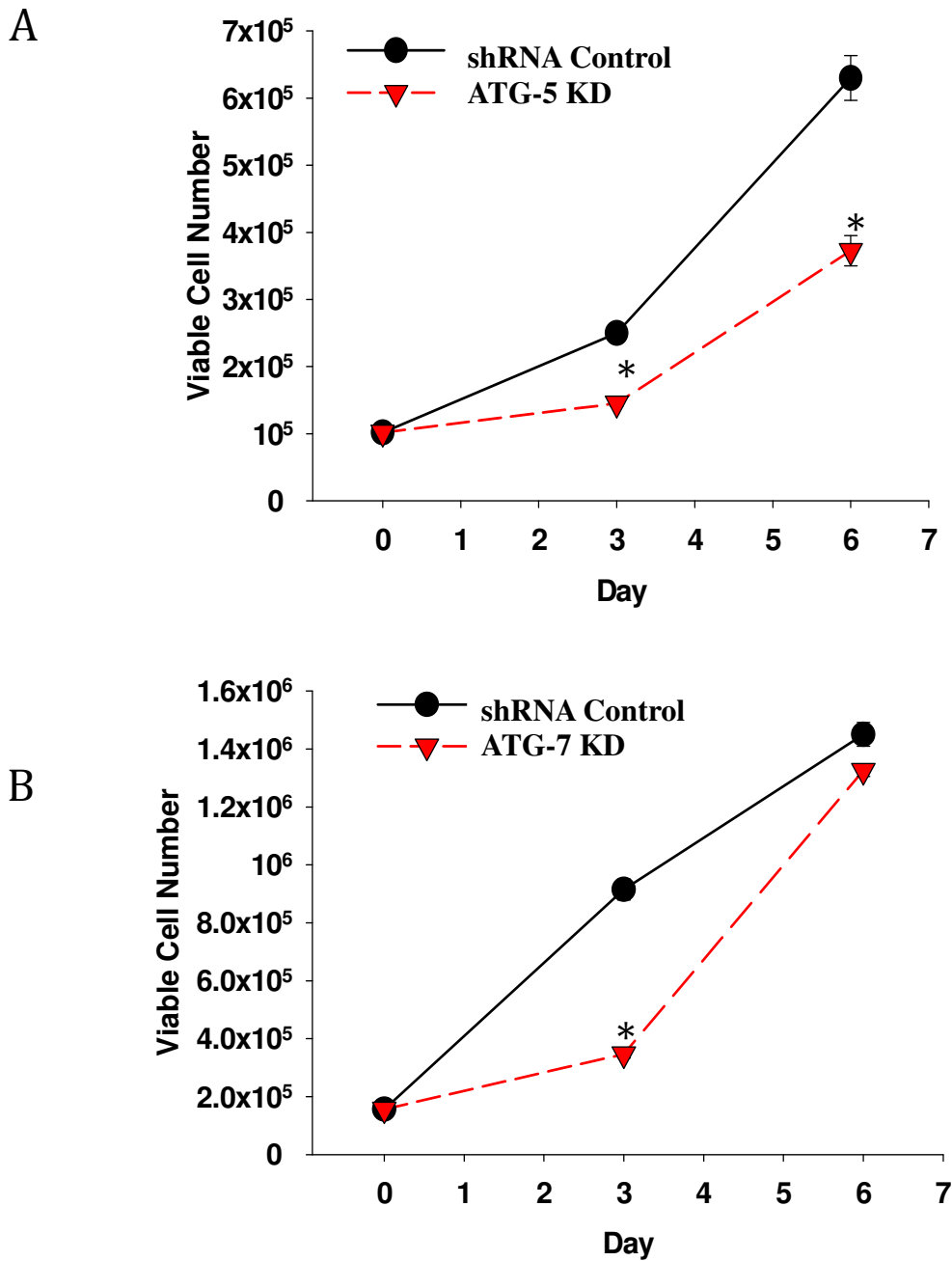
Figure 4.28 _Genetic Inhibition of Autophagy Does Not Interefere With 1,25D3's Ability to Moderately Inhibit Growth._ZR-75-1 breast cancer cells stably transfected with lentivirus for silencing expression of ATG-5 (A) or ATG-7 (B) were exposed to 100nM 1,25D3 continuously. Viable cell number was determined by exclusion of trypan blue at days 3 and 6 following post treatment. * $p < 0.05$ compared to control
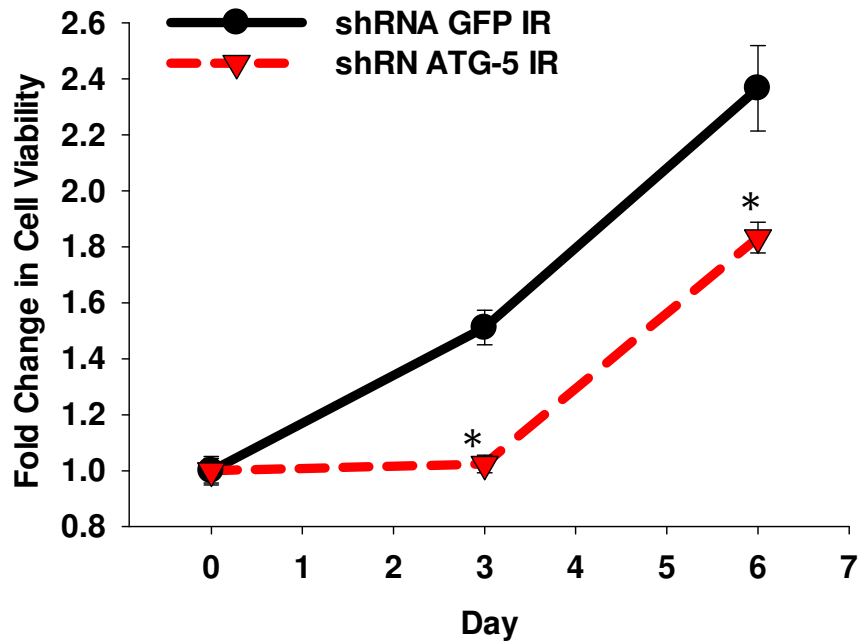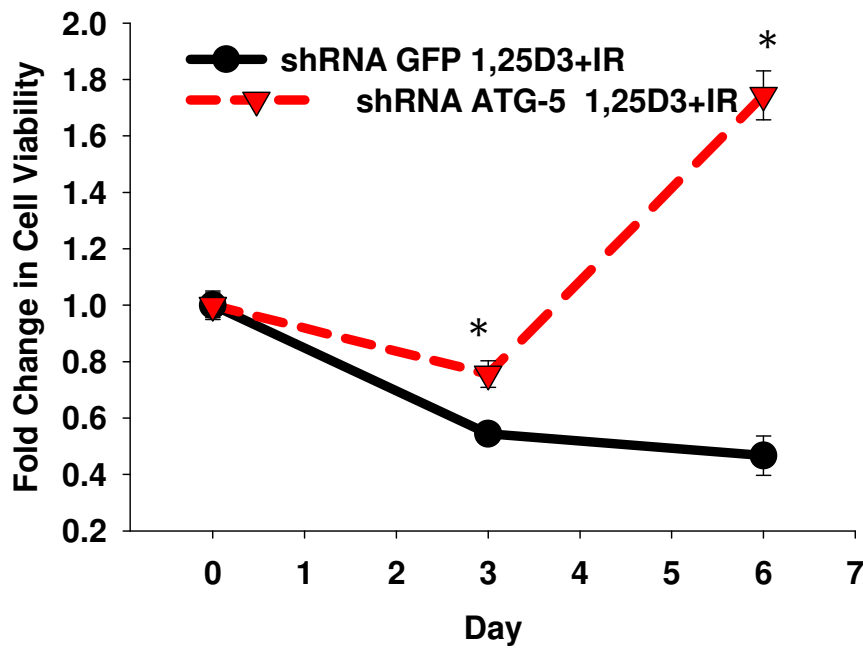
Figure 4.29 *Effects of Genetic Autophagy Inhibition (ATG-5) on Cell Viability by IR and 1,25D3+IR Treatment* ZR-75-1 cells stably transfected with a lentivirus encoding shRNA against GFP (control) or lentivirus for silencing expression of ATG-5 were exposed to a dose of 4 Gy radiation (A) or 4 Gy concurrently with 1,25D3 (B). Viable cell number was determined by exclusion of trypan blue at days 3 and 6 following post treatment. Values shown are from a representative experiment with triplicate samples for each condition. * p<0.05 compared shRNA GFP control
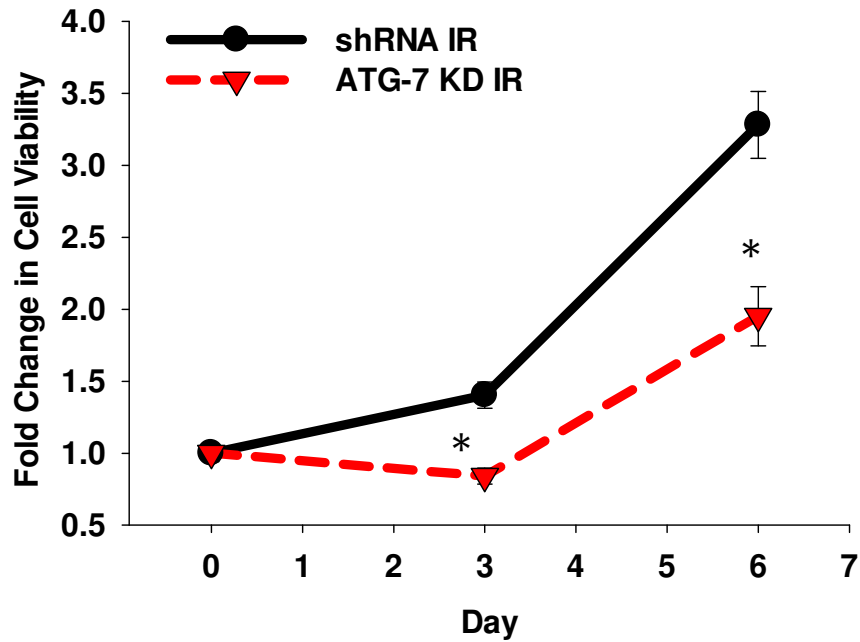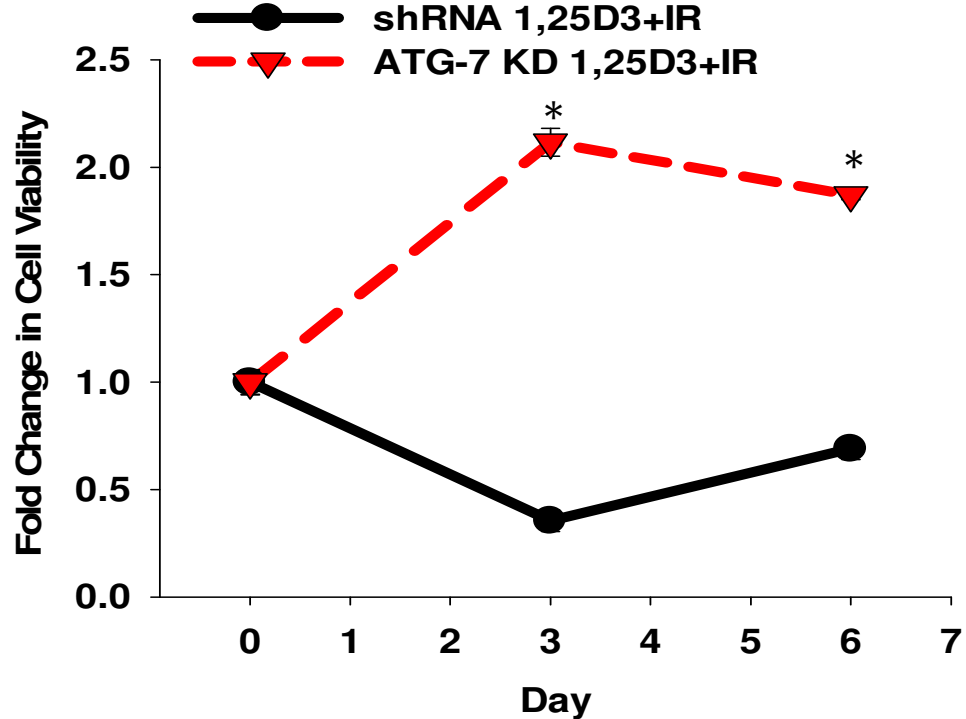
Figure 4.30 *Effects of Genetic Autophagy Inhibition (ATG-7) on Cell Viability by IR and 1,25D3+IR Treatment* ZR-75-1 cells stably transfected with a lentivirus encoding shRNA against GFP (control) or lentivirus for silencing expression of ATG-7 were exposed to a dose of 4 Gy radiation (A) or 4 Gy concurrently with 1,25D3 (B). Viable cell number was determined by exclusion of trypan blue at days 3 and 6 following post treatment. Values shown are from a representative experiment with triplicate samples for each condition. * $p < 0.05$ compared shRNA GFP control

Residual Surviving Cells are in a State of Senescence

In previous studies, we reported that MCF-7 cells undergo a period of growth arrest/senescence following treatment with radiation alone as well as in the residual surviving breast tumor cell population following treatment with vitamin D analog, EB1089 + radiation (Sundaram and Gewirtz, 1999). Based on cell morphology and β-Galactosidase staining, residual surviving cells are indeed in a state of senescence after both radiation alone and 1,25D3 + radiation treatment (Fig 4.31). To further confirm β-Galactosidase activity 5-dodecanoylaminofluoresceindi-β-D-galactopyranoside (C$_{12}$FDG), a fluorogenic substrate for β-galactosidase activity was analyzed by FACS analysis and fluorescence intensity was measured (Debacq-Chainiaux et al., 2009) (Fig 4.32). For both experimental conditions, senescence is most pronounced at 144 hours post-treatment. There was no difference in the extent of senescence induced by IR and 1,25D3+IR. 1,25D3 treatment alone did induce some senescence, but not to the extent of the other treatments.

We were also interested in determining if there was a relationship between senescence and autophagy, since the two processes appear to occur at the same time. There have been some studies in the literature that suggest autophagy and senescence are interrelated (Gewirtz, 2009; Narita et al., 2009; Young et al., 2009; Young and Narita, 2010). Thus, we employed pharmacological inhibition using both Bafilomycin A1 and Chloroquine to evaluate whether senescence might be affected. Senescence was assessed at days 3 and 6 post treatment. Bafilomycin A1 and Chloroquine alone did not generate senescent cells (Fig 4.33). It appears there is a slight increase in senescent cells after IR

and 1,25D3+IR treatments when autophagy is blocked pharmacologically (Figs 4.34 and

4.35).

| Control | 1,25D$_3$ 72hr | 1,25 D$_3$ 144hr |

| IR 72hr | IR 144hr | 1,25D$_3$ +IR 72hr | 1,25D$_3$ +IR 144hr |
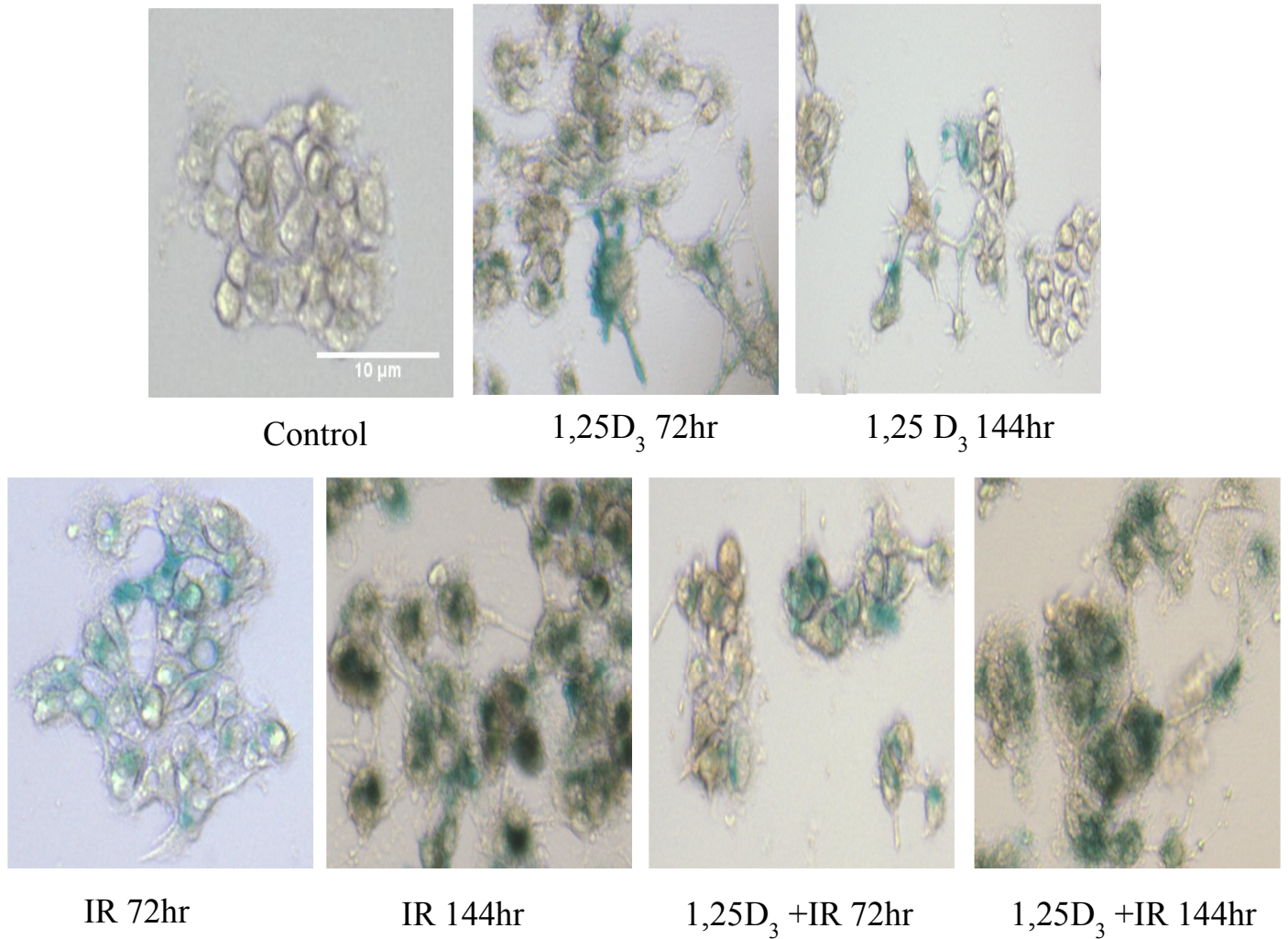
Figure 4.31 *__Residual Surviving Cells are in a State of Senescence.__* ZR-75-1 cells were exposed to 100nM 1,25D3, radiation (4x2 Gy) administered over a period of 2 days or 1,25D3 concurrently with radiation. Cells were stained with β -galactosidase and images were taken at 3 and 6 days post drug treatment or post irradiation.
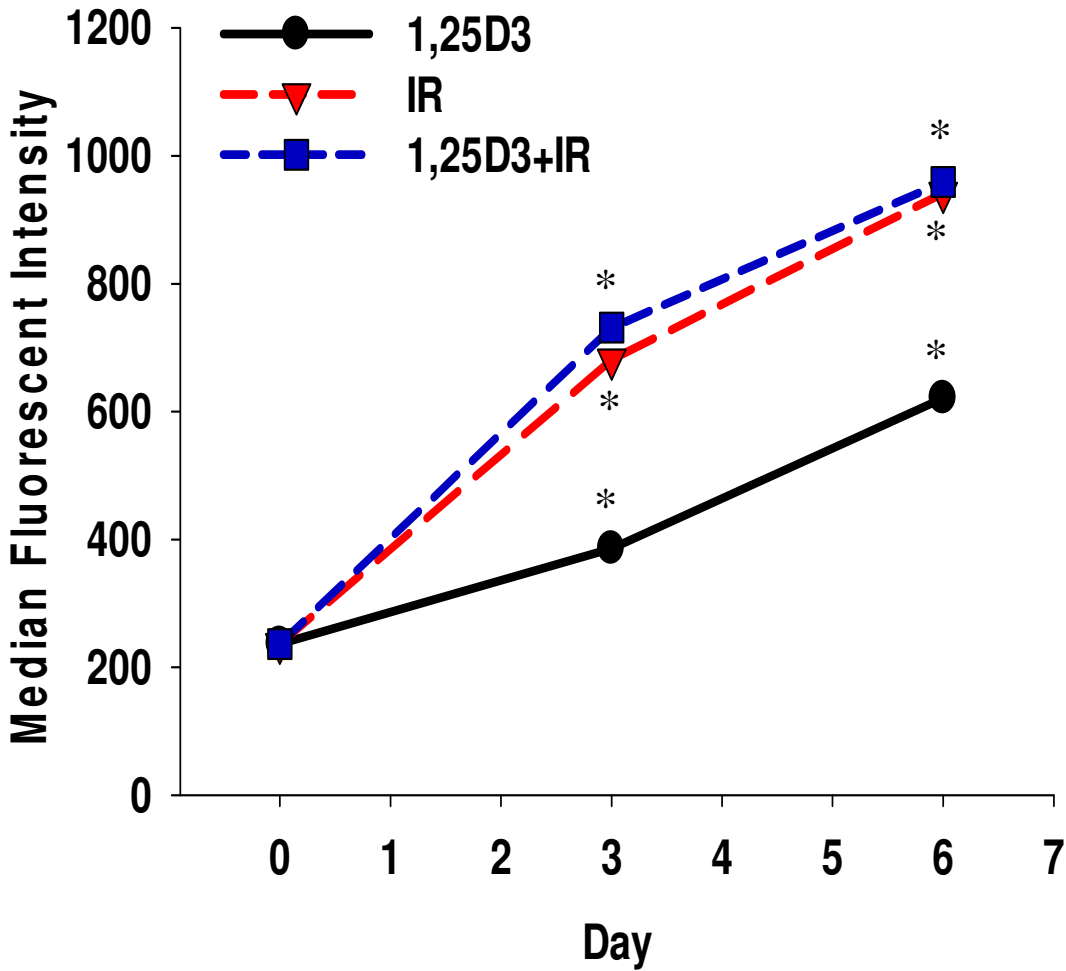
Figure 4.32 *Assessment of Senescence by Flow Cytometry* ZR-75-1 cells were exposed to 100nM 1,25D3, radiation (4 Gy) or 1,25D3 concurrently with 4 Gy radiation and samples were stained as described in materials and methods and analyzed by FACS analysis at days 3 and 6 post treatment. * $p < 0.05$ compared to day 0

BAF 72 hrs

CQ 72 Hrs

BAF 144 Hrs

CQ 144 Hrs
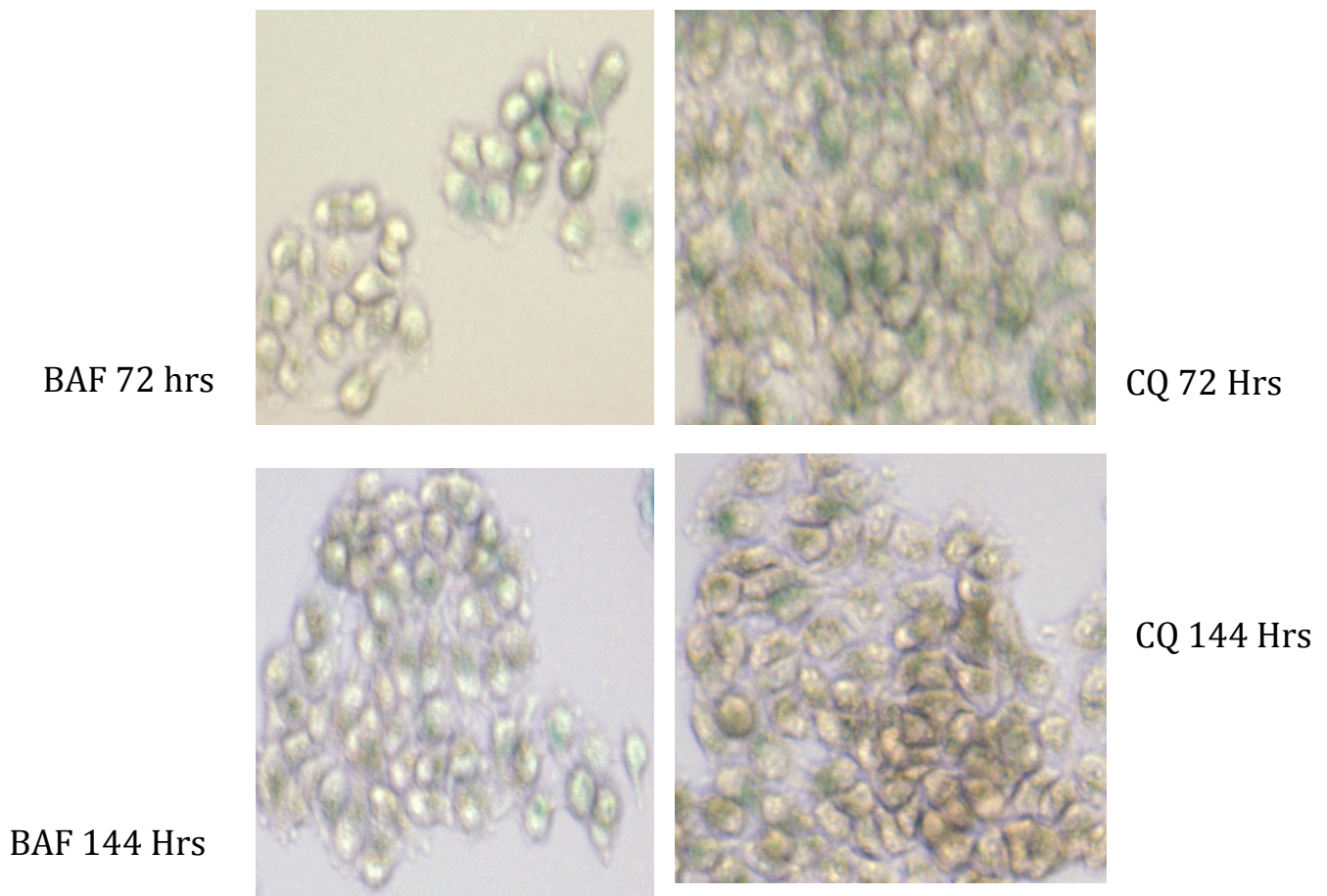
Figure 4.33 *__Minimal Senescence Observed with Pharmacological Autophagy Inhibitors Chloroquine and Bafilomyin A1__* ZR-75-1 cells were exposed to 5uM Chrloroquine or 200nM Bafilomyin A1 and stained with β –galactosidase. Images were taken at 3 and 6 days post drug treatment or post irradiation.

IR 72 hrs
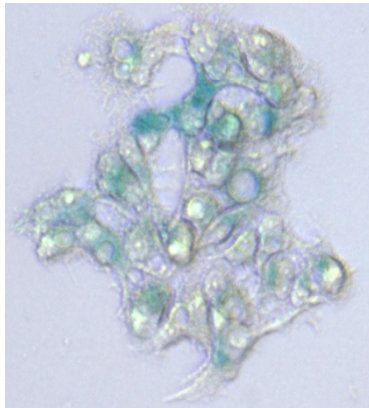
IR 144 hrs
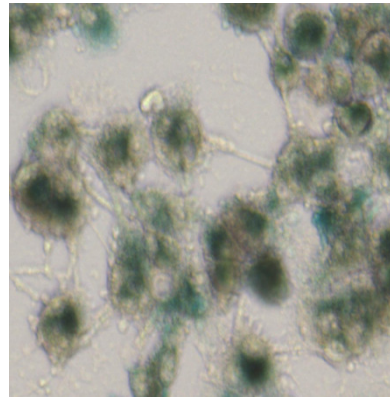
IR+BAF 72 hrs
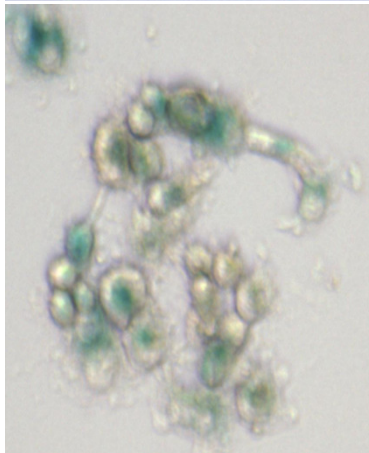
IR+BAF144 hrs
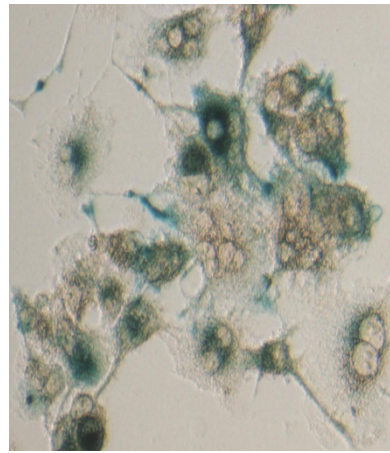
IR+CQ 72 hrs
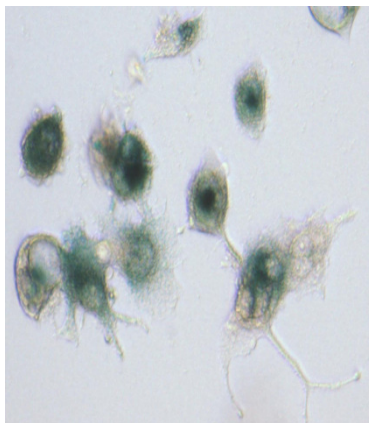
IR+CQ 144 hrs
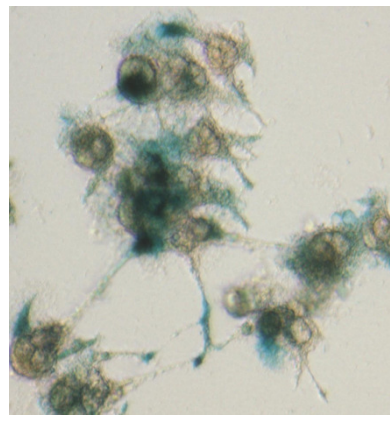
4.34 _**Effects of Pharmacological Autophagy Inhibition on Senescence following IR**_ ZR-75-1 cells were exposed to radiation (4x2Gy) in the absence or presence of either Bafilomycin A1 or Chloroquine and stained with senescence marker, β -galactosidase at days 3 and 6 post treatment.

1,25D3 + IR 72 hrs
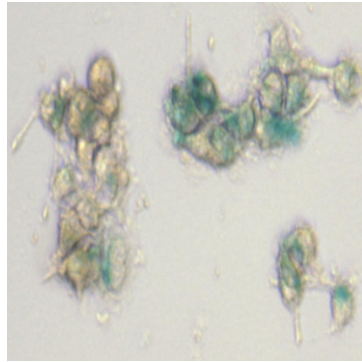
1,25D3 +IR144 hrs
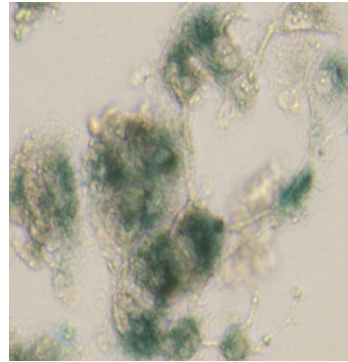
1,25D3+IR+BAF 72 hrs
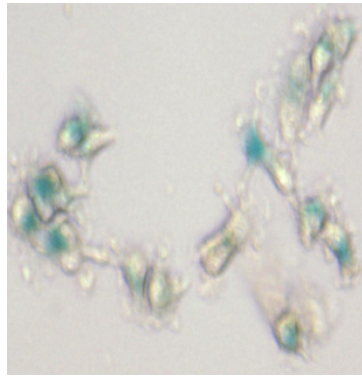
1,25D3 +IR+BAF 144 hrs
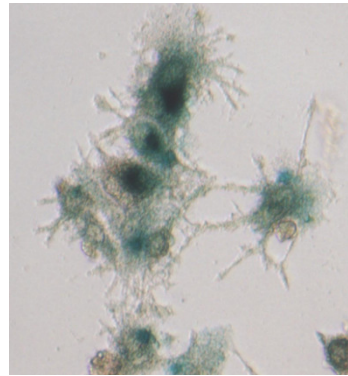
1,25D3 +IR+CQ 72 hrs
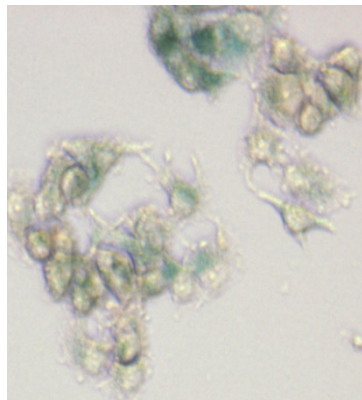
1,25D3 +IR+CQ 144 hrs
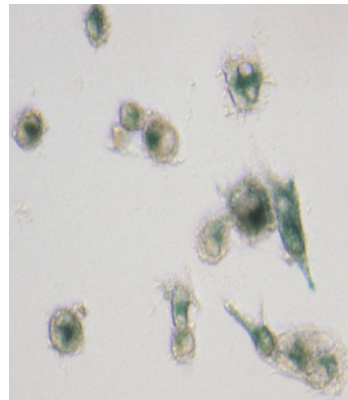
4.35 _**Effects of Pharmacological Autophagy Inhibition on Senescence following 1,25D3+IR**_
ZR-75-1 cells were exposed to 1,25D3 concurrently with 4x2 Gy radiation in the absence or presence of either Bafilomycin A1 or Chloroquine and stained with senescence marker, β - galactosidase at days 3 and 6 post treatment.

Summary

Evaluation of the characteristics of autophagic cell death revealed that 1,25D3 mediated radiosensitization is in fact due to the promotion of autophagic cell death. This conclusion is further supported by data demonstrating the lack of other modes of cell death such as apoptosis, necrosis and mitotic catastrophe. Studies assessing autophagy induction in 1,25D3+IR treatment revealed that autophagy is indeed induced and that the cellular contents are effectively being degraded by the lysosomes, as shown by assays assessing autophagic flux. Furthermore, autophagy inhibition both pharmacologically and genetically attenuated radiation sensitization by 1,25D3. Autophagy was also induced in cells only receiving radiation treatment. Pharmacological and genetic inhibition revealed that the autophagy was different for IR treatment as inhibition resulted in increased sensitivity to radiation treatment, strongly supporting activation of cytoprotective autophagy by radiation alone. Sensitization to radiation by autophagy inhibitors appears to be a result of a combination of apoptotic and mitotic catastrophe cell death mechanisms. Moreover, residual surviving cells are in a state of senescence and autophagy inhibition appears to have a minimal effect on senescence.

Chapter 5

Exploring Intracellular Signaling Pathways Involved in 1,25D3 Mediated Radiosensitization

Studies in this chapter were designed to elucidate the pathways by which 1,25 D3 sensitizes breast cancer cells to radiation in addition to efforts to distinguish the signaling pathways involved in cytotoxic and cytoprotective autophagy. It is known that autophagy is a multi-step process that appears to be regulated by various signaling pathways (Botti et al., 2006; Codogno and Meijer, 2005). The capacity of 1,25D3 to promote radiosensitization may involve its modulation of proteins in the DNA damage response or ER stress and mTOR signaling pathways.

DNA Damage Response

One possible mechanism that could be potentially altered by 1,25D3 to confer enhanced sensitivity to radiosensitization is a change in the DNA damage response. The

DNA damage response is a collection of cellular events by which a cell identifies and repairs DNA upon damage following such stressors as radiation (Zhou and Elledge, 2000). One indicator of DNA damage is γ-H2AX, which is activated in response to double strand breaks in the DNA (Celeste et al., 2003). Initial studies were conducted to determine whether radiation sensitization could be due to 1,25D3's ability to increase DNA damage induction or interfere with DNA repair. ZR-75-1 cells were exposed to 1,25D3, IR or 1,25D3+IR and the increase in γ-H2AX was monitored using immunocytohistochemistry and flow cytometry. Fig 5.1 presents images of γ-H2aX foci taken 1-hour post treatment. Both IR and 1,25D3+IR treatments produced significant increases in γ-H2AX compared to the control and 1,25D3 treatment alone. Images were also taken at 3, 6 and 24 hours post treatment and the results were essentially the same (data not shown). To quantify the extent of DNA damage following treatment, we used an antibody directed toward phosphorylated γ-H2AX and measured fluorescent intensity using flow cytometry at 1 hour and 24 hours post treatment (Fig 5.2). Similar to the images in Fig 5.1, there was a significant increase in median fluorescent intensity in cells treated with IR and 1,25D3+IR at both time points compared to control. However, there was no difference in median fluorescent intensity between IR and 1,25D3+IR at either time point. At 1 hour both IR and 1,25D3+IR treatment resulted in a significant enhancement of γ-H2AX fluorescent intensity, suggesting that both treatments result in the same extent of DNA damage. At 24 hours both treatments resulted in decreased γ-H2AX, suggesting that DNA repair is occurring essentially to the same extent following treatment with IR or 1,25D3+IR. Moreover, these data imply that radiation sensitization by 1,25D3 is likely not due to an alteration in DNA damage induction or repair. This

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observation is consistent with previous studies conducted in the laboratory, which demonstrated an essentially identical response (Demasters, 2006).
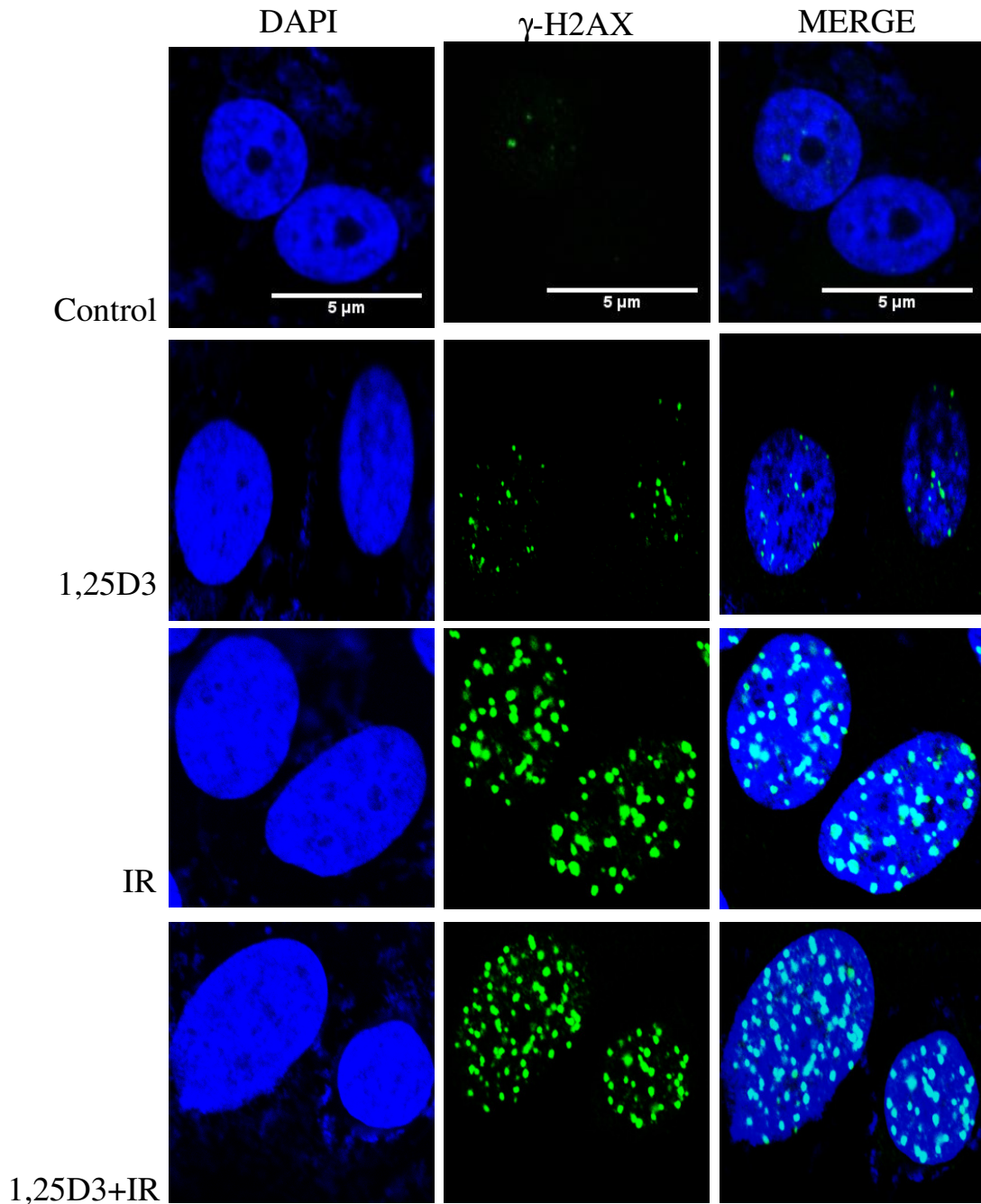
Figure 5-1 *No Difference in γ-H2AX Foci Formation by IR and 1,25D3+IR Treatment. ZR*-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. γ-H2AX foci were monitored 1 hour post treatment
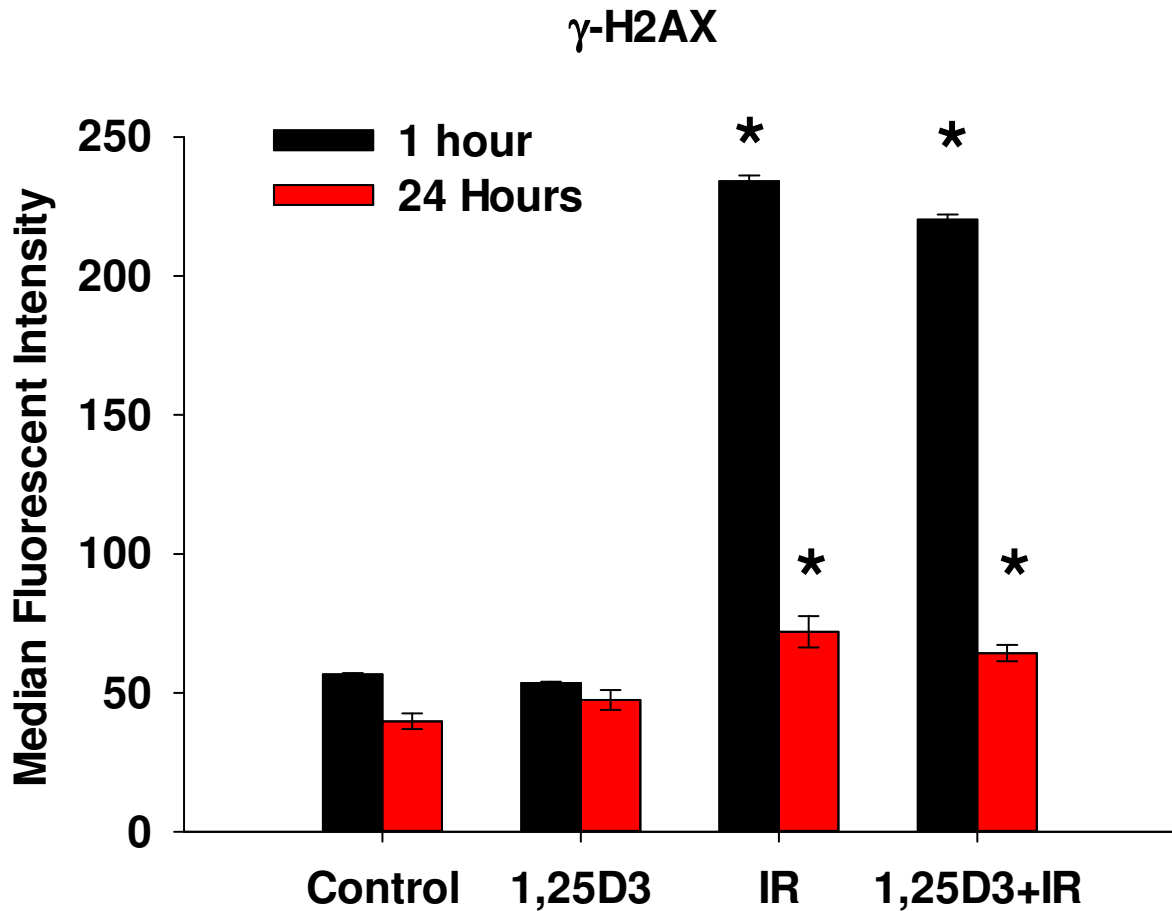
Figure 5-2 *<u>No change in γ-H2AX fluorescence intensity with IR compared to 1,25D3+IR treatment</u>* ZR-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. γ-H2AX fluorescent intensity monitored 1 hour and 24 hours post treatment. 10,000 cells were analyzed for each sample * $p < 0.05$ compared to control

ER Stress

Activation of ER stress has been shown to be involved in autophagy induction (Hoyer-Hansen and Jaattela, 2007a; Qin et al., 2010). Moreover, as both the ER stress pathway and the UPR pathways have been shown to be activated in response to radiation (Kim et al., 2010; Zhang et al., 2010), it seemed logical to explore this pathway as a possible basis for radiosensitization by 1,25D3. Additionally, accumulation of unfolded proteins in the ER in response to diverse cellular insults has been shown to upregulate autophagy in cancer cells via protein kinase-like endoplasmic reticulum kinase (PERK), eukaryotic initiation factor (eif2a) and glucose-regulated protein of 78 kDa (GRP78) (Verfaillie et al., 2010). Therefore, we monitored the upregulation/phosphorylation of these effectors.

We first assessed the activation of PERK using an antibody directed towards the threonine 980 phosphorylation site and measured the fluorescent intensity of this protein at 6, 24 and 48 hours post treatment. PERK is an endoplasmic reticulum resident transmembrane protein that couples ER stress signals to inhibit translation upon activation of ER stress (Hoyer-Hansen and Jaattela, 2007a). Moreover, PERK is a reliable indicator of ER stress as induction of ER stress results in an increase in PERK activity (Hoyer-Hansen and Jaattela, 2007a; Verfaillie et al., 2010). The results presented in Figure 5.3 indicate that both IR and 1,25D3+IR treatments resulted in a significant increase in phosphorylated PERK; 1,25D3+IR produced a slightly more pronounced increase at 6 and 24 hours compared to IR alone (Fig 5.3). The increase of PERK activity by 1,25D3+IR treatment was significant compared to control levels at 6 hours post

treatment, whereas this was not the case with IR alone. 1,25D3 treatment alone did not significantly increase phosphorylated PERK until 48 hours post treatment.

To further delve into this pathway, we assessed the levels of eif2α, which is a downstream target of PERK, by also measuring fluorescent intensity using flow cytometry (Verfaillie et al., 2010). Activation of eif2α ultimately leads to reduced protein translation following ER stress (Verfaillie et al., 2010). At 24 hours and 48, hours both IR and 1,25D3+IR treatment resulted in a significant increase in phosphorylated eif2α compared to control, while no activity was observed with 1,25D3 treatment alone until 48 hours post treatment (Fig 5.4). These data correspond to our PERK data, which demonstrated an essentially identical outcome. By 48 hours there was an approximate two-fold increase in phosphorylated eif2a with all of the treatments compared to control.

Lastly, we assessed levels of GRP78, an endoplasmic reticulum chaperone involved in monitoring protein folding, quality control and calcium signaling (Wang et al., 2010). Analysis of GRP78 levels by western blot analysis failed to demonstrate any change in activity for IR or 1,25D3+IR treatment as there were no significant alterations in protein levels at any of the indicated time points (Figs 5.5). Taken together, these results suggest that the PERK-eif2α arm of the ER stress pathway is indeed activated by treatments with 1,25D3, IR, and 1,25D3+IR while there is no requirement of GRP78 for ER stress induction in our experimental system. In addition, ER stress activation does not appear to be the mode of radiation sensitization by 1,25D3 as there was no difference in PERK or eif2α activation by IR and 1,25D3+IR treatment.
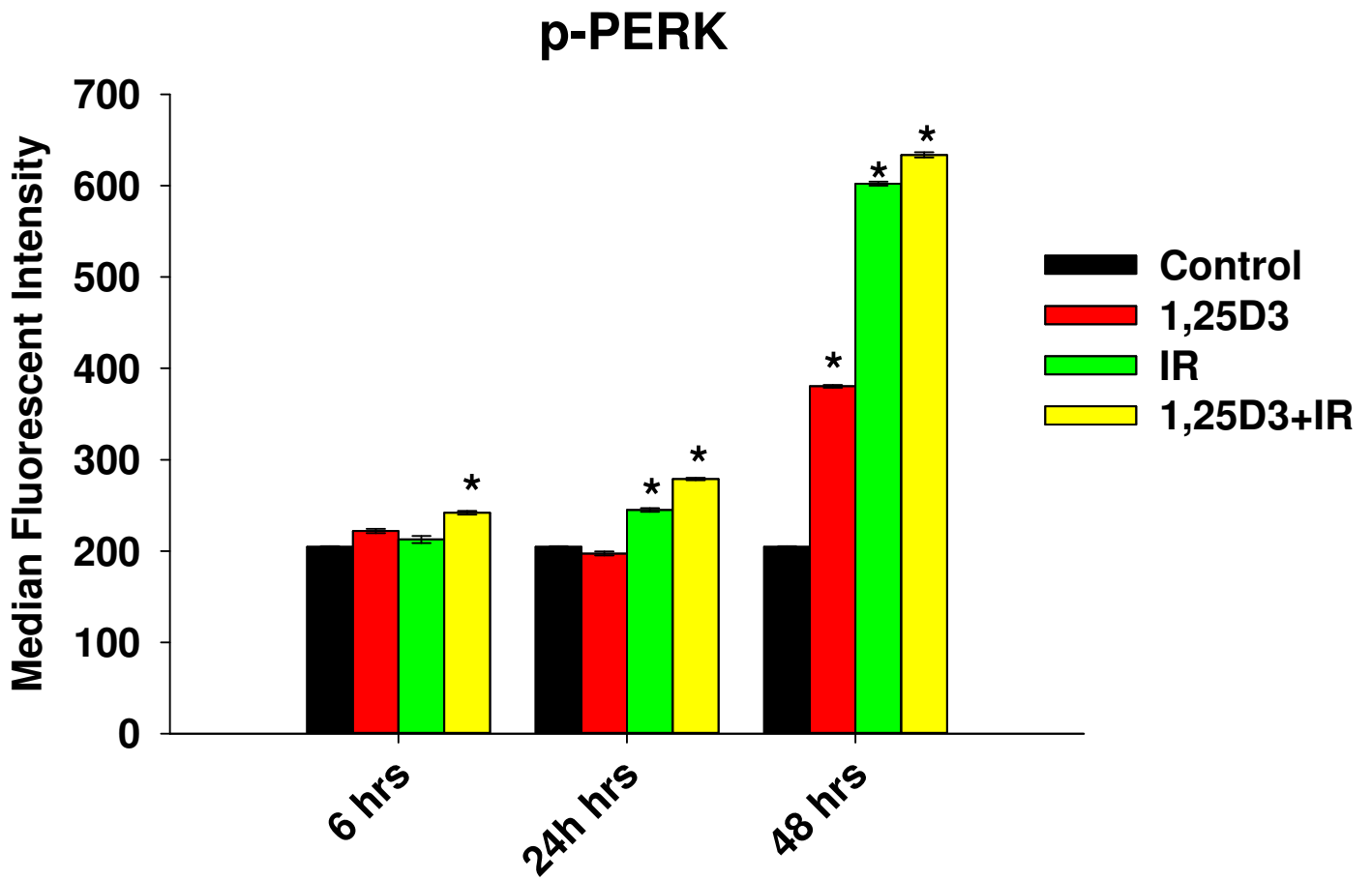
Figure 5-3 *Induction of p-PERK* ZR-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. Fluorescent intensity of p-PERK was monitored at 6 hours, 24 hours and 48 hours post treatment. 10,000 cells were analyzed for each sample. * p<0.05 compared to control

Figure 5-4 *Induction of p-eif2α* ZR-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. Fluorescent intensity of p-eif2a was monitored at 24 hours and 48 hours post treatment. 10,000 cells were analyzed for each sample * p<0.05 compared to control
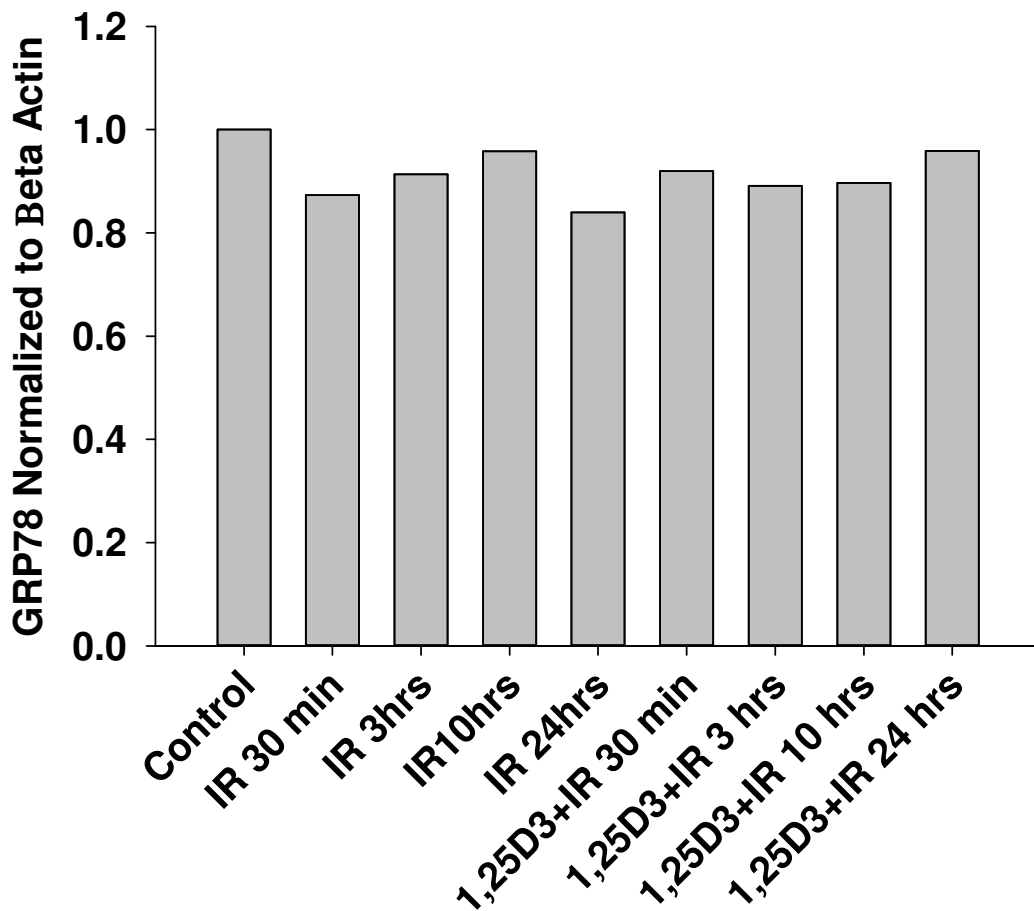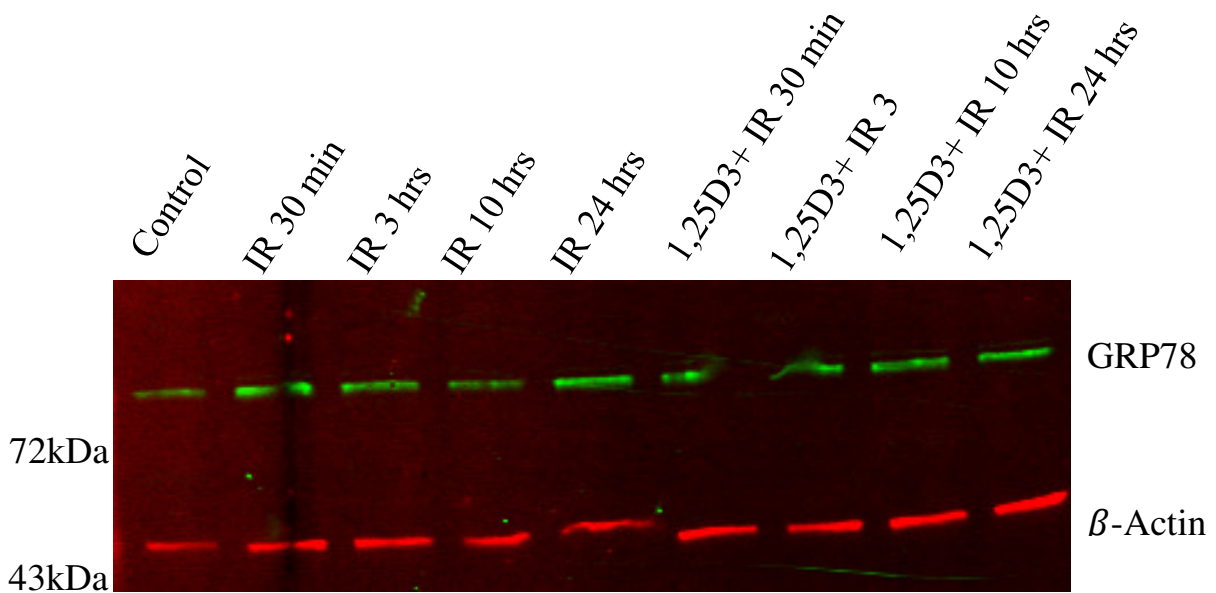
Figure 5.5 *Assessment of GRP78 by Western Blot Analysis (IR and 1,25D3+IR)* ZR-75-1 cells were exposed to a dose of 4 Gy or were treated concurrently with 1,25D3 and 4 Gy radiation. GRP78 levels were monitored at various time points post treatment

AMPK/mTOR

This section will focus on experiments designed to assess the impact of 1,25D3, radiation and 1,25D3+IR treatment on the mammalian target of rapamycin (mTOR), specifically mTORC1. mTORC1 functions as a sensor for nutrients and energy (Guertin and Sabatini, 2005; Jung et al., 2010). As aforementioned, mTOR is the key central negative regulator of autophagy and its downregulation can lead to autophagy (Dowling et al., 2010; Jung et al., 2010). Downregulation of mTOR leads to decreased protein synthesis as a result of low nutrients and growth factor deprivation (Jung et al., 2010).

Initial studies were conducted to determine if 1,25D3, IR or 1,25D3+IR resulted in downregulation of mTOR by evaluating its deactivation using an antibody directed towards the Theronine 2441 phosphorylation site and measuring fluorescent intensity by flow cytometry. The results shown in figure 5.6 indicate a significant decrease in phosphorylated mTOR following 1,25D3, IR and 1,25D3+IR at 24 hours post treatment compared to control levels. However, treatment with 1,25D3+IR resulted in an even a more pronounced decrease in p-mTOR at 24 hours compared to 1,25D3 and IR treatment alone at the same time point.

Subsequent studies assessed AMP activated protein kinase (AMPK) activity, which is an effecter directly upstream of mTOR (Chen and Klionsky, 2011; Motoshima et al., 2006). Activated AMPK inhibits mTOR complex via tuberous sclerosis complex 1 & 2 (TSC 1&2) and its substrate Rheb (an increase in p-AMPK results in a decrease in p-MTOR) (Chen and Klionsky, 2011). Initial studies measured the impact of 1,25D3, IR and 1,25D3+IR on AMPK activity by monitoring its phosphorylation at the Threonine 172 site by visual assessment using confocal microscopy and flow cytometry. Confocal

microscopy revealed that only 1,25D3+IR treatment resulted in a significant increase in phosphorylated AMPK post treatment at 3, 6 and 24 hours, which is indicated by intense green staining in the cytoplasm as well as the nucleus (Fig 5.7A). A similar increase of p-AMPK was observed when p-AMPK fluorescent intensity was monitored by flow cytometry 24 hours post treatment (Fig 5.7B). An approximate 1.5 fold increase was shown for 1,25D3+IR compared to control, suggesting a potential role of AMPK in 1,25D3 mediated radiosensitization. The increase in p-AMPK for 1,25D3+IR was also significant compared to IR treatment alone.

Since there appeared to be an involvement of the AMPK pathway in radiosensitization by 1,25D3, we investigated whether inhibition of AMPK resulted in decreased autophagy induction by 1,25D3+IR. The pharmacological inhibitor, Compound C, was employed to determine if there was a relationship between AMPK activity and autophagy induction. (Gormand et al., 2011; Hoyer-Hansen et al., 2007). Autophagy was monitored by assessing redistribution of RFP-LC3 using confocal microscopy at 24 hours post treatment (Fig 5.8). Treatment with Compound C completely attenuated RFP-LC3 redistribution in cells treated with 1,25D3+IR. Moreover, Compound C reversed 1,25D3 mediated radiosensitization based on evaluation of cell viability by trypan blue exclusion (Fig 5.9). In fact, results of the studies where AMPK was inhibited were very similar to studies where Bafilomycin A1 or Chloroquine were used to suppress autophagy. As expected, there was no effect of Compound C on cell viability with IR treatment alone as no increase in AMPK activity was observed in our previous studies where cells were exposed to IR treatment.

Due to the fact that there was a clear distinction in response among 1,25D3, IR and 1,25D3+IR, we decided to evaluate changes in calcium. An increase in free cytosolic calcium has been shown to activate autophagy via the Ca2+/ calmodulin-dependent kinase kinase-Beta (CaMKKβ)/AMPK pathway (refer to introduction for diagram) (Chen and Klionsky, 2011; Hoyer-Hansen and Jaattela, 2007a). CaMKKβ is directly upstream of AMPK and is one of the three upstream kinases responsible for its initiation (Chen and Klionsky, 2011; Hoyer-Hansen and Jaattela, 2007a). This pathway is mediated by slow release of calcium from the ER to the cytosol, resulting in autophagy (Hoyer-Hansen and Jaattela, 2007a; Hoyer-Hansen et al., 2007). Hoyer-Hansen et al demonstrated that inhibition of AMPK or CaMKKβ by siRNA or pharmacological inhibition attenuated autophagy induced by calcium mobilizing agents in MCF-7 cells (Hoyer-Hansen et al., 2007). Thus, it seemed logical to explore the Ca-AMPK signaling pathway since vitamin D3 and its analogs are calcium-mobilizing agents; furthermore previous studies have shown that long exposure to 1,25 D3 or its analog EB1089 indeed induce a slow increase in cytosolic calcium and autophagy followed by autophagic cell death in MCF-7 breast cancer cells (Hoyer-Hansen et al., 2007).

Changes in free cytosolic calcium levels were evaluated following treatment with 1,25D3, IR or 1,25D3+IR treatment using the calcium indicator, Fluo-3am. The intensity of Fluo-am was assessed one hour post treatment (Fig 5.10). There was indeed a significant increase in free cytosolic calcium following treatment with 1,25D3+IR. In contrast, there was actually a decrease in cytosolic calcium following treatment with either 1,25D3 or IR, suggesting that calcium levels are differentially regulated.
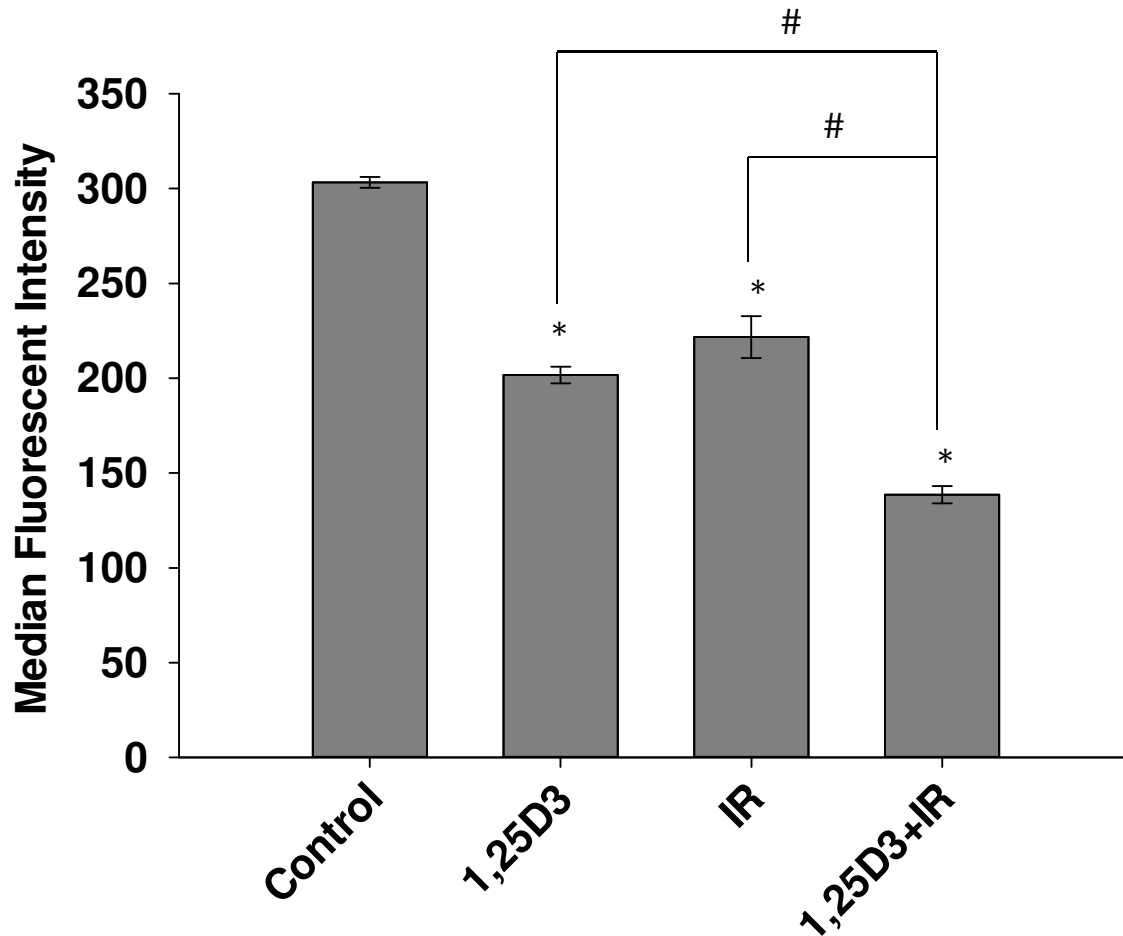
Figure 5-6 _Assessment of mTOR phosphorylation_ ZR-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. Fluorescent intensity of p-MTOR was monitored at 24 hours post treatment. 10,000 cells were analyzed for each sample* $p < 0.05$ compared to control # $p < 0.05$ compared to 1,25D3 and IR alone
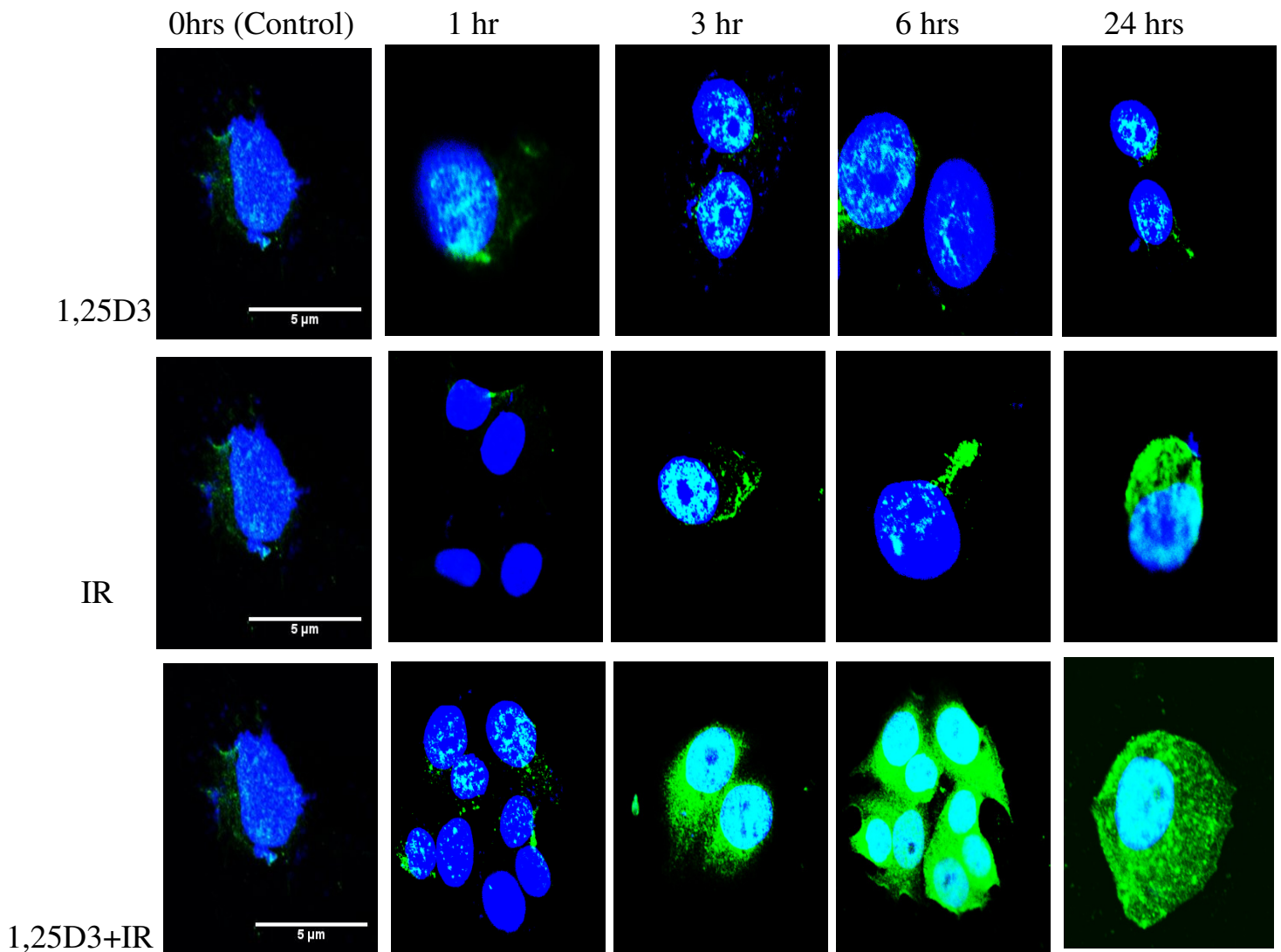
**Figure 5-7A** *Assessment of Changes in Phosphorylated AMPK by Confocal Microscopy(Timecourse)* ZR-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. Induction of p-AMPK was monitored visually by confocal microscopy. p-AMPK is represented by the green staining.
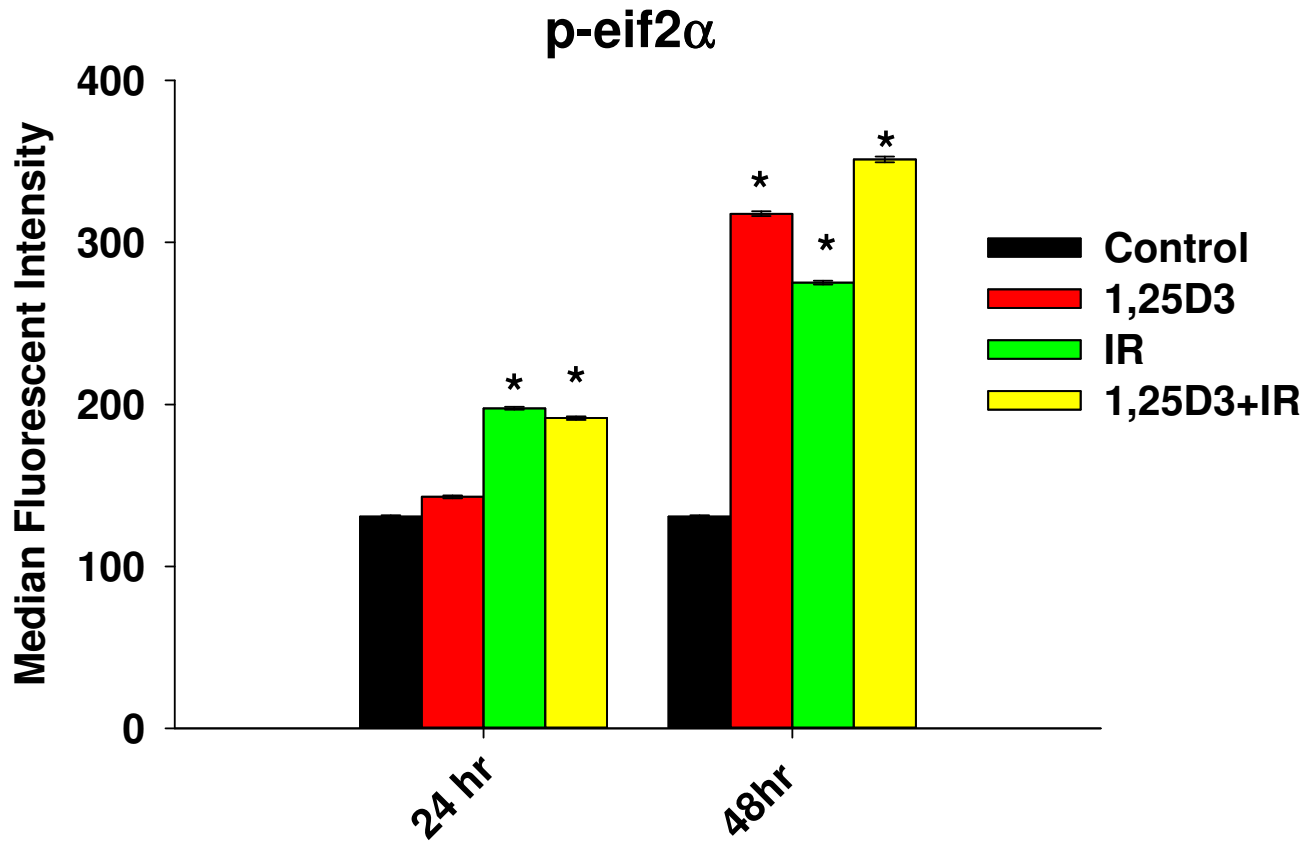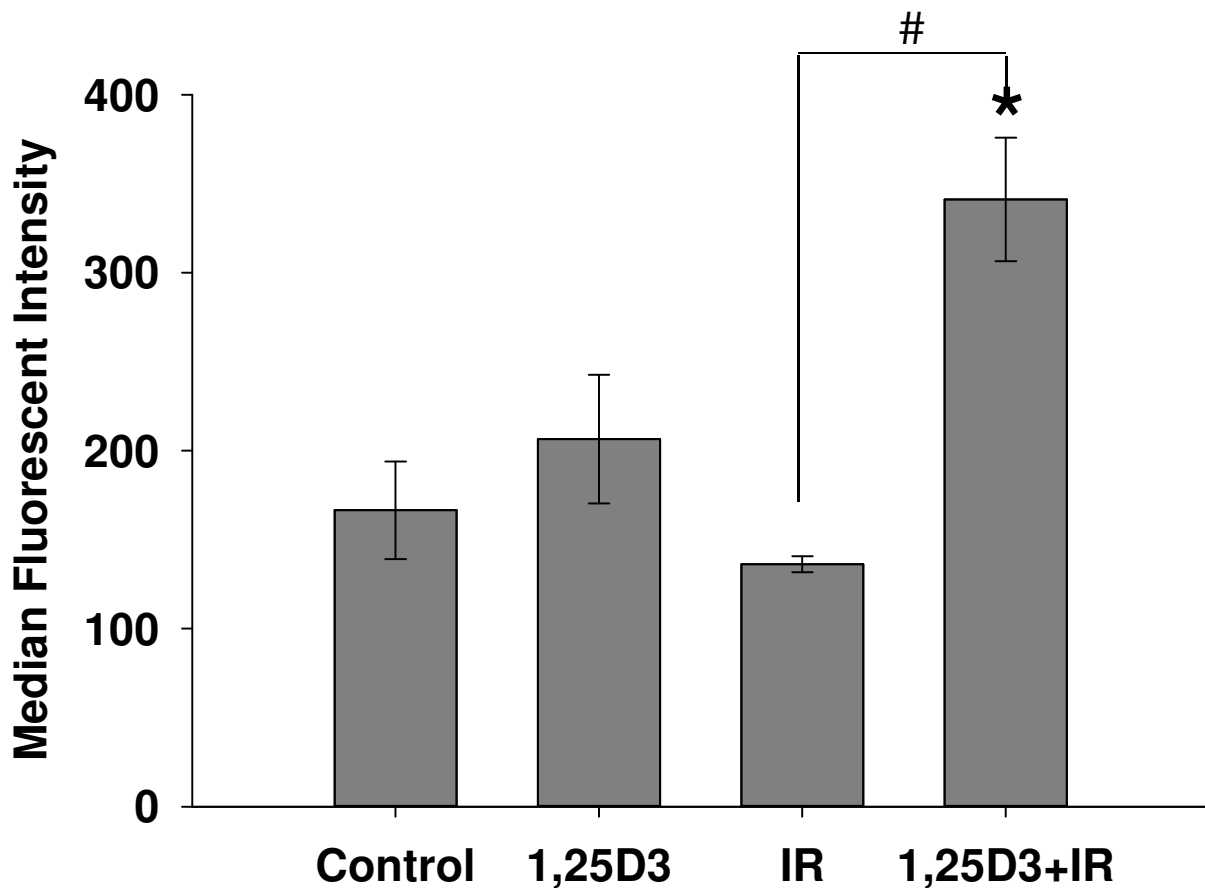
Figure 5-7B *Assessment of Changes in Phosphorylated AMPK by Flow Cytometry* ZR-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. Fluorescent intensity of p-AMPK was measured using flow cytometry at 24 hour post treatment. 10,000 cells were analyzed for each sample *p <0.05 compared to Control #p <0.05 compared to IR
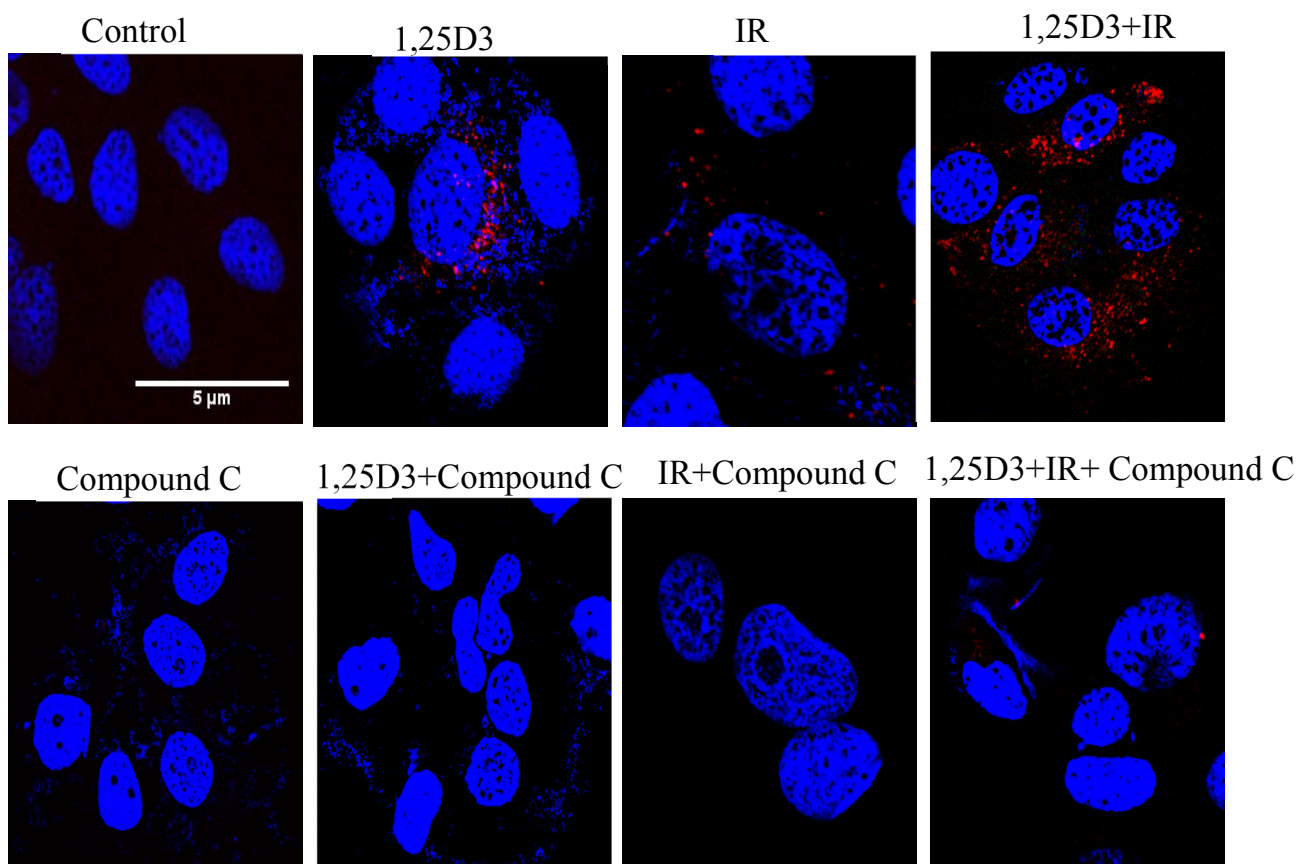
Figure 5.8 *Assessment of Redistribution of RFP-LC3 Following Inhibition of AMPK.* ZR-75-1 cells stably transfected with RFP-LC3 were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy or were treated concurrently with 1,25D3 and 4 Gy radiation in the presence or absence of Compound C (5uM). At 24 post treatment cells were counterstained with DAPI and visualized using Confocal Microscopy.
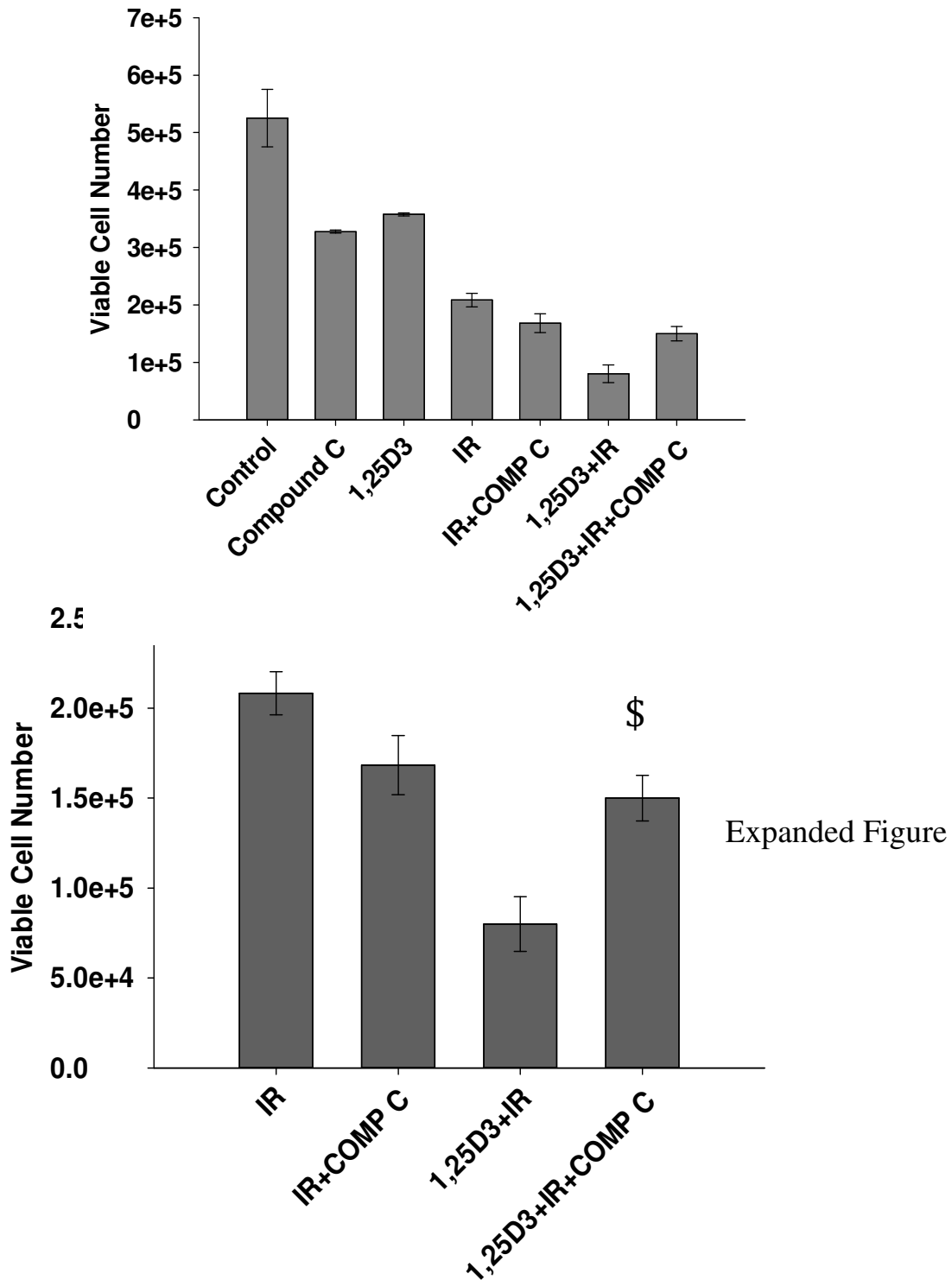
Figure 5-9 *Effects of Pharmacological Inhibition of AMPK on Cell Viability.* ZR-75-1 breast cancer cells were exposed to 5uM Compound C, 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation with or without Compound C. Viable cell number was determined by exclusion of trypan blue at 72 hours post treatment. Values shown are from a representative experiment with triplicate samples for each condition. $ $p<0.05$ compared to 1,25D3+IR
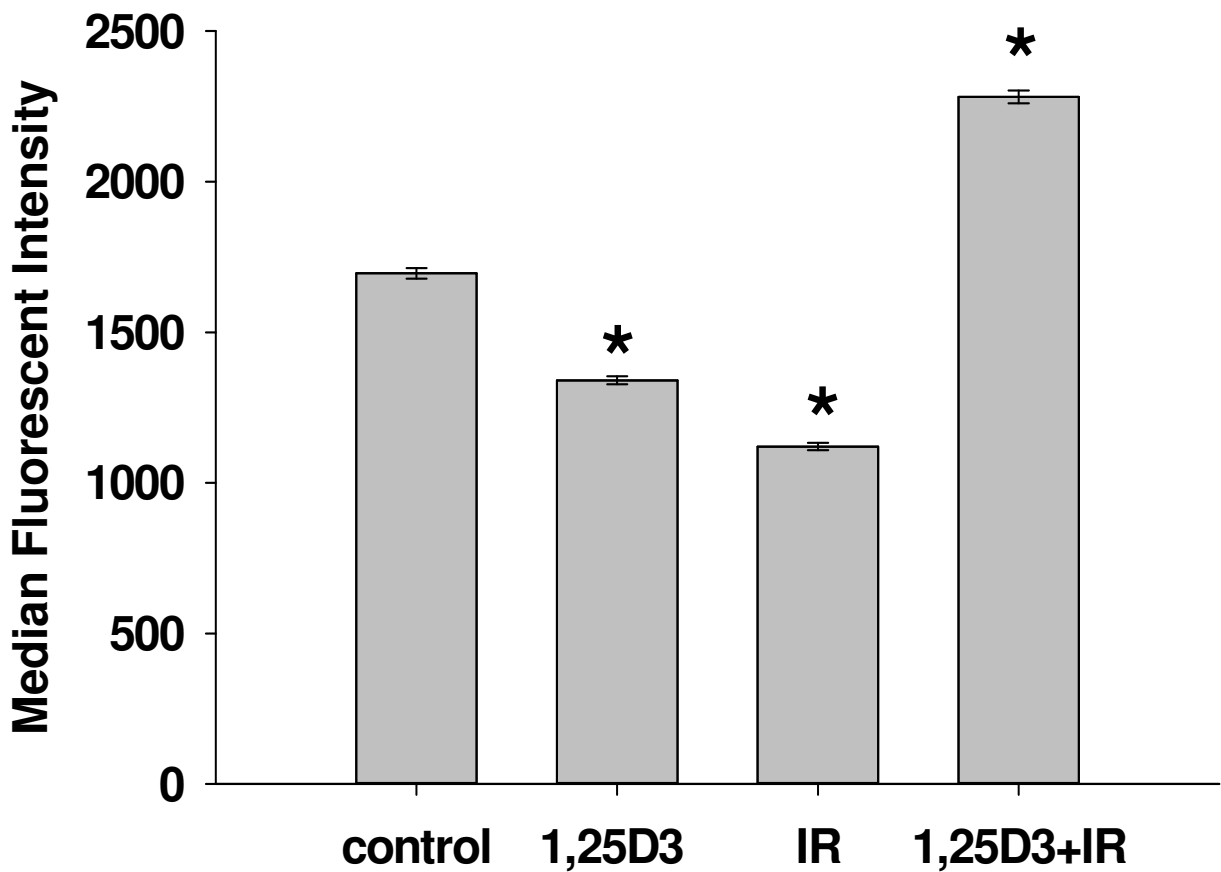
Figure 5-10 *Assessment of Changes in Calcium Levels using Fluo-3am* ZR-75-1 breast cancer cells were exposed to 100nM 1,25D3 continuously, a dose of 4 Gy radiation or concurrently with 1,25D3 and 4 Gy radiation. Fluorescent intensity was measured by flow cytometry at 1 hour post treatment. *p <0.05 compared to Control

<u>Summary</u>

Sensitization to radiation by 1,25D3 does not appear to be a consequence of increased DNA damage or decreased DNA repair as γ-H2AX studies demonstrated essentially identical levels of DNA damage induction following IR or 1,25D3+IR treatment. While ER stress was activated by 1,25D3, IR and 1,25D3+IR, there was no difference in the extent of ER stress induced by IR and 1,25D3+IR as indicated by PERK assessment. Similar to PERK studies, an increase of phosphorylated eiF2a was observed with all treatments. However, phosphorylation was delayed following 1,25D3 treatment. The fact that 1,25D3 does not enhance ER stress signaling following radiation treatment suggest that this pathways is unlikely to be the mode of radiosensitization Furthermore, GRP78 did not appear to play a significant role in ER stress activation following treatment with 1,25D3, IR or 1,25D3+IR.

Studies assessing mTOR phosphorylation revealed that there is a significant decrease in mTOR activity following treatment with 1,25D3, IR and 1,25D3+IR compared to control levels, but the decrease observed with the combination of 1,25D3 and IR treatment was significantly reduced compared to either treatment alone. The fact the all treatments resulted in decreased mTOR activity seems logical as each treatment is a stress to the cell and a halt in proliferation is necessary to deal with these insults.

AMPK activity appeared to be exclusively activated by 1,25D3+IR treatment. Treatment with the pharmacological inhibitor, Compound C, resulted in decreased autophagy and consequently attenuation of radiosensitization observed by 1,25D3. Lastly, due to the fact that 1,25D3+IR treatment results in increased AMPK and

subsequent decrease in mTOR suggest that the AMPK-mTOR pathway may play a critical role in mediating the promotion of autophagic cell death by 1,25D3+IR. Studies using a genetic approach to inhibit AMPK are in progress to validate these findings.

Chapter 6

Discussion

As mentioned in the introduction, breast cancer is one of the most common cancers diagnosed in women and approximately 1 in 8 women will succumb to the disease (U.S. breast cancer statistucs). As result, there is a need for continued research efforts to help develop new treatment strategies to decrease breast cancer mortality rates. The studies conducted in this dissertation research project were designed to build upon and extend previous work from this laboratory focused on determining the nature of cell death when vitamin D was combined with ionizing radiation in MCF-7 breast cancer cells. We sought to extend these studies to other breast cancer cell lines believed to be highly resistant to radiotherapy such as those that are mutant in p53. The current studies further establish the potential utility of vitamin D as an adjuvant therapy for the treatment

of breast cancer and furthermore provide evidence that sensitization to radiation by 1,25D3 occurs through the promotion of autophagy.

Sensitization to Radiation

We hypothesized that drug- and radiation-resistant breast cancer cell lines would be sensitized to radiation therapy following treatment with 1,25-di hydroxyl vitamin D3 (1,25D3). To test our hypotheses, we assessed the effect of vitamin D on sensitivity to radiation in the p53 wild type breast cancer cell line, ZR-75-1, as well as in breast cancer cells lines that were mutant in p53 such as HS587t and 231-BR. Our studies confirmed earlier findings which demonstrated that treating MCF-7 breast cancer cells with 1,25D3 or Vitamin D analogs in combination with radiation results in a significant decrease in viable cell number compared to radiation treatment alone. In contrast, IR treatment alone did not result in actual cell killing but rather growth arrest while treatment with 1,25D3 alone had a modest impact on cell growth. Clonogenic survival assays demonstrated a significant decrease in the ability of ZR-75-1 cells to form colonies when 1,25D3 was combined with either single dose or fractionated radiation.

In a similar fashion to radiation treatment in MCF-7 breast cancer cells, treatment with radiation alone led to growth arrest followed by a recovery in the cells' ability to proliferate. We believe that this proliferative recovery serves a as model of disease recurrence in the clinic. As mentioned before, breast cancer cells respond relatively well to radiation therapy, but the real issue is disease recurrence. Once disease recurs, it is often very aggressive and difficult to treat (American Cancer Society, 2011a). Thus it is very important to develop strategies to help prevent disease recurrence. The fact that treatment with 1,25D3 led to actual cell killing and prevented proliferative recovery is

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very encouraging and suggests that 1,25D3 could be a good candidate for potential use in the clinic as a potential radio sensitizer.

In contrast to studies conducted in ZR-75-1 cells, treatment with 1,25D3 did not sensitize Hs587T and 231-BR breast cancer cells, both lacking p53. In fact, 1,25D3 treatment alone did not have any effect on growth or viability of HS587t cells. Despite that fact that western blot analysis revealed that these cells do express the vitamin D receptor, this observation suggests that sensitization by 1,25D3 is likely related to one of the genomic actions in this cell line. Studies conducted in 231-BR cells actually revealed a modest but significant radioprotection effect following treatment with 1,25D3+IR. This effect was also observed in HS587t cells, but the protection did not reach statistical significance. A similar protective effect was also observed in studies conducted by Stambolsky et al., where 1,25D3 was used in combination with Cisplatin or Etoposide in SKBR3 breast cancer cells (Stambolsky et al., 2010).

The findings that 1,25D3 does not sensitize p53 mutant breast cancer cells are quite consistent with previous studies in our laboratory by Sundaram et al which demonstrated that MDA-MB-231 and T47D breast cancer cells, both lacking functional p53, were not sensitized to radiation by 1,25D3 or the vitamin D analog EB1089 (Sundaram and Gewirtz, 1999). These studies suggested a clear requirement for functional p53 in order to observe sensitization to radiation by 1,25D3. Reports in the literature suggest that p53 status determines the biological impact of vitamin D on tumor cells (Stambolsky et al., 2010). Furthermore, studies have demonstrated that mutant p53 can render vitamin D anti-apoptotic (Stambolsky et al., 2010). It appears possible that mutant p53 can convert vitamin D into a cytoprotective agent and our findings as well as

other studies in the literature suggest an important functional relationship between p53 and 1,25D3 (Stambolsky et al., 2010). Microarray data conducted by Dr. Matthew Beckman supports a requirement of functional p53 to observe radiosensitization by vitamin D.

Enhancement of Radiosensitization by 1,253 is a Consequence of Promotion of Autophagic Cell Death

Based on previous studies in the laboratory, we also hypothesized that autophagy is responsible for radiosensitization of breast tumor cells by 1,25-di hydroxy vitamin D3 and that pharmacological and genetic inhibition of autophagy would block 1,25-di hydroxy vitamin D3 mediated radiosensitization. Thus, our next set of studies were designed to substantiate that autophagy is the mode of radiation sensitization in breast tumor cells treated with 1,25D3. As autophagy has both cytoprotective and cytotoxic actions, we performed rigorous studies to prove our hypothesis. These studies included: evaluating the characteristics of autophagic cell death which include ruling out other forms of cell death such as apoptosis, necrosis and mitotic catastrophe, demonstrating an increase in autophagic flux and demonstrating that both pharmacological and genetic approaches to inhibit autophagy resulted in an increase in cell viability following 1,25D3+IR treatment.

Apoptosis induction was assessed visually using DAPI and TUNEL staining, by western blot analysis for PARP and Caspase-3 cleavage, assessment of the sub-G1 population in cell cycle analysis and by measuring the intensity of apoptotic markers Annexin V and Propidium Iodide using flow cytometry. Evaluation of the characteristics

of apoptotic cell death revealed that the increase in cell death by treatment with 1,25D3 +IR was likely not a consequence of increased apoptosis. This conclusion was based on the fact that none of the assays showed an increase in morphological or biochemical markers associated with apoptotic cell death following treatment with 1,25D3, IR or 1,25D3+IR. There was also no significant increase in the percentage of necrotic cells, which was shown with propidium iodide staining. Furthermore, mitotic catastrophe was assessed visually using Hoechst dye to stain cell nuclei. Morphological changes associated with mitotic catastrophe such as enlarged nuclei, formation of micronuclei and binucleation were not observed when cells were exposed to 1,25D3, IR or 1,25D3+IR. Taken together, these studies demonstrated that apoptosis, necrosis and mitotic catastrophe were not likely to be the mode(s) of cell death responsible for 1,25D3 mediated radiosensitization of breast cancer cells. Further, the data obtained satisfy the first characteristic of autophagic cell death, which is cell death occurring in the absence of apoptosis, necrosis, or mitotic catastrophe.

We next assessed the induction of autophagy following treatment with 1,25D3, IR and 1,25D3+IR. Initial studies assessed autophagy induction by staining cells with acridine orange or MDC and visually monitoring the formation of punctate staining as well as assessment of fluorescent intensity using flow cytometry. Studies revealed that autophagy was being induced by 1,25D3+IR treatment as early as 24 hours post treatment. Autophagy induction was also observed at 48 as well as 72 hours and even as late as 5 days post treatment. In contrast to 1,25D3+IR, treatment with radiation did not induce autophagy until 72 hours, and a significant increase in autophagy was not observed at earlier time points (24 and 72 hours). While there was still an increase in

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autophagic markers at day 5, it was significantly reduced as compared to 1,25D3+IR treatment. Interestingly, acridine orange staining indicated a significant increase in fluorescent intensity by 1,25D3 treatment alone compared to control cells at 24 hours, where the extent of autophagy induced appeared to be essentially identical to that for 1,25D3+IR treatment. But, unlike 1,25D3+IR treatment, autophagy decreased and returned to basal levels at 72 and 120 hours post treatment. These data are actually consistent with reports in the literature, as previous studies have demonstrated treatment with 1,25D3 or its analogs alone can induce autophagy (Hoyer-Hansen et al., 2005; Hoyer-Hansen et al., 2010; Wu and Sun, 2011). Assessment of autophagy induction visually using light microscopy, transmission electron microscopy and confocal microscopy to monitor vacuolization, the formation of autophagasomes and RFP-LC3 redistribution respectively, confirmed that there is an increase in autophagic markers 72 hours following treatment with both IR and 1,25D3+IR. One of the most interesting observations, however, is the fact that treatment with IR appears to result in delayed autophagy.

Additional studies conducted to assess autophagic flux revealed that the contents of the autophagosome were effectively being degraded by the lysosomes, based on p62 degradation and colocalization of RFP-LC3 with Lysotracker Green. Although an increase in autophagic markers was observed in cells treated with 1,25D3 alone, there was no p62 degradation, which suggests that 1,25D3 alone was unable to increase autophagic flux. p62 degradation was observed for both IR and 1,25D3+IR treatment. Again, p62 degradation occurred earlier in cells treated with 1,25D3+IR compared to radiation alone. This observation was confirmed by confocal microscopy, where

increased autophagic flux was detected at 24 hours following exposure to 1,25D3+IR compared to IR treatment alone. Furthermore, although the amount of autophagy induced appears to be essentially the same for both IR and 1,25D3+IR treatment at 72 hours, we speculate that 1,25 D3 increases the rate and extent of autophagic flux in radiation treated cells and that this increase mediates the radiosensitizing effects of 1,25D3. Furthermore, the fact that autophagy induction and flux occurs much earlier in cells treated with 1,25D3+IR suggests that cells are dying by autophagic cell death as a result of intense, prolonged autophagy. The concept of prolonged autophagy leading to autophagic cell death has frequently been reported in the literature (Denton et al., 2012; Levine and Yuan, 2005).

As mentioned previously, cellular proteins are degraded by the lysosomes during autophagy to recycle amino acids (Codogno and Meijer, 2005; Geng and Klionsky, 2008). Experiments assessing the actual rate and extent of degradation are required to test the hypothesis of increased autophagic flux and should provide greater insights into how 1,25D3 sensitizes breast cancer cells to radiation therapy. One such experiment would be to measure protein degradation by labeling proteins with radioactive Valine and quantification using liquid scintillation counting (Bauvy et al., 2009). The rate of degradation is calculated from the ratio of the acid-soluble radioactivity in the medium to that in the acid-precipitable cell fraction (Bauvy et al., 2009). However, since amino acids are physiological inhibitors of autophagy it is important not to choose an amino acid such as leucine, which is a potent inhibitor of autophagy. Valine is frequently used because it does not interfere with autophagy in most cell types (Bauvy et al., 2009).

One of the more exciting findings was that pharmacological inhibition of autophagy

using Bafilomycin A1 and Chloroquine as well as genetic inhibition through knockdown of ATG-5 and ATG-7 completely attenuated radiation sensitization by 1,25D3 in ZR-75-1 breast cancer cells, supporting our hypothesis which was promotion of cytotoxic autophagy by the combination treatment as the mode of radiation sensitization. Pharmacological inhibition of autophagy in MCF-7 breast cancer cells resulted in a similar outcome, which was reversal of 1,25D3 mediated radiosensitzation as indicated by increased cell viability. Surprisingly, pharmacological and genetic inhibition revealed that the autophagy was actually different in cells treated with IR than 1,25D3+IR, as inhibition of radiation induced autophagy switched the cellular response from growth arrest to actual cell death. This increase in sensitivity to radiation treatment strongly supports activation of cytoprotective autophagy. A number of literature reports indicate that autophagy can represent a mechanism of resistance to radiation therapy and autophagy inhibition has frequently been shown to increase sensitivity to radiation therapy (Apel et al., 2008; Ito et al., 2005; Lomonaco et al., 2009). However, to our knowledge the coexistence of cytoprotective autophagy and cytotoxic autophagy in the same experimental system has not been reported in the literature.

Studies examining the actual mode of radiosensitization by autophagy inhibition with IR alone revealed that sensitization is likely due to the promotion of apoptotic cell death. Approximately 20% of cells were apoptotic when autophagy was inhibited using Chloroquine as indicated by Annexin V/ Propidium Iodide staining 72 hours post irradiation and there was no significant increase in the percentage of necrotic cells. In addition, assessment of Mitotic Catastrophe following IR using Hoechst stain showed an increased in giant nuclei and binucleation with both autophagy nhibitors, Bafilomycin A1

and Chloroquine. Sensitization to radiation by autophagy inhibitors appears to be a result of a combination of cell death mechanisms, apoptosis and mitotic catastrophe. A similar observation of increased radiosensitization was evident when autophagy was blocked in MCF-7 cells post radiation; however the sensitization was much more pronounced (an approximate 80% reduction). A dose of 25uM Chloroquine was used in these studies instead of a 5uM dose, which might explain why there was more pronounced sensitization to radiation following autophagy inhibition.

In dramatic contrast to autophagy inhibition following IR treatment alone, no increase in other modes of cell death was observed in cells treated with 1,25D3+IR. A lack of increase in other modes of cells death mechanisms would be the expected outcome if 1,25D3 does in fact sensitize breast cancer cells to radiation by inducing cytotoxic autophagy. Thus, this data is quite consistent with our previous studies demonstrating increased cell viability with autophagy inhibition and this data further supports the promotion of autophagic cell death as the mode of sensitization to radiation by 1,25D3.

Finally, 1,25 D3 appears to switch the cells from a cytoprotective to a cytotoxic mode of autophagy. Although either cytoprotective or cytotoxic actions of autophagy have been reported in multiple publications, to our knowledge these are the first studies in the literature to provide evidence for this type of "autophagic switch." It is important to emphasize that this experimental system should provide the appropriate model system to address the question of what factors might distinguish cytoprotective from cytotoxic autophagy, which is currently a fundamental question in this field (Kroemer and Levine, 2008; Shen and White, 2001). In fact, a recent report by Kroemer et al argues that

autophagic cell death does not exist and autophagy is actually just a precursor to other modes of cell death (Kroemer and Levine, 2008). Further, the authors pose the question whether autophagic cell death is 'cell death with autophagy' or 'cell death by autophagy'. This is an interesting question but we believe our studies provide one more piece of evidence for the existence of autophagic cell death.

Residual Surviving Cells are in a State of Senescence

Senescence had been frequently observed in cells exposed to radiation treatment in our laboratory as well as others (Roninson et al., 2001; Roninson, 2003; Sundaram and Gewirtz, 1999). Thus, we were interested to determine whether growth arrest is a consequence of promotion of senescence by IR or 1,25D3+IR treatment. Assessment of senescence associated β-galactosidase activity both visually and by flow cytometry revealed that both IR and 1,25D3+IR produced a significant increase in senescence at 3 and 6 days post treatment. 1,25D3 treatment alone resulted in senescence as well but not to the extent of IR or 1,25D3+IR. Moreover, this data is consistent with the literature and suggest that residual surviving cells are in a state of senescence. Furthermore, senescence does in fact appear to be the reason for the growth arrest observed with IR and 1,25D3+IR. Senescence might also in part be the reason for the slowed growth of breast cancer cells exposed to 1,25D3 alone.

Reports in the literature have suggested that autophagy is required for senescence (Gewirtz, 2009; Narita et al., 2009; Young et al., 2009). Thus, we were interested in determining whether autophagy inhibition had any effect on senescence. Pharmacological inhibitors, Bafilomycin A1 and Chloroquine, did not promote senescence alone. Pharmacological inhibition of autophagy in ZR-75-1 breast cancer cells following

treatment with IR or 1,25D3+IR did not result in decreased senescence, suggesting that these processes occur independently of one another.

Involvement of the DNA Damage Response, ER Stress and mTOR Signaling

Our last hypothesis was that the ER stress and mTOR pathways would be involved in the autophagic signaling cascade and may be critical signaling elements in the radiosensitization of breast tumor cells by 1,25-di hydroxy vitamin D3. Consequently, studies were designed to elucidate the autophagic signaling mechanisms by which 1,25-di hydroxy vitamin D3 sensitizes breast cancer cells to ionizing radiation therapy by assessing various effectors in both pathways as well as evaluating the DNA damage response.

We first assessed DNA damage induction and repair by monitoring changes in as γ-H2AX following treatment with 1,25D3, IR, and 1,25D3+IR. Assessment of γ-H2AX demonstrated that there was a significant increase in γ-H2AX foci in confocal microscopy images and an increase in the intensity of an antibody directed towards the Serine 139 phosphorylation site of γ-H2AX following treatment with IR and1,25D+IR compared to control and 1,25D3 treatment alone. However, when comparing IR and 1,25D3+IR treatment, there was no significant difference in γ-H2AX foci or fluorescent intensity at 1 or 24 hours post treatment. Consequently we conclude that sensitization to radiation by 1,25D3 did not appear to be a consequence of increased DNA damage or decreased DNA repair. Similar studies conducted in the laboratory by Gerald DeMasters demonstrated no significant changes in γ-H2AX following treatment with 1,25D3, IR or 1,25D3+IR. Future studies to confirm these data would involve monitoring 53-BP1, a DNA damage checkpoint protein that mediates the DNA damage response or conducting

a comet assay to assess DNA damage (Collins, 2004; Wang et al., 2002).

There is accumulating evidence that indicate ER stress is a potent inducer of autophagy and there is also evidence that both 1,25D3 and IR lead to ER stress induction (Hoyer-Hansen and Jaattela, 2007a; Hoyer-Hansen et al., 2007). We explored the ER stress pathway to determine if sensitization to radiation by 1,25D3 is a consequence of ER stress induction. PERK has been implicated as one of the main mediators of ER stress-induced autophagy in mammalian cells and consequently we initially monitored PERK activity. By 48 hours post treatment, PERK phosphorylation was significantly increased by all treatments, 1,25D3, IR and 1,25D3+IR. Of interest, is that there was no significant difference in phosphorylated PERK between IR and 1,25D3+IR treatment as well as delayed PERK activation by, 1,25D3 treatment alone (no increase in p-PERK was observed at 24 hours post treatment). Similar results were obtained when eiF2α, an effector directly downstream of PERK, was assessed; more specifically increased eiF2α activity was detected following treatment with 1,25D3, IR or 1,25D3+IR at 48 hours. Again no eif2α was observed at 24 hours following 1,25D3 treatment alone, which corresponds to the PERK studies. Furthermore, another ER stress marker, GRP78, did not appear to play a significant role in ER stress activation following treatment with 1,25D3, IR or 1,25D3+IR as there was no change in protein levels post treatment. While these data suggest that ER stress signaling is likely not the mode of radiosensitization by 1,25D3, it does provide some insight into the signaling pathways that might be activated leading to autophagy by IR and 1,25D3+IR. It would be interesting to evaluate whether inhibition of PERK or eif2a has any effect on autophagy induction. Another ER stress pathway associated with autophagy is IRE1-TRAF2-JNK (Verfaillie et al., 2010). Studies

by Ogata et al demonstrated a requirement of this pathway for thapsigargan induced autophagy in MEFS and it is possible that effectors in this pathway are required for 1,25D3 mediated radiosensitization (Ogata et al., 2006).

Our last sets of studies were designed to monitor the mTOR signaling pathway. mTOR is thought to be the key central negative regulator of autophagy (Dowling et al., 2010; Jung et al., 2010). Cellular stressors such as nutrient deprivation and energy depletion have been shown to lead to decreased phosphorylated mTOR, consequent growth inhibition and autophagy induction (Jung et al., 2010).

We first conducted studies to see if treatment with 1,25D3, IR or 1,25D3+IR had any effect on mTOR activity. Studies assessing mTOR phosphorylation revealed that there was a significant decrease in mTOR activity following treatment with 1,25D3, IR and 1,25D3+IR compared to control levels. Interestingly, levels of phosphorylated mTOR were significantly decreased following 1,25D3+IR treatment compared to 1,25D3 or IR treatment alone, suggesting that the combination treatment enhances the deactivation of this pathway. The pronounced decreased in mTOR phosphorylation following treatment with 1,25D3+IR might be important in the promotion of autophagic cell death and the radiation sensitization observed in ZR-75-1 breast cancer cells by the combination treatment.

Subsequent studies were conducted to see if there were any differences in an effector directly upstream of mTOR, AMPK.AMPK activation is inversely related to mTOR activity (an increase in AMPK results in decreased mTOR) (Chen and Klionsky, 2011; Hardie, 2011). Assessment of AMPK activity revealed that phosphorylation of AMPK appears to be exclusively activated by 1,25D3 +IR treatment. Confocal

microscopy images of an antibody directed toward the Threonine 172 phosphorylation site of AMPK demonstrated an increase in AMPK activity as early as 3 hours post 1,25D3+IR treatment and AMPK activity was sustained throughout the 24 hour time course. Furthermore, data collected to quantify the extent of AMPK activity by flow cytometry, demonstrated that 1,25D3+IR treatment significantly increases phosphorylated AMPK compared to control levels and IR treatment alone. These results substantiated previous data where AMPK was monitored visually, which showed increased AMPK activity following 1,25D3+IR treatment; again suggesting that AMPK activation is an important regulator of 1,25D3 mediated radiosensitization.

One important observation was what appeared to be nuclear localization of AMPK following treatment with 1,25D3, IR and 1,25D3+IR in confocal microscopy images. Interestingly, only treatment with 1,25D3+IR resulted in a significant increase in both nuclear and cytoplasmic localization of AMPK. Moreover, a review of the literature revealed that the intracellular distribution of AMPK complexes (α1 and α2) is due to shuttling between the nucleus and the cytoplasm in response to specific stimuli (Kazgan et al., 2010; Kodiha et al., 2007). Violett et al reported that AMPK translocates to the nucleus upon stimulation by agents inducing cellular stress in Hela cells (Viollet et al., 2010). Additionally, the two AMPKα subunits, α1 and α2, have been shown to have different localization patterns in mammalian cells, with the α1 subunit being localized to the non-nuclear fraction (cytoplasm) and the α2 subunit localized to both the nucleus and the non-nuclear fractions (Kazgan et al., 2010; Kodiha et al., 2007; Viollet et al., 2010). AMPKα1 is hence likely to phosphorylate cytosolic and plasma membrane substrates, whereas AMPKα2 may be primarily involved in the conversion of metabolic signals into

transcriptional regulation (Viollet et al., 2010) . The antibody used for these experiments detects both α1 and α2 isoforms and thus we speculate that a change in the nucleocytoplasmic redistribution of AMPK might be required to observe sensitization to radiation by 1,25D3. Specifically, the α1 and α2 subunits might be differentially regulated by treatment with 1,25D3+IR since only treatment with this combination resulted in *both* intense nuclear localization as well as cytoplasmic localization. We have adenoviruses that express dominant negative α1 and α2 isoforms. Inhibition of each isoform will be used to test this hypothesis and determine the involvement of each subunit in the sensitization to radiation by 1,25D3.

To determine if AMPK activity has a direct effect on autophagy, we pharmacologically inhibited AMPK using Compound C. Assessment of autophagy by RFP-LC3 redistribution revealed that treatment with Compound C completely attenuates autophagy induction by 1,25D3+IR treatment. In a similar manner to classical autophagy inhibitors, Bafilomycin A1 and Chloroquine, Compound C decreased sensitization to radiation when breast cancer cells were treated concurrently with 1,25D3 and radiation. These data suggests that AMPK may play a critical role in mediating the promotion of autophagic cell death by 1,25D3+IR. Studies using a genetic approach to inhibit AMPK are in progress to validate these findings. A proposed mechanism by which vitamin D sensitizes breast cancer cells to radiation treatment is presented in figure 6. 1.

Lastly, it is possible that 1,25D3+IR treatment increases autophagy and leads to the promotion of cytotoxic autophagy by activating multiple autophagy signaling pathways. Our future studies will be designed to continue to elucidate the pathways by which 1,25 D3 sensitizes breast cancer cell to ionizing radiation. It is very exciting to

have discovered that AMPK activity can possibly be one of the factors that determine whether autophagy will be cytotoxic or cytoprotective. However, there are a number of studies that need to be conducted to elucidate the complete mechanism by which 1,25D3+IR sensitizes breast cancer cells to radiation therapy and these findings should provide the foundation to answer this question.

A relatively new kinase, ULK1, has been shown to be an important regulator of autophagy. It can be activated by decreased mTOR or directly by AMPK. A study by Kim et al demonstrated a molecular mechanism for regulation of the mammalian autophagy-initiating kinase, Ulk1 (Jung et al., 2010; Kim et al., 2011a; Roach, 2011) . Under glucose starvation, AMPK promotes autophagy by directly activating Ulk1 through phosphorylation of Ser 317 and Ser 777. Under nutrient sufficiency, high mTOR activity prevents Ulk1 activation by phosphorylating Ulk1 Ser 757 and disrupting the interaction between Ulk1 and AMPK. It has been suggested that this coordinated phosphorylation is important for Ulk1 in autophagy induction (Fig 6.2). It is possible that the combination of 1,25D3 and radiation promote autophagy and increase radiation sensitization via the AMPK-ULK1 signaling pathway.
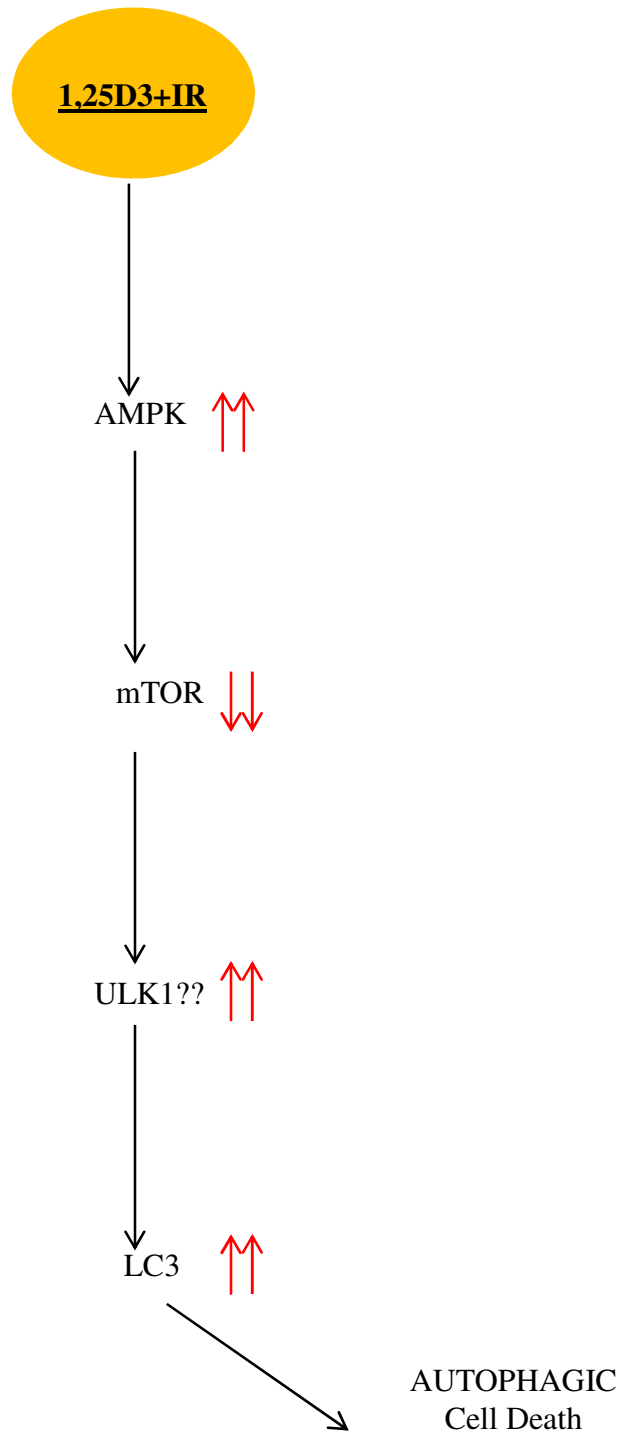
Figure 6.1 Proposed Mechanism for Radiation Sensitization by 1,25D3 in ZR-75-1 Breast Cancer Cells
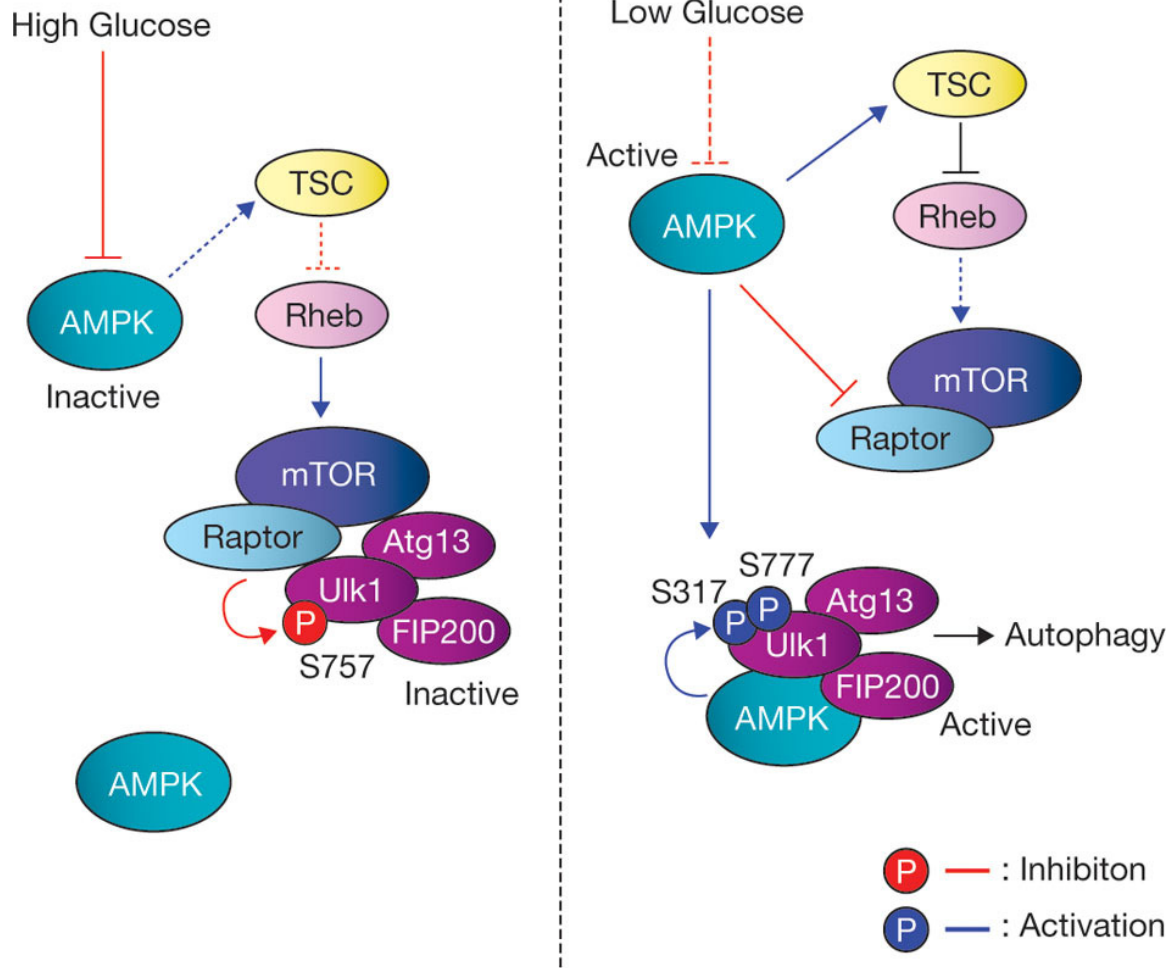
Figure 6.2 AMPK –mTOR- ULK1 Signaling Cascade (Kim et al., 2011a)

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<u>Vita</u>

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<u>**SUMMARY OF QUALIFICATIONS**</u> Highly motivated with extensive research experience as well as outstanding written and oral communication skills. Demonstrated ability to retrieve, analyze, interpret and present complex data.

- *Biomedical Scientist*: Experience interpreting and applying advanced procedures/techniques relative to study of drug actions on living systems. Expertise in Pharmacology, Toxicology, Cancer Biology and Cellular Biology. Data analysis and exploration using advanced statistics including multivariate analyses.
- *Reviewer:* Evaluate application of scientific principles to critique technical manuscripts and determine validity of data to recommend rejection, revision or publication in peer reviewed journals. Compose literature reviews
- *Writer and Public Speaker*: Author and edit grant proposals, status reports, protocols, technical summaries, and scientific papers. Summarize written materials orally by presenting complex scientific concepts and data in a clear concise manner to scientific and non-scientific audiences using visual aids such as multimedia PowerPoint presentations and scientific posters

EDUCATION

Virginia Commonwealth University School of Medicine   **May 2012**
Doctor of Philosophy in Pharmacology and Toxicology
National Institutes of Health Ruth L. Kirschstein Pre-Doctoral Fellow (F31)
Richmond, Virginia
Dissertation Title: Radiation Sensitization of Breast Cancer Cells by Vitamin D through the Promotion of Autophagic Cell Death

Howard University   **May 2007**
Bachelors of Science in Chemistry
Minor in Allied Sciences
Washington, District of Columbia
GPA: 3.3/4.0 Cum Laude

WORK EXPERIENCE

Graduate Research Associate   **2007-2012**

Laboratory of David A. Gewirtz, PhD Department of Pharmacology and Toxicology
Virginia Commonwealth University School of Medicine, Massey Cancer Center

- **PLANNED, ORGANIZED, PRIORITIZED AND COORDINATED WORK TO CONDUCT RESEARCH** focused on utilizing vitamin D as an adjunct treatment for breast cancer patients. Apply fundamental principles, concepts and techniques associated with Pharmacology, Cancer Biology in addition to Cellular and Molecular Biology to assess the role of vitamin D in the sensitization of breast tumor cells to radiation therapy. Specifically, evaluate the role of Autophagy and the intracellular signaling mechanisms involved in vitamin D mediated radiosensitization.

- **AUTHORED NATIONIAL INSTITUTES OF HEALTH RESEARCH GRANT PROPOSAL** and obtained graduate school funding from the National Cancer Institute as a National Institutes of Health (NIH) Ruth Kirschstein Pre-Doctoral Fellow (F31). Furthermore, assist academic advisor in preparation and development of grant proposals as well as devise research strategies related to cell signaling

- **PRODUCED WRITTEN TECHNICAL MATERIAL** as the primary author on a research manuscript based on my dissertation project and published findings in peer-reviewed research journal, *Hormones and Cancer*, which was one of the most highly downloaded papers in the months subsequent to its publication.

- **CONTRIBUTED TO A REVIEW ARTICLE** focused on Autophagy and how natural compounds can lead to autophagic cell death in cancer cells. In addition, I contributed my expertise in cell death analysis to assist in authoring a book chapter focused on protocols used to monitor p53 and its role in Apoptosis (*p53 Protocols*)

- **COMMUNICATED ORALLY TO MAKE CONCISE AND CONVINCING PRESENTATIONS** at numerous seminars in a suitable manner to audiences of either laypersons or professionals. Consistently received excellent evaluations in departmental seminar series where I presented multimedia PowerPoint presentations to convey results of my research findings. Additionally, I have represented the Pharmacology and Toxicology Department at national conferences such as the American Association for Cancer Research annual meeting where I presented and discussed scientific posters to diverse audiences (scientists and general public).

- **ANALYZED AND INTERPRETED WRITTEN MATERIALS** as an ad hoc peer reviewer for the *Journal of Experimental Pharmacology and Therapeutics* and determined if manuscripts were suitable for publication by evaluating: quality of data, validity of conclusions based on data presented and significance/novelty of research findings

- **ESTABLISHED AND MAINTAINED EFFECTIVE WORKING RELATIONSHIPS** which fostered research collaborations with fellow scientists

in diverse disciplines. Moreover, I contributed a seminal piece of data to a manuscript by a fellow laboratory member to overcome the main concern raised by reviewers during the peer review process, which ultimately led to acceptance in the journal *Autophagy*. The development of mutually beneficial relationships as well as collaborations and my research contributions ultimately led to the publication of 2 additional manuscripts in peer-reviewed research journals
- **PROVIDED TRAINING AND SUPPORT** to incoming graduate students on the principles and techniques required to conduct successful Pharmacology and Cellular Biology laboratory research. Furthermore, employed creative thinking to troubleshoot numerous assays in the laboratory to monitor cell fate as well as developed and implemented new protocols to assess autophagic flux in addition to kinase activation using flow cytometry.

Undergraduate Research Associate   **Summer 2006**
Laboratory of Nicolas Farrell, PhD Department of Chemistry
Virginia Commonwealth University,
Research for Undergraduates Summer Chemistry Program

- **CONDUCTED ELECTRONIC REFERENCE SEARCHES** to examine relevant chemistry literature to design experimental approaches to determine potential modifications to anti-cancer drug, Cisplatin
- **PLANNED AND CONDUCTED RESEARCH TO DEVELOP** analogs of Cisplatin in order to improve clinical anti-cancer effects. This was accomplished by using various chemistry based assays to synthesize polar trans-platinum derivatives. Formulations were characterized using *Nuclear Magnetic Resonance* Spectroscopy.
- **ANALYZED AND INTERPRETED** data generated to convey research findings orally in a poster presentation as well in a seminar

PUBLICATIONS

Wilson EN, Bristol ML, Di X, Maltese WA, Koterba K, Beckman MJ, Gewirtz DA. A switch between cytoprotective and cytotoxic autophagy in the radiosensitization of breast tumor cells by chloroquine and vitamin D. Horm Cancer. 2011 Oct;2(5):272-85

Zhang X, Haney KM, Richardson AC, **Wilson E**, Gewirtz DA, Ware JL, Zehner ZE, Zhang Y. Anibamine, a natural product CCR5 antagonist, as a novel lead for the development of anti-prostate cancer agents. Bioorg Med Chem Lett. 2010 Aug 1;20(15):4627-30

Goehe RW, Bristol ML, **Wilson EN**, Gewirtz, DA. p53 in autophagy, senescence, and apoptosis. p53 Protocols: Second Edition, Springer Protocols. Humana Press. (2011)

Gewirtz DA, Hilliker ML, **Wilson EN**. Promotion of autophagy as a mechanism for radiation sensitization of breast tumor cells. Radiother Oncol. 2009 Sep;92(3):323-8.

Bristol ML, Di X, Beckman MJ, **Wilson EN**, Henderson S, Maiti A, Fan Z, Gewirtz DA The role of autophagy in the sensitization of breast tumor cells to radiation.

FELLOWSHIPS, HONORS AND AWARDS

Medical College of Virginia Alumni Associate Award	2012
NIH Ruth **L**. Kirschstein Pre-doctoral Fellowship F31	2009-2012
National Society of Collegiate Scholars	2004-2007
Howard University Trustee Scholar	2004-2005
Howard University Michigan Alumni Association Scholarship Recipient	2003-2004

NATIONAL CONFERENCE PRESENTATIONS

The role of AMPK in the radiosensitization of breast cancer cells by vitamin D American Association for Cancer research 103rd Chicago, Illinois (April 2012)

Radiosensitization of breast cancer cells by vitamin D or chloroquine through modulation of the dual cytoprotective and cytotoxic functions of autophagy American Association for Cancer research 102nd Annual Meeting, Orange County Convention Center (April 2011)

Radiosensitization of breast cancer cells by vitamin D American Association for Cancer research 101st Annual Meeting, Washington Convention Center (April 2010)

PROFESSIONAL MEMBERSHIPS

American Association for Cancer Research	2010-Present
American Chemical Society	2007-2010