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# Relationship between Autophagy, Senescence, and DNA Damage in Radiation Sensitization by PARP Inhibition

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**Relationship between Autophagy, Senescence, and DNA Damage in Radiation Sensitization by PARP Inhibition**

A thesis submitted in fulfillment of the requirements for the degree of Master of Science in

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by

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## Abstract

Radiotherapy continues to be a primary modality in the treatment of cancer. DNA damage induced by radiation can promote apoptosis as well as both autophagy and senescence, where autophagy and senescence can theoretically function to prolong tumor survival. A primary aim of this work was to investigate the hypothesis that autophagy and/or senescence could be permissive for DNA repair, thereby facilitating tumor cell recovery from radiation-induced growth arrest and/or cell death. In addition, studies were designed to elucidate the involvement of autophagy and senescence in radiation sensitization by PARP inhibitors and the re-emergence of a proliferating tumor cell population. In the context of this work, the relationship between radiation-induced autophagy and senescence was also determined. Studies were performed using DNA repair proficient HCT116 colon carcinoma cells and a repair deficient Ligase IV (-/-) isogenic cell line. Irradiation promoted a parallel induction of autophagy and senescence that was strongly correlated with the extent of persistent H2AX phosphorylation in both cell lines; however inhibition of autophagy failed to suppress senescence, indicating that the two responses were dissociable. Irradiation resulted in a transient arrest in the HCT116 cells while arrest was prolonged in the Ligase IV (-/-) cells; however, both cell lines ultimately recovered proliferative function, which may reflect maintenance of DNA repair capacity. The PARP inhibitors (Olaparib) and (Niraparib) increased the extent of persistent DNA damage induced by radiation as well as the extent of both autophagy and senescence; neither cell line underwent significant apoptosis by radiation alone or in the presence of the PARP inhibitors. Inhibition of autophagy failed to attenuate radiation sensitization, indicating that autophagy was not involved in the action of the PARP inhibitors. As with radiation alone, despite sensitization by PARP inhibition, proliferative recovery was evident within a period of 10-20 days. While inhibition of DNA repair via PARP inhibition may initially sensitize tumor cells to radiation via the promotion of senescence, this strategy does not appear to interfere with proliferative recovery, which could ultimately contribute to disease recurrence.

# Chapter 1: Introduction

## *1.1 Cancer:*

Cancer is a general terminology that reflects a group of diseases resulting from uncontrolled growth of abnormal cells. The abnormal cells start to grow inside human bodies due to genomic instability. In normal cells, DNA damage caused by inefficient DNA replication or an increase in reactive oxygen species (ROS) levels can be either repaired or lead to cell death if the damage is not properly repaired. On the other hand, cancer cells can resist the DNA damage and do not undergo cell death, which then lead to production of a new abnormal cell. When a group of cancerous cells continue growing out of control, they form a mass called a tumor. However, not all tumors are cancerous; some can be non-malignant and slowly growing benign tumors that are unable to invade surrounding tissues. Benign tumors are incapable of traveling throughout the blood stream to develop as metastases, which is a well-known characteristic of malignant tumors. During metastasis, malignant tumor cells spread via the blood and lymphatic system to other organs such as liver, brain, and lungs. (American Cancer Society)

## *1.2 Statistics:*

Cancer is one of the leading causes of death in the United States as well as in other parts of the world. According to the American Cancer Society, 1,658,370 new cases are expected to be diagnosed by cancer in 2015 [1]. Among these diagnosed cases, about 589,430 patients are expected to die from cancer . In addition, 10380 children are also expected to be diagnosed with cancer in 2015 and 1250 will die. The annual increase in the diagnosis of cancer and the mortality of certain types of cancer are leading causes for the study of cancer therapeutics.

The most common type of cancer diagnosed in men is prostate cancer, whereas the most common type of cancer diagnosed in women is breast cancer [1]. However, there are three more kinds of cancers that are commonly occurring in both genders beside breast and prostate such as lung, pancreas, and colorectal cancers. The survival of patients differs based on the type of cancer and gender. However, from a statistical viewpoint, the lifespan probability for men is usually longer than it is for women. Also, the occurrence of cancer depends on race and ethnicity. Non-Hispanic black men seem to be the most prone subpopulation diagnosed with cancers, whereas Asian/Pacific Islander men are the least [1]. Possible reasons for this disparity of cancer incidence between various races could be due to different genetic backgrounds. Also, the socioeconomic status of a subpopulation may play a major role due to the lack of early detection and lack of access to the advanced clinical approaches. For the past two decades, the rate of cancer related deaths has been reduced by ~25%.

***1.3 Colorectal cancer:***

Colorectal cancer, sometimes called colon carcinoma, is one of the most common types of cancer in both men and women globally. The most recent statistics have indicated that about 1.2 million new cases are being discovered and almost 600,000 deaths by colorectal cancer per year worldwide [2]. Colorectal cancer incidence in Europe and North America is higher than Africa and some parts of Asia [3]. Also, there is an increasing trend of the incidence of colorectal cancer in other countries such as Spain, East Asia, and Eastern Europe due to what is so-called the “western lifestyle” [4].The mortality associated with colorectal cancer has been decreasing in United States and Europe due to the early detection and improved treatment. Unlike other types of cancers, there is no single or common risk factor for colorectal cancer. Several studies have shown many factors that may lead to colon carcinoma beside age and gender such as family

history [5], obesity [6], inflammatory bowel disease [7], smoking [8], and excessive intake of alcohol [9]. The more risk factors the person has, the greater risk of developing colon cancer.

Colon cancer is usually preceded by the formation of noncancerous polyps coating the inside layer of the colon. These polyps can be categorized into different types, and the type most likely to develop into cancer is the adenomatous polyps. The management and treatment of colon cancer include surgery, chemotherapy, and radiation under certain circumstances such as rectal cancer. Although colorectal cancer could be managed by surgery and chemotherapy during early stages, the late stage of this disease is somehow resistant to current therapeutics. In the last years, the use of bevacizumab, aflibercept, cetuximab, and panitumumab to target VEGF-related pathways and EGFR, repectively, has improved the therapeutic outcomes in colon cancer patients [10]. Nevertheless, the prognosis of these patients is still poor due to the existence of primary and/or development of acquired resistance mechanisms. Mechanisms of resistance to these targeted therapy agents including mutations in KRAS, BRAF, and NRAS genes [11-14]. Thus, the choice of appropriate therapeutic regimens depends on the stage of the disease, age of the patient, comorbidities, and extent of disease. For example, in patients with distant metastases, combination therapy of vascular endothelial growth factor inhibitor (bevacizumab) and epidermal growth factor receptor inhibitor (cetuximab) and kinase inhibition (regorafenib) has increased the survival rate to more than 20 months in some cases [15]. Also, unresectable metastasized tumor cells at the time of diagnosis became resectable after intensive combination therapy [16]. However, treatment of colorectal cancer patients with combinational chemotherapeutic agents is associated with severe side effects such as neuropathy, severe diarrhea, mucositis, and hair loss, especially since most patients are elderly. Hence, searching for new effective anticancer agents is necessary to overcome these major side effects.

### *1.4 DNA repair:*

A study by Theodor Boveri in 1902 suggested that the chromosomal structure of cancer cells is abnormal [17]. Alterations in chromosomal structure "mutations" were defined later as genomic instability, which is considered one of the main characteristics of solid tumors [18]. It is noteworthy to mention that the types of mutated genes affect the response of tumor to therapy [19-20].

Due to the potential for tumor development arising from genomic instability, cellular systems are programmed to maintain genomic integrity using different and sometimes overlapping DNA repair mechanisms (i.e. the DNA damage response (DDR). The repair of DNA lesions generally involves the following components: recognition of damaged site, accumulation of DNA repair enzymes and end processing, DNA synthesis and resolution, and ligation. In addition, mechanisms of DNA repair include base excision repair (BER), nucleotide excision repair (NER), double-strand breaks (DSBs) repair, mismatch repair, and reversal repair.

In base excision repair (BER), the damaged nucleic bases are excised and substituted by the newly synthesized bases [21]. The process of base excision repair (BER) is highly regulated by two enzymes poly (ADP-ribose) polymerase 1 and 2 (PARP1 and PARP2). In addition to the single-base repair mechanism, another single-strand repair mechanism has been identified and characterized, that is termed nucleotide excision repair (NER) [22]. The nucleotide excision repair (NER) usually occurs during transcription and the damaged bases is detected by RNA polymerase whose progression is blocked by the distortion of the DNA double helix. In nucleotide excision repair (NER), not only the damaged bases but also several surrounding undamaged bases are excised by Excision repair cross-complementing protein 1 (ERCC1), followed by the synthesis of new sequences by DNA polymerase as in the normal DNA

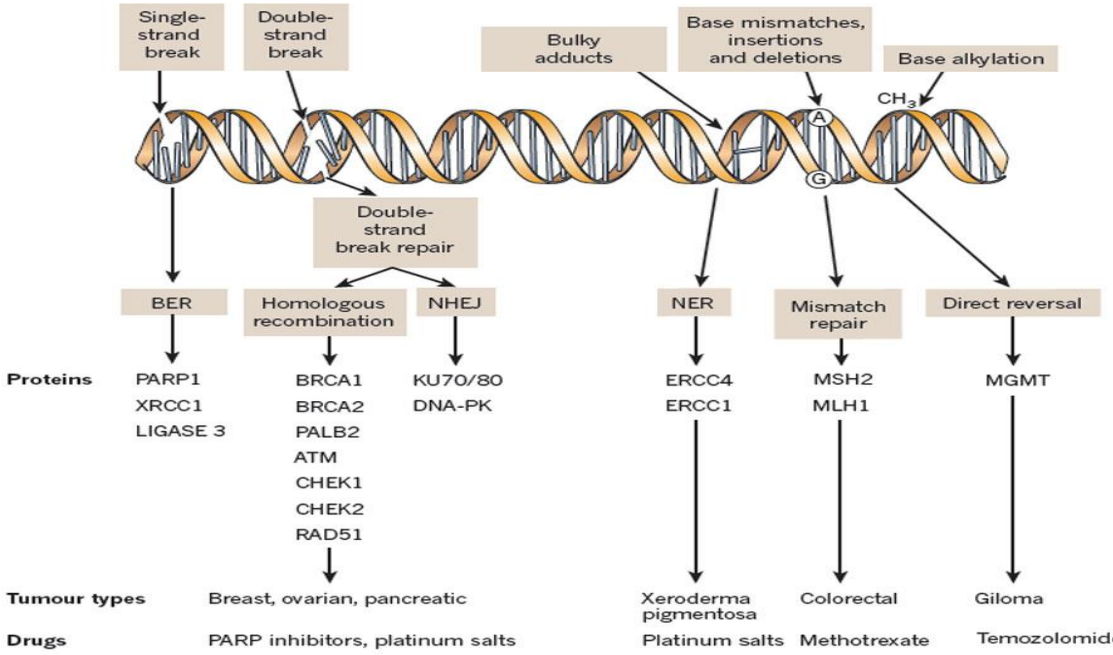
replication process. Another important repair pathway is called mismatch repair [23], in which the repair of misplaced nucleotides occurs. During normal DNA replication, some nucleotides may mistakenly be added to the wrong site, forming a mismatched pair. The mispairing of nucleotides may result in distortion of the DNA double helix structure, which is considered as another form of DNA lesion. Two crucial proteins involved in the repair of mispaired DNA are MSH2 and MLH1.

In addition to the single-strand breaks (SSBs), double-strand breaks (DSBs) are also generated when cells are exposed to radiation, radiomimetic drugs, or topoisomerase inhibitors [24]. In the field of cancer therapy, induction of DSBs is meant to halt proliferating cancer cell growth. However, radiation and radiomimetic drugs can harm normal cells as well, but due to the proper repair mechanisms in normal cells, these injuries to DNA are perfectly repaired. There are two different mechanisms that contribute to the repair of DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ). The differences between these two pathways lie in the genetic distinction, accuracy of repair, and the requirement for a sister chromatid to pursue repair. The NHEJ pathway can occur during all phases of the cell-cycle as it does not require homology, but at the same time it is less accurate than HR. NHEJ is initiated when the site of damage become recognized by the binding of two heterodimeric subunits of KU, KU70 and KU80, at the edges of DNA damage site [25]. The binding of KU subunits allows for the recruitment of the catalytic subunit DNA-PKcs, forming a holoenzyme called DNA-PK [26]. After formation of the DNA-PK holoenzyme, the subtype of histone H2AX become phosphorylated by DNA-PK at serine residue 139 to form γ-H2AX foci, an indicator of a DNA damage site. The auto-phosphorylation of DNA-PK along with other phosphorylating enzymes facilitates the disassociation of DNA-PK from the site of damage and allows other DNA repair

factors to bind. One of the stages of NHEJ is DNA end processing. In this process, damaged nucleotides are replaced by polymerases (pol μ and pol λ) to create ligatable DNA ends [27]. After formation of the ligatable DNA ends, a ligation complex consisting of DNA Ligase IV, X-ray cross complementing 4 (XRCC4), and XLF (cernunos) mediate the final step ligating the DNA ends to restore the DNA integrity.

The second pathway of DSBs is homologous recombination repair (HRR). This pathway is genetically distinguished from NHEJ, as it requires a sister chromatid to function as a template. Thus, the HR repair pathway occurs most likely in S and G2 phases where DNA replication occurs and template DNA is available, which make HR repair much more accurate than NHEJ [28-29]. Unlike other DNA repair pathways, the DNA ends initially resected [30]. Resection of DNA ends allow for the search for the required homology to start the process of HR repair. The resection of DNA ends is mediated through a complex called MRN composed of different proteins including MRE11-RAD50-NBS1 [30]. This complex is recruited at the DNA damage site and cooperates with CtIP to start resection [31-32]. The final result of resection enzyme will end up in forming single-stranded 3’ extensions. The formation of these single-stranded regions allow for the binding of heterotrimeric complex called replication protein A (RPA), which has high affinity to the single strand break sites that occur during normal replication [33]. RPA protein complex will be then substituted by RAD51 recombinase, in a step that requires other mediator proteins such as RAD51 paralogs and (BRCA2) to form RAD51 nucleoproteins [30]. Many enzymes are involved in the regulation of RAD51 filament formation either positively facilitating the binding such as RAD51 paralogs and BRCA2, or negatively such as BLM and RTEL1 that activate the dissociation process of RAD51 nucleoproteins [34]. As the RAD51 nucleoprotein filaments have been formed, the RAD51 invades the intact double strand DNA of

the sister chromatid of the exact homology forming a synapse complex named displacement loop (D-loop), and starts elongating the DNA strand at the 3'-end. To continue the step of elongation, RAD51 in the invading strand has to be removed by RAD54 and its partner RAD54B in order to expose the 3'-OH for priming [35]. At this point, HRR can take different pathways to continue the process of repair. The first route, which is the most frequent route, is called synthesis-dependent strand annealing (SDSA), in which the elongation of the invading strand at 3'-end continues over a limited distance is then followed by the re-ligation with its original DNA strand in a process called (non-crossover). The other route is more complex and involves formation of double Holliday Junctions (dHJ). The repair of DSB by this specific route will require some resolving enzymes such as GEN1 and MUS81/EME1 that lead to the resolution of Holliday junctions and ultimately the repair of the DSB [36-38]. This step may ultimately lead to crossover or non-crossover, depending on the resolving enzymes involved of the double Holliday junctions resolution.



Lord C. J & Ashworth A. The DNA damage response and cancer therapy. *Nature* **481**, 287–294 (19 January 2012)

***1.5 DNA repair and carcinogenesis:***

One of the most consistent features of cancer cells is their genomic instability [39]. Colorectal carcinoma cells are known to be deficient in their ability to repair newly formed errors during DNA replication due to the lack of mismatch repair genes, MLH and MSH [23]. Similarly, other tumors have shown defects in other DNA repair pathways. For example, integrated analysis of around 500 samples of high-grade serous ovarian adenocarcinoma revealed that homologous recombination was defective in nearly half of these samples [40]. Previous reports have suggested that formation of neoplastic tissues is associated with an elevation of the DNA damage marker γH2AX foci (a histone phosphorylation event that occurs on chromatin surrounding a DSB), possibly due to defective DNA damage responses (DDR) [41-42]. One hypothesis proposes that cancer development is through the activation of oncogenes such as MYC and RAS [41, 43-44]. The activation of oncogenes leads to formation of many replication forks in order to enhance the proliferation of precancerous cells. Those replication forks may rapidly stall, collide, and form DSBs, leading to the activation of DDR. In precancerous tissues, it is thought that major DNA repair proteins, such as p53 and ATM, are still efficient and contribute to the repair of these replication forks collapses. When those proteins become mutated or inactivated, the cells may replicate with some ineffectively repaired DNA lesions [45].

### *1.6 Radiation*

In 1895, a German scientist discovered X-ray as a new useful clinical tool to treat cancer. A few years later, Marie Curie, who won the second Nobel prize, established her research into radiation oncology and improved the usefulness of radiation as a therapeutic agent for cancer treatment. During the last century, radiotherapy went through various stages of development as it became one of the most important modalities in cancer therapy beside surgery and chemotherapy. In fact, radiotherapy accounts for about 40% of cases of cure of cancer [46]. Furthermore, approximately 50% of the cancer patients will undergo radiotherapy during their treatment course [47-48]. Although it is highly used in clinic, the cost of radiotherapy composes only 5% out of the total cost of cancer therapy [49]. Therefore, radiotherapy remains a highly cost effective modality in cancer treatment.

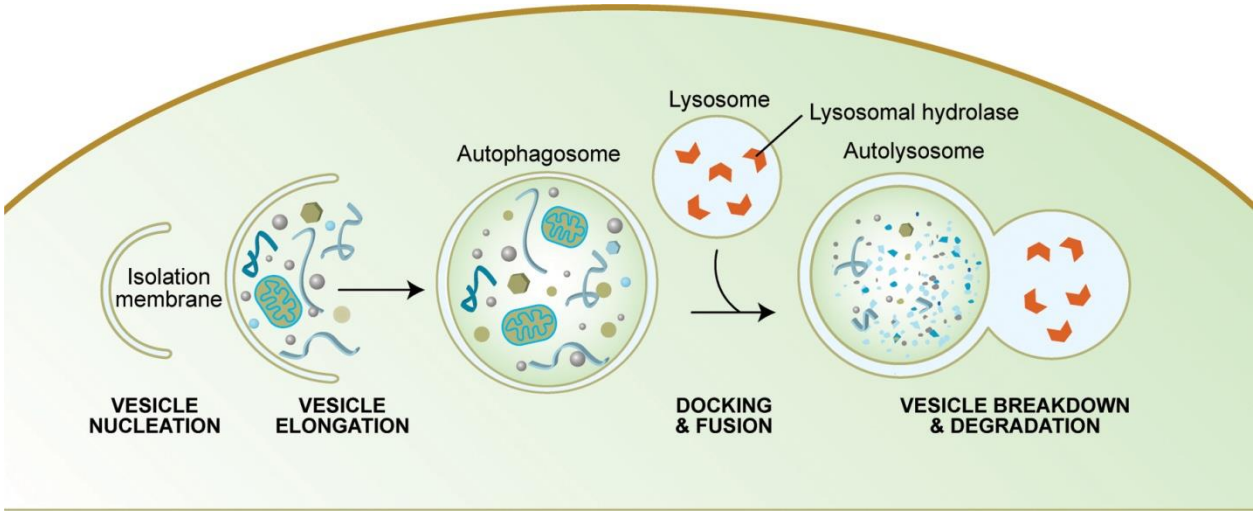
The mechanism of action of ionizing radiation is through forming ions and depositing energy inside the cells of tissues that radiation passes through in order to kill these cells or to damage their genetic materials, leading to cell death. Radiation toxicity is mediated through formation of ions accompanied with application of high energy in the cells it passes through. This high-energy deposition results in instability of the genetic materials (DNA), and may lead to cell death [50]. The main goal of radiotherapy is to maximize the dose to the cancerous tissues with minimum damage to the normal cells adjacent to them. Generally, normal cells are more efficient in repairing their damaged DNA than cancer cells [48]. Several tumors have been found to have mutations in their recombinational DNA repair [51-52]. In some tumors, mutations in major proteins in recombination repair make them sensitive to DSBs, whereas in normal cells such a mutation is absent and will not affect repair. Thus, radiotherapy is useful in the treatment of cancer.

When using ionizing radiation, single- and double-strand breaks can be formed. Due its lethality, DSBs, which are breaks in the phosphodiester backbone in both strands within 10 base pairs or less, are considered to be the toxic lesions induced by ionizing radiation [53-54]. In addition, radiation-induced DSBs generally occur at the 3'end [55], and the number of formed DSBs increases linearly with the extent of dose of radiation [56]. Also, the complexity of the induced DSB depends on the transcription activity of the DNA; wherein the relaxed DNA, the DNA damage is more complexed and sophisticated [57-58]. Generally, promptly-induced DSBs or radiation-induced DSBs are commonly repaired by NHEJ [51, 59]. However, the majority of radiation-induced DSBs are joined within few hours whereas ~20% seem to persist for more than a day, probably due to the complexity of these lesions [60-62]. Thus, the higher the dose of radiation, the more persistent foci being formed, which eventually lead to cell death.

### *1.7 Autophagy*

"Autophagy" is a Greek word that means "self-eating", which is a catabolic process by which the cells can catabolize and reuse its damaged organelles and misfolded proteins [63]. The process of autophagy begins when parts of cytoplasm and other organelles are sequestered within a double-membraned structure called the autophagosome. During autophagy, cellular constituents that need to be recycled are collected into the autophagosomes selectively by autophagy adaptors (SQSTM1/p62, NBR1, NDP52 and optineurin) [63-64]. This formed autophagosome fuses with lysosomes to construct another enclosed compartment called the autolysosome. Inside the autolysosomes, proteins are degraded by hydrolases and recycled to be reused by the cell [64-65]. The process of autophagy is very important during metabolic stresses to either provide the cell with the energy or to provide basic building blocks to synthesize new molecules.

At the molecular level, autophagy is highly regulated by different proteins known as autophagy-regulating genes (ATGs), which control the formation of the phagophore, elongation of the membrane, and formation of autophagosome. The first step of autophagy in eukaryotic cells involves the activation of a complex protein known as ULK1 [66]. The isolated phagophore is then regulated by other proteins called phosphatidylinositol 3-kinase (PI3K) and Beclin 1 that control the shape and the closure of the autophagosomes. Also, this protein complex PI3K – Beclin1 generates phosphatidylinositol 3-phosphate (PI3P) to then recruit PI3P-binding proteins WIPI1/2 and two ubiquitin-like conjugation systems ATG12–ATG5-ATG16L and LC3-PE [67]. At the final step, newly formed autophagosomes fuse with lysosomes to form autolysosomes in a step that requires Rab GTPases and LAMP2 [68]. Inside the autolysosomes, proteins are degraded by hydrolases and recycled to be reused by the cell [65].




Meléndez A. & Levine B. **Autophagy in C. elegans**.

### *1.8 Senescence*

Senescence, or aging, is generally defined by the inability of somatic cells to proliferate and divide, while remaining alive and metabolically active. In 1961, Hayflick and Moorhead first

hypothesized that somatic cells have a so-called “Hayflick limit”, which means that somatic cells have limited replicative capacity [69]. During replication, telomeres become gradually shortened at every cellular division due to the lack of a telomere maintenance mechanism. Consequently, somatic cells with short telomeres undergo replicative senescence. In contrast, malignant cells are capable of undergoing an infinite number of divisions due to the expression of the telomerase enzyme, which maintains the length of telomeres and immortalizes cancer cells. Although the vast majority of cancer cells express telomerase in order to have an infinite lifespan, senescence could be triggered in those cells in response to chemotherapeutic and radiotherapeutic modalities [70].

The induction of replicative senescence is generally thought to be a consequence of telomere attrition. When the telomere is shortened to the end, the telomeric caps at the ends of DNA become compromised. Once the telemeric ends are exposed, the cell recognizes it as a DNA damage site, and consequently activates the DNA damage response (DDR). The activation of DDR is initiated when ATM kinase is first activated, followed by the subsequent activation of p53 and then p53 downstream targets, most importantly p21 [71]. Induction of DNA damage in cancer cells may result in accelerated senescence through the ATM kinase – p53 – p21 pathway but is not thought to be associated with telomere shortening [72-73].

The induction of accelerated senescence is not fully understood but many reports suggested that it could be induced under any condition of stress through two pathways: p14ARF/p53/p21 pathway and INK4/CDK/pRb pathway [74-75]. In the first pathway, the protein p14ARF acts upstream of MDM2, a negative regulator of the tumor suppressor p53 by ubiquitination. The activation of p14ARF indirectly activates the tumor suppressor p53 and its downstream target CDK inhibitor p21, leading to growth arrest and promotion of senescence [76]. The other

pathway involves the activation of p16INK4a, which inhibits the cyclin-dependent kinases (CDKs). Inhibition of CDKs in turn leads to dephosphorylation of pRb by PP1 phosphatase [77]. Activation of pRb by dephosphorylation inhibits the E2F activity, which is essential for the cell cycle progression and proliferation [78]. Cross-talk between those two pathways may occur through the inhibition of cyclin-dependent kinases (CDKs) activity by p21, which then leads to the activation of pRB.

### *1.9 Significance and Background*

**Significance**

Radiotherapy is used along with other modalities such as surgery, chemotherapy, and immunotherapy to either shrink tumors before surgery or kill surviving tumor cells post surgery. Treatment of cells with radiation leads to multiple forms of cell death such as apoptosis, autophagy, mitotic catastrophe, and necrosis [79]. Although radiation (and chemotherapy) may induce different modes of cell death, recurrence of cancer may occur. Unfortunately, the recurrent cancers generally show lower sensitivity to the conventional anticancer agents than the original tumors [80-82]. In response to treatment, tumor cells can also undergo a form of growth arrest that is termed stress-induced senescence. Although anticancer drugs and ionizing radiation frequently cause senescence of cells, it is not necessary that these cells are going to undergo cell death due to several reasons. First, senescence may be permissive for DNA damage repair and subsequent proliferation of cells after exposure to chemotherapeutic agents or radiation. Second, senescence may allow treated cells to upregulate certain enzymes in order to inactivate the anti-cancer agents either by upregulating a drug-metabolizing enzyme or effluxing the out of the targeted cell via some transporters. Third, the dose that is used to induce senescence or the time of cellular exposure to anticancer agents is not enough to enforce cells to undergo cell death; in

other words it is nonlethal doses for the tumor cells whereas increasing the dose could be harmful to normal cells. Fourth, some cancerous cells that lack the activity of proteins involved in senescence might escape the induced senescence and proliferate again.

It has been shown that DNA damage can also induce cells to undergo a state of senescence associated with autophagy [83-85]. Autophagy can function as a pro-survival mechanism or as pro-death mechanism, depending on the agents used and the experimental systems [86-88]. The relationship between autophagy and the DNA repair system is unclear, but several studies showed that autophagy might play a role during exposure to DNA damaging agents. Disruption of autophagy by bafilomycin A1, an autophagy inhibitor, sensitized glioma cells to the alkylating agent telmozolamide by inducing apoptosis [89]. However, 6-thioguanine-induced autophagy enhanced the survival of human colorectal and endometrial cells, indicating that autophagy may also play a protective role against DNA damage [90]. Robert et al have recently found that autophagy and protein acetylation are important in DNA damage repair via activating check points and influencing homologous recombination repair (HRR) [91]. Another study has also shown that PARP-1 might link the response of DNA damage to autophagy through the depletion of ATP and (NAD+), which may indicate that a cytoprotective autophagy was promoted to supply the cell with energy [92]. Also, interference with autophagy led to an impaired DNA repair system according to another study [93]. On the other hand, human malignant glioma cells were shown to undergo autophagic cell death upon the inhibition of DNA dependent protein kinase catalytic subunit (DNA–PKcs), a protein involved in non-homologous end joining [94]. Furthermore, inhibition of DNA-PKcs in resistant prostate cancer cells radiosensitizes these cells by inducing autophagy [95]. It seems likely that autophagy has an important function in the enhancement of DNA repair system in the cells during exposure to genotoxic stress, but the role

of autophagy may differ according to the status of DNA repair. Thus, it is of importance to study the kinetics of DNA repair during the use of DNA damaging agents along with autophagy inhibitors and address whether autophagy would assist DNA-damaged cells to retain their capacity for proliferation.

In addition, some studies have demonstrated that senescence is also induced upon exposure to a DNA-damaging agent such as radiation, suggesting that DNA damage mediates senescence [96-97]. It has been generally thought that senescence is an irreversible process whereby cells lose their capacity to grow, but studies have shown that telomerase enzyme can be reactivated in replicative senescence and by then cells can immortalize [98]. Similar to the telomere-associated foci of replicative senescence, it has been shown that persistent foci lead to senescence [99]. In this study, high doses of IR and Bleomycin showed an increase in the number of DNA damage foci and the majority of the foci were transient, which disappeared within 24 hours post-treatment; however some persistent foci remained for months. Therefore, the repair of these persistent DSBs in senescent cells may result in recovery and regrowth of senescent cells. The basis for the recovery of senescent cells is still unknown but it is of particular importance to our proposed work that studies have indicated that senescent cells can repopulate after exposure to chemotherapeutic agents and radiation [96, 100-102]. One primary aim of this project was to understand the contribution of autophagy to the induction of DNA repair, and the extent of persistent DNA-damage foci that are required to lead to senescence. In addition, this work was designed to investigate whether radiation-induced autophagy and radiation-induced senescence play a cytoprotective function by which cells overcome the persistent DNA damage and recover.

**Background:**

Radiotherapy is one of the most widely used anti-tumor strategies. Radiotherapy can be delivered to the tumor by two different ways, externally and internally. In the external delivery, radiation is administered from outside the body to the site of interest. The internal radiation delivery is through targeting the tumor from inside the body by using catheters seeded with radioactive materials. However, the response of tumors to radiation varies, where in some types of cancers, radiation is quite effective while other tumors show resistance to radiation. For example, treatment with radiation after surgery or along with chemotherapy significantly reduces recurrence and improves the outcomes of breast and head and neck cancer therapy, respectively [103-104]. On the other hand, several types of cancers such as glioblastoma and lung cancer show high resistance to radiation, even in combination with chemotherapeutics, and recurrence is common within a few years after therapy [105-107].

***DNA damage and autophagy.*** Unlike apoptosis, radiation-induced autophagy can result in either survival or death. A number of studies have shown that autophagy can mediate the action of some therapeutics [108-109]. Cells that undergo autophagy are characterized by enlarged and swollen nuclei accompanied with formation of vacuoles in the cytoplasm [110]. The relationship between DNA damage and autophagy is still unclear, but many studies have shown that DNA-damaging agents promote autophagy [111-116].

One of the mechanisms of toxicity of radiation is through production and accumulation of reactive oxygen species (ROS) that cause oxidative stress and lead to autophagy via oxidizing damaged DNA [117-119]. However, inhibition of chemotherapy and radiation-induced autophagy often (but not always) promotes DNA damage-induced apoptosis, which indicates that autophagy functions as a protective mechanism against DNA damage-induced apoptosis [92,

120-122]. Another mechanism by which radiation may lead to the promotion of autophagy is through poly(ADP-ribose) polymerase-1 (PARP-1). When cells undergo DNA damage (DDR), particularly SSBs, the activated nuclear PARP-1 consumes NAD+ and depletes ATP. This energetic disturbance will lead eventually to the activation of AMPK and subsequent autophagy regulating enzymes [92, 123].

One of the proteins that is upregulated in response to DNA damage is ATM, which is regulated by FOXO3a [124]. Both ATM and FOXO3a control autophagy via downregulation of the mTORC1 pathway and transcription of autophagy-regulating proteins [125-130]. Other studies have shown that autophagy can be mediated by p53 through transcriptional activation of DRAM (damage-regulated autophagy modulator) [131-132]. Therefore, p53-induced autophagy may become involved in the growth arrest and the repair of damaged DNA. All of these observations suggest that autophagy plays an essential role in the DNA repair system.

***DNA damage and senescence.*** Senescence occurs as a result of oncogenic stress, DNA damage, or cytotoxic drugs [133]. Senescence can be telomere-dependent or –independent, in which cells remain metabolically active. Telomeres are gradually lost in each S phase of cell cycle, resulting in loss of DNA at the very end of chromosomes. Also, some cells lack the ability to reactivate telomerase, the enzyme responsible for the addition of DNA sequence repeats at the end of chromosomes [134-135]. Existence of erosions at telomeric sites leads to the generation of a persistent DNA damage response and growth arrest [136-138]. In addition, non-telomeric DNA breaks can induce senescence and growth arrest via persistent DNA damage response [139]. Ionizing radiation can cause DNA damage directly by producing single- and double-strand breaks or indirectly through generating reactive oxygen species (ROS) in the cells. Although the

majority of DNA breaks become repaired upon induction of DNA repair pathways, many DNA damage foci appear to be persistent [99]. It is yet unclear why these foci do not undergo DNA repair as other bulk of double-strand breaks (DSBs), but it could be either due to the repression of DNA repair system in the cells or due to the unique properties that persistent foci have.

The early step of detection of DNA damage repair is via the complex MRN (MRE11, RAD50, NBS1) that act as sensors for DSBs. This complex recruits and activates ATM and ATR protein kinases, which in turn connect signaling pathways between DNA damage response and senescence via activating Chk1, Chk2, and p53 [140-141]. In the case of complicated and non-repairable DSBs, cells may either undergo apoptosis or senescence [142-143]. Since apoptosis is known as a programmed cell death, the ability of DNA-damaged cells to avoid apoptosis and enter a state of senescence may indicate that senescence could act as a permissive mechanism for persistent DNA damage foci to be eventually repaired.

# Chapter 2: Poly(ADP-ribose) polymerase (PARP) Inhibitors (Overview).

## *2.1 Introduction (Function and Structure):*

Poly(ADP-ribosyl)ation, or the so-called (PARylation), is a post-translational modification process in which proteins are catalyzed by a family of polymerase enzymes called Poly(ADP-ribose) polymerase (PARP). The main function of these enzymes is to detect the site of single-strand breaks (SSBs) and recruit the required enzymes of single-strand break (SSB) repair pathway or base-excision repair (BER) to remove the induced breaks. The immediate response of PARP enzymes could be due to metabolic, chemical, or radiation-induced single-strand breaks (SSBs). When single-strand breaks (SSBs) are formed, PARP enzyme detects the site of damage, binds to the DNA strand, and initiates the repair process via the synthesis of a poly (ADP-ribose) chain (PAR), as a sign of single-strand break [144]. After the detection step, other repairing enzymes can be recruited such as DNA ligase III, DNA polymerase β, and X-ray cross-complementing gene 1 (XRCC1) to continue repair steps [145]. Once repair is complete, poly(ADP-ribosyl) chain is degraded by Poly(ADP-ribose) glycohydrolase (PARG) [146]. It is noteworthy to mention that Nicotinamide adenine dinucleotide (NAD+) is a substrate to generate (ADP-ribose) monomers. Once PARP is overactivated, the energy source (ATP) of the cell will be depleted, and necrotic cell death will be the final fate. However, PARP enzymes have other functions inside the cellular systems such as expression of inflammatory genes and expression of genes required for the response of smooth muscles to the TNF [147-149]. In this work, we will discuss the role of PARP enzymes in the DNA damage response (DDR).

PARP enzymes are greatly conserved proteins. There are different forms of PARP enzymes known as PARP-1, PARP-2, and PARP-3, where the most abundant form is PARP-1 [150]. The

PARP-1 enzyme is composed of three main domains. The first domain is called the DNA-binding domain that consists of two zinc-finger motifs, which bind to DNA breaks [151]. The second domain is known as the automodification domain (16 kDa) that is located in the central part of the enzyme. The third domain of the enzyme is located at the C-terminal and named the catalytic domain (55 kDa), which has the most conservative part of the enzyme called "PARP signature" [152]. The last domain of the enzyme, the catalytic domain, is the region that has been targeted by a class of anti-cancer drugs called "PARP inhibitors".

### *2.2 Targeting PARPs and the usefulness of PARP inhibition in cancer therapy.*

Knowing the main role of PARP enzymes in the repair of SSBs, it would be important to understand how interference with SSB repair sensitizes tumor cells to DNA damaging agents. When cells are exposed to a DNA damaging agent e.g. Ionizing radiation, single-strand breaks (SSBs) and double-strand breaks (DSBs) are formed. Treated cells will eventually repair the SSBs via PARP activation in the SSB repair pathway, and the DSBs via homologous recombination (HR) and non-homologous end joining (NHEJ). When cells are exposed to PARP inhibitors, cells will be unable to repair the SSBs. During DNA replication, the single-strand breaks (SSBs) would be converted to double strand breaks (DSBs). If cells have efficient DSB repair pathways, especially the homologous recombination (HR) that dominates during the DNA replication, the radiation- and PARP inhibition-induced DSBs would be repaired. Once cells have a mutation in one of the main proteins involved in the homologous recombination (HR) pathway, radiation- and PARP inhibition-induced DSBs would unlikely be repaired, and the deleterious lesion will affect cell growth. Thus, tumors that show aberrant mutations in BRCA1,

BRCA2, and MRE11 proteins will likely show sensitivity to PARP inhibitors when combined with DNA damaging agents. Many types of cancer tumor show mutations in BRCA1 proteins such as breast, ovarian, prostate, and colorectal tumors [153-154]. Also, other types of tumors have abnormal BRCA2 function such as buccal cavity and pharynx, stomach, pancreas, melanoma of the skin, and gallbladder and bile ducts [155-156]. Therefore, inhibition of PARP activity is useful in tumors that show mutations in major proteins regulating the homologous recombination (HR) pathway.

In addition to the role of PARP enzymes in the repair of single-strand breaks, recent reports have demonstrated further functions of PARP enzymes in other DNA repair pathways. Both isoforms PARP-1 and PARP-3 were found to be activated and play roles during the formation of DSBs [157-160]. Emergence of PARP enzymes into the repair of double-strand breaks (DSBs) could be due to the necessity of DSB repair factors to bind to PARylated DSBs. Also, PARylation of repair factors may enhance the efficiency of repair and facilitate the repair of unrepaired DSBs [161].

Several studies have proven the involvement of PARP-1 activity in the homologous recombination (HR) pathway. During DNA replication, DNA replication forks stall when a single-strand break is still unrepaired, leading to fork collapse. The persistent unrepaired single-strand breaks (SSBs) results in double-strand breaks (DSBs). In this phase, homologous recombination is the overwhelming DSB repair pathway due to the existence of the sister chromatid. PARP-1 has been shown to activate MRE11, one of the proteins involved in HR pathway, via PARylation and recruit it during the DNA end-resection [160]. However, another report indicated that PARP-1 may limit the activation of several repair factors involved in NHEJ, favoring toward the homologous recombination (HR) pathway [162]. Another function of

PARPs, particularly PARP-1 and PARP-2, is related to the heterchromatin structure. During the induction of DNA damage, the nucleus undergoes heterochromatic structure of the nucleus is to ease the binding and accessibility of repair factors to DNA damage site. Some studies suggested that PARPs, in cooperation with other proteins, might be an essential factor in this step [163-164]. Taken together, PARPs show some connection with homologous recombination (HR) pathway.

In addition, some studies showed that PARPs may also enhance some repair factors in classical non-homologous end joining (NHEJ) pathway, but this is still in debate [165-168]. DNA-PK enzyme, an enzyme involved in the NHEJ pathway, was found to be PARylated, which then lead sto the enhancement of its kinase activity [169-170]. Also, a structural study has demonstrated formation of complex of (DNA-PKcs/PARP-1) [171]. More interestingly, recent evidence suggested that PARP-1 is involved in a newly discovered DNA repair pathway called "Alternative end joining". When the classical NHEJ pathway is defective, the cells tend to switch the repair mechanism to the backup mechanism, the alternative end joining. The evidence of involvement of PARP-1 in this pathway is based on the delay in end-joining in cells lacking Ku70 or Ku80 while inhibition of PARP [167, 172]. Furthermore, PARP-3 has also been shown to interact with some proteins involved in classical NHEJ pathway such as DNA–PKcs, Ku70, Ku80, and DNA ligase IV [173-174]. PARP-3 also participates in the balance of DNA resection step [175]. This study revealed that PARP-3 works as a protective factor against extensive end resection mediated by MRE11/CtIP proteins. Thus, targeting PARP-3 may result in a clastogenic effect in cancer cells.

In conclusion, all isoforms of PARP are essential components of DNA repair mechanisms. These proteins play a pivotal role in the maintenance of genetic stability and integrity. Loss of

PARPs activity deteriorates the attempt of DNA repair mechanisms to rescue in treated cells. The major functions of PARPs that have been discussed previously point toward the validity of inhibiting the actions of these enzymes in order to target cancer cells via enhancing the DNA damaging effect of currently used therapeutic modalities such as radiation and chemotherapy.

### *2.3 The pharmacokinetic properties of PARP inhibitors.*

Preclinical studies of anticancer agents have poor prediction on the safety and effectiveness of the tested agents in cancer therapy. The high failure rate of many recently designed targeted agents for cancer treatment led to the development of new regulations named Expolratory Investigational New Drug (IND) Guidance to avoid the long-term clinical studies of these agents. Clinical phase 0 was designed to conduct a clinical study in limited number of patients using nontoxic doses for short period of exposure to better understand the pharmacokinetic distribution of these drugs. Pharmacokinetics is the study of drugs movement in the body systems including the absorption, distribution, metabolism, and elimination. The efficacy of drugs mainly depends on the pharmacokinetic characteristics in which the drugs distribute and become delivered to the site of action. In many in vitro studies, PARP inhibitors showed radio- and chemo-sensitizing effects in sub lethal concentrations [176-180]. Therefore, evaluating the clinical efficacy using clinically relevant doses is paramount to continue investigating the usefulness of these agents.

The first clinical trial conducted using a PARP inhibitor was Phase 0 to investigate the pharmacokinetic properties of ABT-888 [181]. In this study, different doses (10, 25, 50 mg) of ABT-888 were orally administered in patients with advanced malignancies, and blood samples as well as tumor biopsies were taken to measure the activity of this drug to inhibit PARP activity before and after the administration of the drug. Doses of 25 and 50 mg showed well tolerability

with an acceptable oral bioavailability of ABT-888 in these patients. Also, another PARP inhibitor, AZD 2281, was evaluated in Japanese patients as a Phase I clinical trial and the pharmacokinetic aspects of this drug were monitored. In line with the findings in western patients [182], the study in Japanese patients failed to identify any serious safety concerns [183]. In addition, the administration of a single or multiple doses of AZD 2281 (Olaparib) demonstrated a relatively rapid rate of absorption where the C*max* was achieved approximately after 2 hrs of treatment, and the half-life ranging from 7-11 hrs. This study strongly confirmed the previous findings of phase I clinical studies on Olaparib in a group of patients of different demographic background, indicating no inter-ethnic differences. Another technique was also used to study the distribution of PARP inhibitor (AZD 2281) scaffold with a boron-dipyrromethene (BODIPY) N-hydroxysuccinimide ester in single tumor cells in vivo to ensure the delivery of these inhibitors to the tumor site [184]. Data in this study clearly demonstrated the distribution of the drug into cancerous and normal cells, entering the nucleus, and binding to their target. On the other hand, a study conducted by Shiv K. Gupta1 et al, explained why the Temozolamide-resistant glioblastoma cell line does not show sensitivity with PARP inhibitor (ABT-888) in xenograft models compared to the in vitro studies [185]. In fact, the distribution of PARP inhibitor in this particular resistant cell line in vivo does not reach the required concentration to sensitize tumor cells to Temozolamide. Another newly developed PARP inhibitor named Rucaparib was found to be retained in the cancer cells for long time and reaching an intracellular concentration 10 times higher than the extracellular [186].

In conclusion, the pharmacokinetic studies have demonstrated a tolerated and rapid distribution of some PARP inhibitors in both in vivo and phase 0 clinical trials. As we explained the importance of the potency of every single PARP inhibitor in the previous section, we here

discussed the importance of the delivery of PARP inhibitor sites. The data presented in different studies indicated that PARP inhibitors distribute easily in different types of tumors except few ones. Until now, there is no study compared between the pharmacokinetic properties of PARP inhibitors in human patients. Also, in the resistant glioblastoma cell line study, authors used one of the least effective PARP inhibitors, possibly due to poor pharmacokinetic issues. Thus, it would be very important to address whether potent PARP inhibitors would show similar pharmacokinetic properties or not.

### *2.4 The implications of PARP inhibitors in cancer therapy.*

Since there has been a great deal of interest in the role of PARPs in the functionality of DNA repair pathways, several compounds have been synthesized in order to target this particular protein and potentiate the chemotherapeutic and radiotherapeutic approaches. The discovery of these synthetic lethal drugs was meant to interfere with the main function of PARP inhibitors and lead to the increase aggregates of unrepaired SSBs and DSBs. We would speculate that PARP inhibition would sensitize cells to radiotherapy and chemotherapy to an extent similar to PARP deletion. Surprisingly, several studies have indicated that PARP inhibition shows higher toxicity in wild-type cells when exposed to DNA damaging agents than PARP-1 – deficient mouse cells [187-189]. Also, PARP inhibitors were found to delay the repair of SSBs longer than PARP knock out cells [145]. This observation led to another question: would PARP inhibitors enhance chemo- or radio-sensitivity via more than pathway? Murai et al have addressed this question by trapping of PARP-1 and PARP-2 on DNA using clinical PARP inhibitors [190-191]. These studies have demonstrated that PARP inhibitors can exert their poisoning effect via two different

mechanisms. The first mechanism is through competition with NAD+ at NAD+ binding site, in which inhibitors show similar potency. The other mechanism is via inhibition of the PARylation activity of PARPs, which in turn lead to stronger binding of PARP to the DNA forming PARP-DNA complex. Different PARP inhibitors vary in their potency in forming PARP-DNA complexes, which explain the difference in toxicity between these drugs. For example, Niraparib (MK-4827) and Olaparib (AZD-2281) revealed stronger potency in trapping PARPs on DNA more than Veliparib (ABT-888).

Several anti-PARP drugs are currenty being investigated in the clinical trials, specifically Iniparib, olaparib, Niraparib, Rucaparib, BMN-673 and ABT-888 (Veliparib) [192]. Recently, Olaparib has been approved by the US food and drug administration (FDA) and European commission for the treatment of ovarian cancer as a combination therapy due to its limited toxicity [193]. Also, several studies suggested that PARP inhibition could be a useful approach in the treatment of prostate cancer tumors [194-195]. The promising results of utilizing PARP inhibitors as chemo- and radio-sensitizer agents led to the development of clinical trials of PARP inhibition along with other chemotherapeutic agents that leads to DNA damage including carboplatin, cisplatin, oxaliplatin; alkylating agents like Temozolomide and topoisomerase inhibitors [176, 196-199].

The topoisomerase-I enzyme is also an important target in cancer therapy. The main resistant mechanism by which cancer cells overcome the toxicity of Topotecan, an inhibitor of topoisomerase-I, is via PARylation of DNA damage by PARPs. Thus, adding PARP inhibitor along with Topotecan enhances the chemotherapeutic effect via antagonizing PAR formation [199]. Several chemotherapeutic medications are being tested in combination with PARP inhibitors as described in (www.clinicaltrials.gov).

The preclinical data strongly suggested that PARP inhibitors are promising chemo- and radio-sensitizer agents in multiple types of tumors. The main mechanism of these drugs was thought to be through interfering with SSBs repair. However, other studies have revealed that PARP inhibitors may form PARP-DNA complexes that lead to additional mechanism of cytotoxicity. Current clinical studies are being conducted in patients of different tumors including malignant glioma, head and neck, and breast cancers. Although the preclinical and early phases of clinical studies show the desired effect, it is necessary to perform extensive optimizations and characterizations of given doses and scheduled treatments to ensure the optimum therapeutic effect of the combination.

### *2.5 Drawbacks and limitations*

Similar to other anti-cancer agents, development of resistance in cancer cells toward PARP inhibitors is possible. Some studies have reported that cancer cells may acquire resistance to PARP inhibitors via three possibilities: (i) upregulation of PgP transporter (ii) loss-of-function of PARP (iii) restoration of functional BRCA2 gene [200-201]. As is the case with other drugs, PARP inhibitors can be pumped out of the cell via PgP transporter. In addition, excessive inhibition of PARP may lead to loss of PARP expression, which in turn leads to resistance of the treated cells to PARP inhibitors to 100 fold resistance [200]. A third mechanism by which cells acquires resistance to PARP inhibitors is via a mutation in the BRCA2 gene that leads to retaining its activity and restoration of the open reading frame (ORF).

In addition, it is important to discuss the challenges that may negatively impact PARP inhibition use in cancer therapy. The major challenge in PARP inhibitors development is the

poor delivery of the drug to the tumor site due to certain pharmacokinetic properties of water insoluble inhibitors. However, PARP inhibitors are usually recommended to be used in combination with other chemotherapeutic drugs. Therefore, there is a possibility of development of drug-drug interaction between PARP inhibitors and the used drugs. Furthermore, this undesired possibility may affect the dosing and the timing of treatment. Thus, it would be of importance to optimize the doses and the timing of drugs administration to avoid the failure of treatment.

In summary, the approval of the first clinical PARP inhibitor opens a new era of anticancer therapy. Although initial pre-clinical and clinical data prove the efficacy of the used drugs, the resistance to PARP inhibitors is possible. From a clinical perspective, the possibility of sensitization to radiation (and chemotherapy) through the administration of PARP inhibitors to interfere with DNA repair continues to be an area of active inquiry [197, 202-204]. Interestingly, sensitization to radiation has been shown to lead primarily to an increase in senescence with minimal apoptosis [205-206]. Furthermore, the potential involvement of autophagy in radiation sensitization via PARP inhibition has not been investigated; this is relevant as autophagy and senescence have been shown to be closely associated responses in some studies [83, 207]. The primary aim of the current work was to understand the involvement of autophagy and senescence in the response to radiation-induced DNA damage, and the interplay between these responses and DNA repair by employing PARP inhibitors.

# Chapter 3: Materials and methods

### *3.1 Cell lines.*

HCT116 colon cancer cells were purchased from ATCC and HCT116 Ligase IV-deficient were kindly provided from Dr. Hendrickson laboratory [208]. HCT116 Ligase IV-deficient and Ligase IV proficient cells lines were maintained as subconfluent cultures in RPMI 1640 medium with 5% fetal bovine serum, 5% bovine calf serum, 2 mM L-glutamine, and penicillin/ (GIBCO Life Technologies, Gaithersburg, MD) and incubated at 37ºC, 5% $CO_2$, in a humidified environment. In every experiment, cells were cultured under identical conditions and incubated overnight to allow for adherence prior to irradiation.

HCT116 *shCon*, HCT116 *shATG5*, and HCT116 *shATG7* cells lines were generated in our laboratory using bacterial stocks from Sigma Aldrich. A packaging mixture composed of Lipofectamine (Invitrogen, 11668–019), psPAX2 and pMD2.G packaging constructs (Addgene, 12260, 12259), and a vector carrying the genetic material were introduced into HEK 293 packaging cells. After two days, lentivirus was collected from the medium and used to infect the HCT116 cells. Infected cells were then maintained under puromycin (2 µg/mL) as a selection marker.

### *3.2 Time course of radiation-induced effects on cell viability.*

Cells were plated in 6-well plates (generally 200,000 cells/well) and allowed to adhere overnight. The next day, cells were treated with radiation and the number of viable cells was counted at indicated time points for 5 days. In case of co-administering a drug (PARP inhibitors, autophagy inhibitors, or Apoptosis inhibitor Z-VAD) with radiation, cells were pretreated with the drug 3 hours before radiation and drug was washed away 24 hr post radiation. At each time point, medium was removed and cells were washed one time with 1X PBS. 500 µL of 0.25% trypsin was added to each well for harvesting and incubated for 5 minutes, then deactivated by 500 µL of fresh medium, to make up 1 mL of cell suspension. Cells were collected in 1 mL conical tubes

(Eppendorf) and 10 µL of cell suspension was added to 10 µL of trypan blue, placed onto chamber slides of a hemacytometer (Hausser Scientific) and counted under a microscope.

### *3.3 Clonogenic survival assay.*

200 cells were plated in 6-well plates and allowed to adhere overnight. After 24 h, cells were pre-incubated with the indicated drug for 3 h and then exposed to the indicated dose of radiation. The following day, drug-containing medium was removed, cells were washed and supplemented with fresh medium that was replaced every other day for two weeks. On the day of staining, cells were fixed with 90% methanol for 10 min, and then stained with 1% crystal violet for another 10 minutes. Colonies were then washed with PBS three times to eliminate excessive crystal violet staining and counted manually.

### *3.4 Assessment of autophagy by acridine orange staining.*

50,000 cells were seeded in 6-well plates, permitted to adhere overnight and exposed to radiation the following day. At the various time points, medium was removed and cells washed once with 1X PBS. Acridine orange dye was diluted in PBS in a ratio of 1:10,000 (prepared in the dark), added to cells for staining, and incubated for 15 min. Dye-containing medium then was aspired, plates were washed with 1X PBS and fresh medium was added. Photographs were taken with an Olympus 1X 70 microscope and an Olympus SC 35 camera.

The cell population positively stained with acridine orange was quantified by flow cytometry. Treated cells were trypsinized, collected, and centrifuged at speed of 1500 rpm for 5 min. Supernatant was removed and pellets were resuspended in 990 µL of 1X PBS. The cell suspension was filtered through standard flow cytometry 40 micron filter (BD Falcon). A 10:1000 dilution of acridine orange in 1X PBS was prepared (in the dark) and protected from light until ready for use. For flow cytometry, 10 µL of acridine orange solution was added to each sample to make the dilution of 1:10000 and allowed to mix for 15 min. Acridine orange is excited at wavelength 525 nM for green flourescence and 620 nM for red flourescence.

### *3.5 Transfection of HCT116 cells with RFP-LC3.*

The RFP-LC3 construct was generated by the Tolkovosky laboratory [208]. $1x10^6$ HCT-116 cells were collected in a pellet, centrifuged, and resuspended with the construct in 100 µL of the Amaxa Nucleofector Kit V. A microgram of the RFP-LC3 vector was added to the suspension. The cell suspension was collected in a cuvette, and then placed in nucleofector device to run program D-032. 500 µL of medium was added to the transfected cells and to transfer them to a Petri dish where cells were maintained under Gentamycin (8 ng/mL) to maintain the stable transfection.

### *3.6 Cell cycle analysis.*

At the indicated time points, cells were trypsinized, collected, and centrifuged at 1500 rpm. The supernatant was aspired, pellets washed in PBS and recentrifuged at 1500. The supernatant was removed, 0.2 mL of PBS was added and pellets were gently mixed to form a single cell suspension. 1.8 mL of cold 70% ethanol was added gradually into the cell suspension; cells were vortexed, centrifuged, ethanol was aspired, and cells were washed with PBS prior to addition of a staining solution (0.1% (v/v) Triton-X-100 in 10 mL PBS, 2 mg of DNase free RNase A, and 0.2 mL of the propium iodide stock (1 mg/ml)) 2 hours prior to flow cytometry.

### *3.7 Evaluation of senescence by β-galactosidase staining.*

β-Galactosidase staining was utilized as a marker of senescence. Cells were washed once with 1X PBS and fixed with 2% formaldehyde/ 0.2% glutaraldehyde for 5 min, again washed with PBS and finally incubated overnight in a staining solution composed of 1 mg/mL 5-bromo-4-chloro-3-inolyl-β-galactosidase in dimethylformamide (20 mg/mL stock), 5 mM potassium ferricyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, 2 mM MgCl2, at pH 6.0 in CO2 at 37ºC. The following day, cells were washed twice with PBS and pictures were taken.

For β-Galactosidase detection by flow cytometry, cells were washed and incubated for 1 h in complete medium containing 100 nM of bafilomycin A1 to induce lysosomal alkalinization. After incubation, $C_{12}$FDG working solution was added to each well in amount to make the final concentration 33 µM and incubation was continued for another 1 h. Medium was then aspired and cells were washed twice with PBS. Cells were harvested, collected by centrifugation at 1500 rpm, resuspended in PBS and analyzed by flow cytometry as above but with excitation at 490 nm and a 514 nm emission filter. $C_{12}$FDG is hydrolyzed by upregulated β-galactosidase enzyme and becomes fluorescent at wavelength of 500–510 nM.

### *3.8 Detection of γH2AX foci as a marker of DNA damage.*

5,000 cells were seeded in 4-chamber coverglass slides (Lab-Tek II) and allowed to adhere overnight. On the following day, cells were irradiated and fixed with 4% formaldehyde for 5 min at indicated time points. Cells were washed with 1X PBS twice, incubated at room temperature in 0.05% trition-X for 15 minutes, washed, and incubated with 1X PBS containing 1% of BSA for 30 min to prevent non-specific binding of the antibody. Finally, cells were incubated in a 1:10 dilution of γH2AX antibody (BD Pharmingen) in 1% BSA for 1 h. Images were taken using an LSM 700 confocal microscope (Zeiss).

Alternatively, for flow cytometry, cells were at harvested at the indicated time points, fixed with 90% ethanol and maintained at -20 °C until the day of experiment. Cells then were centrifuged at 3,000 rpm for 5 min and resuspended in 1% BSA for 30 min. γH2AX antibody (BD Pharmingen) was added to the cells in a dilution of (1:200) and incubated at room temperature for 1 hr. Cells were then analyzed by flow cytometry at an excitation wavelength of 488. Raw data were normalized according to the intensity of control samples (normalized mean intensity = intensity of the sample / the intensity of the correspondent control sample within the same experiement)

### *3.9 Evaluation of DNA damage extent by the comet assay.*

200,000 cells were plated in 6 $cm^2$ dishes and treated as indicated. After 72 hours, cells were gently scraped from the plates and 100,000 cells were mixed with molten LMAgarose (at 37 ºC) at a ratio of 1:10 (v/v). The mixture of cells and LMAgarose was then pipetted onto Comet Slides (Trevigen) and incubated for 30 min at 37 ºC. The slides were kept at 4 ºC for 10 min prior to being immersed in Lysis Solution (Trevigen) overnight. On the following day, the slides were immersed in 1X Neutral Electrophoresis Buffer for 30 minutes at 4 ºC, set onto an electrophoresis tray for 45 minutes and subjected to voltage at 1 volt per cm. Slides were then immersed in DNA precipitation solution for 30 minutes at room temperature, followed by 70% ethanol for another 30 min. Samples were dried at 37 ºC for 30 min and stained with the working dilution of SYBR Green (Trevigen).

### *3.10 Evaluation of apoptosis.*

Following the indicated treatments, cells were harvested at the indicated time points and collected on a cytospin slide, fixed with formaldehyde (4%) for 5 min and washed with 1X PBS twice. Slides were fixed with acetic acid/ethanol (1:2) for 5 min and washed twice with 1X PBS. For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, cells were blocked with BSA (1 mg/ml for 30 min) at room temperature, washed twice in PBS, and incubated with enzyme mixture (terminal transferase, 25 mM $CoCl_2$, fluorescein-12dUTP) for 1 h at 37º to allow the enzymatic reaction. After washing with PBS, cells were stained with DAPI and images were taken using a fluorescence microscope.

For the PI/Annexin assay, adherent and non-adherent cells were harvested in Eppendorf tubes, and pellets resuspended in 100 µL of binding buffer (BD Biosciences). 5 µL of Annexin-FITC (BD Biosciences) and 5 µL of PI at 10 µg/mL (BD Biosciences) were added to cell suspension and incubated for 15 min in the dark at room temperature. 400 µL of Annexin V binding buffer 1X

(BD Pharmingen) was added to each sample, and samples were analyzed by flow cytometry at 530 nM.

### *3.11 Western blotting.*

At the indicated time points, cells were harvested and mixed with lysis buffer (1 M Tris-HCl, pH 6.8, 10% SDS) containing protease and phosphatase inhibitors (Sigma-Aldrich). Proteins were separated on 12% gels using SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked using nonfat dry milk and PBS for 30 min at room temperature, then incubated with the primary antibody overnight at 4ºC. Primary antibodies used were anti-p62 (SQSTM1–Santa Cruz sc-28359), anti-ß actin (Santa Cruz sc-47778), anti-ATG5 (Cell Signaling – 12994S), and anti-ATG7 (Cell Signaling – 8558S). All primary antibodies were used at a 1:1,000 dilution. The following day, membranes were incubated with correspondent secondary antibodies for 1h. Secondary antibodies used were goat anti-mouse IgG (Amersham, GE Healthcare) and monkey anti-rabbit IgG (Amersham, GE Healthcare). Membranes were then washed three times and bands were detected using enhanced chemiluminescence detection reagents (Pierce, Rockford, IL).

### *3.12 Statistical analysis.*

Statistics were conducted using Statview statistical software (SAS Institute, Cary, NC). The data were expressed as means ± S.E. Comparisons were made using two-way analysis of variance followed by the Bonferroni post hoc test. P values $<0.05$ were considered as statistically significant.

# Chapter 4: Results:

### *4.1 Response of DNA repair-competent and DNA repair-deficient cell lines to radiation.*

Radiation sensitivity in the HCT116 and the HCT116 Ligase-IV-deficient cell lines was determined by clonogenic survival. **Figure 1A** shows that HCT116 cells lacking Ligase IV were significantly more sensitive to radiation than the Ligase IV wild-type cells as the clonogenic survival was significantly decreased at lower doses of radiation compared to wild type cells. This finding is consistent with previous reports in the literature indicating that DNA repair-deficient cell lines are more sensitive to radiation than DNA repair-proficient cells [209-211].

Radiation sensitivity was further compared by performing temporal response studies in which the HCT116 and the HCT116 Ligase-IV deficient cell lines were exposed to a radiation dose of 2 Gy and viable cell number was monitored over time. **Figure 1B** shows that growth of the HCT116 cells was inhibited only transiently followed by relatively rapid recovery of proliferative capacity whereas radiation produced a sustained growth inhibition (with a slight decline in viable cell number between days 3 and 5) in the HCT116 Ligase IV-deficient cells.

The difference in sensitivity of the two cell lines to radiation is likely to be a consequence of the extent and persistence of DNA damage [211-212]. **Figure 1C (**staining with γH2AX antibody) and **Figure 1D** (H2AX phosphorylation) indicate that the number of DNA damage foci in HCT116 Ligase IV (-/-) cells was increased compared to the Ligase IV proficient cells. That is, over a range of radiation doses, the extent of residual damage (i.e., γH2AX staining) at 96 h was significantly higher in the Ligase IV-deficient HCT116 cells than in the Ligase IV-proficient cells.

As an additional confirmation of the increased DNA damage, results of Comet assay experiments presented in **Figure 1E** show more extensive formation of tails in the HCT116 Ligase IV (-/-) cells compared to the HCT116 WT cells at low dose of radiation (2 Gy). Furthermore, cell cycle analyses indicated that nearly 45% of the HCT116 Ligase IV (-/-) cells were arrested at the G2/M phase in comparison to 20% of the HCT116 WT cells 72 hours after exposure to 2 Gy of radiation **(Figure 1F)**.

### *4.2 Induction of autophagy and senescence by ionizing radiation in Ligase IV-proficient HCT-116 and Ligase IV-deficient HCT-116 cell lines.*

We and others have reported that a primary response of tumor cells to radiation is autophagy [213-216]. **Figures 2A** presents images of irradiated cells stained with acridine orange, which is indicative of autophagy while **Figures 2B** provides quantification of the extent of autophagy over a range of radiation doses. While the extent of autophagy was significantly greater in the Ligase IV deficient cells compared to parental cells at lower doses of radiation, essentially the entire cell population had entered a state of autophagy for both cell lines at the higher doses.

As senescence has been closely associated with autophagy in a number of studies [83-84], the induction of senescence by radiation was also monitored. Both cell lines demonstrated physiological markers of senescence such as granulation, flattening, and spreading as well as β-galactosidase staining, a hallmark of senescence (**Figure 2C**). In parallel with the findings relating to autophagy, senescence was more pronounced in the Ligase IV deficient cells compared to the Ligase IV proficient cells at the lower doses of radiation while higher doses yielded maximal senescent populations in both cell lines (**Figure 2D**).

Although radiation-induced autophagy, senescence and persistent H2AX phosphorylation were greater in the HCT116 Ligase IV-deficient cells than in Ligase IV-proficient cells at the lower doses of radiation, the fraction of cells showing autophagy and senescence, at any given level of γH2AX, was very similar for the two cell lines (**Supplementary Figures 1A and 1B**). **Supplementary Figure 1C** also indicates a direct correspondence between the extent of autophagy and senescence (as a function of the dose of radiation) in both HCT116 tumor cell lines. Thus, both senescence and autophagy correlate with, and are likely triggered by, persistent double-strand breaks.

### *4.3 The relationship between autophagy and senescence in irradiated cells.*

Our studies are indicative of a close correspondence between the induction of autophagy and senescence by radiation in both the Ligase IV deficient and the Ligase IV proficient cell lines (**Supplementary Figure 1C)**, which is also the case for oncogene and chemotherapy-induced autophagy and senescence [83-84]. To more rigorously investigate the potential association between autophagy and senescence in response to radiation, both cell lines were either pre-incubated with the pharmacological inhibitors of autophagy, chloroquine (5 μM) and bafilomycin (5 nM), for 3 h prior to irradiation, or infected with lentivirus to induce a knockdown of the essential autophagy factors ATG5 and ATG7. (**Supplementary Figure 2A**). **Supplementary Figures 2B and 2C** confirm that autophagy has been inhibited by chloroquine and bafilomycin in both cell lines based on the interference with radiation-induced degradation of p62/SQSTM1. Similarly, **Supplementary Figures 2D and 2E** confirm that autophagy has been inhibited by the genetic silencing approaches. **Figure 3** indicates that pharmacological and genetic inhibition of autophagy had no effect on the promotion of radiation-induced senescence in these cell lines, as

the extent of senescence was essentially identical in the absence and presence of functional autophagy, strongly indicating that autophagy and senescence in response to radiation are dissociable. This has, in fact, proven to be the case for both oncogene-induced senescence and senescence induced by doxorubicin [83-84].

### *4.5 Cells induced to undergo autophagy/senescence by irradiation retain the capacity for proliferative recovery and are capable of repairing persistent double strand breaks (DSBs).*

We have shown proliferative recovery after induction of senescence by radiation as well as doxorubicin in breast tumor cells [83, 96, 213, 216]. The HCT116 Ligase IV proficient and HCT116 Ligase IV deficient cells were exposed to radiation doses of 6 Gy and 3 Gy, respectively; cells were sorted based on staining with the senescence marker, $C_{12}$FDG, 96 hours post-radiation by flow cytometry (**Figure 4A and Figure 4B)**. Both sub-populations (i.e., positively stained and negatively stained cells) were replated at subconfluent density. **Figure 4C** confirms that recovery occurs after radiation-induced autophagy and senescence in both the HCT116 Ligase IV-proficient and Ligase IV-deficient cells. These findings are consistent with studies where proliferative recovery was observed after irradiation of MCF-7 breast tumor cells [96, 213].

The capacity for proliferative recovery suggests that DNA repair is likely to be functional in the autophagy/senescent cells. To address this question, HCT116 cells were exposed to a dose of radiation (8 Gy) that induces ~75% of both autophagy and senescence; the cells were then allowed to undergo repair for 4 days, followed by re-irradiation with 4 Gy. Repair intensity were measured after 30 min, 3 h, 24 h, and 4 days based on γH2AX cytometry. Four days after the first dose of radiation (8 Gy), the level of γH2AX remained high. At the second dose of radiation (4 Gy), the intensity of γH2AX was further elevated for 3 h. However, the intensity of γH2AX was reduced by 24 and 96 h after the second dose, suggesting that, despite the persistence of initial

DNA damage, these cells were still generally proficient in DNA repair capacity (**Figure 4D, left panel**). Similarly, HCT116 Ligase IV (-/-) were treated initially with 4 Gy, a dose that induces ~75% of senescence and autophagy, followed four days late by 2 Gy of radiation. **Figure 4D, right panel** indicates that even these ostensibly repair-incompetent cells show the capacity to repair the newly induced DNA damage.

### *4.6 Radiosensitization by PARP inhibitors correlates with increased autophagy and senescence, but not apoptosis.*

There has been a great deal of interest in utilizing DNA repair inhibitors in combination with chemotherapeutic drugs and radiation to enhance the efficacy of cancer therapy. In the context of this work, it has been reported that radiation sensitization by PARP inhibitors is accompanied by increased senescence [206, 217]. Given the evidence for correspondence between autophagy and senescence in the current work, we proceeded to investigate whether sensitization by PARP inhibitors could be mediated through the promotion of autophagy as well as senescence. Two different PARP inhibitors, AZD-2281 (Olaparib) and MK-4827 (Niraparib), were utilized to investigate whether the PARP inhibitors could sensitize both Ligase IV deficient cells and Ligase IV proficient HCT116 cells to radiation. **Figure 5A** shows that PARP inhibitors conferred profound radiation sensitization in the Ligase IV-proficient HCT116 cells. However, while Ligase IV deficient cells were also sensitized, the degree of sensitization was clearly less than in the Ligase IV proficient cells. Temporal response data **(Figure 5B)** also showed a more pronounced radiosensitization in Ligase IV proficient than in the Ligase IVdeficient cell lines when using equitoxic doses of radiation. As would have been expected, this sensitization was associated with an increase in DNA damage based on the intensity γH2AX formation **(Figure 5C)** and the Comet assay **(Supplementary Figures 3A and 3B)**.

Sensitization to radiation by the PARP inhibitors is also associated with an increase in senescence. Quantification of the intensity of β-galactosidase staining by flow cytometry indicated that between 55-60% of the Ligase IV proficient HCT116 cells had entered a state of senescence when the PARP inhibitors were used in combination with radiation, whereas radiation alone induced ~20% senescence (**Figures 5D, left panel, and Supplementary Figure 3C).** Similarly, the HCT116 Ligase IV deficient cells showed an increase in the senescent population from less than 20% to between 45-55% when the PARP inhibitor was administered along with radiation **(Figure 5D, right panel, and Supplementary Figure 3D)**. Consistent with the increased senescence, cell cycle analysis results demonstrated that ~ 45% of the population in both cell lines underwent growth arrest at the G2/M phase when cells were treated with the combination compared to ~ 20% when cells were exposed to radiation alone **(Supplementary Figure 3E)**.

The combination of AZD-2281 or MK-4827 with radiation also resulted in increased autophagy. Quantification of the intensity of autophagy by flow cytometry showed an increase in the number of autophagic cells to 70-80% of the population for the combination treatment in the Ligase IV-proficient cells, whereas radiation alone promoted approximately 30% autophagy **(Figure 5E, left panel, and Supplementary Figures 4A and 4B)**. Similarly, the HCT116 Ligase IV-deficient cells show an increase in the autophagic population to 75% when the PARP inhibitor was administered along with radiation compared to 35% when exposed to radiation alone **(Figure 5E, right panel and Supplementary Figure 4C)**. Overall, the PARP inhibitors appear to produce comparable enhancement of H2AX phosphorylation, autophagy and senescence in wild-type and Ligase IV-deficient cells, but less radiosensitization of Ligase IV-deficient cells, particularly as measured by clonogenic survival.

Although co-treatment with PARP inhibitors enhanced the radio-sensitivity of HCT116 cells and HCT116 Ligase IV-deficient cells, it was critical to determine whether the cells would retain their proliferative recovery after the exposure to the combination treatment by monitoring cell viability over an extended period post treatment. **Figure 6** demonstrates that both cell lines recovered proliferative capacity on days 10, 15, and 20 post-treatment both in the case of radiation alone as well as when radiation was combined with the PARP inhibitors.

### *4.7 Lack of involvement of apoptosis in sensitization by PARP inhibitors in HCT116 cells*

Use of PARP inhibitors has generally been shown to radiosensitize cells through the induction of senescence, but not apoptosis [206, 217-218]. To rule out the potential involvement of apoptosis in radiation sensitization, apoptotic cell death was monitored by Annexin V staining. **Supplementary Figure 5A** indicates that apoptosis is unlikely to be involved in sensitization of both cell lines to radiation by PARP inhibition as apoptosis was minimal and not increased by the PARP inhibitors. The minimal involvement of apoptosis in radiosensitization by PARP inhibition was confirmed by assessment of apoptosis 72 h post-treatment using the TUNEL assay **(Supplementary Figures 5B and 5C).** To further confirm these results, irradiated HCT116 cells were treated using the Pan Caspase Inhibitor Z-VAD-FMK (10 μM) and viable cell numbers were monitored over a period of five days. **Supplementary Figure 5D** shows that interference with apoptosis via inhibition of caspases did not interfere with radio-sensitization by the PARP inhibitors, indicating that apoptosis does not appear to be involved in mediating the observed effects. Consistent with these observations, cell cycle analysis demonstrated that administering PARP inhibitors along with radiation does not increase the sub-G1 population (data not shown), confirming that apoptosis is not occurring in cells exposed to radiation + PARP inhibitors.

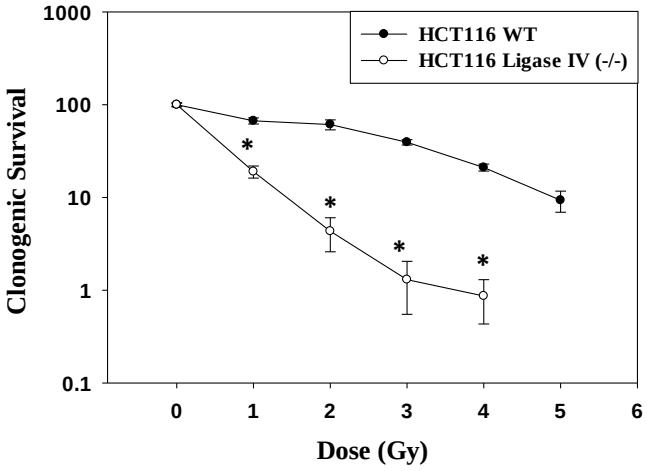
### *4.8 Effects of autophagy inhibition on radiosensitization by PARP inhibition.*

The studies presented above clearly show that autophagy was increased in association with senescence when PARP inhibitors are used in combination with radiation. Several studies, including our own, have demonstrated that autophagy can act as a cytotoxic or cytostatic process through which cells die or undergo prolonged growth arrest [213, 216, 219]. To address whether inhibition of autophagy would interfere with the radiosensitization by PARP inhibitors, HCT116 cell lines where autophagy was genetically silenced or pretreated with choroquine were exposed to radiation in the absence and presence of the PARP inhibitors. **Figures 7A, 7B, and Supplementary Figure 6** demonstrate that genetic interference with autophagy does not rescue either of the cell lines from radiosensitization by PARP inhibition, indicating that radiosensitization does not occur via the promotion of autophagy.

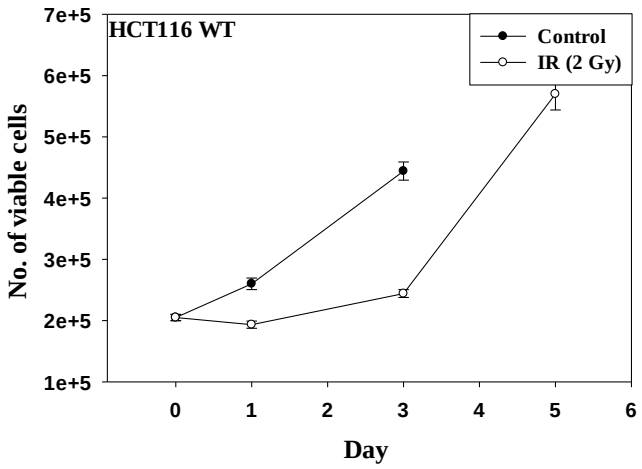
## *Figures and Legends*

**Figure 1**

**A.**




**B.**



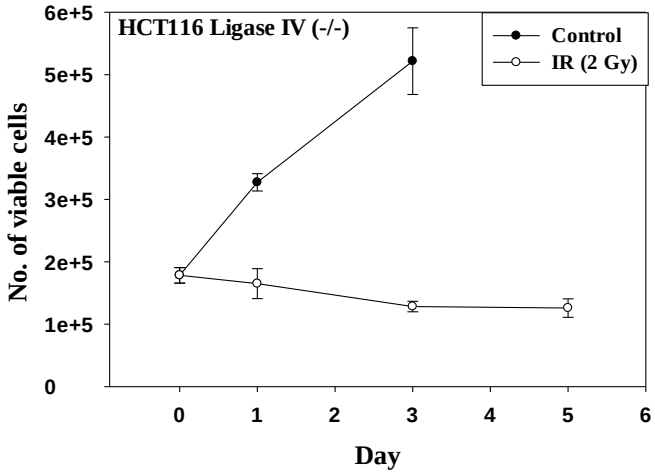
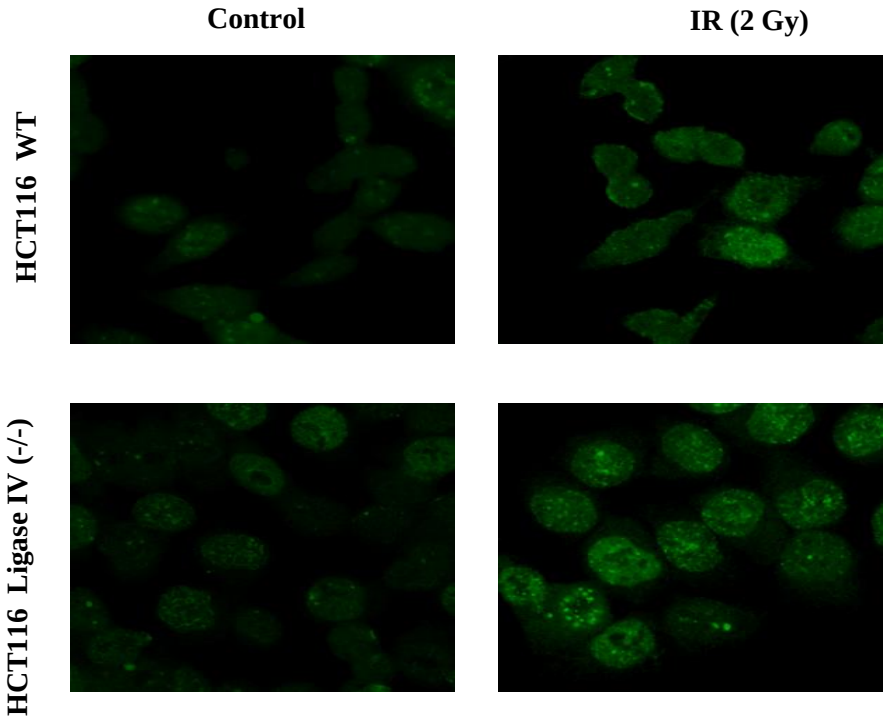



**Figure 1**

**C.**


Control
IR (2 Gy)
HCT116 WT
HCT116 Ligase IV (-/-)

**D.**

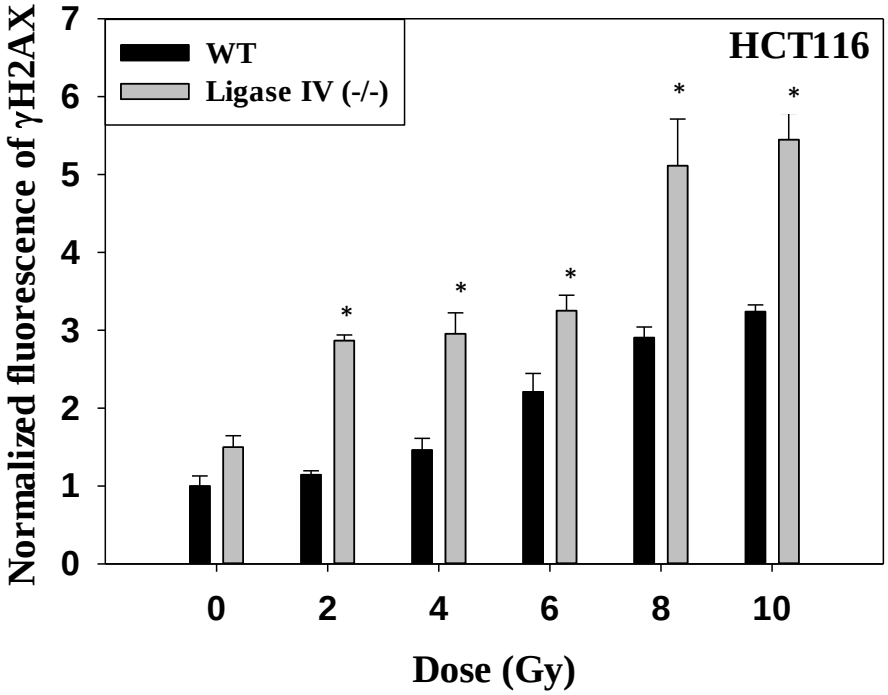
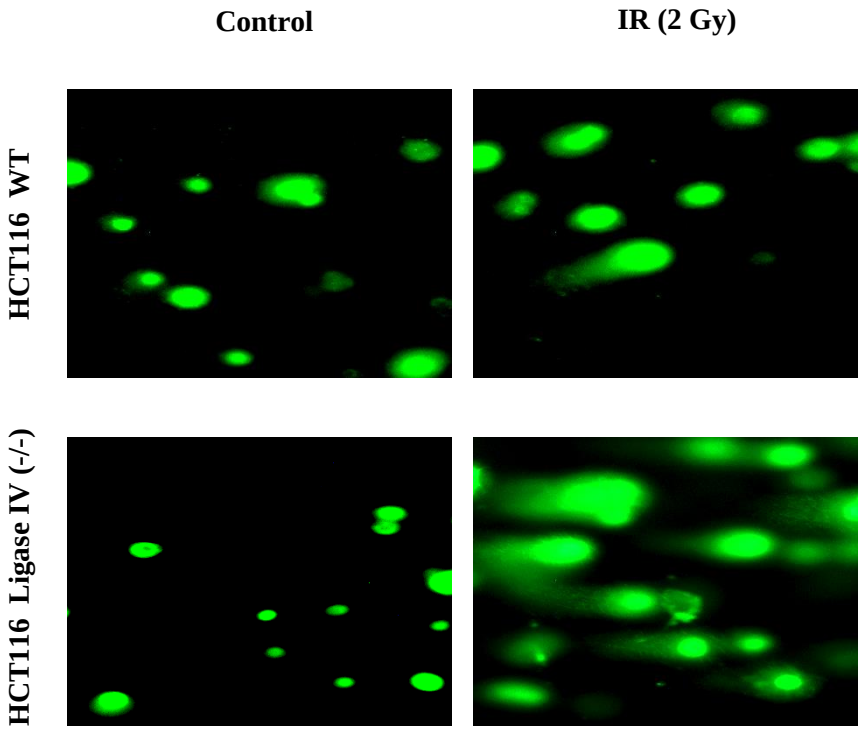

HCT116
WT
Ligase IV (-/-)
Normalized fluorescence of γH2AX
Dose (Gy)
0
1
2
3
4
5
6
7
0
2
4
6
8
10
*

**Figure 1**

**E.**


Control
IR (2 Gy)
HCT116 WT
HCT116 Ligase IV (-/-)

**F.**

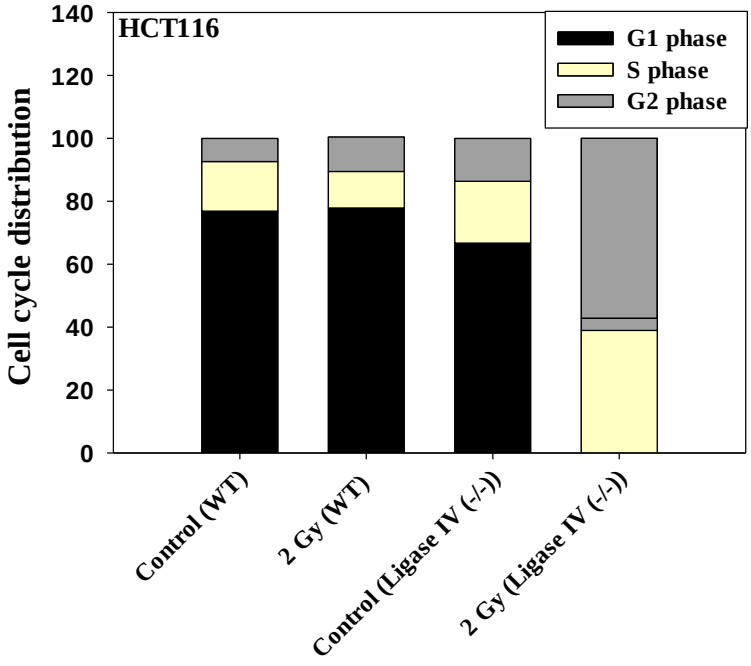
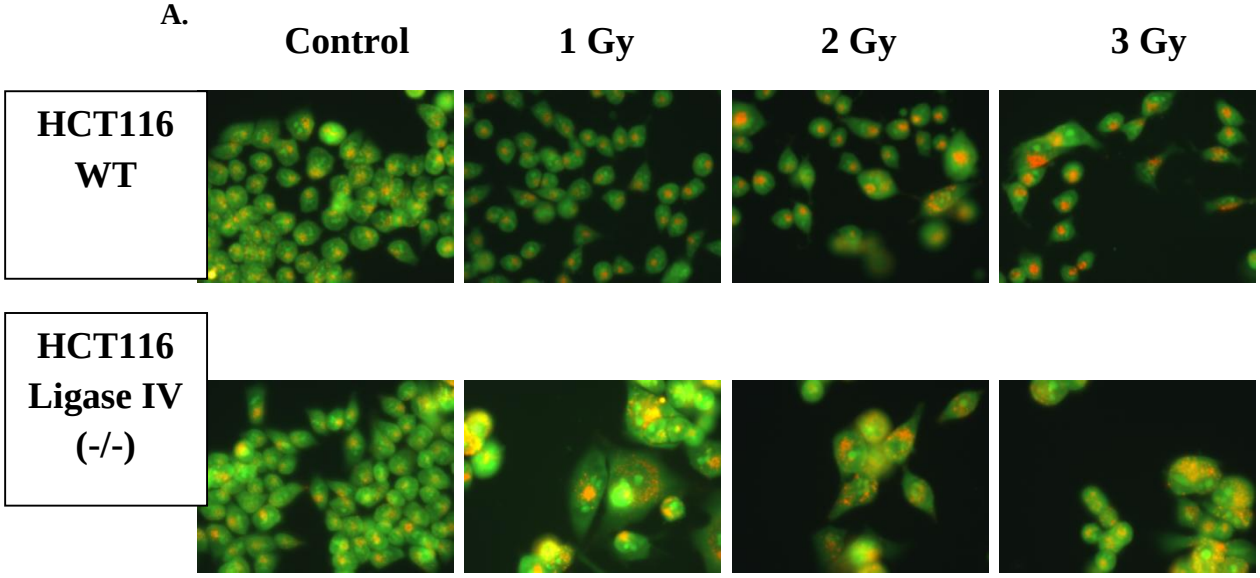

HCT116
G1 phase
S phase
G2 phase
Cell cycle distribution
140
120
100
80
60
40
20
0
Control (WT)
2 Gy (WT)
Control (Ligase IV (-/-))
2 Gy (Ligase IV (-/-))

**Figure 1. Radiation responses of DNA-repair-proficient and Ligase IV deficient HCT116 cell lines.** **A.** Radiosensitivity of HCT116 wt and HCT116 Ligase IV-deficient cells as determined by clonogenic survival (n=3). **B.** Impact of radiation on cell growth. Cells were exposed to 2 Gy of radiation and the number of viable cells was determined on days 0, 1, 3, and 5 (n=3). Graphs represent pooled data from three replicate experiments. **C.** Confocal microscope imaging of γH2AX foci formation at 2 Gy irradiation at 72 h post-treatment. **D.** Mean intensity of γH2AX as determined by flow cytometry 96 hr post treatment (n=3). **E.** Comet assay. Fluorescent microscope imaging of both cell lines 72 hours post treatment with 2 Gy. **F.** Cell cycle analysis after exposure of HCT116 Ligase IV (-/-) colon cancer cells to 2 Gy of irradiation at 72 hr post-treatment.

**Figure 2**

**A.**


Control
1 Gy
2 Gy
3 Gy
HCT116 WT
HCT116 Ligase IV (-/-)

**B.**

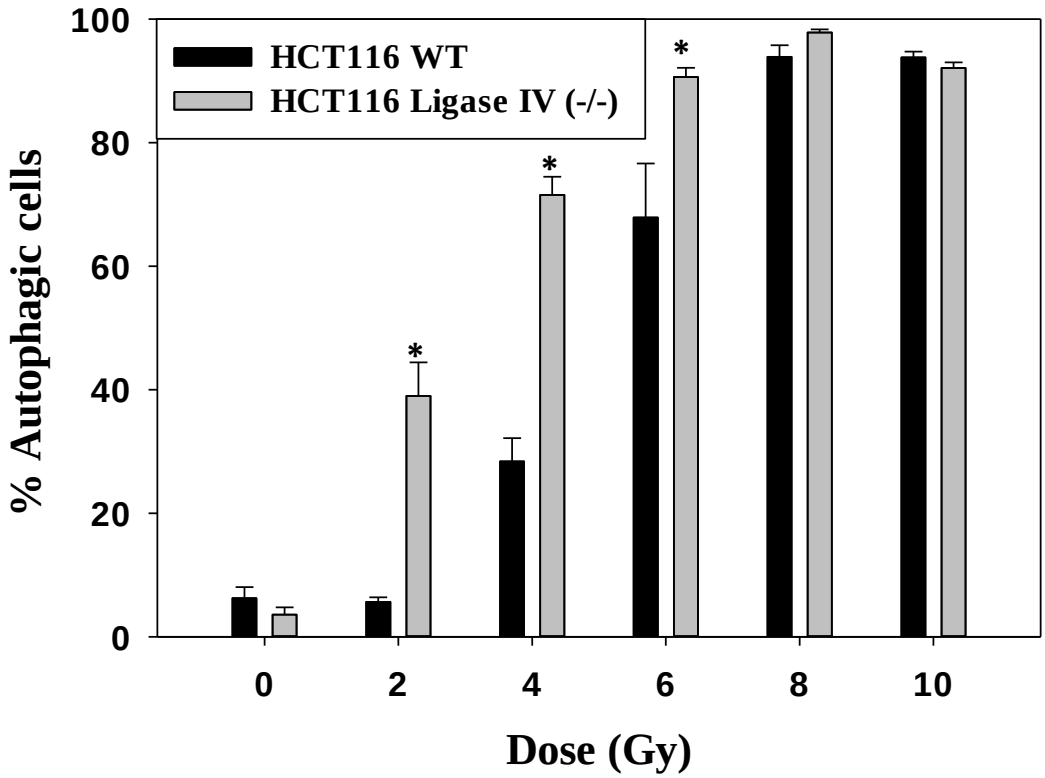
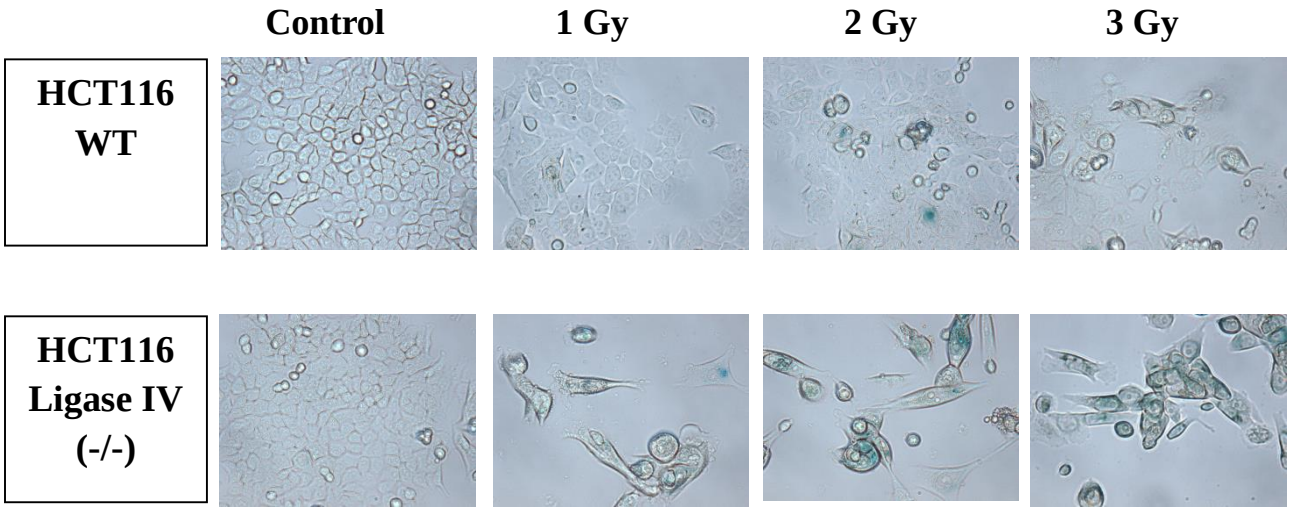

HCT116 WT
HCT116 Ligase IV (-/-)
% Autophagic cells
Dose (Gy)
0
20
40
60
80
100
0
2
4
6
8
10
*
*
*

**Figure 2**

**2C.**




**2D.**

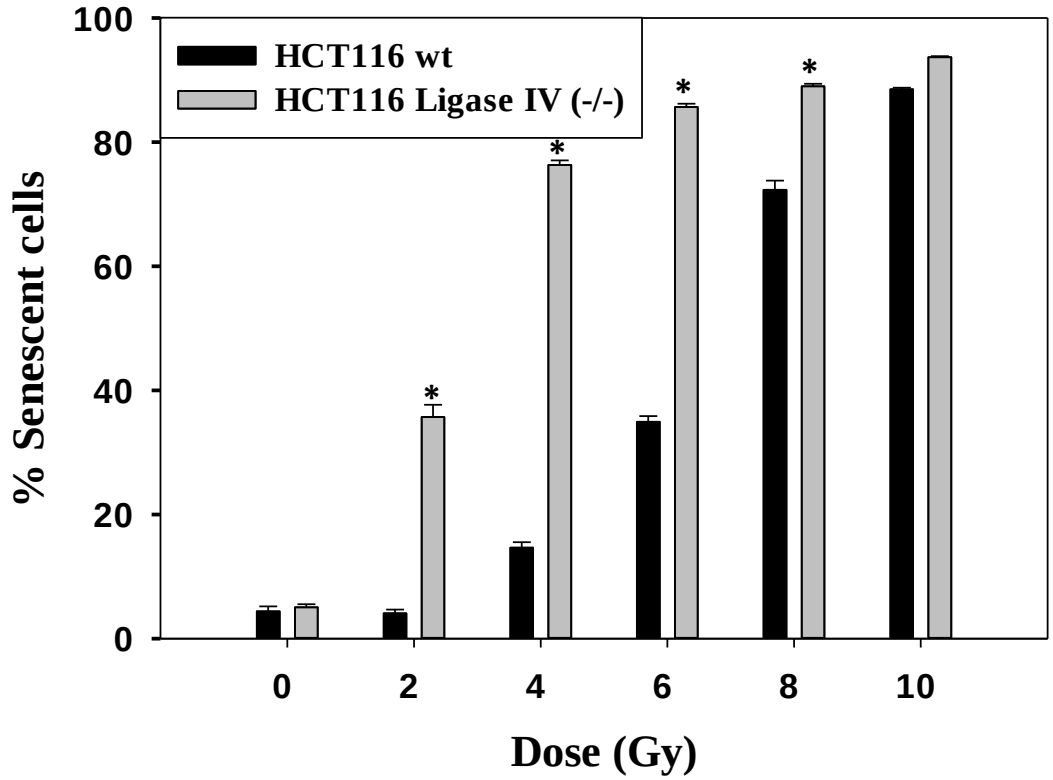
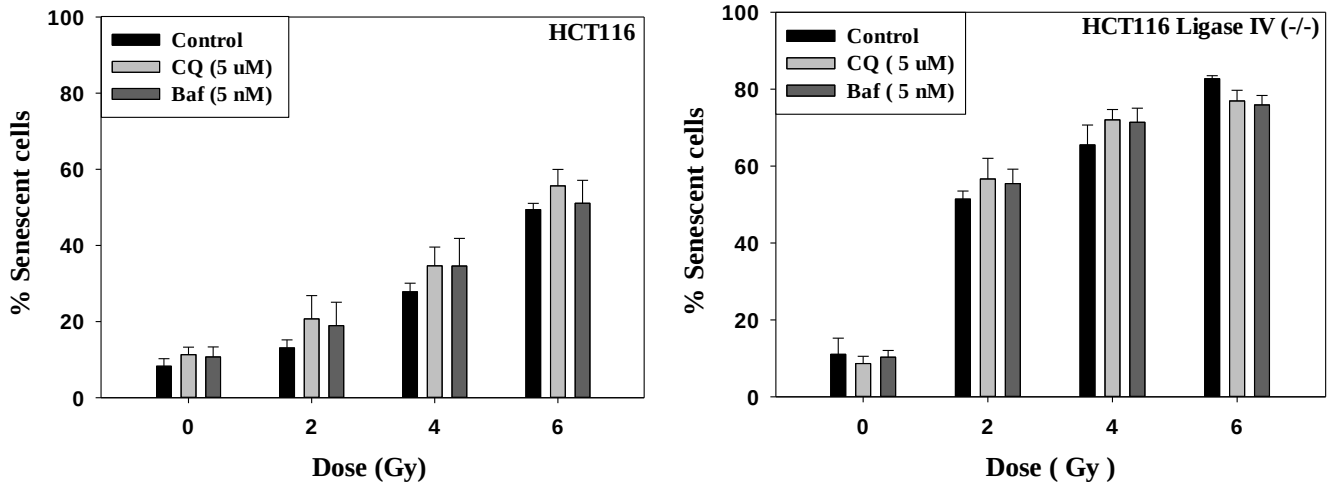


**Figure 2. Promotion of autophagy and senescence by radiation in HCT116 cells. A.** Acridine orange staining of HCT116 wt and HCT116 Ligase IV-deficient cells 96 hrs post treatment. Images shown are representative of three replicate studies. **B.** Quantification of autophagy by acridine orange flow cytometry 96 hr post treatment. Error bars represent standard error (* $p<0.05$ compared to the correspondent dose of HCT116 wt) (n=3). **C.** Promotion of senescence based on βgalactosidase staining. **D.** Quantification of β-galactosidase by flow cytometry at 96 hr (n=3). Error bars represent standard error (* $p<0.05$ compared to HCT116 wt cells as the same dose) (n=3).

**Figure 3**

**A.**


HCT116
Control
CQ (5 uM)
Baf (5 nM)
% Senescent cells
Dose (Gy)
HCT116 Ligase IV (-/-)
Control
CQ ( 5 uM)
Baf ( 5 nM)
% Senescent cells
Dose ( Gy )

**B.**

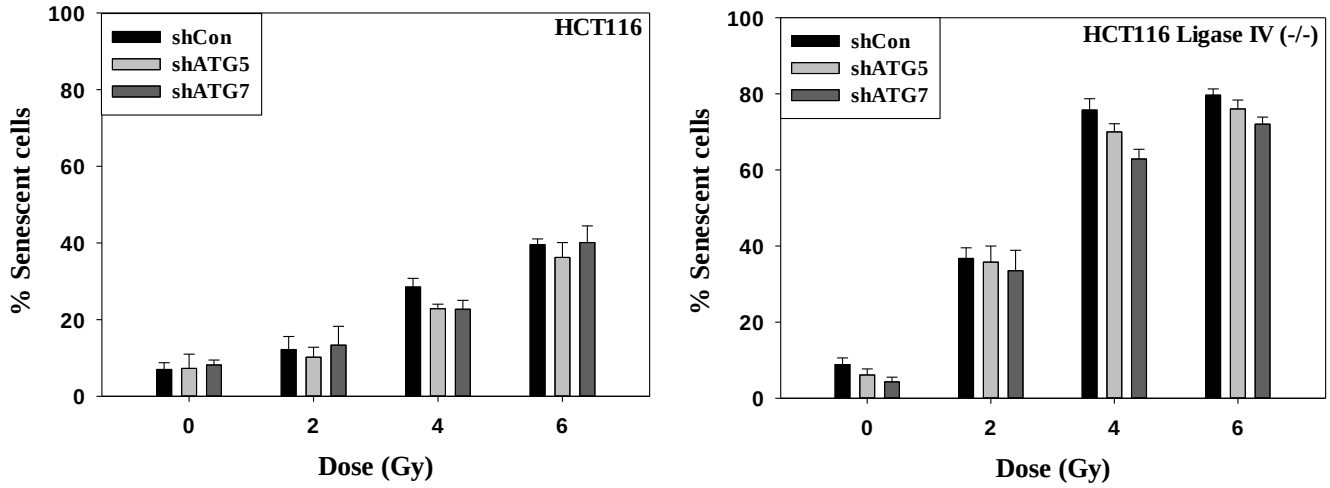
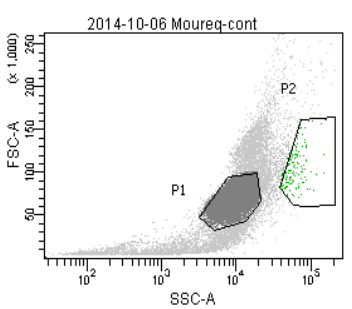

HCT116
shCon
shATG5
shATG7
% Senescent cells
Dose (Gy)
HCT116 Ligase IV (-/-)
shCon
shATG5
shATG7
% Senescent cells
Dose (Gy)

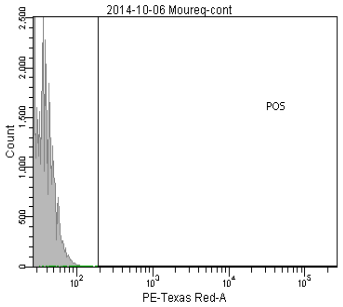
**Figure 3. Inhibition of autophagy fails to suppress radiation-induced senescence. A.** HCT116 cells and HCT116 Ligase IV-deficient cells were pretreated with chloroquine (5 uM) or Bafilomycin (5 nM) and exposed to the indicated doses of radiation. Senescence was assessed after 96 h by flow cytometry (n=3). **B.** HCT116 cells and HCT116 Ligase IV-deficient cells with silencing of ATG5 or ATG7 were exposed to the indicated doses of radiation and senescence was assessed after 96 h by flow cytometry (n=3).

**Figure 4**

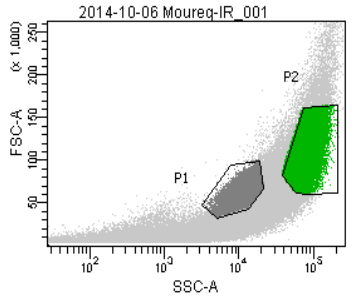
**A.**

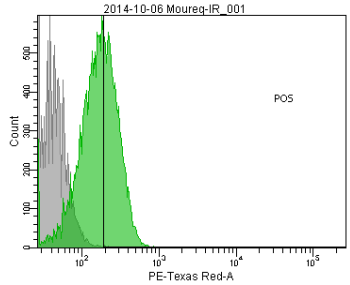
**Control**




**6 Gy**



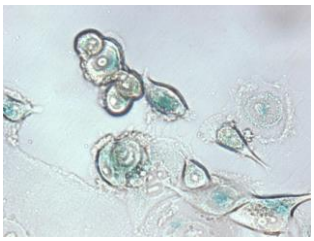

**B.**

**Negatively-stained cells**

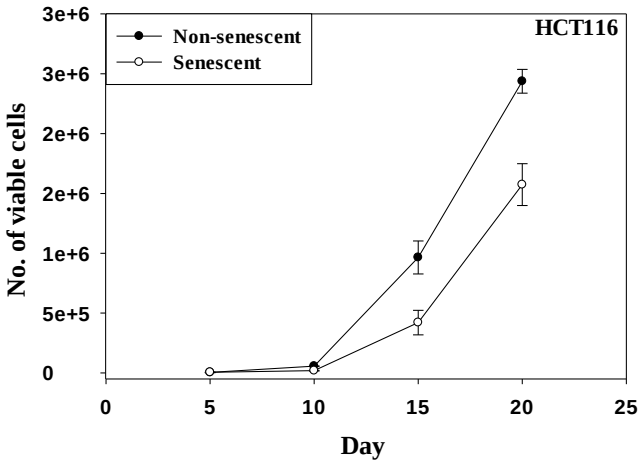
**(Non-senescent)**

**Positively-stained cells**

**(Senescent)**



**C.**



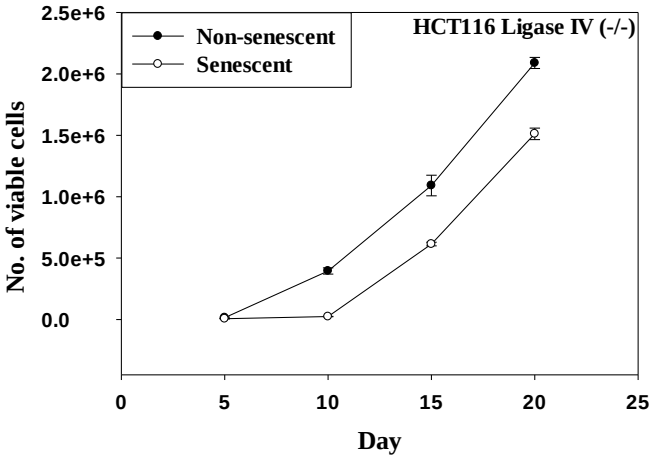



**Figure 4**

**D.**

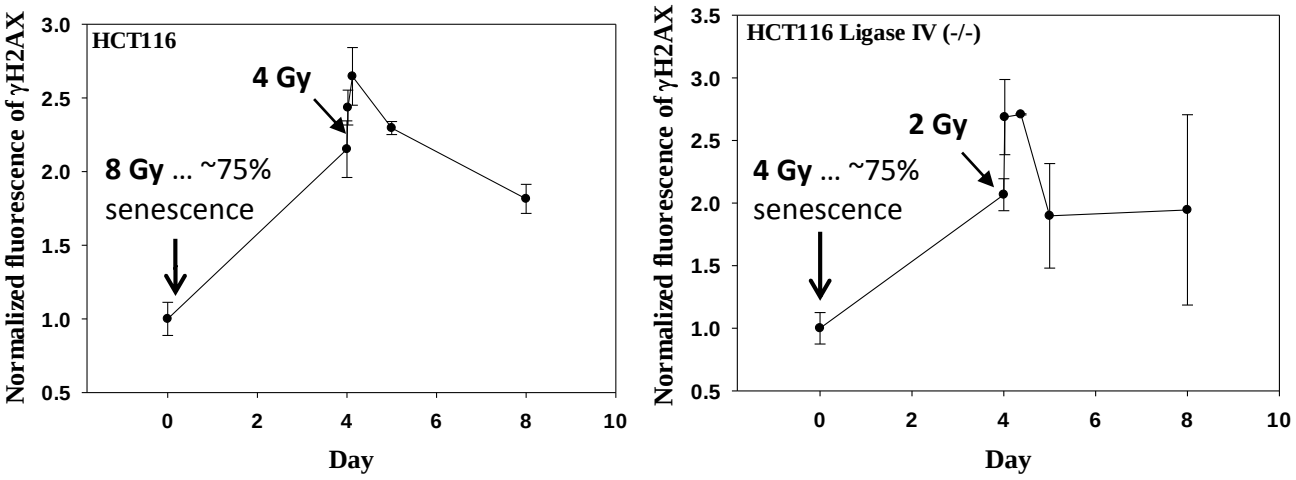
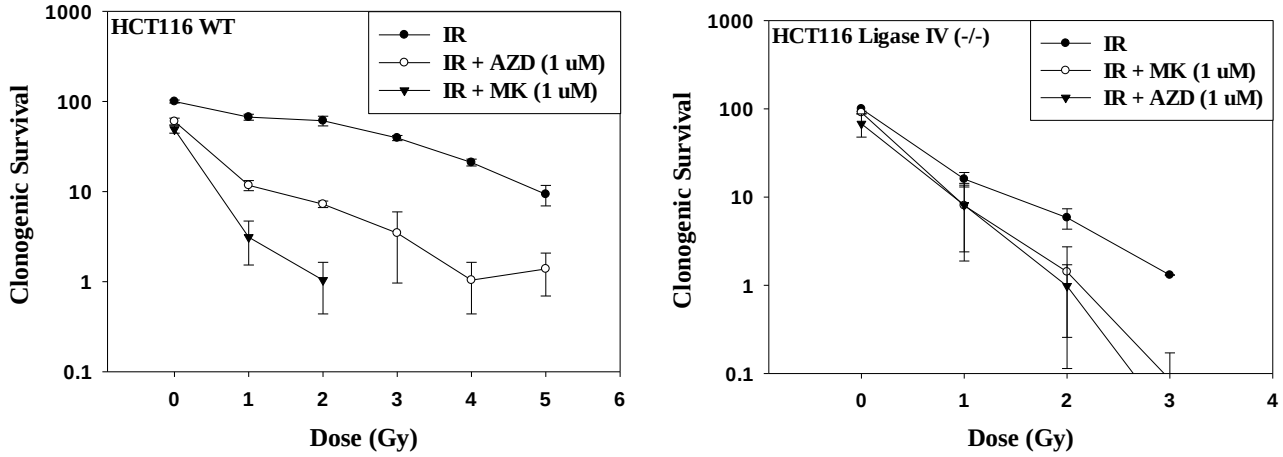


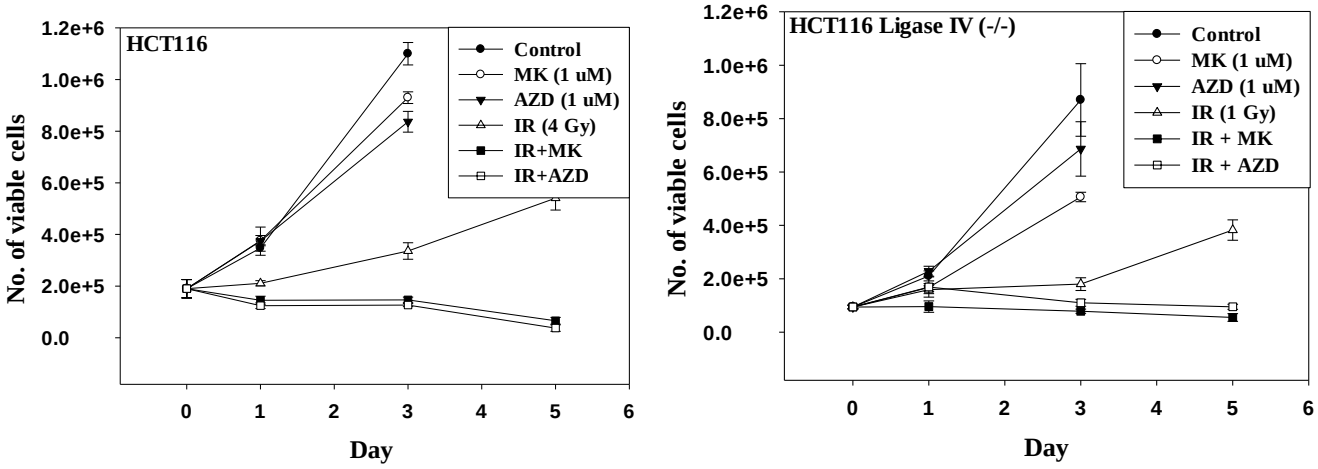

**Figure 4. DNA repair capacity in senescent cells.** HCT116 cells were stained with β-galactosidase substrate (C12-FDG) 96 hours post-treatment, and subjected for sorting by flow cytometry machine. **A.** An example of raw data obtained from flow cytometry for both conditions. Upper demonstrating the distribution of HCT116 WT and HCT116 Ligase IV-deficient cells when were exposed to 6 Gy and 3 Gy of radiation, respectively. **B.** HCT116 cells were stained with β-galactosidase substrate ($C_{12}$FDG) 96 hours post-treatment, and subjected for sorting by flow cytometry at excitation/emission wavelengths of 490⁄514 nm. Left panels show gating of cells based on forward scatter vs side scatter; right panels show gating applied to data from 488-610/20 channel to detect β-Gal fluorescence. Both subpopulations were stained with β-galactosidase to ensure that cells were successfully sorted according to size and fluorescence. **C.** Senescent and non-senescent sub-populations were replated separately in 6-well plates, and viable cell number was monitored at the indicated time points by trypan blue exclusion. **D.** HCT116 cells and HCT116 Ligase IV (-/-) cells were exposed to 8 Gy or 4 Gy radiation followed by a 96 h interval for DNA repair and subsequent re-exposure to 4 Gy or 2 Gy radiation, respectively. Intensity of γH2AX fluorescence was measured by flow cytometry at the indicated time points (n=3).

**Figure 5**

**A.**


HCT116 WT
IR
IR + AZD (1 uM)
IR + MK (1 uM)
Clonogenic Survival
Dose (Gy)
HCT116 Ligase IV (-/-)
IR
IR + MK (1 uM)
IR + AZD (1 uM)
Clonogenic Survival
Dose (Gy)

**B.**


HCT116
Control
MK (1 uM)
AZD (1 uM)
IR (4 Gy)
IR+MK
IR+AZD
No. of viable cells
Day
HCT116 Ligase IV (-/-)
Control
MK (1 uM)
AZD (1 uM)
IR (1 Gy)
IR + MK
IR + AZD
No. of viable cells
Day

**C.**

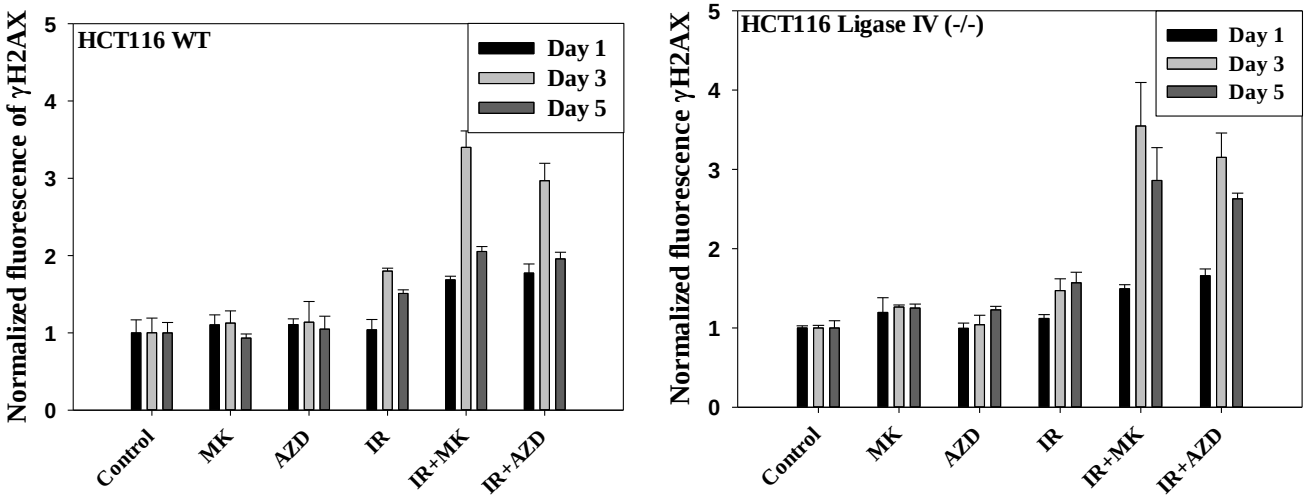
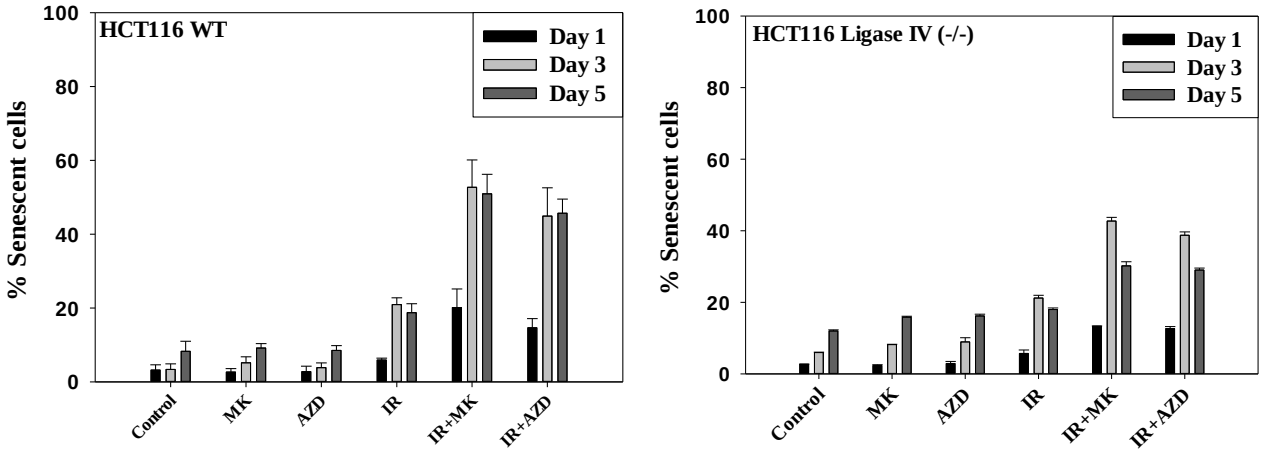

HCT116 WT
Day 1
Day 3
Day 5
Normalized fluorescence of γH2AX
Control
MK
AZD
IR
IR+MK
IR+AZD
HCT116 Ligase IV (-/-)
Day 1
Day 3
Day 5
Normalized fluorescence γH2AX
Control
MK
AZD
IR
IR+MK
IR+AZD

**Figure 5**

**D.**




**E.**

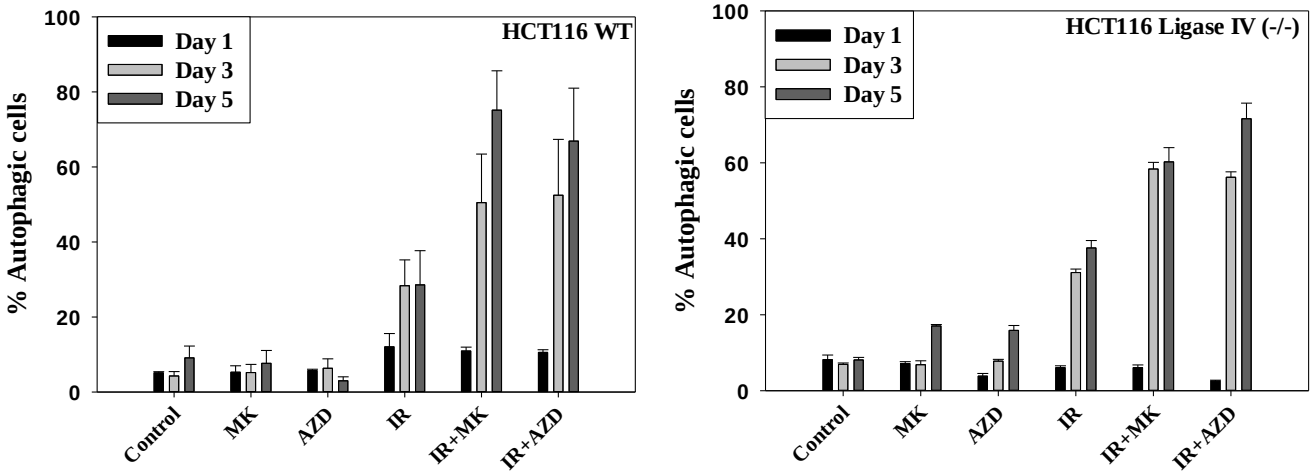



**Figure 5. Cell survival, DNA damage, autophagy and senescence in irradiated cells exposed to PARP inhibitors.** HCT116 WT and HCT116 Ligase IVdeficient cells were incubated with AZD-2281 (1 µM) or MK-4827 (1 µM) for 3 h before irradiation. **A.** Number of colonies was determined after 14 days (n=3). **B.** Number of viable cells was counted at the indicated time points (n=5). **C.** γH2AX intensity was measured at the indicated time points by flow cytometry in both cell lines (n=3). **D.** Quantification of senescence by flow cytometry at the indicated time points (n=3). **E.** Quantification of autophagy by flow cytometry at the indicated time points (n=3).

**Figure 6**

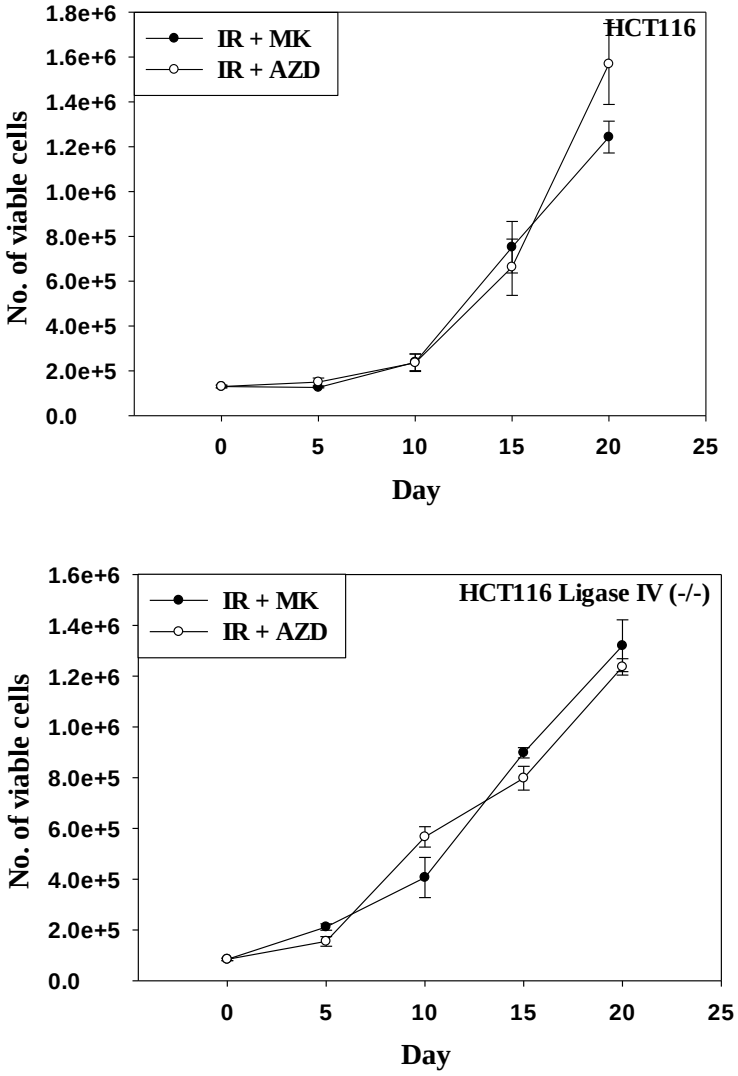
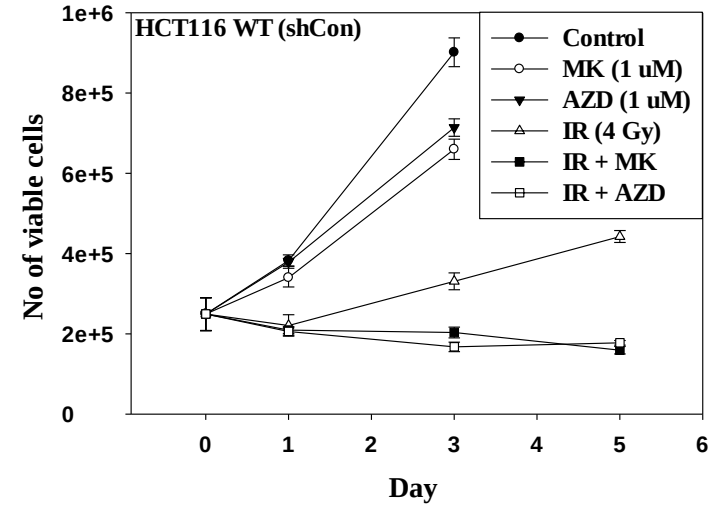
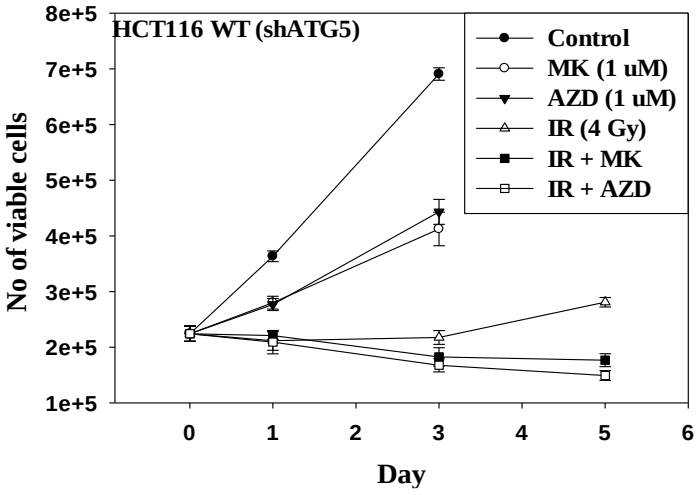


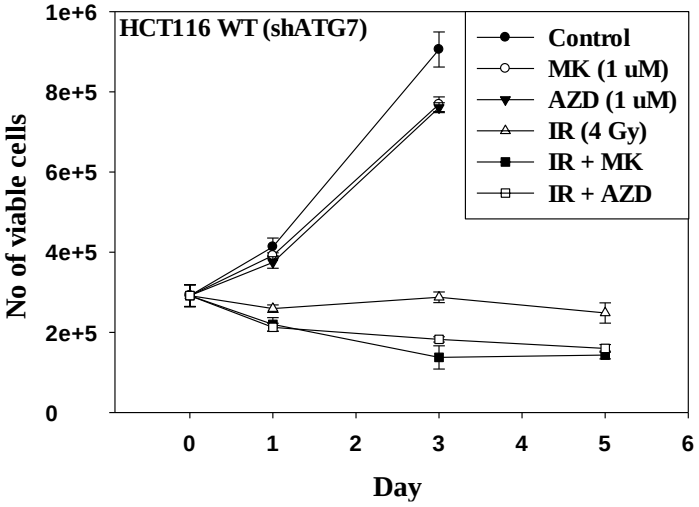

**Figure 6. Proliferative recovery after radiation and PARP inhibition.** HCT116 wt and HCT116 Ligase IV-deficient cells were incubated with AZD-2281 (1 μM) or MK-4827 (1 μM) for 3 h before exposure to radiation 4 Gy and 1 Gy, respectively. Viable cell number was monitored over a period of 20 days.

**Figure 7**

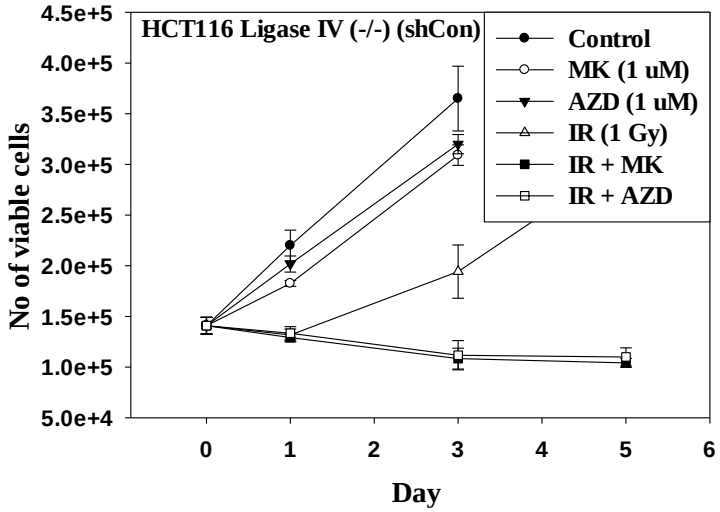
**A.**

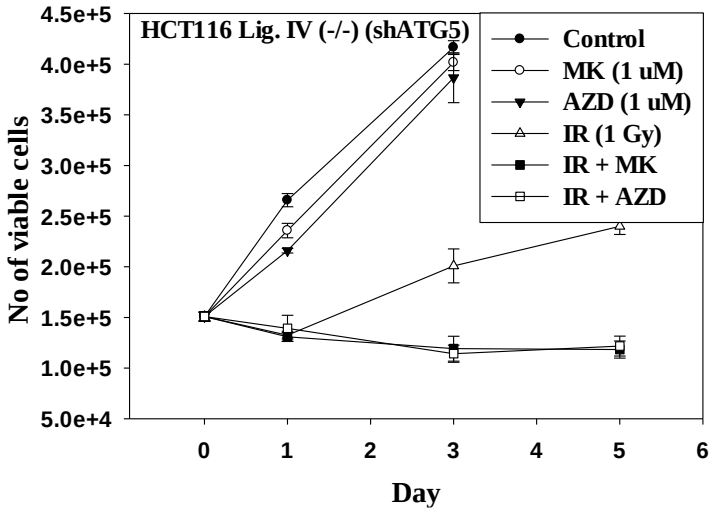

HCT116 WT (shCon)
Control
MK (1 uM)
AZD (1 uM)
IR (4 Gy)
IR + MK
IR + AZD
No of viable cells
Day
0
2e+5
4e+5
6e+5
8e+5
1e+6
0
1
2
3
4
5
6


HCT116 WT (shATG5)
Control
MK (1 uM)
AZD (1 uM)
IR (4 Gy)
IR + MK
IR + AZD
No of viable cells
Day
1e+5
2e+5
3e+5
4e+5
5e+5
6e+5
7e+5
8e+5
0
1
2
3
4
5
6


HCT116 WT (shATG7)
Control
MK (1 uM)
AZD (1 uM)
IR (4 Gy)
IR + MK
IR + AZD
No of viable cells
Day
0
2e+5
4e+5
6e+5
8e+5
1e+6
0
1
2
3
4
5
6

**B.**


HCT116 Ligase IV (-/-) (shCon)
Control
MK (1 uM)
AZD (1 uM)
IR (1 Gy)
IR + MK
IR + AZD
No of viable cells
4.5e+5
4.0e+5
3.5e+5
3.0e+5
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0
1
2
3
4
5
6
Day


HCT116 Lig. IV (-/-) (shATG5)
Control
MK (1 uM)
AZD (1 uM)
IR (1 Gy)
IR + MK
IR + AZD
No of viable cells
4.5e+5
4.0e+5
3.5e+5
3.0e+5
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0
1
2
3
4
5
6
Day

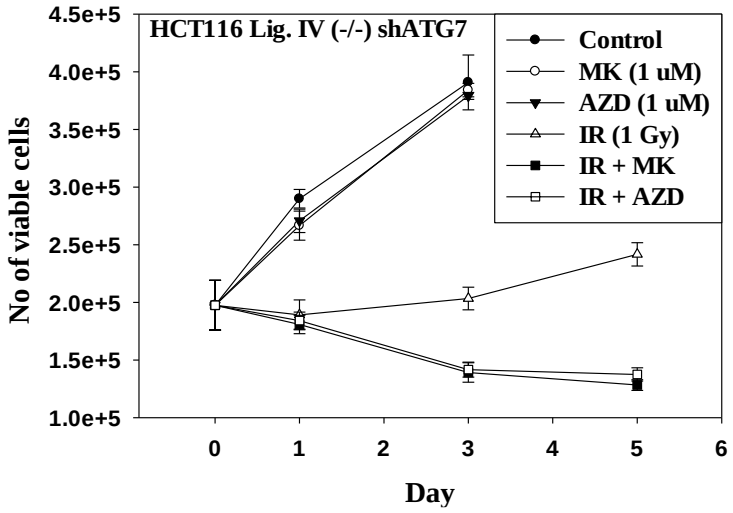
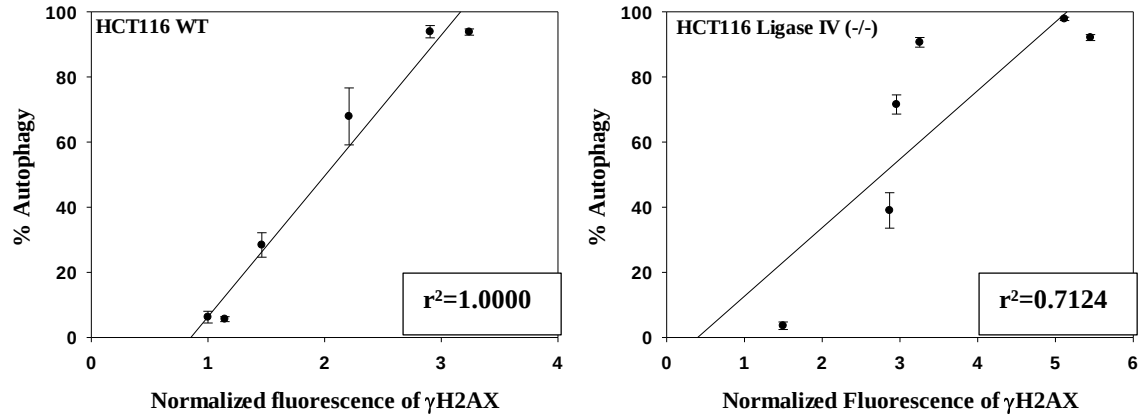

HCT116 Lig. IV (-/-) shATG7
Control
MK (1 uM)
AZD (1 uM)
IR (1 Gy)
IR + MK
IR + AZD
No of viable cells
4.5e+5
4.0e+5
3.5e+5
3.0e+5
2.5e+5
2.0e+5
1.5e+5
1.0e+5
0
1
2
3
4
5
6
Day

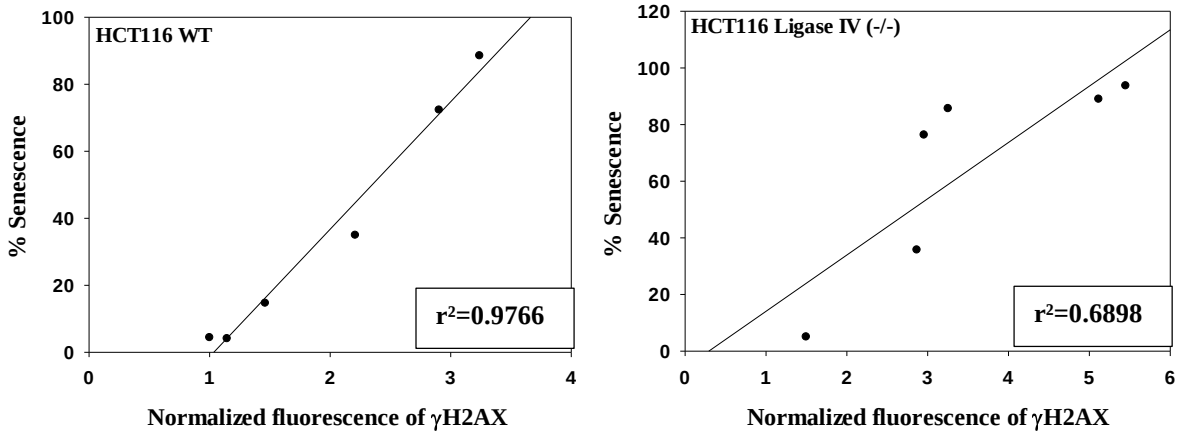
**Figure 7. Inhibition of autophagy does not alter sensitization to radiation by PARP inhibition.** Autophagy regulated genes were silenced in the HCT116 wt and HCT116 Ligase IV deficient cell lines using short hairpin RNA (shRNA) for *ATG5* and *ATG7*. **A.** Autophagy-deficient and autophagy-proficient HCT116 cells were irradiated with and without exposure to the PARP inhibitors. **B.** Autophagy-deficient and autophagy-proficient HCT116 Ligase IV-deficient cells were irradiated with and without exposure to the PARP inhibitors.

**Supplementary Figure 1**

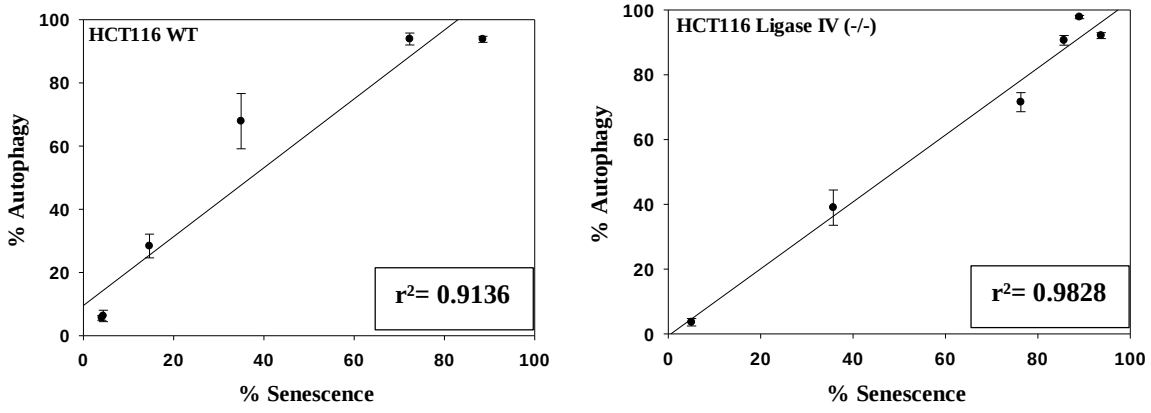
**A.**




**B.**

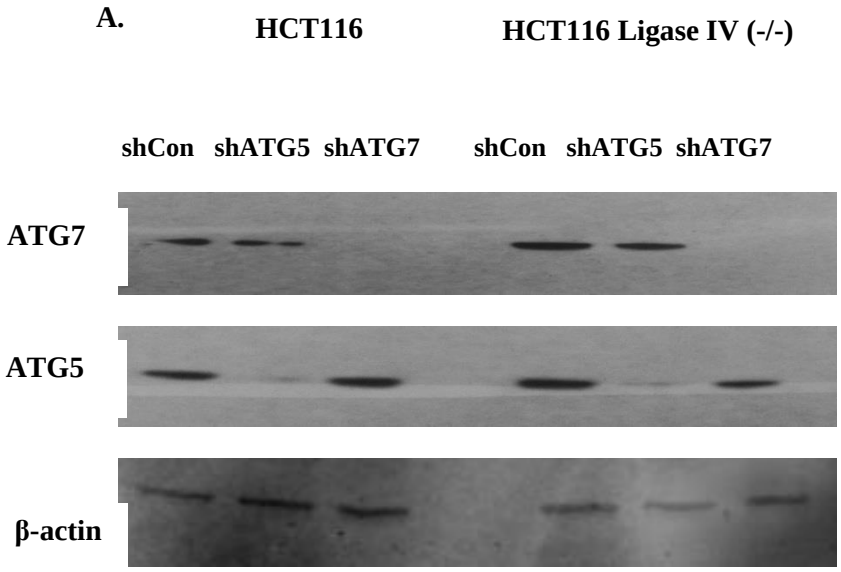


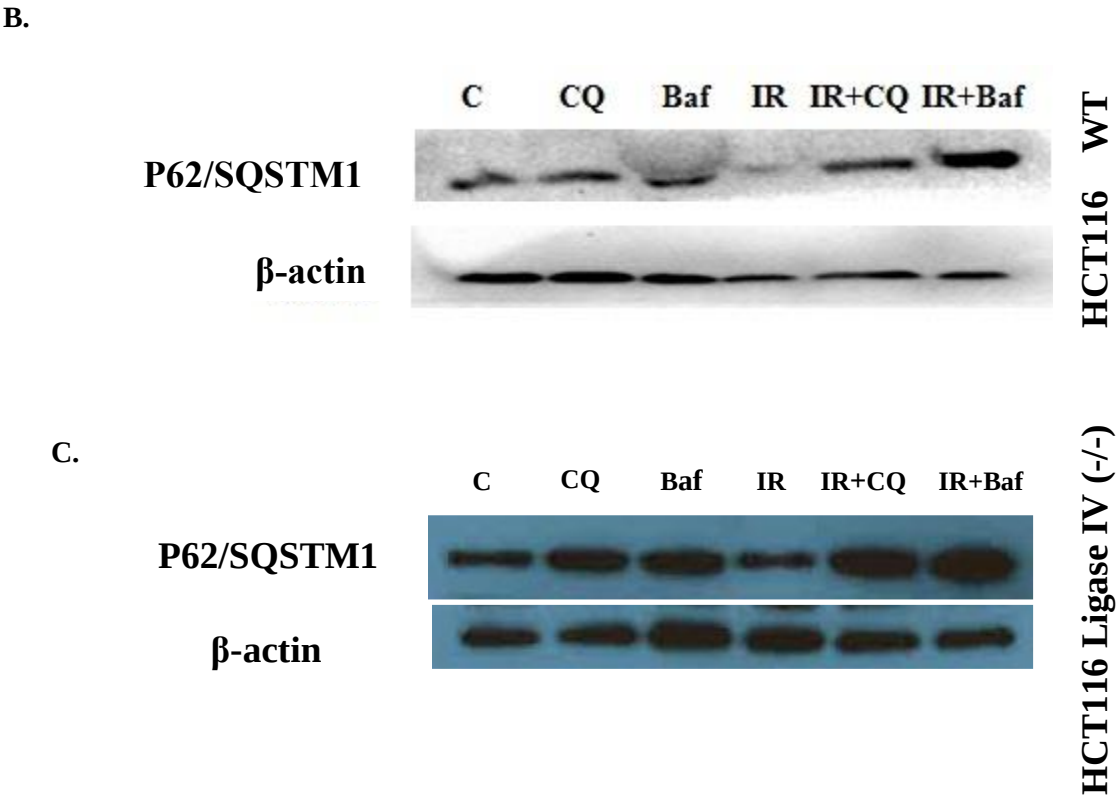

**C.**

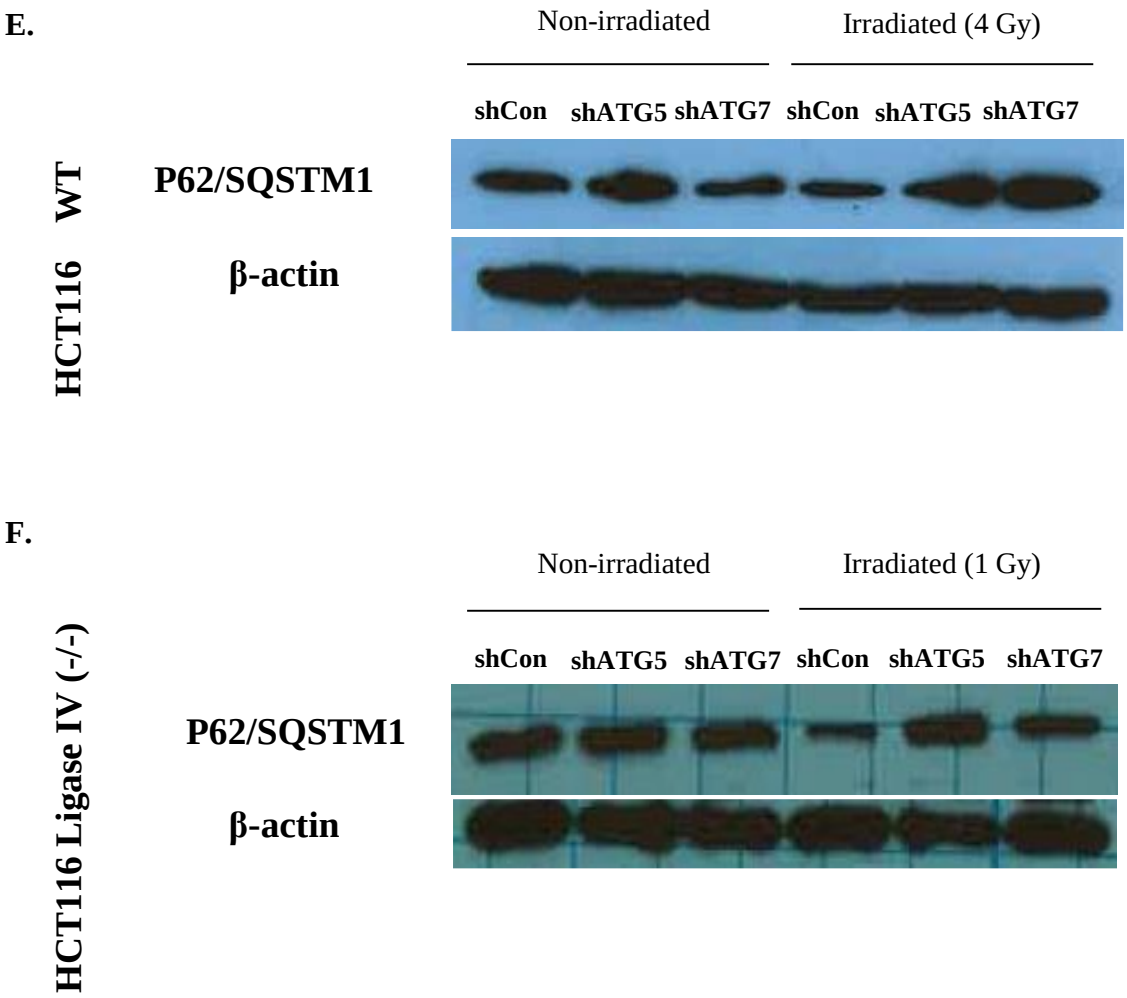


**Supplementary Figure 1. Relationship between autophagy, senescence, and DNA damage.** intensity of γH2AX staining in Ligase IV-proficient and -deficient cells. The extent of autophagy, senescence, and DNA damage (γH2AX staining) were obtained from flow cytometric analyses (n=3). Data were normalized to the value measured in unirradiated HCT116 wt cells. The fraction of cells showing evidence of autophagy by (acridine orange staining as determined by flow cytometry) was plotted against either the fraction of senescent cells as determined by $C_{12}$FDG staining and flow cytometry or the intensity of γH2AX. $r^2$ values are shown for each correlation.

**Supplementary Figure 2**

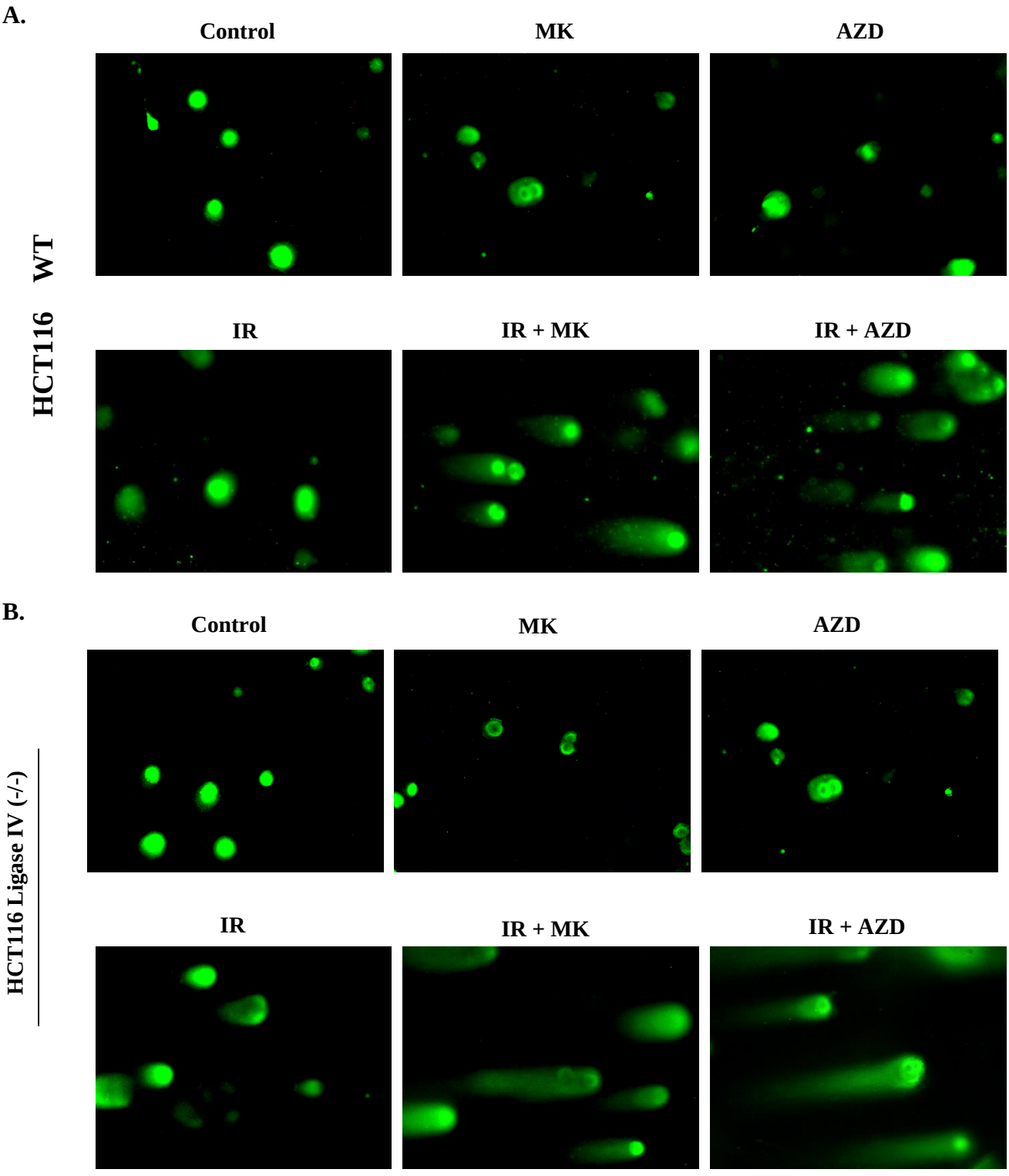


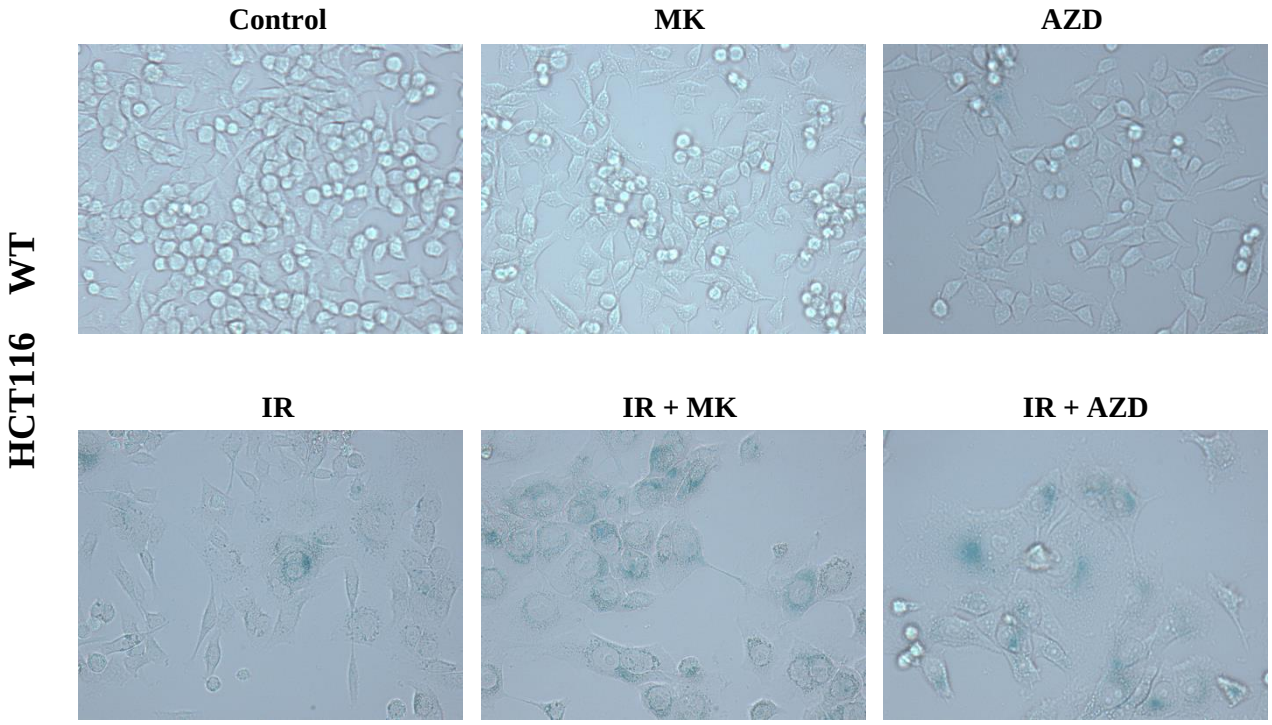



**Supplementary Figure 2. Interference with autophagy in both cell lines by pharmacologic and genetic approaches. A.** HCT116 WT and HCT116 Ligase IV-deficient cells were transfected with shRNA of *ATG5* and *ATG7* or scrambled controls to suppress autophagy via genetic silencing. **B/C.** Western blotting for degradation of p62/SQSTM1, a marker of autophagic flux was performed to confirm blockade of autophagy in cells pretreated with chloroquine (CQ, 5 μM) or bafilomycin (Baf, 5 nM) 3 hr before exposure to radiation. **D/E.** Western blotting for degradation of p62/SQSTM1 was performed to confirm blockade of autophagy by silencing of ATG5 and ATG7.

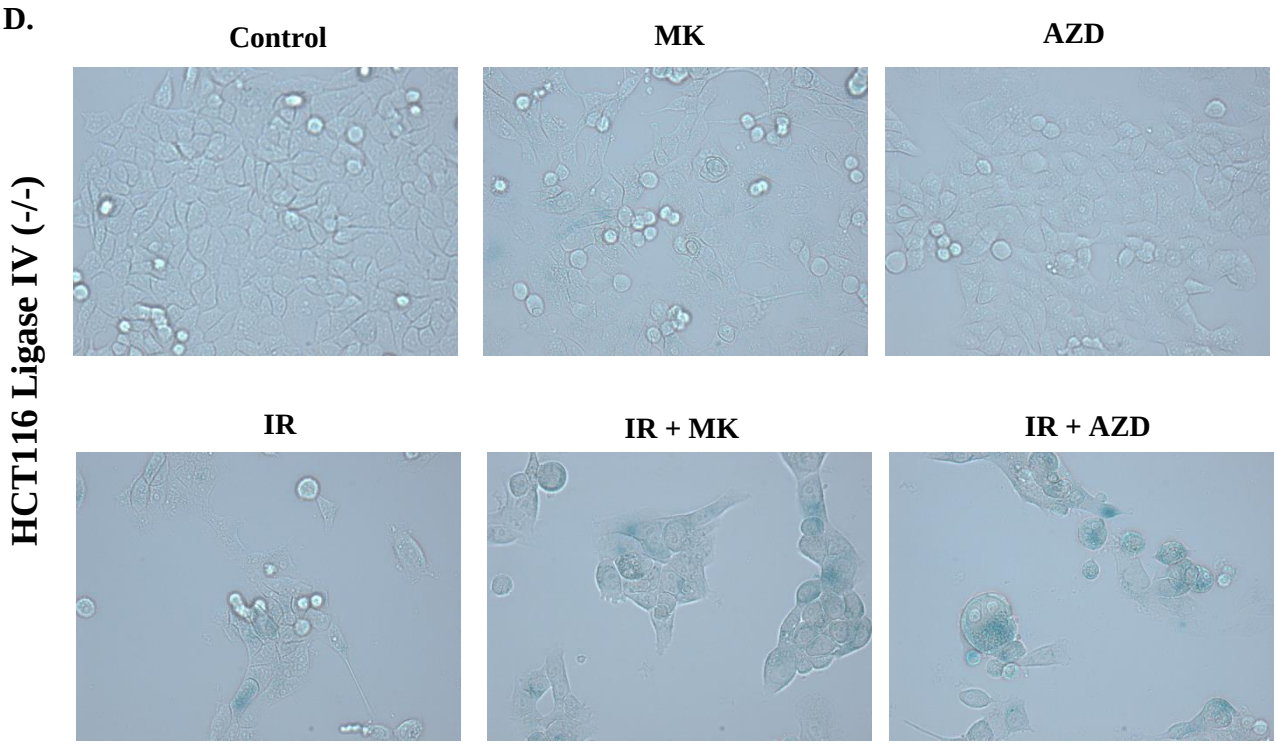
**Supplementary Figure 3**



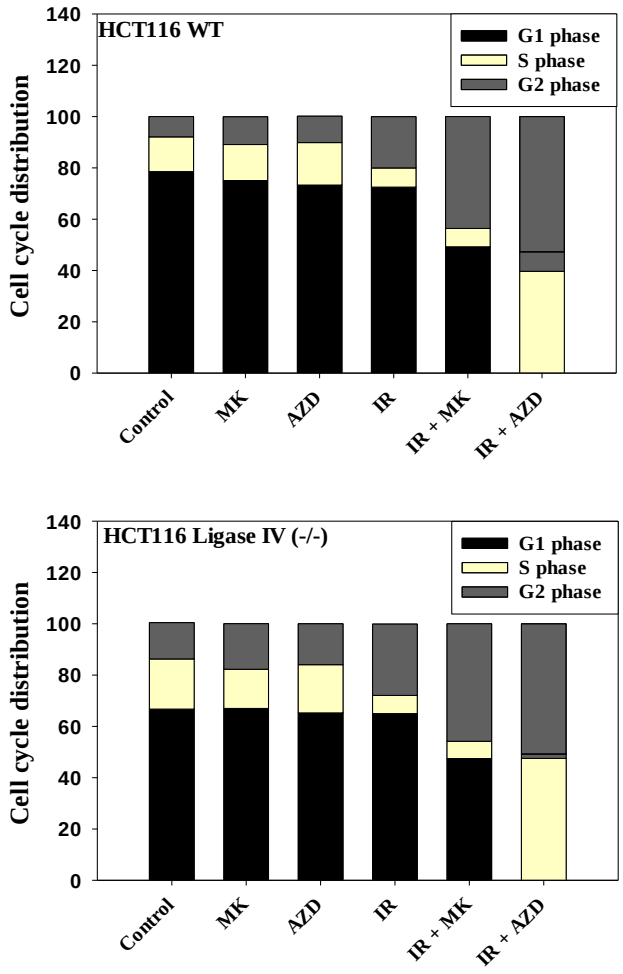
C.


HCT116 WT
Control
MK
AZD
IR
IR + MK
IR + AZD

D.


HCT116 Ligase IV (-/-)
Control
MK
AZD
IR
IR + MK
IR + AZD

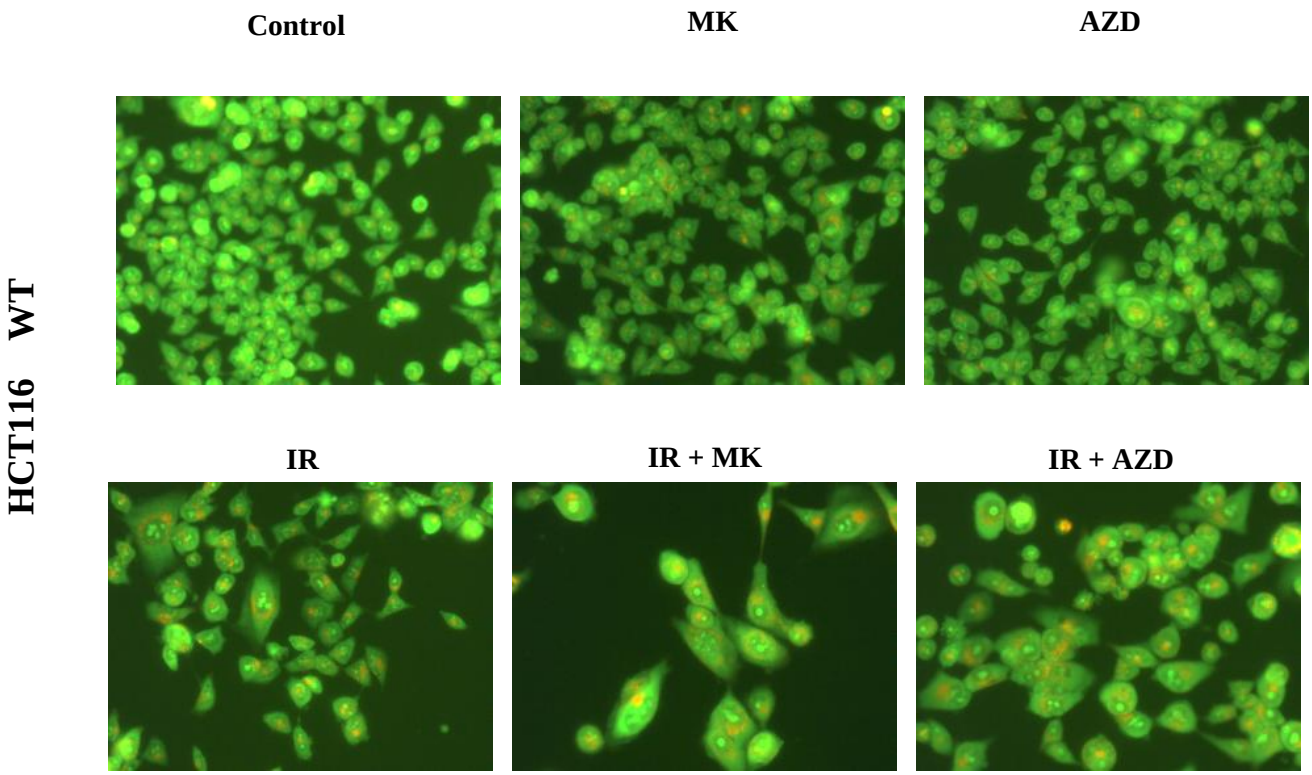
**E.**



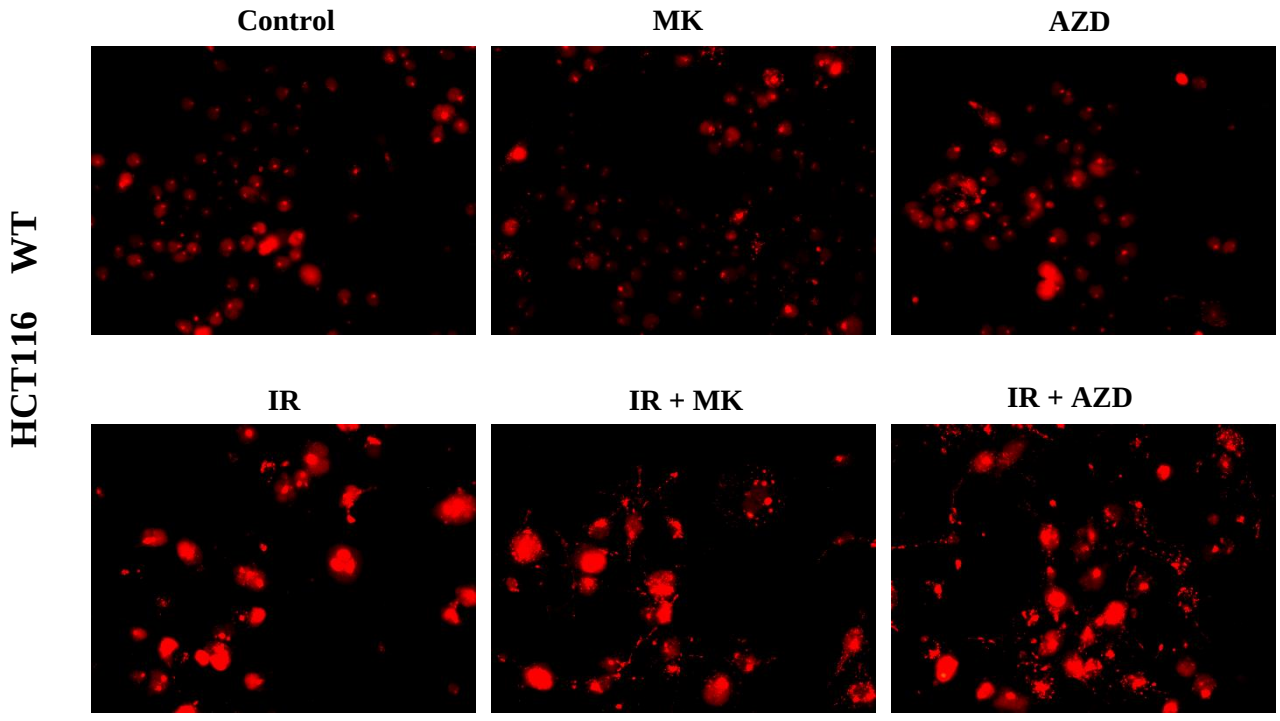

**Supplementary Figure 3. Comet assay, senescence and cell cycle analysis.** DNA damage by the Comet assay and senescence based on βgalactosidase staining were monitored in HCT116 WT and HCT116 Ligase IV (-/-) cells after the exposure to PARP inhibitors and radiation 72 hours post treatment. **A/B.** Images of comet assay taken by fluorescent microscope at magnification 20X. **C/D.** Promotion of senescence based on βgalactosidase staining. **E.** Cell cycle analysis in HCT116 WT and HCT116 Ligase IV (-/-) colon cancer cells after exposure to 4 Gy and 1 Gy, respectively, with or without PARP inhibitors.

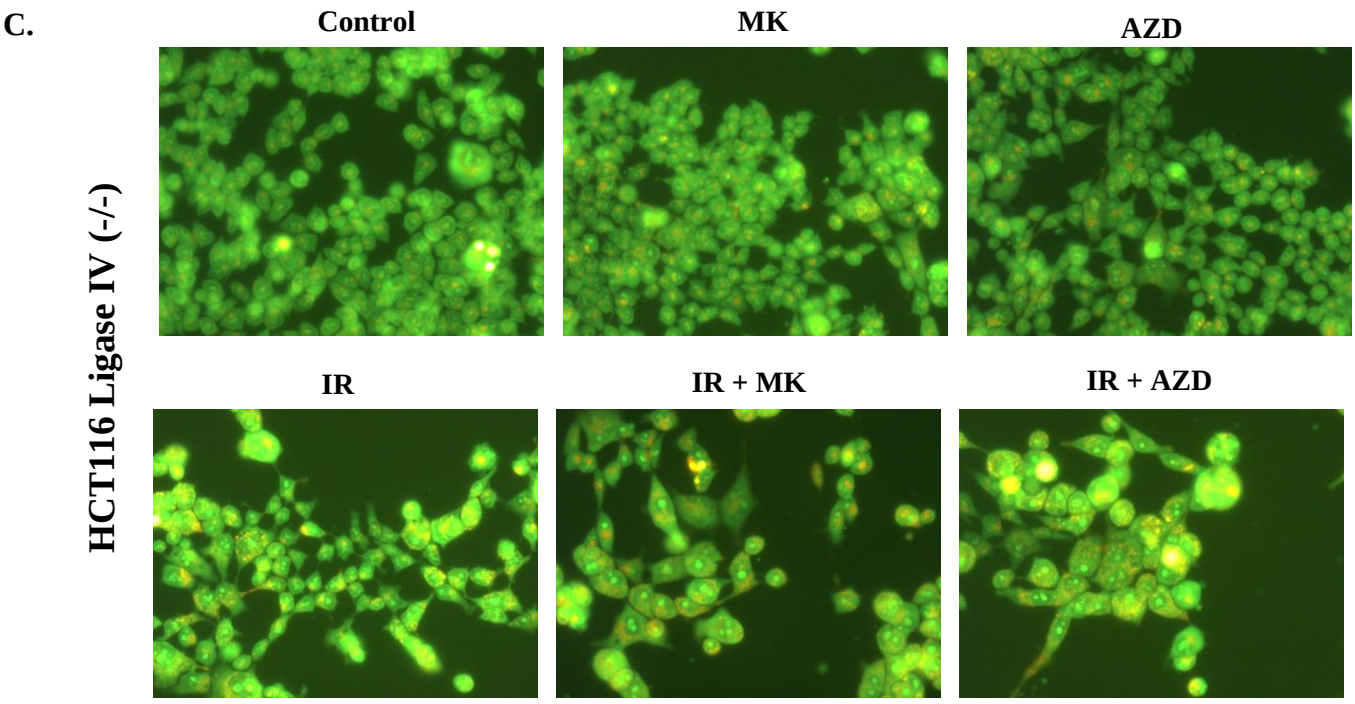
**Supplementary Figure 4**

**A.**




**B.**

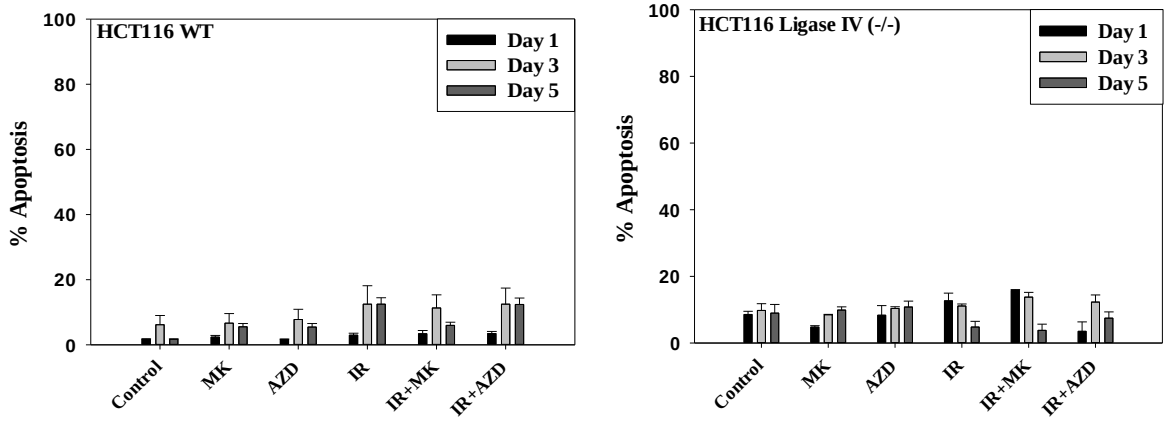




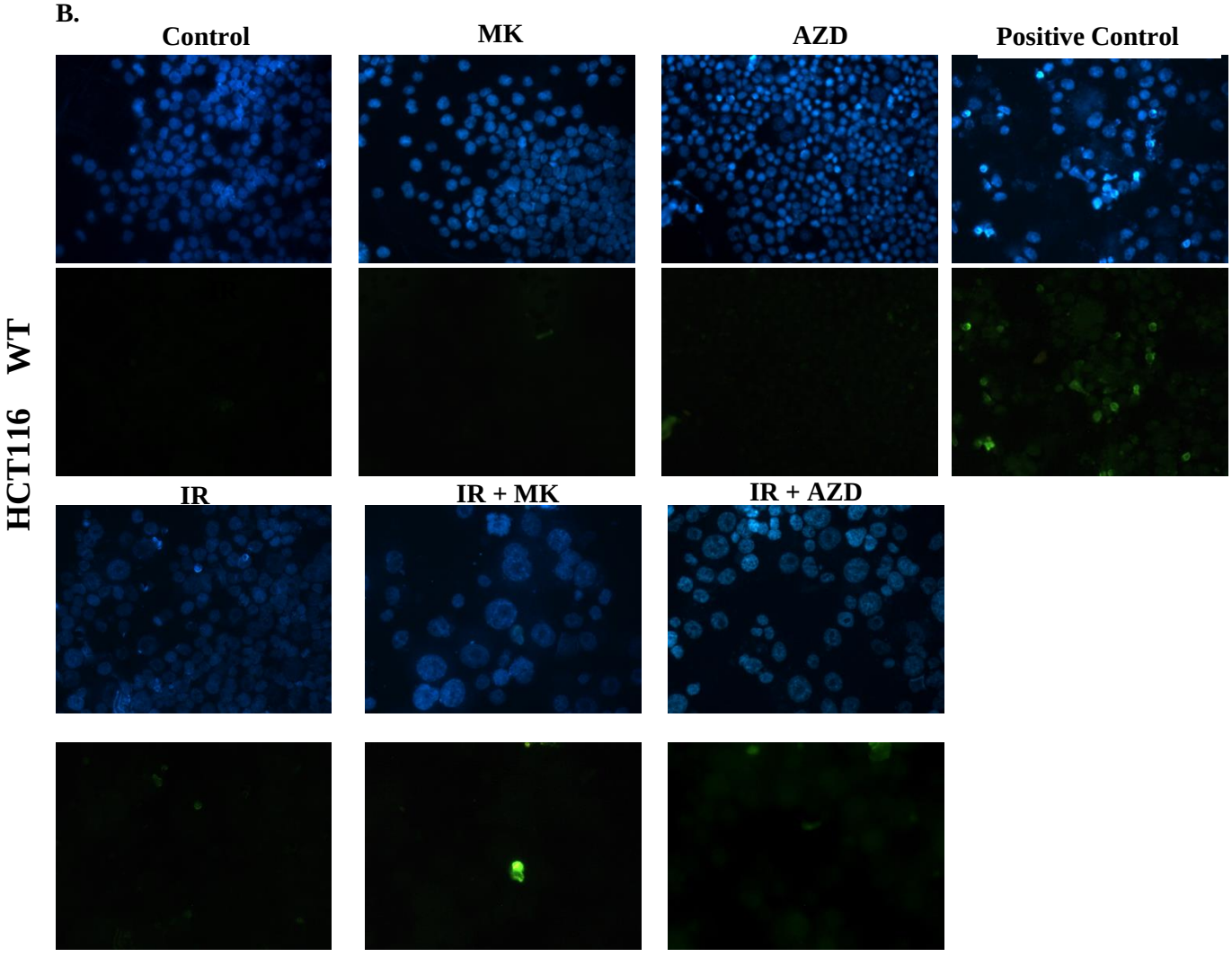

**Supplementary Figure 4. Autophagy in irradiated HCT116 WT and HCT116 Ligase IV (-/-) cells exposed to PARP inhibitors. A.** Acridine orange staining in HCT116 cells as an indication of autophagy 72 hours post radiation treatment with and without PARP inhibitors. **B.** HCT116 WT cells were transfected with RFP-LC3 construct as an indication of autophagy 72 hours post treatment. **C.** Acridine orange staining of irradiated HCT116 Ligase IV-deficient cells with and without co-administration of PARP inhibitors.

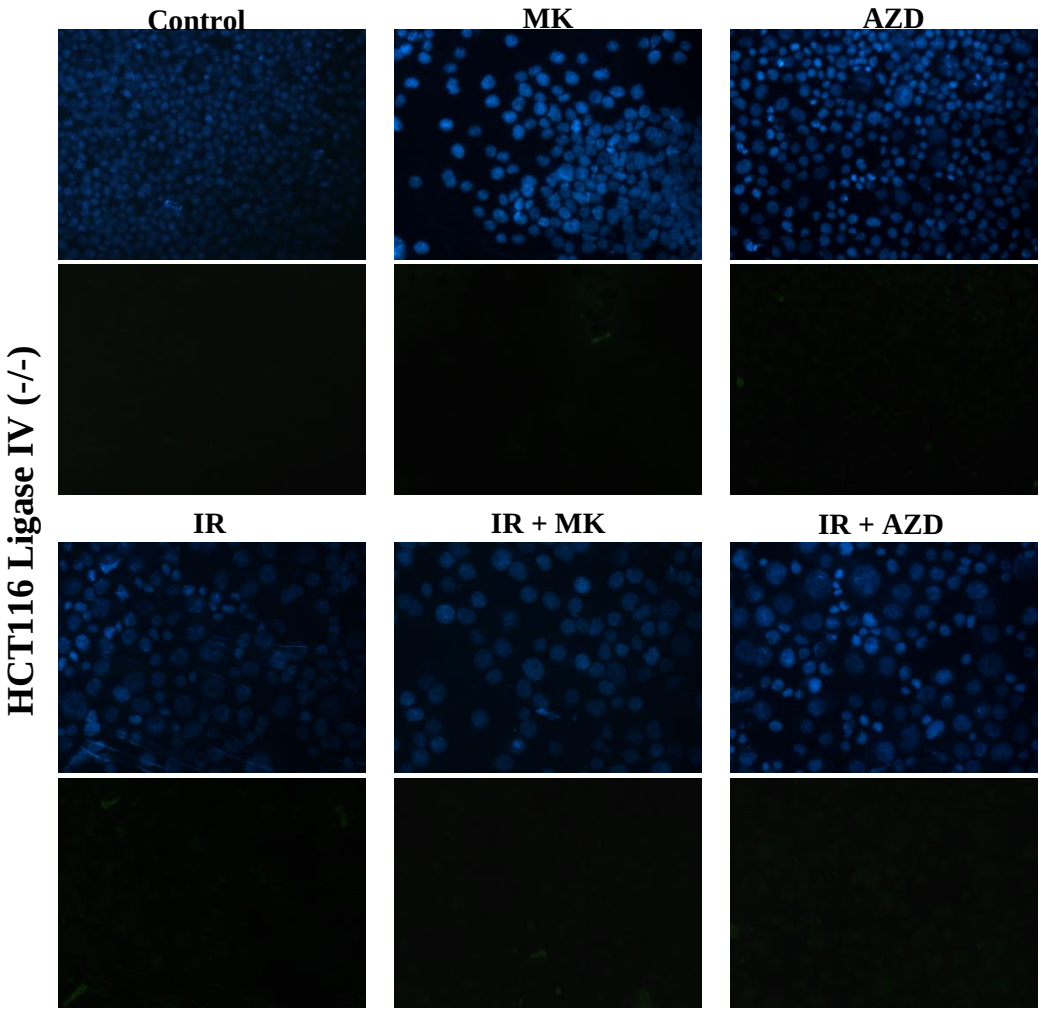
**Supplementary Figure 5**

**A.**

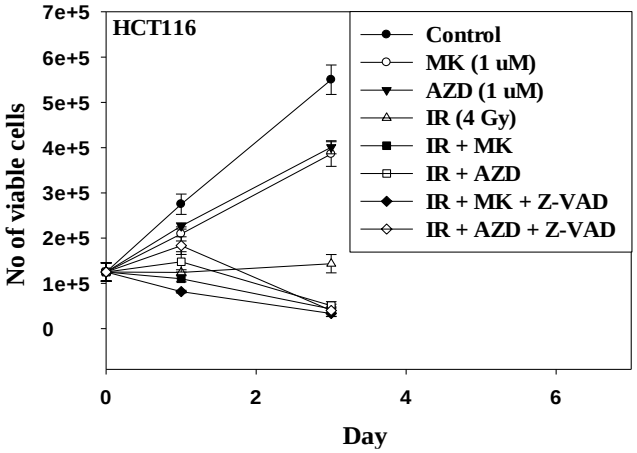



**B.**




Control
MK
AZD
HCT116 Ligase IV (-/-)
IR
IR + MK
IR + AZD

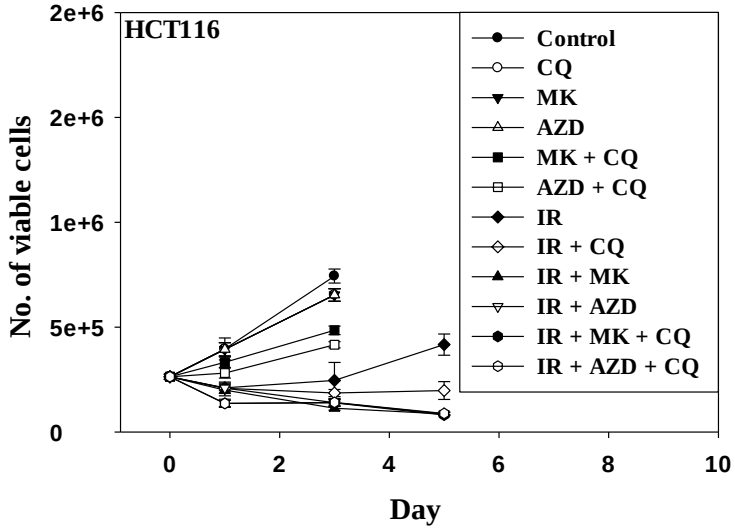
**C.**


HCT116
No of viable cells
Day
Control
MK (1 uM)
AZD (1 uM)
IR (4 Gy)
IR + MK
IR + AZD
IR + MK + Z-VAD
IR + AZD + Z-VAD
7e+5
6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0
0
2
4
6

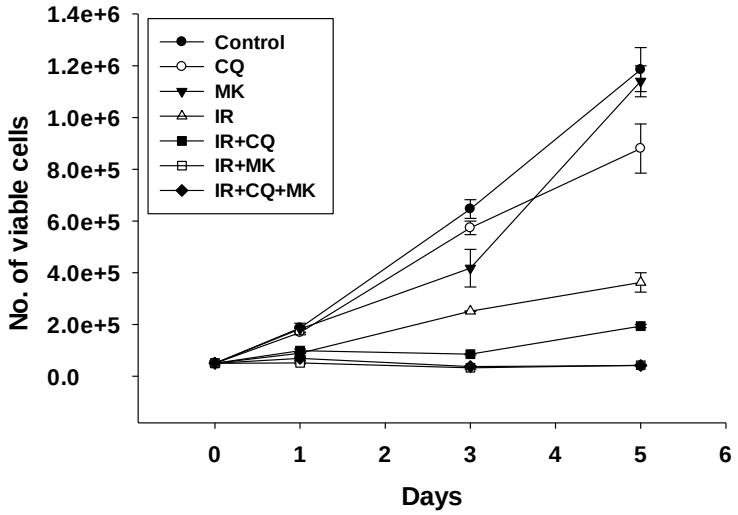
**Supplementary Figure 5. Apoptosis is not involved in the radio sensitization of HCT116 cells by PARP inhibitors. A.** Induction of apoptosis evaluated by staining with Annexin 5 (n=3). **B/C.** Apoptosis evaluated in HCT116 WT and HCT116 Ligase IV (-/-) cells based on the TUNEL assay **D.** HCT116 cells were pretreated with caspase inhibitor Z-VAD (10 µM) and exposed to radiation alone and radiation with PARP inhibitors. Number of viable cells was monitored over a period of 3 days.

## Supplementary Figure 6

**A.**




**B.**



**Supplementary Figure 6. Inhibition of autophagy does not alter sensitization to radiation by PARP inhibition.** HCT116 cells and H460 cells were exposed to radiation + PARP inhibitor with or without chloroquine. **A.** HCT116 cells were pretreated with chloroquine (5 uM), PARP inhibitor (1 uM), or both, 3 hr prior to radiation. Number of viable cells was monitored at the indicated time points. **B.** H460 cells were pretreated with chloroquine (10 uM), PARP inhibitor (Niraparib (1 uM)), or both, 3 hr prior to radiation. Number of viable cells was monitored at indicated time points.

# Chapter 5: Discussion

## 5.1 Introduction.

Radiotherapy is one of the most widely used anti-tumor strategies. However, the response of tumors to radiation varies. In some types of cancers, radiation is quite effective while other tumors show resistance to radiation. For example, treatment with radiation after surgery or along with chemotherapy significantly reduces recurrence and improves the outcomes of breast and head and neck cancer therapy, respectively [103-104]. On the other hand, glioblastoma and lung cancer show high resistance to radiation, even in combination with chemotherapeutics, and recurrence is common within few years after therapy [105-107].

Radiation toxicity is mediated through formation of ions accompanied with application of high energy in the cells. This high-energy deposition results in instability of the genetic material, and may lead to cell death [50]. The main goal of radiotherapy is to maximize the dose to the cancerous tissues with minimum damage to the normal adjacent cells. Generally, normal cells are more efficient in repairing their damaged DNA than cancer cells [48]. This may be due to the fact that some tumors have been found to have mutations in their recombinational DNA repair [51-52]. Mutations in major proteins involved in recombination repair make the tumors more sensitive to DSBs, whereas in normal cells such a mutation is absent and will not affect repair. This is one basis for the relative selectivity of radiotherapy in the treatment of cancer.

Ionizing radiation can cause DNA damage directly by producing single- and double-strand breaks or indirectly through generating reactive oxygen species (ROS) in the cells. Although the majority of DNA breaks become repaired upon induction of DNA repair pathways, many DNA damage foci appear to be persistent [99]. It is yet unclear why these foci do not

undergo DNA repair as is the case for the bulk of double-strand breaks (DSBs), but it could be either due to the repression of DNA repair system in the cells or due to the unique properties of these persistent foci.

In response to treatment, tumor cells can undergo a form of growth arrest that is termed stress-induced senescence. The ability of DNA-damaged cells to avoid apoptosis and enter a state of senescence may indicate that senescence could act as a permissive mechanism for persistent DNA damage foci to be eventually repaired. Although anticancer drugs and ionizing radiation frequently cause senescence of tumor cells, it is not necessary that these cells are going to undergo cell death due to several reasons. First, senescence may be permissive for DNA damage repair and subsequent proliferation of cells after exposure to chemotherapeutic agents or radiation. Second, senescence may allow treated cells to upregulate certain enzymes in order to inactivate the anti-cancer agents. Third, the dose that is used to induce senescence or the time of cellular exposure to anticancer agents may not be sufficient to enforce cells to undergo cell death; in other words, it is nonlethal doses for the tumor cells whereas increasing the dose could be harmful to normal cells.

### 5.2 Differences in response to low doses of radiation between Liagase IV-proficient and Ligase IV-deficient cell lines.

Non-homologous end joining (NHEJ) is considered the major pathway for repair of the double-strand breaks in DNA induced by ionizing radiation in mammalian cells. In studies by Katsube *et al,* differences in sensitivity to radiation were observed for HCT116 cells lacking proteins involved in the NHEJ pathway such as XRCC4 and Artemis [209]. In this study, the XRCC4 and Artemis loci were inactivated by gene targeting and the response of these

genetically modified cell lines to radiation was evaluated in terms of cell viability and other markers of DNA damage. The function of XRCC4 is known to be associated with Ligase IV, forming a complex (XRCC4/Ligase IV) to rejoin the broken DNA ends [220]. Also, Artemis has a related function in the same pathway (exonuclease activity) where it trims some types of broken DNA before the rejoining process by XRCC4/Ligase IV complex occurs [221]. Although both proteins were found to be essential for the NHEJ pathway, *Artemis -/-* showed less sensitivity to radiation than *XRCC4 -/-* cell lines; sensitivity was also reduced for other different DNA-damaging agents [209]. Furthermore, the level of DNA damage was significantly higher after treatment with radiation in repair deficient cell lines than the parental cells (based on the DNA damage marker γ-H2AX), which indicates a compromised DSB repair mechanism in these cell lines.

Similarly, in the current work, we used two HCT-116 cell lines differing in their capability in repairing radiation-induced DSB's in order to assess the potential involvement of autophagy and senescence in DNA damage repair. Exposure of both cell lines to radiation showed significant differences in the promotion of autophagy and senescence. The repair-deficient cell line demonstrated higher levels of autophagy and senescence at the low dose of 2 Gy accompanied by a significant increase in the DNA damage marker γ-H2AX foci at each dose of radiation. Confocal microscopy comparing the formation of DNA damage foci between both cell lines confirmed that the Ligase IV-deficient cell lines significantly showed more γ-H2AX foci than the proficient cell line.

The results of our experiments were also consistent with studies in the literature showing that DNA damage can induce cells to undergo a state of senescence associated with autophagy [83, 222]. Our data show a direct correlation between the % of autophagy and % of senescence

in each cell line, indicating that DNA damage may play a role in the interplay between autophagy and senescence. Taken together, increased generation of radiation-induced DSB's promotes more senescence and autophagy, which might explain the increased radiation sensitivity in HCT116 Ligase IV-deficient cells.

### 5.3 The nature of irradiation-induced autophagy and irradiation-induced senescence in HCT116 cells.

Autophagy may have multiple cellular functions including the elimination of misfolded proteins and damaged organelles [223]. During carcinogenesis, autophagy plays dual roles. During formation of the tumor, autophagy can significantly delay tumorigenesis as studies have shown that Beclin1 can work as a haploinsufficient tumor suppressor and inhibit tumor progression [224-226], as well as tumors show mutations in autophagy regulating genes, indicating that tumor progression requires escaping autophagy [227]. On the other hand, autophagy may act as a cytoprotective manner in the fully formed tumor against some genotoxic stress factors, including radiation [228-229]. In this work, we sought to understand the role of autophagy in tumor response to DNA damage induction using radiation. A study by Li et al showed that inhibition of autophagy in p53 WT HCT116 cell sensitized the cells to topotecan [230]. In addition, inhibition of autophagy in HCT116 colon carcinoma cells led to the impairment of proliferation [231]. In the current work parental HCT116 cells became more sensitive to radiation when blocking autophagy was blocked either pharmacologically or genetically. Thus, in these cells autophagy was radioprotective [231-232]. In Ligase IV-defective cells, however, most if not all of this radioprotective effect was lost, suggesting that radiopreotection was mediated at least in part by an effect on NHEJ.

A recent study by Aleission *et al* investigated the relationship between autophagy and DNA repair pathways of DSBs in human bone marrow mesenchymal stromal cells (MSC) [233]. Their data suggested that inhibition of autophagy affects the activity of the NHEJ repair pathway rather than the HR, indicating that part of the cytoprotection of autophagy is via NHEJ. During irradiation, cells usually enter a state of growth arrest and non-cycling phase. During this stage, NHEJ pathway is the main DSB repair pathway that would be activated due to the lack of sister chromatid that is important for the Homologous recombination. Thus, inhibition of autophagy in a cell line that has an intact NHEJ would affect the cell viability of autophagic cells, whereas inhibition of autophagy in NHEJ – deficient cells would not show sensitization due to the absence of NHEJ and probably the dependence of these cells on an alternative repair pathway other than the classical NHEJ.

We then tried to investigate the function of radiation-induced senescence. One of our main questions we are aiming to address is the reversibility of radiation-induced senescence. Several reports in literature have indicated that fibroblasts entered a state of replicative senescence have escaped senescence and re-entered the cell cycle, forming pre-transforming cells [234-236]. Similarly, many laboratory studies have shown the recovery of the cells treated with radiation and chemotherapy [96, 101, 237-238]. Although some studies suggested the principle of the reversibility of senescence, none of these studies have successfully isolated the senescent and non-senescent cells. Therefore, we isolated senescent and non-senescent cells to study their recovery separately to address the question whether senescent cells are undergoing an irreversible or reversible growth arrest. Even though non-senescent cells show higher rate of growth, senescent cells also show late recovery in both cell lines. In addition, our data also suggest that when cells treated with dose of radiation that causes ~ 75% of senescence and

autophagy; the cells tend to repair their newly formed DNA damage foci. These data together suggest that autophagy plays a cytoprotective role when HCT116 cells were exposed to radiation while senescence seems to be reversible, possibly due to repairing the DSBs, and permissive to DNA-damaged cells.

In conclusion, radiation-induced autophagy and radiation-induced senescence lead ultimately to the survival of the treated cells. Although our data showed different degrees of cytoprotection of autophagy in both cell lines, the data of senescent cells of both cell lines also demonstrated permissive function of senescence in these cells.

### 5.4 The connection between autophagy and senescence. Are they directly linked?

In addition, the relationship of autophagy and senescence is still complicated and unclear. Several studies have shown that induction of senescence was dependent on autophagy [83-84], whereas other studies concluded that senescence is independent of autophagy [222, 239]. In contrast, several reports demonstrated that inhibition of autophagy leads to the promotion of senescence. The explanation of these contradicted viewpoints is difficult, but it seems that the outcomes depend on the experimental systems, the stress inducer, the role of autophagy, and the cell lines used in each study. However, it is still mysterious whether the connection between autophagy and senescence are linked via a defined signaling pathway. Furthermore, a study performed in our laboratory demonstrated that suppression of senescence may lead to attenuation of autophagy using chemotherapeutic agents [83]. Interestingly, studies have shown that serum starvation-induced autophagy in HCT116 cells is not associated with senescence in a p53 proficient cell line, whereas remarkable senescence was observed with p53- deficient HCT116 cell line [240]. Thus, it was important to understand the relationship between radiation-induced

autophagy and radiation-induced senescence in HCT116 cells and Ligase IV-deficient cells, since we noticed similarity between both phenomena in function and extent in response to radiation. As we found a correlation between the % of autophagy and % of senescence in response to DNA damage in both cell lines, it was noteworthy to determine whether the function of autophagy and senescence was also similar. Surprisingly, pharmacological and genetic inhibition of autophagy in both cell lines did not seem to affect the promotion of senescence even at high doses of radiation, indicating that promotion of senescence is independent of autophagy in our model. Earlier in this work, we have shown that autophagy and senescence were closely promoted in response to DNA damage, but does not necessarily indicate a common signaling pathway. It has been demonstrated that DNA damage response can lead to senescence and autophagy, possibly via induction of ATM [241-242]. The up-regulation of ATM leads to the activation of its downstream target p53, which by then leads to the promotion of senescence via p21-pRb pathway [241]. Also, p53 can activate the autophagy promoter AMP-activated protein kinase during the genotoxic stress [243], which in turn phosphorylates tuberous sclerosis (TSC) complex proteins TSC1 and TSC2. Both proteins TSC1 and TSC2 downregulate mTOR protein, which eventually lead to the promotion of autophagy [125]. In this study we used cell lines that have a wild-type p53. Thus, we would predict p53 to be functioning in both pathways and none of them would be compromised. Our data suggest that persistent DNA damage response (DDR) is maintained via upregulation of the ATM-p53 axis. Then both pathways diverge at p53 wherein senescence the pathways could be p53-p21-pRb, whereas in autophagy the pathway is p53-AMPK-TSC-mTOR. If these are indeed the primary signaling pathways, inhibition of DNA damage-induced autophagy would not attenuate senescence in HCT116 cells due to promotion of different downstream effectors.

### 5.5 Targeting DNA repair via inhibiting PARP

Given the observation that irradiated cells appear to undergo proliferative recovery after a period of growth arrest, we sought to sensitize both cell lines to radiation via interfering with DNA repair to combat the recovery. PARP inhibitors are considered one of the promising radio-sensitizing agents that have been tested in clinical trials [180, 244-248]. The poly (ADP-ribose) polymerase (PARP) enzyme is involved in repair of single-strand breaks (SSBs), and lack of this enzyme in knockout mice incresed sensitivity to radiation and alkylating agents [249]. It has been shown that PARP inhibition converts the SSBs to be DSBs at the S phase in cell cycle, which in turn leads to the activation of homologous recombination repair (HRR). In cells lacking the protein BRCA, which is a major protein involved in repair pathway (HRR), PARP inhibition can be lethal during exposure to radiation [250]. Due to the microsatellite instability in colorectal cancer cells, the expression of protein MRE11, another protein involved in (HRR) pathway, is reduced [251]. Thus, administration of PARP inhibitors may lead to radio-sensitization in HCT116 cells.

PARP inhibitors increased the number of γ-H2AX foci and the number of irradiated cells undergoing autophagy and senescence, but not apoptosis. These data were consistent with our findings that both autophagy and senescence were directly correlated with induced DNA damage. In parental HCT116 cells, this increased DNA damage was associated with a dramatic reduction in clonogenic survival of irradiated cells, especially with MK. Remarkably, however, there was little or no further radiosensitization of the already radiosensitive Ligase IV-deficient cells, despite markedly higher levels of γ-H2AX, autophagy and senescence. This result suggests that NHEJ is required for PARP inhibitor-mediated radiosensitization, and is thus consistent with the premise that radiosensitization results from inappropriate channeling of

replication-associated one-sided double-strand breaks into NHEJ. This mechanism has been invoked previously to explain the similar dependence of PARP inhibitor sensitivity on NHEJ in BRCA1-deficient cells [252], except that in those cells the initial single-strand breaks would be spontaneous rather than radiation-induced.

In any case, apoptosis does not seem to be involved in the cytotoxicity of combination therapy in either cell line, indicating that the radiosensitization of these cell lines by PARP inhibitors might be mediated by promoting autophagy and senescence. In our previous results, we have shown that irradiation-induced autophagy and irradiation-induced senescence seem to be permissive to the irradiated cells to undergo repair and recover. Since the autophagy and senescence promoted after the co-administration of PARP inhibitors, it was very important to understand whether suppression of autophagy and senescence would change the sensitivity of HCT116 cells to the combination therapy.

**5.6 Inhibition of autophagy in HCT116 cells treated with irradiation and PARP inhibitors.**

Studies performed in our lab indicated that inhibition of autophagy may confer the radio-sensitization by vitamin D and its analogue in breast and non-small lung cancer cell lines, respectively [213, 216, 219]. Our strategic treatment seems to only induce minimal apoptosis, indicating that the main mode of toxicity of PARP inhibitors is through promoting prolonged growth arrest. Since markers of apoptosis were not observed in this model, inhibition of autophagy was important to understand whether autophagy is crucial for the sensitization. Interestingly, inhibition of autophagy did not interfere with the cytotoxicity of our therapeutic strategy. Taking into the consideration that radiation-induced senescence and radiation-induced autophagy are not linked in our system, this disassociation may explain why inhibition of

autophagy does not confer the sensitization in these cells, indicating that these cells can undergo senescence independently.

### 5.7 Effect of PARP inhibitors on the recovery of senescent and non-senescent cells.

Recent study showed that PARP-1 is involved in a newly identified back up pathway named PARP1-dependent end joining (PARP1-EJ) [253]. This new finding adds another aspect of the lethality of our combination therapy in HCT116 cell lines when main repair pathways are blocked, i.e., it would explain more about the radio-sensitizing effect of PARP inhibitors in the cells that lack Ligase IV. However, another NHEJ-like back up repair mechanism, called mutagenic NHEJ pathway, can be activated when PARP1- mediated and HR pathways are inactivated [254]. In line with this suggested pathway, our presented data showed that radio-sensitivity by PARP inhibition in both cell lines can be reversible as in **(Figure 6),** possibly due to the alternative pathway (mutagenic NHEJ).

### 5.9 Conclusion:

There is no consensus as to whether senescence induced by radiation or chemotherapy is reversible [235, 255]. We demonstrate that both in the case of radiation alone and in the studies combining PARP inhibition with radiation, growth arrest is followed by proliferative recovery. These observations clearly suggest that tumor cells that enter a state of autophagy/senescence have the capacity to re-emerge into a proliferative state. If these findings can be extrapolated to clinical cancer, this may explain why radiation is not fully effective in the treatment of some types of malignancies. Furthermore, it is likely that the use of PARP inhibitors will result in only transient radiosensitization.

We conclude that the extent of radiation-induced DNA damage is accompanied with an increase of autophagy and senescence. The extent of autophagy and senescence induced at different doses of radiation was more pronounced in the ligase IV deficient cells, which is correlated with increased levels of DNA damage. Autophagic/senescent HCT116 cells demonstrated the ability to repair the newly formed DSBs. These data may indicate that promoting senescence alone would not have an effect on overall DNA repair system efficiency. However, radio-sensitized cells ultimately recover proliferative capacity, indicating that the inclusion of PARP inhibitors with radiation may not interfere with disease recurrence. Currently therapeutic regimens such as radiotherapy generally fail to completely eradicate the tumor cell population; this could be due, in part, to the induction of autophagy and senescence, which may be permissive for DNA repair. While inhibition of DNA repair (PARP inhibition) may initially sensitize cells via increased autophagy and senescence, this strategy does not appear to interfere with proliferative recovery, which is likely to contribute to disease recurrence.

**Future studies:**

In our study, we tried to interfere with recovery via using PARP inhibitors, and investigate the mode of radiosensitization by PARP inhibitors in HCT116 cells. Our results indicated that co-administration of PARP inhibitors with radiation resulted in autophagy and senescence, but not apoptosis. To understand whether this combination therapy would lead to multiple modes of cells death, it would be significant to screen for another mode of cell death called "mitotic catastrophe". Mitotic catastrophe is described as deregulated mitosis results in misaligned chromosomes, clusters of aneuploidy, and creation of multi micronuclei enclosed within a nuclear envelope. The phenomenon of mitotic catastrophe occurs when the process of DNA replication is disconnected from the process of mitosis during cell cycle. Thus, it is unclear yet whether mitotic catastrophe is a cell death mechanism or a protective mechanism by which cancer cells avoid apoptosis [256]. Intriguingly, several studies have shown that promotion of mitotic catastrophe was associated with the remarkable markers of apoptosis, which might indicate that mitotic catastrophe cross-talks to apoptosis [257-260]. In regard to radiosensitization by PARP inhibitors, few studies have claimed that interfering with DNA repair along with DNA damaging agents may lead to differential modes of cell death, including mitotic catastrophe [261-262]. In line with these findings, our DAPI-stained cells demonstrated some mis-segregated macro- and micro-nuclei when cells were exposed to combination therapy. Thus, it is worth conducting more sophisticated experiments to investigate the promotion of mitotic catastrophe extensively by probing cell cycle regulating proteins and different microscopic

assays.

In addition, it is worthwhile to study the sensitizing effects of combining chemotherapeutic agents with radiation and PARP inhibitors. As mentioned earlier in introduction, colorectal carcinoma is mainly treated by chemotherapy. Since we have shown in our studies that combination of PARP with radiotherapy led to sensitization of HCT116 colon cancer cells, it would be important to study the chemoradiation regiment with PARP inhibitors. A recent study by Shelton JW *et al* demonstrated that the PARP inhibitor (ABT-888) synergized the effects chemoradiation treatement [263]. In this study, authors used one of the earliest discovered PARP inhibitors that has less potency than Olaparib and Niraparib, the ones we used in our studies [190]. Thus, testing the synergistic effect of Olaparib and Niraparib in this system might add usefulness to these powerful PARP inhibitors. Similar to other reports, this study did not conduct any further assays to investigate what mode of sensitization these cells undergo during chemoradiation. Therefore, we would try to find out what mode of cell death would sensitize colon carcinoma cells when Olaparib and Niraparib were added to chemoradiation.

Finally, we would evaluate the effectiveness of PARP with either radiation alone or chemoradiation on HCT116 colon cancer cells in vivo, using xenograft model or an orthotopic model [263-264]. In the previous studies of in vivo studies of PARP inhibitor ABT-888 in colorectal carcinoma cells, the PARP inhibitor showed efficacy against HCT116 and HT29 cells in vivo [263]. This drug was given orally at dose of 12.5 mg/kg twice daily in 6-hours interval with or without chemotherapy, and then exposed to radiation 2 (Gy). The PARP inhibitor ABT-888 has shown an inhibitory effect in HCT116 cells in vivo. In addition, co-administration of PARP inhibitor with chemoradiation resulted in a significant inhibition in tumor growth. Therefore, using more potent inhibitors such as Niraparib and Olaparib may provide better tumor

killing results. Also, both drugs were found to be well tolerated in humans at clinically achievable doses [265-266], indicating that these inhibitors could be promising radiosensitizers due to their low toxicity. Taken together, the anti-cancer and the radiosensitizing properties may suggest that PARP inhibitors are effective agents for the therapy colon cancer.

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