2021

The Role of Autophagy and Senescence in the Responses of Non-Small Cell Lung Cancer Cells to Chemotherapy and Radiation

Nipa H. Patel

THE ROLE OF AUTOPHAGY AND SENESCENCE IN THE RESPONSES OF NON-SMALL CELL LUNG CANCER CELLS TO CHEMOTHERAPY AND RADIATION.

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

By

Nipa Patel

Virginia Commonwealth University

Advisor: David Gewirtz, PhD

Professor, Department of Pharmacology & Toxicology

School of Medicine

Virginia Commonwealth University

Richmond, VA

April 2021

Acknowledgements

I would like to begin by thanking my advisor, Dr. David Gewirtz, whose mentorship and guidance have provided me with invaluable wisdom on my project, scientific career and life in general. I would also like to thank him for creating an amazing lab/scientific environment that has cultivated open discussions of scientific thought and ideas, as well as his positivity and inquisitive nature in the face of "negative" data. I would also like to extend my sincerest appreciation to my committee members: Drs. Katherine Nicholson, Eddy Ishac, Ross Mikkelsen and Hisashi Harada for their guidance, encouragement and fresh perspectives into the project.

Throughout the years, I have been fortunate to be surrounded by amazing people and scientist in the Gewirtz Lab (collectively known as "The Wendies"). One of my first mentors within the Gewirtz Lab was Tareq Saleh, who was genuinely passionate about science and teaching others. Thank you for always pushing me forward, whether it was in science or my life, and always lending an ear when I needed to talk or defuse my anxiety. I would also like to thank Lauren Kyte, whose kind heart and patience was appreciated as both a mentor and lasting friend. After joining the laboratory of Dr. Gewirtz, I had the pleasure of working with Dr. Jingwen Xu, who initially started off the studies presented in this dissertation and piqued my interests in autophagy and intracellular signaling in cancer cells. Her mentorship, intelligence and fantastic work ethic were perfectly balanced with her "down-to-earth" easygoing personality. I feel truly honored and humbled to be able to learn from you and continue to learn from you throughout my graduate studies. I'd also like to thank Tammy Tran, an undergraduate student who has become one of my best friends. I will always admire your resilience, hard work and perseverance. I would like to thank Valerie Carpenter, for her sharpness, both in intelligence and candor, and always being a friend whom I could bounce ideas with and my companion in commiseration. I also had the pleasure of working

with Liliya Tyutyunyk-Massey, who was both an elegant and brilliant scientist. Finally, I'd like to thank all of the former and current lab members of the Gewirtz Lab whom I have had the great joy of working alongside, including Dr. Emmanuel Cudjoe, Dr. Moureq Alotaibi, Christina Li, Zara Siddiqui, Ryan Finnegan and Fereshteh Ahmadinejad. It was a joy and honor to work with each and every one of you.

It has been my privilege to collaborate with the laboratory of Dr. Damaj. I'd like to acknowledge Drs. Wisam Toma and Martial Caillaud, as well as Dr. Damaj for allowing the great fortune of working with them on the studies related to chemotherapy-induced peripheral neuropathy. I enjoyed doing science with all of you and learned a great deal about the toxicities associated with chemotherapy. I would like to thank the VCU, Pharmacology & Toxicology Department, as well as Massey Cancer Center, for providing the opportunity to work alongside brilliant scientists, and providing a uniquely collaborative atmosphere for both scientific learning and progress.

Finally, I'd like to acknowledge my family and friends who have carried me through this journey. Thank you to my parents, Himansu and Niru Patel, who have loved and supported us throughout our lives, changing their way of living and adapting to unfamiliar circumstances to ensure my brother and I had a better life filled with opportunities. I'd also like to thank my brother, Neil Patel, and my cousins, Kisha, Ravibhai, Jay and Khushbu Patel, for all the love, wisdom, laughs and encouragement you've given me to strive forward, as well as my niece, Nirmani, for providing me with free "baby therapy". I'd also like to acknowledge the rest of my family, who are too many to name, I feel blessed to be surrounded with people who have so much love and care for my well-being. Most importantly, I'd like to thank Maharaj & Swami and my Gurus, Pramukh Swami Maharaj and Mahant Swami Maharaj. Your letters of encouragement and guidance in life have provided me with stability amidst the uncertainties and pushed me to be the person I am today.

Thank you for imbuing me with the skills and support I needed to succeed. I hope that I may imbibe your quality of “in the joy of others, lies our own” to persevere and help others through the scientific work I achieve.

Table of Contents

Acknowledgements ..... *III*

Table of Contents ..... *VI*

List of Tables ..... *IX*

Abbreviations ..... *X*

Abstract ..... *XV*

Chapter One: General Introduction *1*

1.1 Lung Cancer **1**

1.1.1. Lung Cancer: Overview 1

1.1.2. Stages and Treatment options for lung cancer 2

1.2. Apoptosis **3**

1.3. Autophagy **4**

1.3.1. General Introduction and history 4

1.3.2. Types of Autophagy 5

1.3.3. Autophagic Machinery 6

1.3.4. Autophagy and Disease 10

1.3.5. Autophagy in response to cancer therapy 11

1.3.6. Functional forms of autophagy 18

1.3.7. p53 and autophagy 27

1.3.8. Autophagy and the Immune Response 28

1.3.9. Current modalities to modulate autophagy and clinical trials 30

1.4. Senescence **34**

1.4.5. General Introduction and History 34

1.4.6. Senescence and Apoptosis Evasion 35

1.4.7. Characteristics of senescence 36

1.4.8. Senescence in response to therapy 39

1.4.9. Clinical Implications of Senescence: Utilization of Senolytics 41

Chapter Two: What are the cellular responses and the effect of p53 status on radiosensitivity in H460 NSCLC cells? *43*

2.1 Introduction **43**

2.2. Methods **46**

2.2.2. Cell Lines 46

2.2.3. Cell Culture and Treatment 47

2.2.4. Cell Viability Assay 47

2.2.5. Assessment of Apoptosis 47

2.2.6. Determination of Acidic Vesicle formation through Acridine Orange Staining 48

2.2.7. LC3/LAMP2 Co-localization 48

2.2.8. Western Blot Analysis 49
2.2.9. β-galactosidase and C_{12}FDG staining 49
2.2.10. Extent of DNA Damage 50
2.2.11. Statistical analysis 50

2.3. Results 51
2.3.1. Radiation Sensitivity in p53 Wild-type and p53 Knockout H460 cells 51
2.3.2. Radiation induced apoptosis in H460wt and H460crp53 cells 53
2.3.3. Radiation induced autophagy in both H460wt and H460crp53 cell 55
2.3.4. Nonprotective autophagy induced in response to radiotherapy in H460wt and H460crp53 cells 58
2.3.5. Radiation induced senescence in H460wt and H460crp53 cells 67

2.4 Discussion 69

Chapter Three: What is the effect of p53 status on cisplatin sensitivity and the nature of autophagy in NSCLC cells? 77

3.1 Introduction 77

3.2. Methods 79
3.2.1. Cell Culture and Treatment 79

3.3 Results 79
3.3.1. Cisplatin sensitivity in H460wt and H460crp53 NSCLC cells 79
3.3.2. Cisplatin-induced autophagy in p53 wt and p53 knockout cells 82
3.3.3. Evidence for cytoprotective autophagy in the p53 knock-out cells and nonprotective autophagy in the p53 wild-type H460 cells 84
3.3.4. Cisplatin induced senescence in H460wt and H460crp53 NSCLC cells 88
3.3.5. Inhibition of cytoprotective autophagy shifts the temporal response to cisplatin in the H460crp53 cells 90

3.4 Discussion 93

Chapter Four: What are the contributions of nonprotective autophagy to senescence? 101

4.1 Introduction 101

4.2. Methods 105
4.2.1. Cell Culture and Treatment 105

4.3. Results 106
4.3.1. Radiation induced senescence and autophagy in HCT116 cells 106
4.3.2. Radiation induced nonprotective autophagy in HCT116 colorectal cells 108
4.3.3. Senescence induction and recovery from growth arrest was independent of autophagy in HCT116 cells exposed to radiation 110

4.4. Discussion 114

References 119

List of Figures

Figure 1.1 Schematic representation of the macroautophagic process. 9

Figure 1.2. Autophagy induction in response to therapy. 16

Figure 2.1. Sensitivity to ionizing radiation (IR) in H460wt and H460crp53 NSCLC cells. 52

Figure 2.2. Radiation induced apoptosis in H460wt and H460crp53 cells to a similar extent. 54

Figure 2.3. Radiation-induced autophagy in H460wt and H460crpp53 NSCLC cells. 57

Figure 2.4. 3-MA fails to alter radiation sensitivity in H460wt or H460crp53 NSCLC cells. 60

Figure 2.5. Influence of chloroquine (CQ) on radiation sensitivity in H460wt and H460crp53 cells. 62

Figure 2.6. Influence of Bafilomycin A1 (Baf A1) on radiation sensitivity in H460wt cells. 64

Figure 2.7. Atg5 knockdown fails to alter radiation sensitivity in H460wt and H460crp53 cells. 66

Figure 2.8. Radiation induced senescence in H460wt and H460crp53 cells. 68

Figure 2.9. Tumor cell responses to radiotherapy. 76

Figure 3.1. Cisplatin sensitivity in H460wt cells and H460crp53 cells. 81

Figure 3.2. Cisplatin induces autophagy in H460wt cells and H460crp53 cells. 83

Figure 3.3. Pharmacological autophagy inhibition sensitizes H460crp53 cells, but not H460wt cells to cisplatin exposure. 85

Figure 3.4. Influence of genetic autophagy inhibition on cisplatin sensitivity in H460wt cells and H460crp53 cells. 87

Figure 3.5. Cisplatin induced senescence in H460wt and H460crp53 NSCLC cells. 89

Figure 3.6. Inhibition of cytoprotective autophagy shifts the temporal response to cisplatin in the H460crp53 cells. 91

Figure 3.7. Cellular responses to cisplatin exposure in H460wt and H460crp53 NSCLC cells. 92

Figure 3.8. Radiation and Cisplatin induced DNA damage in H460wt and H460crp53 cells. 97

Figure 4.1. Interconnectivity of cellular senescent and autophagic pathways. 104

Figure 4.2. The induction of senescence and autophagy in HCT116 cells in response to radiation. 107

Figure 4.3. Radiation induced nonprotective autophagy in HCT116 colorectal cells. 109

Figure 4.4. Pharmacological autophagy inhibition did not alter senescence induction or recovery in HCT116 cells exposed to radiation. 111

Figure 4.5. Genetic autophagy inhibition did not alter senescence induction or recovery in HCT116 cells exposed to radiation. 113

List of Tables

Table 1.1. Pharmacological Autophagy Inhibitors. 26

Table 1.2. Clinical Trials manipulating autophagy inhibition to sensitize cancer patients to antitumor therapy. 32

Table 3.1. Tumor responses to radiation and cisplatin exposure in H460wt and H460crp53 NSCLC cells. 100

Abbreviations

3-MA	3-methyladenine
5-FU	5-fluorouracil
ALLN	N-acetyl-leu-leu-norleucinal
AMPK	AMP-activated protein kinase
AO	Acridine Orange
ATG	Autophagy regulatory proteins
ATG	Autophagy-related gene
ATM	Ataxia-Telangiectasia mutant
ATP	Adenosine triphosphate
BafA1	Bafilomycin A1
BAK	Bcl-2 homologous killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-W	Bcl-2-like protein 2
Bcl-X_L	Bcl-2-associated X protein
BECN1	Beclin-1
C_{12}FDG	5-Dodecanoylaminofluorescein Di-ß-D-Galactopyranoside
CDK	Cyclin-dependent kinases
CDKI	Cyclin-dependent kinase inhibitors
CMA	Chaperone-mediated autophagy
CQ	Chloroquine
CRISPR/Cas9	Clustered regulatory interspaced short palindromic repeats/Cas9

CTL	Cytotoxic T lymphocytes
DAMP	Danger-associated molecular pattern
DC	Dendritic cells
DDR	DNA damage response
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DRAM	Damage-regulated autophagy modulator
Drp1	Dynamin-related protein 1
DSB	DNA double-stranded breaks
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	endothelial-to-mesenchymal transition
ER	Estrogen receptor
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green-fluorescent protein
GM	Glioblastoma multiforme
GOF	Gain of function
GSC	Glioma stem cells
Gy	Gray
HCC	Hepatocellular carcinoma
HCQ	Hydroxychloroquine
HDAC	Histone deacetylase

HIF-1 α	Hypoxia-inducible factor-1
HNSCC	Head and neck squamous cell carcinoma
ICB	Immune-checkpoint blockade
ICD	Immunogenic cell death
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IR	Irradiation
KD	Knockdown
KO	Knockout
KRAS	Kirsten rat sarcoma viral oncogene
MAP1-LC3	Microtubule-associated protein light chain 2
MHC-I	Major histocompatibility complex class I
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilization
MPR	Mannose-6-phosphate receptor
mTOR	mammalian-target of Rapamycin
MTX	Mitoxantrone
NAC	N-acetyl-L-cysteine
NCI	National Cancer Institute
NIX	Nip3-like protein X
NSCLC	Non-small cell lung carcinoma
NRB1	Neighbor of BRCA1

OXA	Oxaliplatin
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered solution
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
Rb	Retinoblastoma
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
SAHA	Suberoylanilide hydroxamic acid
SAHF	Senescence Associated Heterochromatin Foci
SASP	Senescent Associated Secretory Phenotype
SA-β-gal	Senescence-Associated β-galactosidase
SCLC	Small Cell Lung Cancer
SD	Standard deviation
SEM	Standard error of the mean
shRNA	Short hairpin RNA
SOD	Superoxide dismutase
SQSTM1	Sequestosome 1/p62
TASCC	TOR-autophagy spatial coupling compartment
TIA	Therapy-induced autophagy
TIS	Therapy-Induced Senescence
TME	Tumor microenvironment

TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
ULK-1	Unc-51 Like Autophagy Activating Kinase 1
VEGF	Vascular endothelial growth factor
Vps18	Vacuolar protein sorting protein 18
Vps34	Vacuolar protein sorting protein 34
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

Abstract

Cancer-associated deaths account for the second-highest mortality rates in the United States. Primary modalities of treatment often include surgery, radiation, and chemotherapy, and may also incorporate targeted therapy and immunotherapy. However, resistance to these treatments remains high, resulting in disease reoccurrence and poor survival rates. While apoptosis or cell death of tumor cells is the ideal outcome for anti-cancer therapy, this is often not the case, and in fact cancer cells may upregulate several pathways, such as autophagy and senescence, as a means to undergo alternative cell fate and evade apoptotic cell death. An essential tumor suppressor gene, *TP53*, regulates all three of these processes, apoptosis, autophagy, and senescence, and loss of function or mutated *TP53* is often implicated in early tumorigenesis and reduced sensitivity to antineoplastic therapy. To assess the effects of p53 status on the functionality of autophagy and cellular responses to radiation and chemotherapy, we utilized a pair of isogenic non-small cell lung cancer cells (NSCLC) expressing wild type p53 (H460wt) or lacking p53 expression generated using CRISPR/Cas9 editing (H460crp53). Exposure to the DNA-damaging agents, cisplatin and radiotherapy, revealed differential sensitivity between H460wt and H460crp53 cells, in which H460crp53 cells were significantly less sensitive to cisplatin and radiation exposure compared to their wild-type counterpart. In response to radiotherapy, apoptosis was induced to similar extents in both cell lines, while autophagy interference identified a *nonprotective* function of autophagy in response in both cell lines, regardless of p53 status. Rather, the differential radiosensitivity exhibited between H460wt and H460crp53 cells was attributed to differences in senescence induction, where H460wt cells demonstrated a significantly greater extent of senescence induction. Of particular interest was the finding that when the same set of isogenic cell lines was exposed to cisplatin, the cells exhibited a similar extent of senescence induction over time; however,

autophagy inhibition revealed two different functional forms of autophagy: *nonprotective* autophagy in H460wt cells and *cytoprotective* autophagy in H460crp53 cells. Blockade of cytoprotective autophagy in H460crp53 exposed to cisplatin was sufficient to restore sensitivity and apoptosis induction to a similar extent as in the H460wt cells, further confirming the existence of an autophagic switch and the role of cytoprotective autophagy in the initial resistance to cytotoxic therapy. Finally, given concomitant activation of both autophagy and senescence in response to chemotherapy and radiation, we also examined the relationship between these two processes. At least in the case of nonprotective autophagy, autophagy inhibition did not interfere with senescence induction or proliferative recovery from growth arrest, indicating these two processes may be dissociated when autophagy is nonprotective in function. Taken together, cancer chemotherapy and radiotherapy activate a number of cellular mechanisms, such as autophagy and senescence, and not solely apoptotic cell death; consequently, further analysis and screening are warranted prior to therapeutic administration of autophagy inhibitors to patients. While autophagy seems to be an attractive therapeutic target under its *cytoprotective* function, autophagy can in fact play multiple functions and switch functional responses. These studies demonstrate that autophagy is contextual in nature and may, in part, depend on the therapeutic modality utilized and the p53 status of the tumor cells.

Chapter One: General Introduction

1.1 Lung Cancer

1.1.1. Lung Cancer: Overview

Lung cancer is the second most common type of cancer and is responsible for the most cancer-related deaths in the United States, yearly (1). There are two major types of lung cancer, which are divided histopathologically: non-small cell lung cancer (NSCLC), which accounts for ~80-85% of new cases, and small cell lung cancer (SCLC), which contributes to ~10-15% of lung cancer cases (2). The most common causes of lung cancer include smoking, family history, and exposure to certain environmental factors, such as asbestos, radon, and other carcinogens (3). SCLC often starts in the bronchi in the middle of the chest but is highly aggressive and grows rapidly, resulting in diagnosis at later stages and poorer survival rates when compared to NSCLC (4). Furthermore, SCLC is strongly associated with smoking (5,6). Differences between SCLC and NSCLC are usually defined by light microscopy through hematoxylin and eosin (H&E) staining and cytology of patient biopsies. SCLC appears as small (< the diameter of 2-3 resting lymphocytes) and round-fusiform shaped cells compared to tumor cells in NSCLC, which appear larger (7–9). SCLC has a higher nuclear/cytoplasm ratio, higher mitotic activity, finely granular nuclear chromatin, and nuclear molding (8,9). There are three major subtypes of NSCLC: adenocarcinoma, squamous cell carcinoma, and large undifferentiated cell carcinoma (10,11). Adenocarcinomas usually encompass the outer parts of the lungs and incorporate cells that normally secrete substances, such as mucus (3). Squamous cell carcinomas initiate from the flattened squamous epithelial cells that line the inside of the airways in the lungs (3).

The most common mutations in lung cancer include *KRAS* (30%), *EGFR* (up to 23%), and *TP53* (50%) (12–15); moreover, tumors with p53 mutations generally have a poor prognosis and exhibit chemoresistance (16,17). Lung cancers have a high p53 mutation rate, of approximately 46% in lung adenocarcinoma and 81% in squamous cells; moreover, lung cancer also has a high percentage of *TP53* mutational hotspot regions (18). *TP53* is an essential tumor suppressor gene coding for the protein, p53, that plays a role in regulating a number of cellular responses, including but not limited to apoptosis, autophagy, and senescence (19–21).

1.1.2. Stages and Treatment options for lung cancer

Treatment for NSCLC is stage specific. For patients in early stages (stage I or II), a lobectomy or a surgical resection is indicated, which can be followed up with adjuvant chemotherapy to clear remaining cancer cells. Radiation prior to surgical resection may also be administered to shrink tumors before surgery. Patients with specific mutations, such as overexpression of epidermal growth factor receptor (EGFR) mutations may qualify for targeted therapy (22).

For patients with Stage IIIA NSCLC, treatment often includes some combination of radiotherapy, chemotherapy, and surgery; targeted therapy may also be given depending on a patient's genomic profile. Primary chemotherapies utilized for the treatment of NSCLC include platinum-based drugs (i.e. cisplatin), etoposide (topoisomerase II inhibitor), and microtubule poisons (i.e. docetaxel and paclitaxel) (23,24) For patients with Stage IIIB NSCLC, the tumors cannot generally be removed by surgery; therefore, chemoradiation may be utilized for treatment, as well as immunotherapies such as pembrolizumab (3).

For NSCLC patients diagnosed at Stage IV, where the disease has spread, treatment options vary depending on the extent of metastasis and patient overall health. Clinical recommendations range to include treatments such as surgery, chemotherapy, radiotherapy, targeted therapy, or

immunotherapy. Depending on the site of spread and the number of sites detected, stereotactic radiation may be given. Furthermore, certain gene mutations, such as E*GFR, BRAF,* or *ALK* genes, may allow for the use of specific targeted therapies (3).

The nature of the tumor cell response to radiation and chemotherapy can vary. The general consensus appears to be that radiation induces delayed cell death, possibly through mitotic catastrophe, and other direct cell death responses including apoptosis and possibly necrosis. Several cell survival mechanisms are also activated as alternative cell fates as the cell attempts to repair damaged DNA and remove injured organelles to evade killing. Tumor cells exposed to ionizing radiation and chemotherapy invariably also undergo autophagy and senescence as possible strategies to escape cell death.

1.2. Apoptosis

Cell death is the desired outcome for anti-tumor therapies. In this regard, most standards of care induce some degree of apoptotic cell death in response to radiotherapy and chemotherapeutic options. Apoptosis is a process of programmed cell death characterized by chromatin condensation, DNA fragmentation, cell shrinkage, membrane blebbing, and formation of apoptotic bodies (25).

There are two major apoptotic pathways: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway (26). The intrinsic pathway is triggered by pore formation in the mitochondria by the Bax and Bak proteins, resulting in mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome C from the mitochondrial intermembrane space (26). Release of cytochrome C promotes apoptosome formation through Apaf-1 and pro-caspase 9, ultimately contributing to cleavage of pro-caspase 9 to caspase 9. Activated caspase 9, then cleaves and activates caspase 3, initiating executioner caspases which activate cytoplasmic

endonucleases and proteases degrading nuclear components and cytoskeletal proteins (26). The extrinsic pathway is triggered by binding of a trimeric ligand to a transmembrane receptor, called the "death receptor", such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and FAS (27). Binding of ligands to the death receptors activates the formation of a multi-protein complex, the Death-Inducing Signaling Complex (DISC), and recruitment of adapter proteins for catalytic cleavage and activation of caspase-8. Activated caspase-8 can cleave and activates caspase 3 (26). Both the intrinsic and extrinsic pathways converge on caspase 3, leading to mass degradation of intracellular components.

Under ideal conditions, clinicians aim to selectively optimize apoptotic cell death in tumor cells, while limiting toxicity and cell death in healthy tissue. However, this is not necessarily the outcome for all patients. Adverse toxicities to cytotoxic anti-tumor therapies often result in lower tolerated doses and reduce the extent of apoptosis, while collaterally inducing sufficient damage for tumor cells to upregulate several cellular survival mechanisms (28,29).

1.3. Autophagy

1.3.1. General Introduction and history

The word autophagy is derived from the Greek words "auto" meaning self and "phagy" meaning eating to describe the cells' distinct self-degradative process designed to maintain organellar and energy turnover during injurious events (30). Accordingly, autophagy is an evolutionarily highly conserved intracellular catabolic process through which proteins, organelles, and pathogens are degraded for waste elimination or repurposed for the anabolic cellular needs (31). Autophagy is frequently activated as part of homeostatic processes in response to cellular stresses, such as hypoxia or nutrient deprivation (32,33).

The term “autophagy” was first coined by Christian de Duve in the 1960s while studying lysosomes, for which he later won the Nobel Prize in Physiology or Medicine in 1974. Using electron microscopy, he observed what appeared to be a double membrane vesicle sequestering elements of the cytoplasm and other cellular organelles (34); thus, de Duve was able to describe the end stages of autophagy and identify autophagy morphologically. It was not until the 1990’s that the mechanistic components of autophagy were demonstrated by Yoshinori Ohsumi and colleagues, who identified the numerous genes involved in the autophagic machinery and phagophore formation by investigating autophagy mutants in yeast for which they also received the Nobel Prize in Physiology or Medicine. These studies provided the backbone for our current understanding of the autophagic molecular mechanism. As of this writing, 31 autophagy-related genes (ATG) have been discovered, coding for various components of the autophagic machinery and essential components required for macroautophagy. Through loss-of-function studies, it has been demonstrated that autophagy is important for multiple aspects of an organism’s lifespan, including maintaining cellular and tissue homeostasis, metabolism, immunity, protection against aging and early differentiation and development, as well as its role in disease states such as cancer (35).

1.3.2. Types of Autophagy

Autophagy often incorporates a broader, more ubiquitous degradative process, but in fact there are multiple types of autophagy. The three primary types of autophagy include: macroautophagy, microautophagy, and chaperone-mediated autophagy (31). In macroautophagy, components of the cytoplasm and dysfunctional organelles are sequestered into the growing phagophore, which is a *de novo* cytosolic double-membrane vesicle that engulfs cytoplasmic proteins and organelles and delivers them to the lysosome (30). Microautophagy occurs when cargo is directly taken up

through the invagination of the lysosomal membrane (36). While macro- and microautophagy can vary in their selectivity for the recycled cellular components, chaperone-mediated autophagy (CMA) is highly specific (37). CMA involves the transport of unfolded proteins directly into the lysosomal membrane by heat-shock proteins, which recognize a specific consensus motif on the target protein (31,37).

Autophagy can be a selective or nonselective degradative process. Various adaptor proteins are involved in the sequestration of specific cargo into autophagosomes through the recognition of cargo tagged with distinct degradative signals, such as neighbor of BRCA1 (NRB1), which works with p62 to recognize mono-ubiquitylated peroxisomes in pexophagy (38,39), and Nip3-like protein X (NIX), which is important in the clearance of mitochondria during mitophagy (39,40). However, for the remainder of this dissertation, we will focus on macroautophagy, which we will refer to as autophagy.

1.3.3. Autophagic Machinery

During the macroautophagic process, a double-membrane compartment (vacuole) is formed, the vacuole fuses with the lysosome, and the degraded contents of the vacuole are released into the cytosol **(Figure 1.1)**. Under normal conditions, mammalian-target of Rapamycin (mTOR) complex 1 negatively regulates autophagy by phosphorylating and binding to Unc-51 Like Autophagy Activating Kinase 1 (ULK-1), thus inactivating ULK-1 (41). Under conditions of cellular stress, ULK-1 becomes dephosphorylated and dissociates from mTOR (41). Initiation of autophagy occurs through the activation of the ULK-1 complex, which triggers the nucleation of the phagophore through phosphorylation of PI3KC3, VPS34, and Beclin-1 (BECN1) (42,43). While the source of the pre-autophagosomal structure is not fully understood, there is literature

evidence supporting phagophore generation from the endoplasmic reticulum (44), mitochondria (45), Golgi apparatus (46), and recycling endosomes (47).

Initial biogenesis of the autophagosome begins with the formation of the phagophore that is further elongated by the recruitment of autophagy regulatory proteins (ATG), such as ATG5 and ATG7. Microtubule-associated protein light chain 2 (MAP1-LC3) is lipidated by ATG3-mediated conjugation and incorporated into the growing phagophore, and is necessary for the closure, fusion, and maturation of the autophagosome (48,49). Cargo is sequestered into the autophagosome by SQSTM1/p62, a sequestrosome that binds ubiquitinated protein and anchors itself to LC3 located on the inner membrane of the autophagosome (49). The growing phagophore extends until the two ends join and fuse together to form a double-membrane vesicle or the autophagosome. In the final step, the mature autophagosome fuses with the lysosome to form the autolysosomes. The acidic hydrolases of the lysosome degrade the cargo within the autophagosome, which is then released into the cytoplasmic space for cellular repurposing (50).

Activation of autophagy is regulated by a plethora of stress factors, including hypoxia, nutrient starvation, ATP/AMP levels, ROS, and microbial infection (51). Increasing reactive oxygen species (ROS) generation results in damaging oxidation of lipids, proteins, and DNA, as well as mitochondrial dysregulation contributing to further accumulation of oxidative stress (51). Metabolic stress deregulating ATP/AMK ratios and mitochondrial dysfunction activates 5' AMP-activated protein kinase (AMPK), which directly and indirectly modulates autophagy. AMPK inhibits mTOR, an inhibitor of autophagy, through TCS2 and raptor phosphorylation; thus, AMPK activation indirectly induces autophagy (52). Furthermore, under stressed conditions, AMPK can directly phosphorylate ULK1 complexes important for the initiation of autophagy (53) and can contribute to autophagosomal maturation and lysosomal fusion (54). Oxidative stress also activates

and stabilizes hypoxia-inducible factor-1 (HIF-1α), which results in its subsequent nuclear translocalization and regulates the gene transcription of essential autophagy machinery (55). HIF-1α can regulate autophagy indirectly by altering glucose metabolism, mTOR regulation, and unfolded protein responses (55).

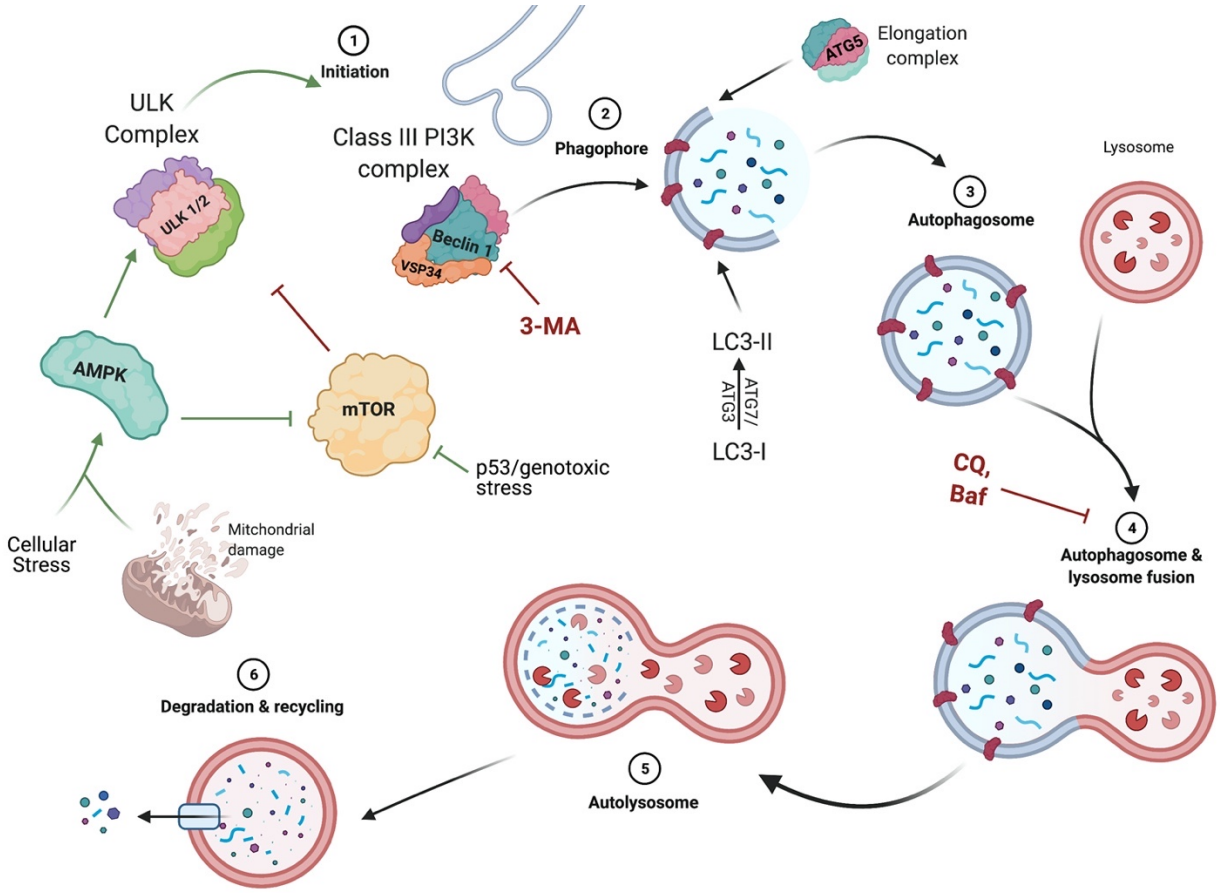

Figure 1.1 Schematic representation of the macroautophagic process.
A number of cellular stresses, including but not limited to hypoxia, nutrient deprivation and therapy-induced damage resulting in mitochondrial and genotoxic stress, activate autophagy as a means to prevent damage accumulation and replenish intracellular nutrients. In this figure, green arrows demonstrate positive regulators of autophagy, while red indicators demonstrate negative regulators. AMPK is activated in cells undergoing stress or bioenergetic dysregulation resulting in mTOR inhibition, which otherwise negatively regulates autophagy by binding and inactivating ULK-1. Under stressed conditions, ULK-1 is dephosphorylated and dissociates from mTOR, and triggers nucleation of the phagophore through activation of beclin-1 and PI3K complexes. Elongation and maturation of the growing phagophore are carried out by essential ATG proteins, including ATG5 and ATG12, and lipidation of LC3-I to LC3-II and its subsequent incorporation into the autophagosome membrane. Cargo sequestration is mediated through p62 recognition of ubiquitinated targets and trafficked into the growing autophagosome where p62 anchors itself to LC3-II. Lastly, the mature autophagosome fuses with the lysosome, and the catalytic hydrolases of the lysosome degrade the autolysosomal cargo, which is exported back out into the cytoplasm for repurposing. Created with BioRender.com.

1.3.4. Autophagy and Disease

Dysfunctional autophagy has been associated with a number of cardiac and neurodegenerative disease states, such as Parkinson disease, Alzheimer Disease, and Huntington disease (56). Alternatively, autophagy has been implicated to have dual functions in cancer development and therapeutics, exhibiting both tumor-promoting and tumor-suppressing roles. Mice with Beclin-$1^{-/-}$ embryonic stem cells were shown to die during embryogenesis, suggesting that autophagy is essential in early development. More intriguingly, a greater number of haplosufficient Beclin-$1^{+/-}$ mice developed spontaneous tumors when compared to wild-type (wt) mice, suggesting that Beclin-1 may serve as a tumor-suppressor gene, and mutations in Beclin-1, consequently resulting in autophagy dysregulation, could contribute to tumorigenesis (57–59). With regard to the tumor-suppressive effects of autophagy, induction of autophagy prevents the accumulation of damaged organelles, protein aggregates and promotes the removal of oncogenic proteins (60–62). Furthermore, autophagy induction in healthy tissue functions as a tumor-suppressive mechanism by removing dysfunctional mitochondrial and ROS that may cause DNA damage, thereby maintaining genomic stability (51,61). Autophagy induction also plays a role in eliciting an immune response through modulation of immunogenic-cell death (ICD) by contributing to the secretion of danger-associated molecular patterns (DAMPs), such as ATP (63), secretion of cytokines, as well as antigen processing (64–71).

In tumor cells, depending on the extent of genomic and cellular damage, autophagy can promote tumor cell survival through clearance of protein aggregates and damaged organelles incurred by cytotoxic therapy to allow tolerance of stress (72–74). The catabolic processes of autophagy also provide metabolic intermediates and raw materials, which feed into the intracellular anabolic processes, permitting the maintenance of cellular bioenergetics (75,76). Studies by Guo *et al*.

demonstrated that RAS-expressing tumors had elevated levels of basal autophagy, and relied on mitophagy-induced clearance of dysfunctional mitochondria and maintenance of metabolically functional mitochondria for survival (75); that is these types of tumors could be described as being autophagy-dependent or autophagy-addicted.

Autophagy can also aid to maintain low intracellular levels of ROS that contribute to activation of pro-survival pathways, such as Src and NfkB (77,78), resulting in tumor promotion (51,79,80). Moreover, HIF-1α induced autophagy may contribute to tumor resistance to anti-cancer treatments, and inhibition of autophagy may restore sensitivity to therapy (81,82). While autophagy is implicated in tumorigenesis, functioning as both a tumor-promoting and tumor-suppressing mechanism, autophagy is also induced in response to therapy as a potential mechanism to prevent damage accumulation (83,84).

1.3.5. Autophagy in response to cancer therapy

Cytotoxic therapies, acting through a multitude of mechanisms, rely largely on extended damage and impaired cellular functions to activate cell death machinery. In an effort to mitigate the cytotoxic effects of cancer therapies, autophagy is often, if not uniformly, activated to remove protein aggregates, nuclear damage, and/or depolarized mitochondria (85). Removal of these damaged moieties serves to evade activation of apoptotic cell death pathways and provides the raw material necessary for metabolic processes (86); however, depending on the extent of damage, excessive autophagy activation can also potentially result in cell death (87).

Extensive pre-clinical and clinical studies have demonstrated autophagy induction in response to therapy (83,88,89). Although the desired outcome of radiation and chemotherapy is tumor cell death by a pathway such as apoptosis, it is not obligatorily the sole or primary response to radiation and chemotherapy. While the effectiveness of clinical therapy in promoting tumor shrinkage may,

of necessity, ultimately involve apoptosis, studies in tumor cells in culture clearly indicate that a consistent and uniform initial response to radiation and chemotherapy is autophagy (90–93). Studies by Ren *et al.* utilizing 30 NSCLC patient tissue samples subjected to 2 Gray (Gy; a clinically relevant dose of radiation), assessed LC3 and SQSTM1/p62, markers of autophagy, by immunohistochemical staining. Of these 30 samples, 26 demonstrated significant upregulation of LC3 and downregulation of SQSTM1/p62, indictive of autophagy induction (88,89,91,94,95). Screening was performed in which U20S osteosarcoma cells were exposed to 80 National Cancer Institute (NCI) anticancer drugs and the extent of apoptosis, autophagy, and necrosis were measured. Of these 80 cytotoxic drugs, 59 drugs induced autophagosome formation and demonstrated autophagic flux in studies where the cells were also exposed to bafilomycin A1 (BafA1), a pharmacological inhibitor of autophagic flux (96). The remaining 21 drugs produced predominantly necrosis and apoptosis, with little autophagic flux. These latter agents were further characterized as microtubule inhibitors, which may in part be due to the necessity of microtubules for autophagosome localization and migration to lysosomes (97). There is an indisputable complexity to the cellular responses activated by chemotherapy, and of those responses, autophagy is clearly induced in tumor cells by many anticancer therapeutics (**Figure 1.2)**.

1.3.5.1.Autophagy in response to DNA damaging therapy.

A primary mechanism whereby a number of anticancer drugs, such as platinum-based compounds and topoisomerase I/II poisons, exert their cytotoxic effects is through DNA damage. DNA double strand breaks (DSB) activate Ataxia-Telangiectasia mutant (ATM), which in turn can activate Chk1/2 as well as AMPK (98). ATM, itself, and activated Chk1/2 can phosphorylate and stabilize p53 (99–101), which, in turn modulates the transcription of key autophagy-related genes as well as damage-regulated autophagy modulator (DRAM), resulting in autophagy induction (98,102).

Autophagy also plays a role in DNA damage repair by providing metabolic precursors for ATP generation required by several DNA damage response (DDR) pathways, maintains dNTPs needed for DNA replication and repair, and plays a role in the turnover of key proteins involved in DDR and DSB processing (103). Lin *et al.* demonstrated that cisplatin, an alkylating agent that generates bulky adducts on DNA, induced autophagy through Beclin-1 activation in human bladder cancer (104). Similarly, Li *et al.* demonstrated that interference with autophagy by 3-MA or siRNA targeting ATG7, both *in vitro* and *in vivo*, sensitized colorectal cells to 5-fluorouracil (5-FU), a pyrimidine analog and antimetabolite (105). Aydinlik *et al*. showed autophagy induction in response to doxorubicin, a topoisomerase II inhibitor, in triple-negative breast cancer cells; furthermore, autophagy inhibition increased doxorubicin-induced cell death and increased sensitivity (106).

Therapies such as radiotherapy induce excessive damage throughout the cell through direct DNA damaging capabilities and through the indirect generation of ROS (81,82,107,108). Both DNA damage and ROS are well characterized as inducers of autophagy. Ito *et al*. showed that radiation exposure in malignant glioma cell lines induced autophagy and cell cycle arrest, instead of apoptosis (95).

1.3.5.2.Autophagy in response to non-DNA damaging therapy

Autophagy is also induced in response to multiple chemotherapeutic modalities which are not considered to directly promote DNA damage. Moreover, as therapeutic approaches, such as targeted therapies and immunotherapy, have become an additional element of anticancer treatment regimens, autophagy has also been shown to be induced in response to a variety of such novel agents. For example, tamoxifen and other anti-estrogens induce protective autophagy in breast

cancer (109). Histone deacetylase (HDAC) inhibitors, such as butyrate and SAHA, were shown to induce autophagy, and more specifically autophagic cell death in cervical cancer (110). The epidermal growth factor receptor (EGFR) represents a major target for a variety of monoclonal antibodies for the treatment of different cancer types. EGFR activates several downstream pro-survival pathways, including Ras/MAPK, Jak/Stat, and PI3K/AKT/mTOR signaling pathways (111). While EGFR inhibitors are often effective, many cancer patients become resistant to these therapies; thus, further understanding of and manipulation of autophagy may serve as a potential therapeutic strategy for sensitization of patients to anti-EGFR therapies. In support of this approach, Li *et al*. demonstrated autophagy induction in response to cetuximab, an EGFR-blocking antibody, via inhibition of mTOR and activation of Beclin-1 in human vulvar squamous carcinoma, colorectal cancer, and NSCLC (112). Autophagy induction was further demonstrated in response to targeted therapy in studies where hepatocellular carcinoma (HCC) cells were shown to undergo autophagy in response to linifanib, a vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) inhibitor, through suppression of PDGFR-β and its downstream signaling pathways, *in vitro* and *in vivo* (113).

1.3.5.3. Non-cell autonomous effects of autophagy.

Autophagy modulation may mediate both cell-autonomous (direct cellular) and cell non-autonomous effects, the latter related to surrounding cells, such as immune, epithelial, and other tumor cells in the TME. Immune evasion by tumor cells remains a major barrier to effective cancer treatment. Tumor cells can develop multiple mechanisms to evade host-mediated immunity, such as impaired/downregulation of antigen presentation and upregulation of immune checkpoints, which downregulate T-cell activity (114). Yamamoto *et al*. demonstrated that major histocompatibility complex class I (MHC-I), a molecule important in antigen presentation, was

selectively degraded through autophagy-dependent mechanisms in pancreatic ductal adenocarcinoma (PDAC) cell lines (115). Furthermore, in a mouse model of PDAC tumor cells expressing a doxycycline-inducible dominant-negative ATG4B knockdown, autophagy inhibition was shown to increase MHC-1 expression and CD8+ T cell activation, enhanced T-cell mediated killing and consequently a reduced tumor cell viability (115). Reduced tumor burden and increase tumor-infiltrating T cells were also evident in a similar model with knockdown of ATG7 (115). Given that PDAC is refractory to immune-checkpoint blockade (ICB), the authors examined whether autophagy inhibition would sensitize tumors to immune-checkpoint inhibitors. Utilizing a syngeneic mouse model with orthotopic tumors, Yamamoto *et al*. demonstrated a significant reduction in tumor volume and increased CD8+ T cell infiltration in the ATG4B knockdown mice compared to autophagy-proficient mice when exposed to dual ICB (anti-PD-1 and anti-CTLA4). Furthermore, administration of CQ significantly sensitized tumors to the ICB therapy. Finally, Liang *et al*. also demonstrated autophagy induction in response to high-dose interleukin-2 (IL-2) treatment in a model of advanced metastatic murine liver tumors (116). Addition of CQ with IL-2 therapy increased immune infiltration, reduced tumor burden, and increased survival. These data suggest that autophagy inhibition may be a potential strategy for the sensitization of patients to immune-checkpoint therapy.

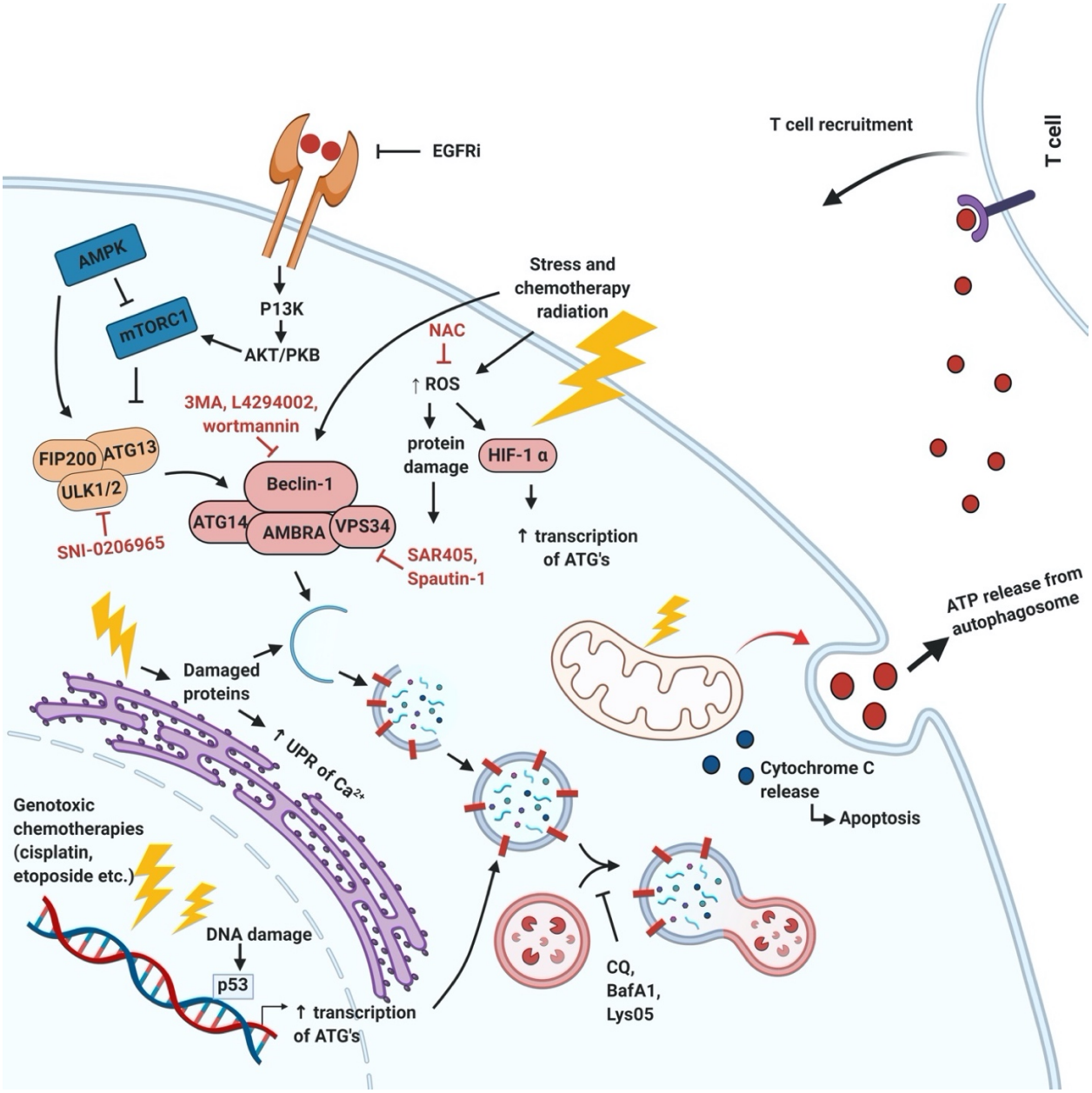

Figure 1.2. Autophagy induction in response to therapy.
Anticancer therapies work through multiple mechanisms of action, such as genotoxic stress, oxidative stress and increase ER and mitochondrial damage. Genotoxic therapies, such as cisplatin and etoposide, incur DNA damage, activating DNA damage response and p53 pathways. p53 activation can promote the transcription of autophagy-related genes (ATG's), which contribute to components of the autophagic machinery. Stress, chemotherapy and radiation can also induce protein and organelle damage, as well as the accumulation of misfolded proteins. Excessive aggregation of unfolded proteins activates the unfolded protein response, UPR, in the ER, which promotes autophagy as a means to alleviate the burden unfolded/misfolded proteins and maintain homeostasis. Aside from increased ER stress, multiple chemotherapies and radiation promote the

generation of ROS levels as well. Increased cellular ROS levels and oxidative stress triggers HIF-1a activation. HIF-1a can translocate into the nucleus and promote transcription of ATG genes; thus, inducing autophagy to prevent the accumulation of oxidative stress. Furthermore, increasing levels of ROS oxidize surrounding proteins resulting in oxidative damage and malfunction, as well as dysregulation of mitochondrial bioenergetics, exacerbating damage and activate intrinsic apoptotic pathways. The resulting metabolic dysfunction increases intracellular ATP, which can be shuttled into the extracellular space, bind to purogenic receptors on T cells, and promote recruitment. Autophagy has been shown to play a role in ecto-ATP release in dying cells and promote immune recruitment and clearance of tumor cells. Lastly, therapies, such as EGFR inhibitors, block tyrosine kinase receptor signaling cascades promoting mTOR activation, a known inhibitor of autophagy; therefore, EGFRi block upstream receptor signaling, resulting in mTOR inhibition and Beclin-1 activation. Taken together, autophagy is involved and induced in response to multiple cytotoxic therapies through a multitude of intracellular pathways as a means to prevent the accumulation of damage incurred by therapy. Created with BioRender.com.

1.3.6. Functional forms of autophagy

While autophagy is routinely observed in tumor cells in response to chemotherapy and radiation, the functional contribution of autophagy to the overall outcome of therapy can vary depending on a number of factors, including but not limited to, the type of therapeutic utilized, time of treatment, dose, type of cancer and cell type. Autophagy can play multiple functions in response to chemotherapy and radiation treatment; however, the functional form of autophagy can only be determined through the impact on drug or radiation sensitivity when the autophagy is inhibited either pharmacologically and/or genetically. Conventional pharmacological inhibition, using agents such as chloroquine (CQ), Bafilomycin A1 (BafA1) or 3-methyladenine (3-MA), or genetic silencing techniques, including siRNA or shRNA knockdown or cell-specific gene knockout of Beclin-1 and essential ATG proteins, can be utilized to determine the functional form of autophagy induced by a particular treatment (**Table 1)**.

1.3.6.1.Cytoprotective Autophagy.

One of the best-known functions of autophagy is its conventionally *cytoprotective* function in response to cellular stresses, such as serum starvation or hypoxia. In this scenario, pharmacological and genetic inhibition of autophagy increases sensitivity to the anticancer treatment that promotes autophagy (117,118). Increased sensitivity is often associated with the activation of cell death pathways, apparently indicating that autophagy was acting as a *protective* cellular mechanism for the evasion of cell death (119,120). In most of the current literature, autophagy is considered to have a cytoprotective function; consequently, inhibition of cytoprotective autophagy would be anticipated to result in radiosensitization and chemosensitization. Early work performed by Chaachouay *et al.* showed that autophagy inhibition with 3-methyladenine (3-MA) and chloroquine (CQ) radiosensitized MDA-MB-231 (MDA-231) and HBL-100 breast cancer cells

(92). Similarly, CQ was also shown to sensitize bladder cancer cells to radiotherapy both *in vitro* and *in vivo* and to promote apoptosis when autophagy inhibition was combined with radiation (121). Additionally, Qadir *et al*. demonstrated that autophagy inhibition sensitized MCF-7 breast tumor cells to tamoxifen. In these studies, sensitization was due to increased mitochondrial dysregulation and caspase 9-mediated apoptosis, indicating that inhibition of cytoprotective autophagy increased cell death (122). Similarly, Selvakumaran *et al*. showed that autophagy was induced in response to oxaliplatin in multiple colon cancer cell lines, and administration of CQ significantly reduced tumor volume in response to oxaliplatin and bevacizumab in HT29-derived orthotopic mouse tumors (123).

A number of miRNAs have also been identified that mediate autophagy (124). The expression of miRNA-30a (miR-30a) is downregulated in several renal carcinoma cell (RCC) lines and patient samples and has been shown to inhibit autophagy through Beclin-1 downregulation. Knockdown of miR-30a significantly increased Beclin-1 expression and interfered with the cytotoxic effects of sorafenib, a multikinase inhibitor, in RCC cells (125). Pharmacological autophagy inhibition through CQ, 3-MA, and BafA1 reduced cell viability and increased cell death in RCC cells treated with sorafenib, indicating that autophagy was playing a cytoprotective function in response to sorafenib (125). Collectively, these studies demonstrate the functional capacity of autophagy to protect tumor cells challenged by anticancer therapy.

Resistance Induction of cytoprotective autophagy has also been implicated in resistance to therapy and can facilitate multidrug resistance (126). Utilizing epirubicin-resistant MCF-7er and SK-BR-3er cells, Sun *et al*. examined the role of autophagy in multi-drug resistance (127). MCF-7er and SK-BR-3er cells were collaterally insensitive to paclitaxel and vinorelbine compared to parental

cells. Genetic silencing of Beclin-1 and ATG7 in MCF-7er and SK-BR-3er restored sensitivity to paclitaxel and vinorelbine. Anthracycline-resistant MDA-MB-231-R8 and SUM159PT-R75 cells exhibited higher basal autophagic flux than parental cells (128). Genetic knockdown of ATG5/7 and pharmacological inhibition of autophagy with CQ or hydroxychloroquine (HCQ) significantly sensitized both epirubicin-sensitive and resistant triple-negative breast cancer cell lines. These studies support the concept of autophagy functioning as a cytoprotective mechanism employed by the tumor cells to avert cell death, and that manipulation of these processes could hold therapeutic potential.

Autophagy induction can also play a role in resistance by contributing to cancer stem cell maintenance. Cancer stem cells are a subpopulation of tumor cells that have self-renewal and differentiation capabilities and can contribute to resistance to anti-cancer therapy and disease reoccurrence (129,130). Lomonaco *et al.* investigated the role of autophagy in radioresistant glioma stem cells (GSC). CD133+ GSC cells exhibited a greater extent of autophagy when exposed to γ-radiation compared to CD133- cells; moreover, exposure to BafA1 or genetic knockdown of Beclin-1 and ATG5 significantly reduced cell viability (93). These data suggest that autophagy is protective in response to γ-radiation in CD133+ GSC cells.

Additionally, acidic lysosomes can trap weakly basic drugs, such as doxorubicin and irinotecan, thereby reducing drug efficacy and conferring resistance. Guo *et al*. examined the role of autophagy in doxorubicin localization as MCF-7 cells acquired resistance to increasing doses of doxorubicin (131). Their studies demonstrated increased autophagy induction and sequestration of doxorubicin within lysosomes as MCF-7 cells developed resistance to doxorubicin treatment; furthermore, autophagy inhibition with CQ was sufficient to restore sensitivity in doxorubicin-resistant MCF-7 cells.

While autophagy inhibition may have beneficial outcomes, in a cell-autonomous manner, the effects of autophagy inhibition on surrounding cells, or the cell non-autonomous aspects, must also be considered. Thorburn *et al*. demonstrated that silencing of ATG5 and ATG7 in MDA-MB-231 TRAIL-sensitive breast tumor cells resulted in increased outgrowth of GFP-tagged TRAIL-resistant cells when compared to autophagy-proficient controls (132). Furthermore, autophagy inhibition through an inducible shATG12 model in a population of GL261 glioma cells sensitive to EGF inhibition promoted outgrowth of GFP-tagged EGF-resistant cells. Through these studies, Thorburn *et al*. also demonstrated that autophagy inhibition in *drug-sensitive* cells induced caspase activation, and the subsequent stimulation of prostaglandin E_2 (PGE_2) signaling promoted tumor repopulation of *drug-resistant* cells (132,133). Taken together, autophagy inhibition may increase cell killing and sensitize drug-sensitive cells to therapy; however, this may promote the selection and growth of a resistant population, which may have detrimental therapeutic outcomes. Thus, there is an intricacy to autophagic function in response to chemotherapy and radiation that must be considered prior to the adoption of autophagy inhibition therapy.

1.3.6.2.Cytotoxic Autophagy.

The generally accepted premise that autophagy is *cytoprotective* in cells in response to stress has spilled over to establishing its *cytoprotective* function in response to anticancer therapy in tumor cells. Extensive preclinical data in a number of experimental tumor models and in response to various chemotherapeutic agents does support the cytoprotective function of autophagy based on the observations outlined in the previous section. However, it should be emphasized that pre-clinical data has, in fact, demonstrated that autophagy can be divergent from this protective function and exhibit both a cytotoxic activity as well as, in certain cases, failing to modulate the tumor response to therapeutics.

Autophagy modulation may appear as a desirable therapeutic target; however, it is important to acknowledge the multi-functional nature of autophagy. In response to chemotherapy and radiation, cells can also undergo a *cytotoxic* form of autophagy, resulting in reduced cell viability and clonogenic survival. Cytotoxic autophagy can kill tumor cells through both apoptosis-dependent and independent mechanisms (117,134,135). When autophagy takes on a cytotoxic function, autophagy inhibition reduces tumor cell sensitivity to therapy and promotes cell viability. Studies by Talarico *et al*. examined the effects of SI113, a selective SGK1 serine/threonine inhibitor, in combination with radiotherapy on human glioblastoma multiforme (GM) cells (136). Administration of SI113 significantly reduced cell viability and oxidative stress in GM cell lines exposed to 5, 8, or 10 Gy of radiation. The authors demonstrated that SI113 induced autophagy and that the addition of CQ abolished the cytotoxicity of SI113, thus indicating that the antitumor effects of SI113 were mediated through induction of cytotoxic autophagy. Kanzawa *et al.* demonstrated autophagy inhibition with 3-MA and genetic silencing ameliorated temozolomide anti-tumor effects (137). Indeed, autophagy inhibition when the autophagy is protective in nature is a potential therapeutic to be exploited as a means of sensitization; however, when the autophagy is toxic in function, this can result in detrimental outcomes, such as tumor survival and drug resistance.

1.3.6.3.Cytostatic Autophagy.

Autophagy can also exhibit a *cytostatic* function in response to cancer therapeutics. Of the functional forms of autophagy, this is perhaps the least understood, although it is logical that cells exposed to stress might arrest and fail to continue to progress normally through the cell cycle.

In the case of cytostatic autophagy, therapy-induced growth inhibition and sensitivity is mediated through autophagy, rather than apoptosis; furthermore, autophagy interference restores cellular

proliferation and ameliorates antitumor therapeutic effects (117,138). For instance, work by Sharma *et al*. demonstrated cytostatic autophagy in response to EB 1089, a vitamin D analog, in NSCLC cells when combined with radiation (139). The authors show that administration of EB 1089 or 1,25-D_3 significantly sensitized H460 NSCLC cells to radiation. Sensitization was not associated with increased DNA damage, apoptosis, or necrosis, or senescence, but rather these cells exhibited a more pronounced growth arrest, which was characterized as a form of cytostatic autophagy. Autophagy inhibition through CQ administration, shATG5 or ShBECN1 knockdown sensitized H460 cells to radiation alone, indicative of radiation-induced cytoprotective autophagy in H460 cells; however, in cells treated with EB 1089 and radiation, autophagy inhibition reversed the sensitization induced by EB 1089.

Studies by Dou *et al*. also demonstrated cytostatic autophagy in breast cancer cells exposed to ivermectin, an antiparasitic drug with anticancer therapeutic potential (140). The authors showed that ivermectin was sufficient to suppress breast cancer cell proliferation and tumor burden, *in vivo*. Breast cancer cells were shown to undergo little or no significant apoptosis in response to ivermectin but did undergo autophagy. Furthermore, autophagy inhibition was sufficient to attenuate the antiproliferative effects of ivermectin in breast cancer cells with no significant impact on the extent of apoptosis, suggesting that ivermectin's antiproliferative effects are mediated through induction of *cytostatic* autophagy (140). Whether cytostatic autophagy sensitizes tumor cells to therapy by contributing to the induction of quiescence or senescence is not well understood, and in fact, there is no literature evidence delineating the differential activation of either senescence or quiescence in the case of cytostatic autophagy.

1.3.6.4.Nonprotective Autophagy.

A novel function of autophagy in response to therapy is the *nonprotective* form. In previous studies from our own laboratory, we identified the "non-protective" form of autophagy (141). This is a functional definition wherein autophagy is induced in response to radiation or chemotherapy but where subsequent inhibition of autophagy fails to alter radiation sensitivity (142). Eng *et al.* demonstrated that both pharmacological inhibition of autophagy with CQ and Lys01, and genetic inhibition by genome editing of ATG7 did not alter sensitivity to radiation (or 30 different chemotherapies) of KRAS mutant tumors *in vitro* and *in vivo*. Further, they were able to show that CQ-mediated sensitization was independent of autophagy, suggesting the antiproliferative effects may be due to modulation of off-target effects (143,144). Similarly, studies performed by Schaaf *et al.* demonstrated that radiosensitization effects of CQ, 3-MA, and ATG7 deficiency were independent of canonical autophagy pathways and may involve effects on lysosomal degradation (145).

In a seminal paper by Michaud et al, autophagy that was induced in colorectal cancer cells by oxaliplatin or mitoxantrone proved to be nonprotective in function, in that silencing of the autophagy gene, ATG7, failed to influence drug sensitivity in the tumor cells *in vitro* (146). Intriguingly, work by Cechakova *et al.* suggested that Lys05, an autophagy inhibitor, could radiosensitize H1299 (p53 null) cells, indicating a cytoprotective autophagic function in these NSCLC cells (147). However, a closer examination of data revealed the observed sensitization is modest in effect, and unlikely to be therapeutically relevant, indicating that the autophagy is likely acting in a nonprotective fashion. This observation serves to confirm the findings from our own laboratory where we reported radiation-induced nonprotective autophagy in the same H1299 (p53 null) cells, i.e. wherein autophagy inhibition likewise failed to alter radiosensitivity (141).

As exemplified through the literature presented in this section, autophagy exhibits a multifunctional nature in response to chemotherapy and radiation. Clinical autophagy inhibition in scenarios where the autophagy is *cytoprotective* in function proves an advantageous avenue for chemo-and radio- sensitization. However, the capacity to determine autophagic function in response to antineoplastic therapy in patients is not considered or screened for prior to the addition of autophagy inhibitors to therapeutic regimens. This is in part due to the lack of clinical biomarkers or determinants of autophagic function; thus, in-depth pre-clinical studies are required to elucidate molecular regulators and conditions modulating the particular functions of autophagy before routine incorporation of autophagy inhibitors into the clinic.

Table 1.1. Pharmacological Autophagy Inhibitors.

Autophagy Inhibitor	Mechanism of Inhibition	Target	Effective Inhibitory Concentration	Reference
CQ/HCQ	Accumulates within lysosome and prevents lysosomal fusion	Lysosomal fusion	5-50 μM	(148,149)
BafA1	V-ATPase inhibitor, interferes with lysosomal acidification	Lysosomal fusion	1-10 nM	(73,150)
Lys05	Dimeric form of CQ, accumulates in lysosome	Lysosomal fusion	10-100 μM	(151,152)
3-MA	Class III PI3K inhibitor, prevents initiation	Autophagosome formation	1-10 mM	(153,154)
SAR405	Vacuolar protein sorting protein 18 & 34 (Vps18 and Vps34) inhibitor	Autophagosome formation	1-10 μM	(155–157)
Spautin-1	Ubiquitin-specific peptidase inhibitor, Vps34 inhibitor, prevents initiation	Autophagosome formation	1-20 μM	(158,159)
LY294002	PI3K inhibitor, prevents initiation	Autophagosome formation	10-100 μM	(160,161)
Wortmannin	PI3K inhibitor, prevents initiation	Autophagosome formation	1-10 μM	(162)
SBI-0206965	ULK1 inhibitor, prevents initiation	Autophagosome formation	10-50 μM	(163,164)
NSC185058	ATG4B inhibitor	Autophagosome formation	1-100 μM	(165)
Mdivi-1	Mitochondrial division inhibitor, inhibits fission by selective dynamin-related protein 1 (Drp1) inactivation	Mitophagy	1-10 μM	(166,167)

1.3.7. p53 and autophagy

A number of molecular pathways have been implicated in regulating autophagy; however, a protein that seems to be upstream of most of these regulators is p53 (19,168). p53 is a well-known key tumor suppressor, which is often aberrant in many tumor cell types and is frequently implicated in early tumorigenesis. Amongst its various cellular functions, p53 plays primary functionals role in regulating apoptotic cell death, cellular growth arrest, and DNA repair. While p53 has been shown to regulate autophagy, the nature of the relationship between p53 and autophagy is not completely established. Depending on spatiotemporal localization, nuclear p53 has been shown to activate autophagy through transcription of upstream inhibitors of mTOR and through more direct means, such as DRAM upregulation, which encodes a lysosomal protein inducing macroautophagy (102). However, on the flip side, cytoplasmic p53 protein inhibits autophagic cell death by inducing BECN degradation via the ubiquitin-specific peptidases USP10 and USP13 and/or inhibiting the AMPK-mTOR-ULK1 signaling pathway (169–171). To add further complexity to this relationship, autophagy can also mediate p53 turnover and degradation (159). Whether p53 activates or suppresses autophagy is not clear; however, this may provide initial insights as to a potential mediator of the various functional forms of autophagy.

Mutant p53 has also been shown to regulate autophagy to confer chemoresistance in lung cancer (172). Mutant p53 has been shown to upregulate Nrf2 activity, a transcription factor that codes for multidrug resistance, antioxidant proteins, and other proteins triggered to protect against oxidative and chemotoxic damage (173). In this study, Tung et al demonstrated that wild-type p53 suppressed Nrf2 promoter activity in NSCLC cells; however, in p53 mutant cells, promoter activity was not suppressed, resulting in increased mRNA levels of Nrf2 and upregulated transcription of the anti-apoptotic proteins, Bcl-2 and Bcl-X_L, ultimately, contributing to cisplatin resistance (173).

Studies by Saini *et al.* examined the effects of p53 GOF (gain of function) mutants on cancer cell resistance to chemotherapy and proteasomal inhibition utilizing H1299 p53 null NSCLC cells transfected with either wild-type p53, R273H mutant GOF, or empty vector (174). They demonstrated that R273H-p53 mutant cells were significantly less sensitive to cisplatin and 5-FU and exhibited multi-drug resistance. While dual treatment with a proteasomal inhibitor, peptide aldehyde N-acetyl-leu-leu-norleucinal (ALLN), and an autophagy inhibitor did not sufficiently promote cell death in R273H-p53 mutant cells, activation of autophagy by serum starvation or rapamycin exposure promoted cytotoxic autophagy and enhanced cell killing through increased autophagosome accumulation and ROS levels. In contrast, Wu *et al.* demonstrated that cisplatin-refractory A549 lung adenocarcinoma cells expressing wild type p53 exhibited greater basal autophagy compared to parental A549 cells; furthermore, treatment with cisplatin increased autophagy induction (175). Autophagy inhibition with CQ in cisplatin-resistant A549 cells induced apoptosis and resensitized these cells to cisplatin, suggesting autophagy was cytoprotective in function in A549 p53 wt cisplatin-refractory cells. Taken together, both NSCLC cell lines expressing either p53 mutant or wt p53 exhibited differential responses to cisplatin exposure, indicating that p53 status may impact autophagic function in response to anti-cancer therapy and that therapy-induced autophagy (TIA) may play a role in the development and maintenance of chemoresistance.

1.3.8. Autophagy and the Immune Response

Therapy-induced autophagy is an integral component of immunogenic cell death and immune modulation in response to anticancer therapy. Autophagy plays a role in the release of ATP in dying cells that act as chemotactic ligands for purogenic receptors on immune cells (176). This interaction is important in the recruitment of dendritic cells (DC) to the tumor microenvironment

(TME) and the subsequent activation of T cells to elicit anti-tumor responses (146,177). Studies by Michaud *et al.* demonstrated that autophagy induction *in vitro* did not significantly alter tumor cell responses to mitoxantrone (MTX) and oxaliplatin (OXA) (68). In dramatic contrast, in immune-competent mice, autophagy-proficient cells exhibited increased DC and T cell infiltration compared to autophagy-deficient cells. Autophagy inhibition suppressed ATP release and subsequent immune recruitment, while increasing exogenous ATP in autophagy-deficient tumors was able to restore immune infiltration and tumor-suppressive responses (68). These data implicate a role for autophagy in the release of ATP, a DAMP, and its function in eliciting anti-tumor immune responses to mitoxantrone or oxaliplatin in mouse colon carcinoma xenografts. Similarly, Ko *et al.* examined the immunomodulatory effects of autophagy in response to radiotherapy (176). These authors demonstrated that shRNA knockdown of ATG5 and Beclin-1 significantly radiosensitized A549 and H460 NSCLC cells and CT26 colon carcinoma cells, *in vitro*, and in immune-deficient mice. However, autophagy depletion reduced radiosensitivity and decreased ATP release in immune-competent mice, suggesting that autophagy-induced ATP release could be critical for anti-cancer immune responses (176). Consistent with the studies from Michaud *et al*., increasing exogenous ATP in autophagy-deficient tumors was able to restore immune infiltration and radiosensitize tumors, *in vivo* (176).

Autophagy modulates immune function through multiple pathways, such as pathogen degradation, antigen presentation, and processing of cytokines (65,178). Autophagy can also regulate the trafficking of receptors to the cell surface, which are required for antigen presentation and complement activation (67,179). Work by Ramakrishnan demonstrated the role of autophagy and tumor cell lysis by cytotoxic T lymphocytes (CTL). They showed upregulation of the mannose-6-phosphate receptor (MPR) in tumor cells exposed to multiple therapies, including cisplatin,

paclitaxel and doxorubicin, as well as multiple myeloma patient tumor samples (179). Furthermore, MPR was implicated in chemotherapy-induced anti-CTL responses, reduced tumor volume, as well as cell killing of bystander tumor cells that did not express tumor antigen. Autophagy inhibition abrogated paclitaxel-induced MPR cell surface expression in melanoma cells and doxorubicin-induced MPR expression in multiple myeloma cells, as well as reducing CTL cytotoxicity (179), implicating autophagy in the regulation of MPR expression on tumor cell surfaces and CTL cell killing. Taken together, these data indicate the immunomodulatory functions of autophagy in both innate and adaptive immune responses to chemotherapy and radiation.

1.3.9. Current modalities to modulate autophagy and clinical trials

Current paradigms for clinical administration of autophagy inhibitors to sensitize patients to chemotherapy and radiation are based on the premise that the autophagy induced is *cytoprotective* in function; however, the clinical utilization of autophagy inhibitors has largely produced inconsistent results (180). Currently, the only clinically approved autophagy inhibitor is hydroxychloroquine (HCQ), a Food and Drug Administration (FDA)-approved anti-malarial drug used for prophylactic treatment of malaria, treatment for lupus or rheumatoid arthritis (181). HCQ acts by inhibiting lysosomal acidification, as well as preventing the fusion of the autophagosome to the lysosome in the final steps of autophagy (149); however, there are also off-target effects of HCQ, which still remain elusive (182).

Significant pre-clinical data indicating the *cytoprotective* function of autophagy in response to chemotherapy and radiation (92,183,184) provided the foundation for the initiation of a number of clinical trials to inhibit autophagy as a strategy for sensitization of patients to therapy **(Table 1.2)**. Studies published by Vogl *et al*. in 2014 examined the effects of HCQ on the efficacy of bortezomib, a proteasomal inhibitor, in patients with relapsed/refractory myeloma. The

combination of HCQ with bortezomib demonstrated partial or minor responses in 6 (28%) of patients and stable disease in 10 (45%) of patients (148). Furthermore, electron micrographs of bone marrow plasma collected from patients exhibited greater autophagic vesicles and increased misfolded protein trafficking with the combination therapy. Patients were given 600mg of HCQ twice daily with a standard dose of bortezomib and only Grade 2 gastrointestinal toxicity and cytopenia were observed. Similarly, studies by Barnard *et al*. examined the effects of HCQ administration in combination with doxorubicin in canine Non-Hodgkin lymphoma in a Phase I clinical trial. Canine patients received HCQ orally 72 h prior to standard dose of doxorubicin treatment. Oral HCQ administration was well tolerated with no significant grade 3 or 4 toxicities. Combination therapy exhibited a 93% overall response rate in canines with lymphoma, with a median progression-free interval of 5 months (185). In two separate studies by Levy *et al.*, they were able to show improved therapeutic efficacy with autophagy inhibitors in combination with vemurafenib, a $BRAF^{V600E}$ inhibitor (186,187). Mulcahy Levy *et al.* examined the effects of HCQ administration in $BRAF^{V600E}$-mutant brain tumors resistant to vemurafenib. Patients received 500 mg CQ orally daily with continued standard dose of vemurafenib treatment. Improved therapeutic efficacy was exhibited in resistant patients who received CQ in combination with vemurafenib (186). Furthermore, increased LC3 II accumulation was seen in peripheral white blood cells in patients given CQ, suggesting autophagy was sufficiently inhibited. In contrast, a Phase I/II clinical trial combining HCQ, temozolomide (an alkylating agent), and radiation were performed in patients with glioblastoma multiforme (89). Doses of 600mg HCQ combined with low dose temozolomide failed to demonstrate improved overall survival in glioblastoma patients. Furthermore, when increasing doses to 800mg of HCQ daily combined with temozolomide, significant Grade 3 and 4 neutropenia and thrombocytopenia were exhibited. Electron microscopic

images indicated increased autophagic vesicles in patients treated with radiation; however, even at the higher doses of HCQ, substantial autophagy inhibition could not be demonstrated.

In addition to the dose-limiting side effects of HCQ, another limitation likely contributing to the inconsistent clinical results is the failure of currently available pharmacological autophagy inhibitors to sufficiently interfere with autophagy in patients. In order to effectively block autophagy in patients, significantly higher doses of HCQ would be required, which may result in undesirable side effects (180). Several other clinical trials have been initiated in order to study the effects of autophagy inhibition on chemo- and radiosensitivity; however, these provided inconsistent results. HCQ as a monotherapy in patients with metastatic pancreatic adenocarcinoma provided inconsistent autophagy inhibition and poor therapeutic efficacy in a phase II trial (188). Combination of HCQ with docetaxel in patients with metastatic prostate cancer was terminated early due to a lack of improved efficacy (Identifier: NCT00786682). However, several clinical trials have been launched utilizing higher doses of HCQ in combination with chemotherapeutics, such as bortexomib, which may show more promising results (148) (Identifier: NCT00568880, NCT01206530, NCT01506973).

Table 1.2. Clinical Trials manipulating autophagy inhibition to sensitize cancer patients to antitumor therapy.

Cancer Type	Treatment	Results	Noted Toxicities	Reference
Relapsed/ refractory myeloma	600mg of HCQ twice daily with a standard dose of bortezomib	Partial or minor responses in 6 (28%) of pts and stable disease in 10 (45%) of patients	Twice daily with a standard dose of bortezomib and only Grade	(148)
Glioblastoma Multiforme	600mg or 800 mg HCQ combined with low dose	No significant improvement in overall survival	At 800mg of HCQ daily combined with temozolomide significant Grade 3	(89)

	temozolomide and radiation therapy		and 4 neutropenia and thrombocytopenia	
Metastatic prostate cancer	HCQ with docetaxel	Early termination due to a lack of improved efficacy		Identifier: NCT00786682
High Grade Glioma	lower doses of HCQ and short course radiotherapy	When compared to radiation alone, combination did not improve patient outcome	Grade 3-5 toxicities	(189)
Metastatic Pancreatic adenocarcinoma (did not respond to conventional therapy)	400 or 600 mg HCQ, twice daily as a monotherapy	10% (2 out of 20 pts) achieved 2-month PFS (poor therapeutic efficacy) and inconsistent autophagy inhibition	2 patients experienced Grade 3/4 lymphopenia and elevated alanine aminotransferase	(190)
Advanced Solid tumors and melanoma	Combined temsirolimus (rapamycin analog) (25 mg, weekly) with 600 mg, twice daily of HCQ	67% of pts reached stable disease. Tumor biopsies demonstrated sufficient autophagy inhibition at 1200 mg HCQ	Tolerated with minor toxicities (7% anorexia, 7% fatigue and 7% nausea)	(191)
Advanced Solid tumors and melanoma	HCQ 200 to 1200 mg daily with dose-intense oral TMZ 150 mg/m (2) daily	Partial response in 14% of pts and stable disease in 27% of patients with metastatic melanoma was observed.	At 600 mg, twice daily of HCQ, treatment (1200 mg HCQ) was tolerated with Grade 2 toxicities: fatigue (55%), anorexia (28%), nausea (48%), constipation (20%), and diarrhea (20%)	(192)
Pancreatic Cancer	gemcitabine and nab-paclitaxel (1,000 mg/m^2 and 125 mg/m^2 combined with	Not powered enough to detect survivor differences, but increased immune infiltration and serum biomarker responses		(193)

	600 mg, twice daily of HCQ	detected in patients treated with HCQ		
Non-small Cell Lung cancer	Paclitaxel with carboplatin, and bevacizumab combined with 200 or 600 mg HCQ daily	21% of pts had stable disease with addition of HCQ and median PFS was 3.7 months (3.2 to 5.8 months) for patients taking HCQ	Grade 3 or higher: neutropenia (35%), anemia (15%), thrombocytopenia (10%) and dehydration (10%)	(194)
EGFR-mut NSCLC	Erlotinib (150 mg/daily) with or without HCQ (1,000 mg/daily)	Combination of HCQ and Erlotinib did not improve PFS compared to Erlotinib alone	Grade 3 Fatigue and nausea	(195)

1.4. Senescence

1.4.5. General Introduction and History

Cellular senescence is a durable growth arrest induced in response to oxidative, genotoxic, replicative, and therapeutic stress. Senescence is originally derived from the Latin word “senex”, which means “old” and was first utilized by Hayflick and Morehead in the early 1960s (196,197). Contrary to the paradigms of the time that cells *in vitro* grow *indefinitely*, Hayflick and Morehead, utilizing these primary fibroblast cultures derived from embryonic tissue, discovered that cells in culture did not have the ability to replicate infinitely, but rather the fibroblasts replicated for a finite number and entered into a growth-arrested, or senescent state (196,197). Although losing their capacity to proliferate, these cells still remained viable and metabolically active. This was later described as the “Hayflick Limit”, in which telomeres, which protect chromosomal ends, progressively shorten during replication cycles until reaching a critical length resulting in a cease in cellular division (198).

Senescence contributes to a number of physiological processes including wound healing and embryonic development and has also been implicated in various aging-related diseases, neurological disorders, and cancer (199–201). Cellular senescence is conventionally viewed as a cell-autonomous tumor suppressor mechanism because it arrests and prevents the propagation of cells with severely mutated DNA or at risk for malignant transformation (201,202). Senescence cells can be identified and targeted for clearance by several elements of the immune system (203), adding to its anti-cancer function. However, a growing body of literature now supports the notion that senescence can also promote tumorigenesis, resulting in yet another cellular program exhibiting a double-edged sword function in cancer development (201). Cell non-autonomous functions of senescent cells through secretion of the senescence-associated secretory phenotype (SASP) can promote the transformation of surrounding cells (204,205) and cause remodeling of the extracellular matrix (ECM), ultimately promoting the spread of malignant tumor cells (206–208).

Senescence may also present as a model of tumor dormancy (209). Damage incurred by the cells from radiation and chemotherapy can induce senescent growth arrest as the cell attempts to repair the damaged DNA. Radiation has been shown to induce a temporary period of growth arrest, followed by a phase of proliferative recovery (210,211) that could theoretically contribute to disease recurrence.

1.4.6. Senescence and Apoptosis Evasion

In response to chemotherapy and radiation, cancer cells can activate multiple cellular responses to evade apoptotic cell death. In regard to this, senescence can be induced in response to therapy as a mode of alternative cell fate to elude cell death, and possibly contribute to disease recurrence. For example, senescent cells exhibit multiple epigenetic modifications, including BCL family

promoters (212,213). Yosef et al demonstrated upregulation of Bcl-W and Bcl-X_L expression, anti-apoptotic proteins of the Bcl-2 protein family, in senescent human fibroblasts and showed that inhibition of these proteins with the pan-Bcl-2 family inhibitor, ABT-737, was sufficient to remove senescent cells (214). Furthermore, Saleh *et al.* demonstrate senescence induction in response to etoposide and doxorubicin in MDA-MB-231 breast cancer cells and A549 NSCLC cells, respectively (215). Administration of ABT-293, a Bcl-2/Bcl-X_L inhibitor, increased apoptosis in response to doxorubicin and etoposide in MDA-MB-231 and A549 cells by interfering with Bcl-X_L interaction with BAX, a pro-apoptotic protein (215). The authors were also able to show sensitization to etoposide and doxorubicin, as well as reduced tumor burden *in vivo*, with the addition of ABT-263; however, ABT-263 as a monotherapy had no significant effect on tumor burden. These studies offer insights into the potential of senescent cells to upregulate anti-apoptotic Bcl-2 family proteins as a means to evade apoptotic cell death and provide potential novel therapeutic targets for cancer treatment.

1.4.7. Characteristics of senescence

Senescent cells present with a myriad of phenotypes, such as morphological changes (enlargement and flattening), expression of a pH-dependent ß-galactosidase activity, secretion of chemokines and cytokines that encompass the senescence-associated secretory phenotype (SASP), and senescence-associated heterochromatic foci (SAHF) appearance (216–218). While senescent cells are growth-arrested, they maintain metabolic activity and exhibit enhanced lysosomal biogenesis (109,219,220). Of note, the Senescence-Associated β-galactosidase (SA-β-gal) enzyme, a lysosomal enzyme that functions at low lysosomal pH ~1-2 in normal cells, demonstrates an altered optimal pH of 6 in the lysosomes of senescent cells; moreover, this has become a key hallmark of senescent cells and is often utilized as the basis of multiple assays screening for

senescence (221). Aside from this functional change, senescent cells undergo several morphological and structural changes. Senescent cells appear enlarged and have flattened morphology due to cytoskeletal changes; moreover, some cell types may develop projections, appearing similar to a neuron-like shape (222,223). Cells undergoing senescence also have altered mitochondrial fusion and function, as well as increased mitochondrial mass and length (224). Cellular senescence exhibits a number of nuclear changes, including the appearance of altered chromatin, termed heterochromatin foci, designed to repress the expression of proliferation-related genes and maintain the growth-arrested state (225,226). While all of these multiple features are characteristics of senescence, senescent cells exhibit a heterogenous expression of these phenotypes and may not present all of these features; therefore, it is necessary to demonstrate multiple phenotypes of senescence to confirm induction.

1.4.7.1.DNA Damage, p53, and senescence

Senescence is a prolonged growth arrest generally associated with the induction of DNA damage and consequent signaling pathways. Therapy-induced DNA damage is detected by ATM, which phosphorylates and activates p53 (99). Activation p53 results in induction of $p21^{Cip1}$ (and sometimes p16), inhibition of cyclin-dependent kinases, dephosphorylation of retinoblastoma (Rb), and the presumed formation of Rb-E2F complexes (227,228). Collectively, these responses interfere with the interaction between cyclin and cyclin-dependent kinases and halt cell cycle progression. Given that cyclin-dependent kinase inhibitors (CDKIs) can drive growth arrest, senescent cells often upregulate key cell cycle regulators, such as $p21^{Cip1}$ and $p16^{INK4a}$ (229,230). Senescent growth arrest can furthermore be considered as an alternative cell fate that can be induced to allow cells to evade apoptotic cell death (231). Luo et al showed that 6 Gy radiation did not induce significant apoptosis in A549 and H460 cells, but rather induced a premature

senescence indicated by increased SA-β-gal staining. Furthermore, knockdown of p53 inhibited radiation-induced senescence, while restoration of p53 expression sensitized cells to radiation and induced senescence (232). Studies performed by Roberson *et al.* demonstrated induction of accelerated senescence in response to camptothecin, a DNA-damaging agent, in p53 null H2199 human lung cancer cells. Of interest was that these investigators were able to show that a subset of cells had the capacity to escape the therapy-induced senescence and these cells resembled parental cells while still exhibiting SA-β-gal activity (210).

1.4.7.2.Senescence-associated secretory phenotype

A key characteristic of senescent cells is the SASP, which encompasses a unique secretion profile including cytokines, matrix metalloproteases (MMPs), growth factors, and several other soluble regulators (206). A number of these factors can alter the TME or promote tumor growth. Additionally, several of these cytokines and soluble factors participate in wound healing processes that modulate ECM remodeling, tissue repair, surrounding cells in the TME, as well as regulate immune infiltration (233). MMPs and other proteases help to remodel the ECM around tumor cells and can promote tumor migration (204,234). Moreover, senescent cells secrete a number of pro-inflammatory cytokines and chemokines, such as interleukin-6 (IL-6) and interleukin-8 (IL-8), which can play a role in promoting tumor growth and migration. Ortiz-Montero et al demonstrated that when MCF-7 cells were treated with conditioned media from senescent cells, IL-6 or IL-8, markers of endothelial-to-mesenchymal transition (EMT) were upregulated, and promoted tumor cell migration and invasion (235). Both of these inflammatory factors can modulate the activation of the NF-κB pathway, resulting in an increase in transcription of anti-apoptotic proteins and promote tumor growth (236). In contrast, secretion of pro-inflammatory cytokines can promote

immune infiltration, and depending on the type of immune infiltrates, can promote or inhibit tumor growth. Studies by Meng et al utilized radiation and the PARP inhibitor, veliparib, to promote premature senescence in B16SIY melanoma cells (237). When these senescent cells were isolated and injected into C57BL/6 mice, a number of cytokines were upregulated and an increase in CD8+ T cell proliferation was observed in coculture studies. Furthermore, vaccination of tumor-bearing mice with senescent cells followed by subsequent radiation was sufficient to elicit immune responses and eliminate established tumors. As exemplified, cellular senescence can play a dual role in tumor development itself, and a verdict on whether these soluble factors produced by senescent cells promote or antagonize tumorigenesis has yet to be achieved (238).

1.4.8. Senescence in response to therapy

1.4.8.1.DNA Damaging Agents

A number of cytotoxic cancer therapies have been shown to induce senescence in multiple different cancer cell types (239). Previous studies have quite conclusively demonstrated growth arrest, characteristic of senescence, in response to radiation therapy and DNA-damaging agents (240–244). Cui et al showed that treatment with 4 Gy radiation in cervical cancer cells induced only 16% apoptosis but did induce a long-lasting G2/M phase arrest (245). Cisplatin, a DNA-alkylating agent, was shown to induce senescence in nasopharyngeal carcinoma and ovarian cell lines through p53 and cdc2 induction (246,247). Etoposide, a topoisomerase II inhibitor, induced senescence in HepG2 hepatocarcinoma and U2OS osteosarcoma cells as exhibited by increased SA-ß-gal activity and p53/p21 protein levels (248). Moreover, doxorubicin, an anthracycline antibiotic, mediates its anti-tumor effects by intercalating DNA base pairs, as well as inhibiting topoisomerase II, causing DNA strand breaks and blocking both DNA and RNA synthesis

(249,250). Doxorubicin has been shown to induce senescence in breast, NSCLC, colorectal cancer (251–254). Taken together, several chemotherapies inducing DNA damage through varying means seem to activate senescent programming in response to damage.

1.4.8.2.Non-DNA damaging Agents

While DNA-damaging agents are more often associated with senescence induction due to their mechanism of action, various cytotoxic cancer therapies not directly associated with causing DNA damage have also been shown to induce senescence (239). For example, methotrexate, an antimetabolite that inhibits tetrahydrofolate synthesis, was shown to activate a premature senescence through p53 acetylation in C85 human colon cancer cells (255). Paclitaxel is a microtubule poison that stabilizes tubulin and prevents its depolymerization, ultimately disrupting cell division (256). Exposure to paclitaxel was shown to induce senescence in bladder cancer and breast cancer (257,258). Furthermore, hormonal deprivation therapy, such as anti-estrogens and anti-androgen therapy, demonstrated senescence induction in breast and prostate cancer models, indicating activation of the senescent phenotype is not reliant on genotoxic stress (259–261). Palbociclib, a CDK4/6 inhibitor, has become a front-line treatment for advanced metastatic estrogen-positive breast cancer patients who developed resistance to prior anti-estrogen therapy (262). Palbociclib works much like a traditional CDKI, such as p21 and p16, by interfering with the interaction between CDK4/6 and cyclin D, resulting in a cell cycle halt (262,263). Palbociclib was also shown to induce both autophagy and senescence in gastric cells and breast cancer cells (264,265).

While several pre-clinical models demonstrate senescence induction in response to a multitude of chemotherapies, radiation, and target therapies (261,265–270), activation of senescence in patient

tumors is poorly understood. Thus, further inquiry and advanced screening techniques are required to enhance our comprehension of patient tumor responses to anti-cancer therapy.

1.4.9. Clinical Implications of Senescence: Utilization of Senolytics

One limitation to efforts to fully understand the role and contributions of therapy-induced senescence in sensitivity to radiation is the absence of specific pharmacologic or genetic approaches to silence the senescence response. Nevertheless, our recent evidence of outgrowth/escape from senescence (271) argues for the likelihood that senescence, like autophagy, maybe a cytoprotective response that allows the tumor cells to escape elimination by radiation and chemotherapy. The prolonged and sustained growth inhibition may be permissive for the ultimate regrowth of the tumor cells and the consequent disease recurrence. Given this possibility, coupled with evidence that the SASP may also promote tumor growth, the recent identification of agents with "senolytic" properties, which promote apoptosis selectively in senescence cells opens the possibility of developing a therapeutic strategy for elimination of the residual surviving tumor cells (272,273).

Work by Yosef and colleagues demonstrated that human fibroblasts induced into senescence by radiation upregulated anti-apoptotic proteins, Bcl-W and Bcl-X_L *in vivo*. Targeting these proteins using a small-molecule inhibitor, ABT-737, was sufficient to eliminate these senescent cells (214). Similarly, Samaraweera *et al.* showed senescence induction and SASP secretion in both NSCLC cells and head and neck squamous cell carcinoma (HNSCC) cells when exposed to cisplatin or taxanes. Furthermore, administration of Panobinostat, an FDA-approved HDAC inhibitor, following chemotherapy exposure suppressed proliferation and induced cell death in both cancer cell types (274). While many agents have been proposed to have senolytic properties (272,275), not all of these compounds are universally effective; consequently, further work will be required

to generate clearer insights as to exactly how these agents act as “senolytics” and why particular agents are effective under certain experimental conditions but not others. Taken together, selectively targeting senescent cells while they are dormant and before they begin to regain proliferative recovery may serve as a therapeutic benefit and prolong patient survival, as well as increasing the delay before disease recurrence.

In response to chemotherapy and radiation, tumor cells can undergo a number of cell fates and processes. While apoptosis is the desired outcome of clinical treatment, tumor cells can often activate various survival mechanisms, such as autophagy and senescence, in hopes to evade apoptotic cell death. It is, therefore, necessary to examine the contributions and function of these alternative cell fates on tumor cell sensitivity to anti-cancer therapy. The studies presented in this dissertation were designed to examine the cellular response of autophagy in radiation and cisplatin exposure, individually, in NSCLC cell lines, as well as the effects of p53 status on these responses. An additional goal was to determine whether autophagy could be modulated to sensitize tumor cells to therapy and to assess the contribution of nonprotective autophagy towards senescence induction.

Chapter Two: What are the cellular responses and the effect of p53 status on radiosensitivity in H460 NSCLC cells?

2.1 Introduction

Radiotherapy

Radiation is an important pillar of cancer therapeutics, exerting its anti-tumor DNA-damaging effects through various direct and indirect mechanisms. The effects of radiation are largely mediated through DNA damage that is both direct and indirect, the latter via free radical generation (276). Thus, the efficacy of radiation is partly dependent on the oxygenation of tumors, which is required to generate ROS and incur damage (277). While cells respond with compensatory mechanisms by antioxidants, such as glutathione and superoxide dismutase (SOD), localization of radiation-induced damage increases ROS levels, tipping redox equilibrium, and ultimately resulting in cell death (278). The impact of radiation-induced damage tends to be delayed, occurring over several cell cycles, resulting in aberrant chromosomes and compromised DNA integrity. Long-term damage to normal cells/tissue at the tumor periphery remains a key issue when injury accumulates in critical organs. The nature of the tumor cell response to radiation can vary. Radiation-induced DNA strand breaks activate a multitude of DNA damage response processes to prevent the propagation of cells carrying the mutated/damaged DNA. Radiation has served as an effective mode of treatment for a number of cancer types, for both curative and palliative treatment; however, resistance to therapy still remains a prevalent issue.

Although tumor cell apoptosis is the desired outcome for radiation and cytotoxic therapy, it is often not the sole or obligatory response to therapy. Tumor cells can activate multiple pathways, such as autophagy or senescence, in attempts to evade cell death pathways, which may contribute

to disease reoccurrence or poor patient response to therapy. Autophagy is induced as a "first responder", aiding to prevent damage accumulation. Cellular stress, metabolic dysregulation, and oxidative stress can have detrimental effects on protein oxidative states, genome stability, and mitochondrial energetics (279). In order to reduce the burden of the damage, autophagy is induced to alleviate stress by aiding in the turnover of damaged proteins and organelles (280). While hypoxia- and starvation-induced autophagy represent largely cytoprotective responses (281,282), the role(s) of chemotherapy- and radiation-induced autophagy are less clear (117). The cytoprotective function of autophagy is often thought to reflect efforts by the tumor cell to prevent the cell from undergoing apoptosis and prolong survival (104,126). However, there are also extensive examples of studies where inhibition of autophagy fails to sensitize the tumor cells to the initiating stress (141,283,284) which we have termed "non-protective autophagy" (117,285). The relevance and potential clinical importance of the non-protective form of autophagy relate to efforts to sensitize malignancies to therapy through autophagy inhibition. That is unless the autophagy induced in the clinic is cytoprotective in function, there is unlikely to be a therapeutic advantage to its inhibition.

Literature evidence also demonstrates the functional autophagic response is not always consistent in tumor cells exposed to radiotherapy (141). Further analyzing these studies by Schaaf *et al.,* pharmacological inhibition with CQ and 3MA *did not* alter radiosensitivity in MDA-MB-231 breast tumor cells when exposed to 5.6 Gy; however, work done by Chaachouay *et al*. showed that autophagy inhibition with similar concentrations of CQ and 3MA was sufficient to radiosensitize the same MDA-MB231 cell line exposed up to 5 Gy radiation (92,145). These contradictory observations present a conundrum within the field when the same cell line, exposed to similar doses of radiation and concentrations of the autophagy inhibitor, can produce two

divergent responses, leading to opposing conclusions relating to the role of autophagy. Thus, the nature of autophagy in response to radiotherapy and mediators regulating autophagic function warrant further interrogation.

Alongside autophagy, recent studies have demonstrated that radiation can also induce a cellular growth arrest characteristic of senescence. Senescence is an alternative cell fate that can be induced to allow cells to evade cell death by halting cell cycle progression (286). Radiation-induced DNA damage can result in a cellular senescence as the cell attempts to repair the damaged DNA; however, depending on the extent of damage, cells can undergo cell death if the damage is too great or repair the damaged DNA and allow a subset of senescent cells to regain proliferative capacity (287,288). p53, an essential tumor suppressor, is a central player in regulating apoptosis, autophagy, and senescence, and tumor cells with loss or mutated p53 have been shown to exhibit reduced susceptibility to radiation-induced apoptosis (289–291). Therefore, utilizing H460 p53 wild type expressing cells (H460wt) and H460 cells with a p53 knockout generated through CRISPR/CAS9 technologies (H460crp53), we further examined the effects of p53 status on tumor cell responses and sensitivity to radiotherapy.

2.2. Methods

2.2.1. Antibodies and reagents

The following primary antibodies were used: SQSTM1/p62 (BD Biosciences, 610497); ATG5 (Cell Signaling Technology, 2630); LC3B (Cell Signaling Technology, 3868); TP53 (BD Biosciences, 554293); GAPDH (Cell Signaling Technology, 2118). Secondary antibodies: Horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling, anti-mouse, 7076S; anti-rabbit, 7074S), and TRITC-conjugated secondary antibodies (Invitrogen, A21424) or FITC-conjugated secondary antibodies (Invitrogen, A11070). Hoechst 33258 was purchased from Thermo Fisher Scientific (H3569).

2.2.2. Cell Lines

H460 cells were acquired from ATCC (NCI-H460). p53 knockout H460 cells were generated by co-transfection (3x106 cells in 10 cm dish) with 1 μg CRISPR-Cas9 plasmid targeting the p53 loci (Santa Cruz Biotechnologies; cat #sc-416469) and 1 μg of a homology directed repair plasmid for p53 (Santa Cruz, cat. #sc-416469-HDR). Cells were transfected using PolyJet reagent (Signagen) following the manufacturers guidelines. After 72 hours, cells were exposed to 2.5 μg/ml puromycin with daily media exchanges to replenish selection agent. After all cells transfected with 1 μg of a control CRISPR/Cas9 plasmid (Santa Cruz Biotechnologies cat#sc-418922) were killed (~96 h), puromycin was removed and the cells allowed to recover and grow as individual colonies, which were then selected and examined for expression of p53 by western blotting.

The ATG5-knockdown was generated as follows: Mission shRNA bacterial stocks for ATG5 were purchased from Sigma Aldrich. Lentiviruses were produced in HEK 293T cells co-transfected using EndoFectinTM Lenti Transfection Reagent (GeneCopoeia, 1001-01) with a

packaging mixture of psPAX2 and pMD2.G constructs (Addgene). Media containing the viruses was used to infect the H460 cells; puromycin (1 μg/ml) was used as a selection marker to enrich for the infected cells.

2.2.3. Cell Culture and Treatment

H460wt and H460crp53 NSCLC cells were cultured in DMEM media supplemented with 10% (v/v) fetal bovine serum (Thermo Scientific), 100 U/ml penicillin G sodium (Invitrogen), and 100 μg/ml streptomycin sulfate (Invitrogen). Puromycin (1 μg/ml; Sigma) was used to maintain the selection of shATG5 and shControl transfected cells. Cells were incubated at 37℃ under humidified 5% CO2.

Cells were seeded on day 0 followed by irradiation (0,2,4,6, or 8 Gy) on day 1 utilizing a ^{137}Cs irradiator. Media was replenished every other day.

2.2.4. Cell Viability Assay

For cell viability assessed by trypan blue exclusion, cells were plated in 6-well plates at a density of 50,000 cells per well and pre-treated with 3MA (0 or 1 mM), CQ (0 or 10 μM), or Baf A1 (0 or 5 nM) 3 h prior to irradiation (0,2,4,6, or 8 Gy). Media was replenished 24 h post-radiation. Cells were trypsinized, stained with 0.4% trypan blue (Sigma, T01282), and counted on the indicated days using a hemocytometer. Cellular growth curves were generated from the collected data.

2.2.5. Assessment of Apoptosis

The extent of apoptosis was monitored by Annexin V-FITC and Propidium Iodide staining. Cells were pre-treated with 3MA (0 or 1 mM), CQ (0 or 10 μM), or Baf A1 (0 or 5 nM) 3 h prior to irradiation (0,2,4,6, or 8 Gy). Media was replenished 24 h post-radiation. On the day of analysis,

cells were trypsinized, washed with 1X PBS and stained according to manufacturer protocol (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences, 556547). Fluorescence was measured using flow cytometry using BD FACSCanto II and BD FACSDiva software at the Flow Cytometry Core Facility at Virginia Commonwealth University. For all flow cytometry experiments, 10,000 cells per replicate were analyzed and three replicates for each condition were analyzed per independent experiment.

2.2.6. Determination of Acidic Vesicle formation through Acridine Orange Staining

Cells were plated in 6-well plates at a density of 50,000 cells per well and pre-treated with 3MA (0 or 1 mM), CQ (0 or 10 μM), or Baf A1 (0 or 5 nM) 3 h prior to irradiation (0,2,4,6, or 8 Gy). Media was replenished 24 h post-radiation. Cells were stained with 1 μg/ml acridine orange at 37 ℃ for 15 min and then washed with PBS. Cells were imaged using an inverted fluorescence microscope (Olympus, Tokyo, Japan). For quantification of autophagic vesicles (AVOs), cells were trypsinized, harvested and washed with PBS. Pellet fractions were resuspended in PBS and analyzed by BD FACSCanto II and BD FACSDiva software. All experimental procedures were performed with cells protected from light.

2.2.7. LC3/LAMP2 Co-localization

For immunofluorescence staining, cells were treated with 6 Gy radiation and staining was performed 72 h post-radiation. Cells were fixed with 100% methanol for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and then blocked with 5% bovine serum albumin (BSA). Cells were incubated with primary antibody (1:100) overnight at 4°C, and then exposed to FITC- or TRITC-conjugated secondary antibody for 2 h at room temperature. Finally, nuclei were

stained by Hoechst 33258. Immunofluorescence was detected by inverted fluorescence microscopy (Olympus, Tokyo, Japan).

2.2.8. Western Blot Analysis

After indicated treatments, cells were trypsinized, harvested, and washed with 1X PBS. Pellets were lysed with CHAPS buffer (Thermo Scientific) containing protease inhibitor (Sigma, P8340) and phosphatase inhibitor Sigma, P576). Protein concentrations were determined by the Bradford Assay (Bio-Rad Laboratories, 5000205 Total protein was diluted in sample buffer and boiled for 15 minutes prior to loading. Protein samples were loaded and subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blocked with 5% milk in 1X PBS with 0.1% Tween 20 (Fisher, BP337). Membrane was incubated overnight at 4°C with indicated primary antibodies at a dilution of 1:1000 in 5% BSA. The membrane was then washed with 1X PBS with 0.1% Tween 20 three times and secondary antibody was added at a dilution of 1:2000 in 5% BSA for 2 h at room temperature. The membrane was washed again with 1X PBS with 0.1% Tween 20 three times. Blots were developed using Pierce enhanced chemiluminescence reagents (Thermo Scientific, 32132) on BioRad ChemiDoc System.
Densitometry analysis of the western blots was performed using NIH software Image J2. Except in the case of LC3, all protein band densities were normalized for their corresponding GAPDH loading control band densities.

2.2.9. β-galactosidase and C_{12}FDG staining

Cells were plated in 6-well plates at a density of 50,000 cells per well and treated with irradiation (0,2,4,6, or 8 Gy). Media was replenished 24 h post-radiation. β-galactosidase staining was utilized to qualitatively visualize senescent cells. Staining was performed as previously described

by Dimri *et al.* (292). Phase contrast images were taken using an inverted microscope (Olympus, Tokyo, Japan).

To quantify ß-gal positive senescent cells, after irradiation, cells were treated with Bafilomycin A1 (100 nM) for 1 h to achieve lysosomal alkalinization, followed by staining with C_{12}FDG (10 μM) for 2 h at 37 ℃. After incubation, cells were collected and analyzed by BD FACSCanto II and BD FACSDiva software. All experimental procedures were performed with cells protected from light.

2.2.10. Extent of DNA Damage

Cells were seeded in 6-well plates and incubated overnight. After exposure to the indicated treatment, cells were fixed with 70% ethanol for 15 minutes and then blocked with 5% bovine serum albumin (BSA). Cells were incubated with γH2AX antibody (1:1000; BD Pharmagen, 560445) for 2 h, and fluorescence was quantified using flow cytometry. For quantification of DNA damage, cells were trypsinized, harvested and washed with PBS. Pellet fractions were resuspended in PBS and analyzed by BD FACSCanto II and BD FACSDiva software. All experimental procedures were performed with cells protected from light.

2.2.11. Statistical analysis

GraphPad Prism 5.0 software was utilized to conduct statistical analysis. Data are shown as mean ± SD from at least three separate experiments, unless indicated otherwise. Statistical comparisons between groups were assessed via one-way ANOVA followed by Bonferroni post-hoc test and two-tailed *t* tests; p-value < 0.05 was considered statistically significant.

2.3. Results

2.3.1. Radiation Sensitivity in p53 Wild-type and p53 Knockout H460 cells

Mutations to p53 or loss of p53, an essential tumor suppressor gene, have been reported in more than 50% of tumor cell types, including lung, breast, colon, and many others (16,293). The status of p53 and its contributions to radiation sensitivity has become an area of important interest over the years; however, the mechanisms underlying the effect of p53 status on radiosensitivity and resistance to therapy still remains elusive. To further interrogate this question, we utilized p53 wild-type H460 NSCLC cells and H460 cells with a p53 knockout generated through CRISPR/CAS9 gene-editing **(Figure 2.1A)**. Radiation sensitivity was assessed in both cell lines through analysis of cell viability over time using trypan blue exclusion. Temporal analysis of radiosensitivity demonstrated that H460wt cells were significantly more sensitive than H460crp53 cells **(Figure 2.1B)**. Furthermore, H460wt cells exhibited growth arrest in response to 6 Gy radiation exposure while H460crp53 underwent a lesser degree of growth inhibition and continued to proliferate.

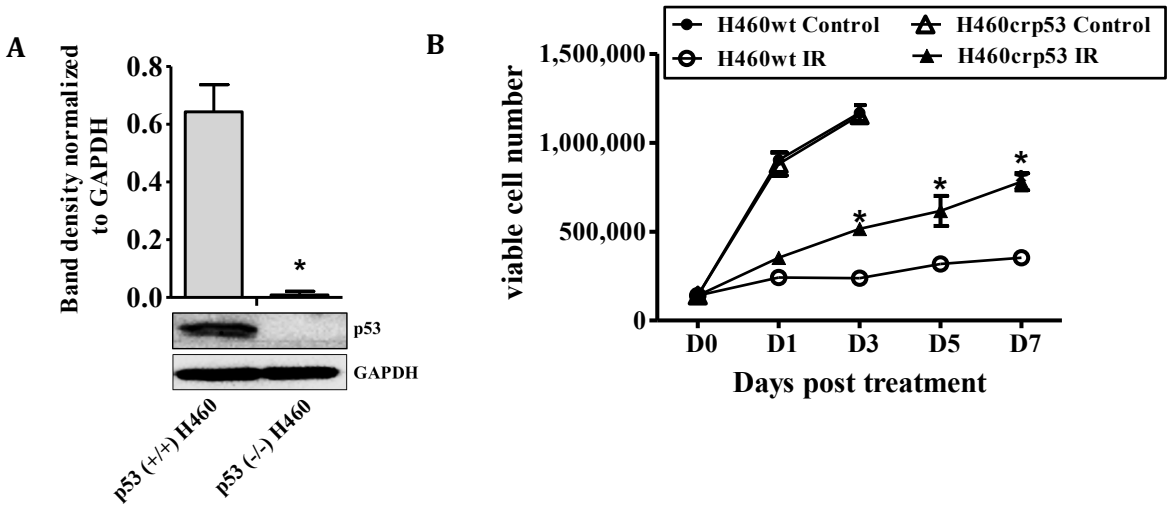

Figure 2.1. Sensitivity to ionizing radiation (IR) in H460wt and H460crp53 NSCLC cells. A. **Western blot analysis.** B. **Temporal analysis of cell viability.** Cells were exposed to 6 Gy radiation, incubated with fresh medium for the indicated number of days and viable cell number determined by trypan blue exclusion. Results were from three independent experiments. *$p < 0.05$, radiation exposure in H460wt cells versus H460crp53 cells.

2.3.2. Radiation induced apoptosis in H460wt and H460crp53 cells.

In response to radiotherapy, H460wt and H460crp53 cells exhibited differential sensitivity. Previous literature has suggested loss of functional p53 can reduce cellular susceptibility to apoptotic cell death (291,294). To further inquire whether the differences in sensitivity were a result of cell death, we examined the extent of apoptosis induced in response to radiation in both of these cell lines. Cells were exposed to varying doses of radiation and the extent of apoptosis was measured through Annexin V/PI staining. The fluorescence was quantified using flow cytometry. **Figure 2.2A** demonstrates the extent of apoptosis in both cell lines was relatively low and induced to similar extents in both H460wt and H460crp53 cells in a dose-response study of radiation-induced apoptosis. Further examining the induction of apoptosis over time, cells were exposed to 6 Gy radiation and radiation-induced apoptosis was assessed on the indicated days. Radiation did moderately increase apoptotic cell death, falling in between ~15-25% with 72 h post-radiation exposure; however, consistent with the dose-response study, the extent of apoptosis was similar in both H460wt and H460crp53 cells **(Figure 2.2B)**. Taken together, these data indicate, the differential sensitivity exhibited between H460wt and H460crp53 cells was not a result of differences in the extent of apoptosis induced by radiation.

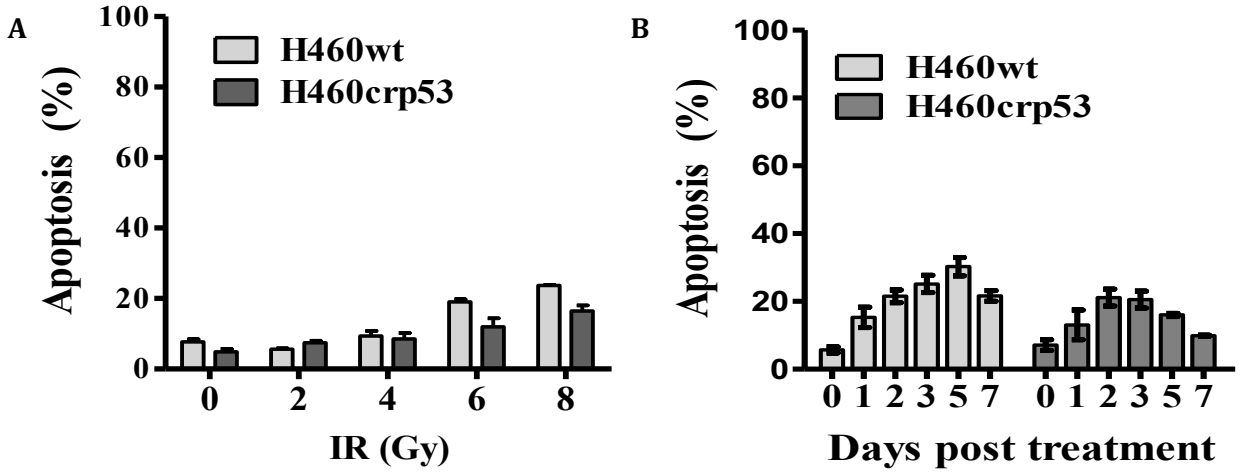

Figure 2.2. Radiation induced apoptosis in H460wt and H460crp53 cells to a similar extent. **A**. **Apoptosis Dose Response.** After exposure to the indicated doses of radiation, cells were incubated with fresh medium for 2 days, and apoptotic cells identified by Annexin V/PI staining and flow cytometry. **B**. **Apoptosis Time Course.** After exposure to 6 Gy radiation, apoptotic cells were identified by Annexin V/PI staining and flow cytometry at the indicated days. Results were from three independent experiments. *$p < 0.05$, radiation exposure in H460wt cells versus H460crp53 cells.

2.3.3. Radiation induced autophagy in both H460wt and H460crp53 cell

Extensive literature evidence indicates that autophagy is often induced in response to radiation-induced damage (91,92). Consequently, we examined autophagy induction in response to radiation exposure in both cell lines, utilizing acridine orange to demonstrate acidic vesicle formation as an initial screen. While acridine orange is not a specific indicator of early autophagosome formation, it can be utilized as an indication for later stages of autophagy, as the autophagosome fuses with lysosomes to form acidic vacuoles termed "autolysosomes" (295). An increase in acidic vacuole formation was demonstrated 72 h post-6 Gy radiation exposure in both H460wt and H460crp53 cells **(Figure 2.3A)**. A dose-response in acridine orange fluorescence was further quantified using flow cytometry. Dose-dependent acidic vesicle formation was exhibited in both H460wt and H460crp53 cells 72 h post-radiation exposure; however, acridine orange fluorescence was greater in H460wt cells compared to H460crp53 cells **(Figure 2.3B)**. To further confirm autophagy induction, western blot analysis was utilized to determine lipidation of LC3 (LC3 I to LC3 II conversion) and degradation of p62/SQSTM1 protein levels. Figure 3C demonstrates autophagic induction and autophagic flux (i.e., autophagy going to completion) in both cell lines, as indicated by LC3 I-II conversion and p62 protein degradation in both H460wt and H460crp53 cells exposed to 6 Gy radiation. Interestingly, p62 degradation occurs on Day 4 in H460crp53 cells compared to H460wt cells where significant p62 degradation is evident on Day 1, suggesting that autophagy may be delayed in H460crp53 cells. The difference in the rate of autophagic flux between the two cell lines may contribute to differences seen in the acridine orange quantification since the acridine orange studies were performed on Day 3. Finally, **Figure 2.3C** demonstrates co-localization of LC3 (green), an autophagosome marker, to LAMP-2 (red), a lysosomal marker, indicating fusion (yellow) of autophagosomes with lysosomes in the final steps of autophagy completion. In

response to 6 Gy radiation exposure, both H460wt and H460crp53 cells exhibited increased LC3 (green) fluorescence, indicating autophagy induction. Furthermore, merging of LC3 and LAMP-2 demonstrated the formation of autolysosomes (yellow) in both H460wt and H460crp53 cells 72 h post-IR exposure, indicating autophagy was going to completion. Interestingly, untreated H460crp53 exhibited higher LC3 immunofluorescence compared to H460wt cells, suggesting basal autophagy was higher in H460crp53 cells compared to their p53 wt counterpart. Collectively, these data indicate autophagy was induced and autophagic flux was occurring in response IR exposure in both H460wt and H460crp53 cells, and autophagic that flux is delayed in H460crp53 cells.

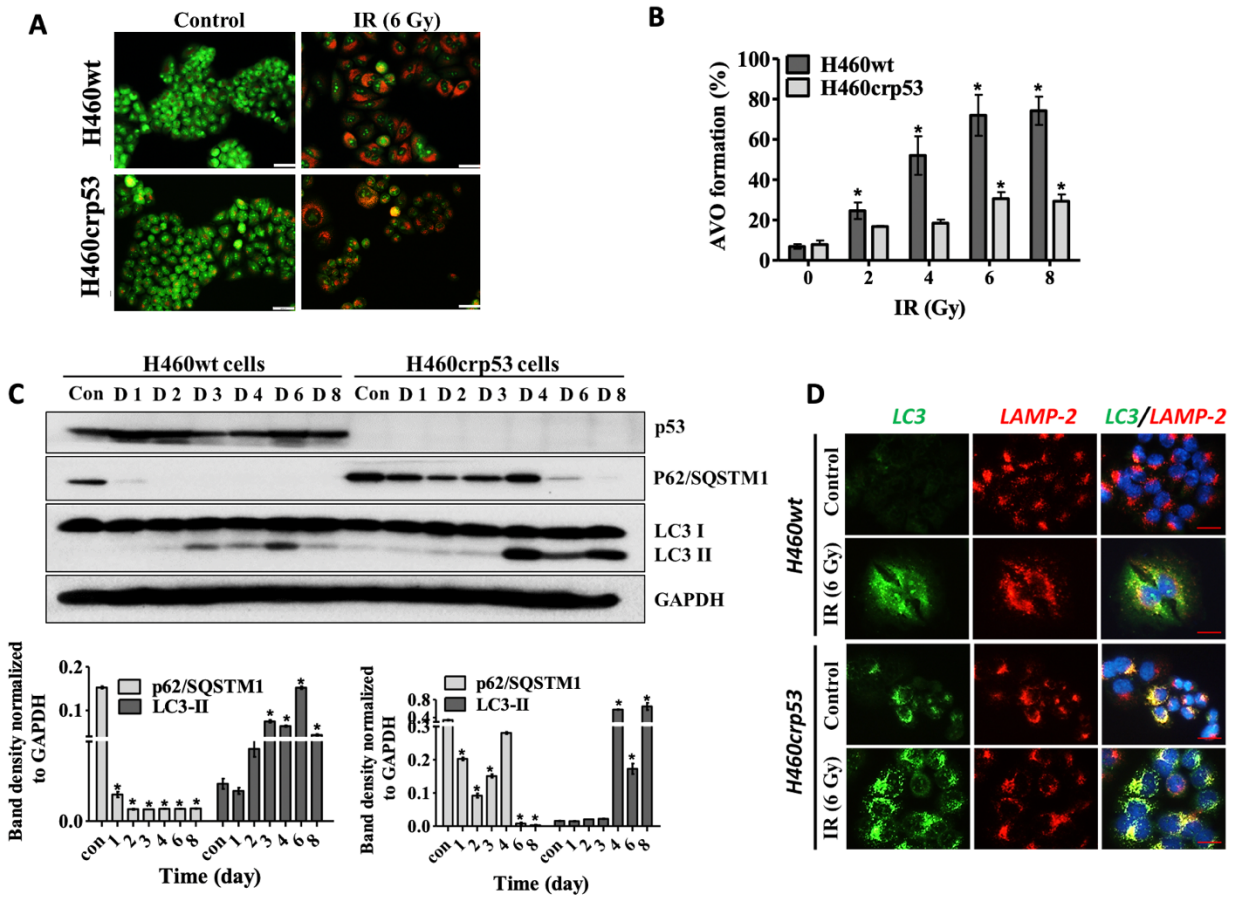

Figure 2.3. Radiation-induced autophagy in H460wt and H460crpp53 NSCLC cells.
A. **Acridine orange staining.** Three days after exposure to 6 Gy radiation, cells were stained with acridine orange; images were taken at the identical magnification (scale bar = 200 μm, n = 2). **B**. **Quantification of acridine orange staining.** Autophagy was quantified based on acridine orange staining as measured by flow cytometry. **C**. **Western blotting for levels of relevant proteins.** The status of p53 in both H460 cell lines was confirmed by western blotting. Autophagy was assessed based on p62/SQSTM1 degradation and the conversion of LC3 I to LC3 II. The bar graph in each panel indicates the relative band intensity generated from densitometric scans of three independent experiments in arbitrary densitometric units. **D**. **Co-localization of LC3 and LAMP.** Fluorescence microscopy showing LC3 and LAMP2 co-localization in response to 6 Gy radiation. Imaging was performed 3 days after radiation exposure (scale bar = 20 μm, n = 2). Unless stated, otherwise data were from three independent experiments. *p < 0.05, control cells versus irradiated cells.

2.3.4. Nonprotective autophagy induced in response to radiotherapy in H460wt and H460crp53 cells

In response to chemotherapy and radiation, autophagy is often thought to elicit its conventional cytoprotective function in order to remove damaged organelles and proteins and prevent toxic accumulation (296,297). While autophagy can play a cytoprotective function in response to anti-cancer therapy, autophagy can also be multifunctional in its role. Therefore, we interrogated the functional role of autophagy in response to radiotherapy to identify whether functional differences in autophagy contributed to the differential radiosensitivity exhibited between H460wt and H460crp53 cells. The pharmacological autophagy inhibitor, 3-methyadenine (3-MA), was utilized to examine the effects of autophagy inhibition on radiosensitivity of these two isogenic cell lines. 3-MA is a class III PI3K inhibitor, which is essential for induction and initiation of autophagosome formation (153). To ensure autophagy was sufficiently inhibited, acridine orange staining and western blot analysis was utilized. Cells were pre-treated with 3-MA (1 mM) for 3 h followed by 6 Gy IR and stained with acridine orange or protein lysates were collected 72 h post-radiation for Western blotting. **Figure 2.4A** demonstrated reduced acidic vacuoles when cells were exposed to 3-MA prior to IR treatment in both H460 and H460crp53 cells. Furthermore, autophagy inhibition with 3-MA was confirmed through western blot analysis. **Figure 2.4B** shows that administration of 3-MA in H460wt and H460crp53 cells resulted in a reduced LC3 II/I conversion ratio, suggesting 3-MA in combination with radiation was sufficient to inhibit autophagy in both cell lines. Next, the effects of pharmacological inhibition of autophagy on radiosensitivity in H460wt and H460crp53 cells were examined. Cells were pre-treated with 3-MA (1 mM) for 3 h followed by 6 Gy radiation. Autophagy inhibition through 3-MA exposure prior to 6 Gy IR *did not* significantly alter sensitivity to radiation when examining cell viability over time in both of these

cell lines and did not interfere with radiation-induced cell death **(Figure 2.4C-D)**. Taken together, pharmacological autophagy inhibition did not significantly alter radiosensitivity or radiation-induced cell death in H460wt and H460crp53 cells, indicating that autophagy was ***nonprotective*** in function in response to radiation.

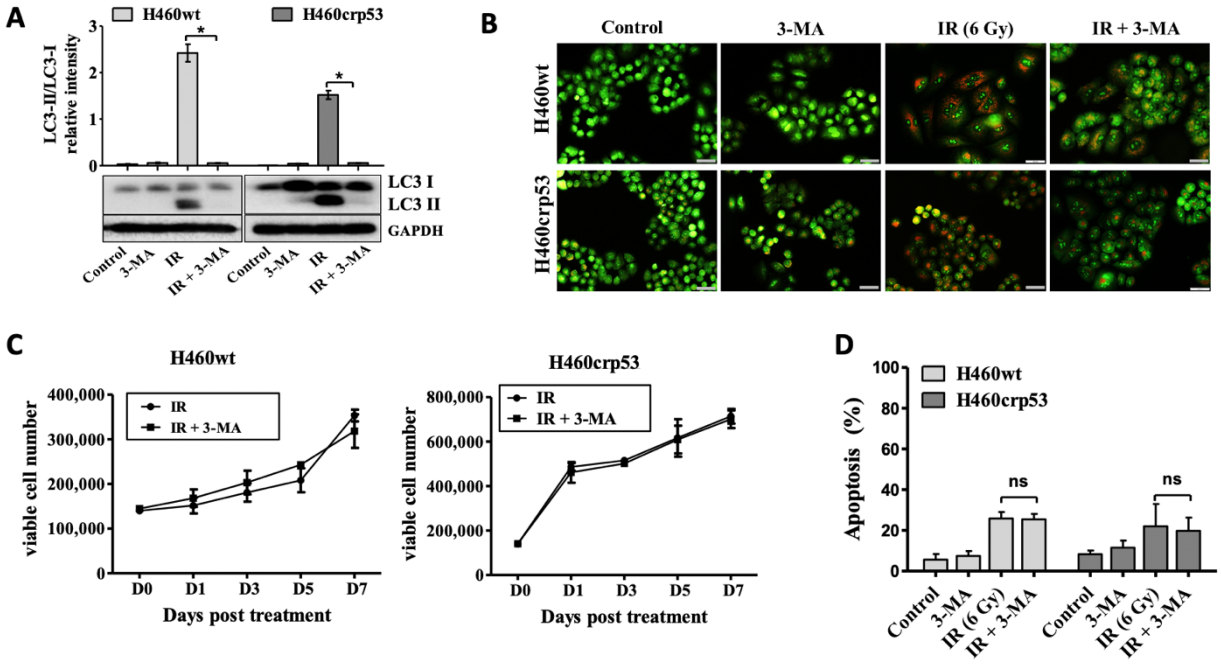

Figure 2.4. 3-MA fails to alter radiation sensitivity in H460wt or H460crp53 NSCLC cells. **A**. **Inhibition of autophagy by 3-MA.** Western blot showing autophagy blockade by 3-MA (1 mM) based on levels of LC3 II. Cells were pretreated with 3-MA for 3 h prior to radiation and protein was collected three days after irradiation. The bar graph in each panel indicates the relative band intensity generated from densitometric scans of two independent experiments in arbitrary densitometric units. **B**. **Inhibition of autophagy by 3-MA.** Cells were pretreated with 3-MA for 3 h prior to radiation and cells were stained with acridine orange three days after irradiation (scale bar = 200 μm, n = 2). **C**. **Influence of 3-MA on radiation sensitivity.** Cell viability assay indicating that 3-MA has no effect on radiosensitivity in either H460wt or H460crp53 cells. Cells were pretreated with 3-MA for 3 h followed by radiation. **D**. **Influence of 3-MA on radiation induced apoptosis**. Annexin V/PI staining showing that 3-MA has no effect on radiation induced apoptosis in either cell line. Cells were pretreated with 3-MA for 3 h prior to radiation and apoptosis was assessed after 2 post-treatment. Unless stated, otherwise data were from three independent experiments. *$p < 0.05$, radiation treated vs radiation + 3-MA treated.

CQ is a late-stage autophagy inhibitor, which accumulates within lysosomes and increases lysosomal pH and prevents fusion of the autophagosome to the lysosome (149). **Figure 2.5A** demonstrated lysosomal alkalization (yellow puncta in basic conditions rather than orange/red puncta in acidic conditions) when cells were exposed to CQ prior to IR treatment in both H460 and H460crp53 cells. Furthermore, autophagy inhibition with CQ was confirmed through western blot analysis. **Figure 2.5B** shows administration of CQ in H460wt and H460crp53 cells resulted in an increase accumulation of LC3 II/I ratio and prevented p62 protein degradation, suggesting CQ in combination with radiation was sufficient to inhibit autophagic flux in both cell lines. Next, the effects of pharmacological inhibition of autophagy on radiosensitivity in H460wt and H460crp53 cells were examined. Cells were pre-treated with CQ (10 μM) for 3 h followed by varying doses of radiation, colonies were allowed to form, and the number of colonies were counted. **Figure 2.5C** shows that CQ sensitized the H460wt cells to radiation while failing to influence radiation sensitivity in the H460crp53 cells. **Figure 2.5D** presents a temporal analysis of cell viability in both cell lines, confirming that the combination of 6 Gy radiation and CQ enhanced growth inhibition in the H460wt cells, but not in the H460crp53 cells. Furthermore, annexin V/PI staining showed that CQ significantly (but modestly) increased radiation-induced apoptosis (from ~ 24% to 37%) in the H460wt cells but did not alter apoptosis in the H460crp53 cells (**Figure 2.5E**). Taken together, CQ inhibition of radiation-induced autophagy suggests that autophagy provides a modest (albeit statistically significant) survival advantage to cells with functional p53, which is however *inconsistent* with the more pronounced radiation sensitivity evident in the H460wt cells.

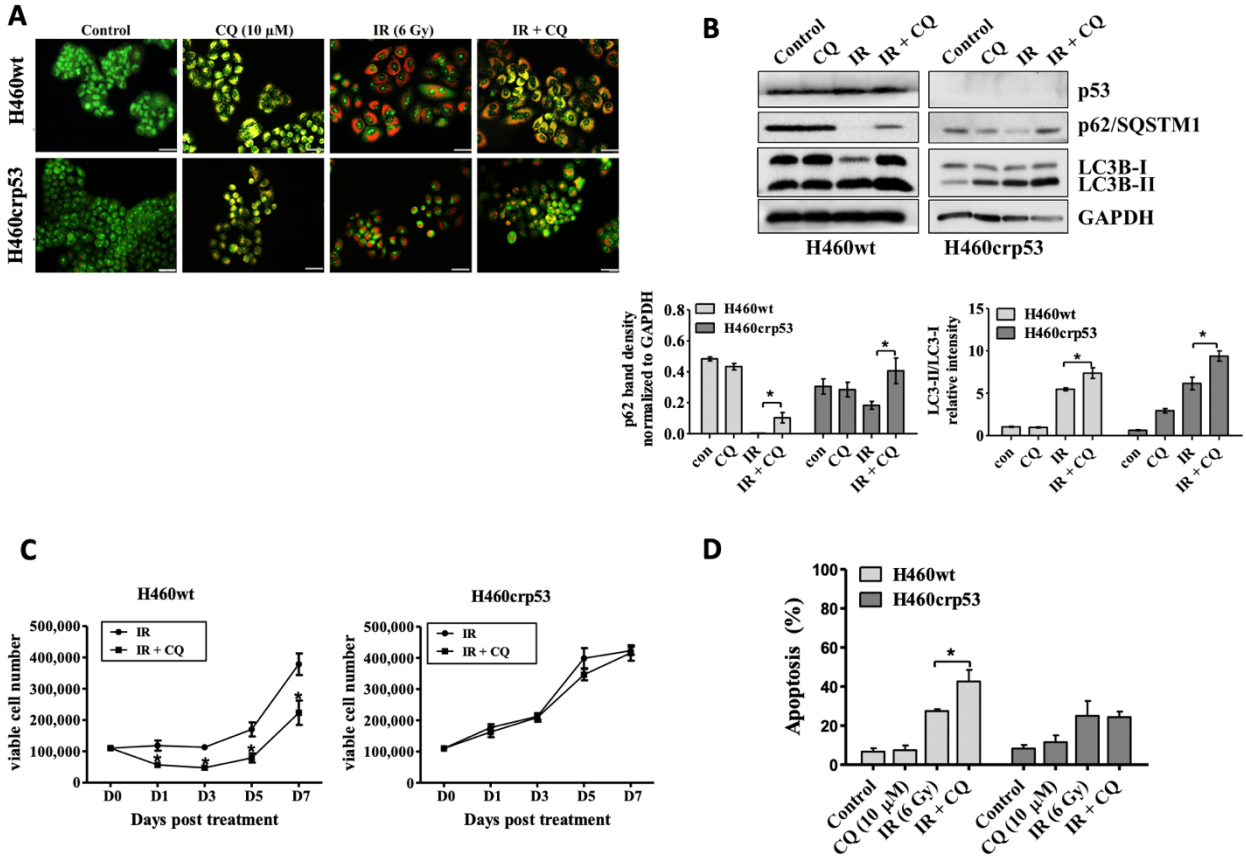

Figure 2.5. Influence of chloroquine (CQ) on radiation sensitivity in H460wt and H460crp53 cells.

A. **Inhibition of autophagy by CQ.** Fluorescence microscopy showing acridine orange-stained vacuoles induced by 6 Gy radiation alone or with CQ (10 μM) treatment (scale bar = 200 μm). **B**. **Inhibition of autophagy by CQ.** Western blot showing autophagy blockade by CQ (10 μM) based on levels of p62/SQSTMQ and LC3 II. Cells were pretreated with CQ for 3 h prior to irradiation and protein was isolated after 3 days. The bar graph in each panel indicates the relative band intensity generated from densitometric scans of three independent experiments in arbitrary densitometric units. **C**. **Influence of autophagy inhibition on radiation sensitivity.** Cell viability assay indicating that CQ increased sensitivity of H460wt cells to radiation (6 Gy), but not H460crp53 cells. **D**. **Influence of autophagy inhibition on radiation induced apoptosis**. Annexin V/PI staining indicating that CQ (10 μM) increased radiation-induced apoptosis (after 2 days) in H460wt cells, but not in H460crp53 cells. Results were from three independent experiments. *$p < 0.05$, radiation treated cells vs cells treated with radiation + CQ.

Further, these data conflicted with the findings of the early-stage pharmacological inhibition data in H460wt cells, which demonstrated lack of radiosensitization in both H460wt and H460crp53 cells when 3MA was administered. One possibility to the differences exhibited in autophagic function in H460wt cells is that the autophagy was not actually cytoprotective and that the sensitization observed in the H460wt cells might have been due to off-target effects of the chloroquine, as has been proposed by the Thorburn laboratories (298). Finally, to confirm these findings when late stage autophagy is inhibited, we used bafilomycin A1, which disrupts autophagic flux by inhibiting V-ATPase-dependent decrease in lysosomal pH and, thus, autophagosome-lysosome fusion (150). Bafilomycin A1 interfered with the completion of radiation-induced autophagy in H460wt cells based on failure of lysosomal acidification (**Figure 2.6A**) and upon p62 accumulation (**Figure 2.6B**). Again, in agreement with the previous observations, bafilomycin A1 had no impact on radiation-induced growth inhibition (**Figure 2.6C).** These results further confirm that CQ effect in H460wt was likely non-specific, that late-stage autophagy inhibition does not interfere with radiation sensitivity in this cell model, and that autophagy plays a *non-protective* role in H460 cells regardless of p53 status.

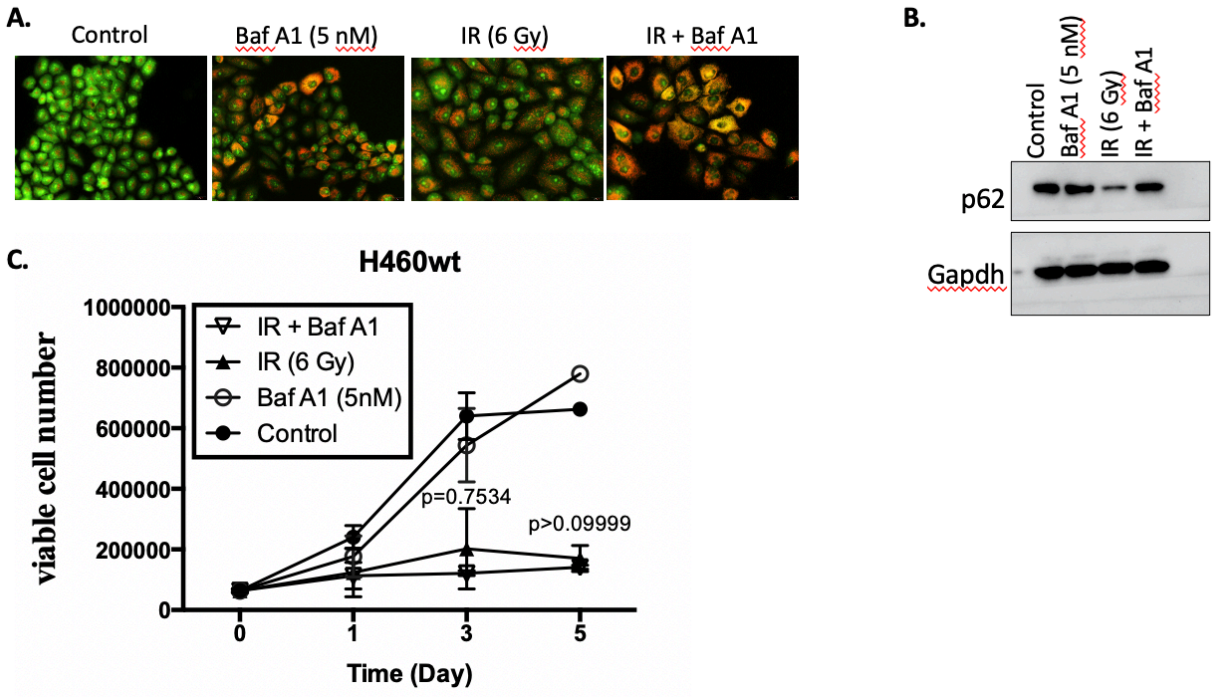

Figure 2.6. Influence of Bafilomycin A1 (Baf A1) on radiation sensitivity in H460wt cells. **A. Inhibition of autophagy by Baf A1.** Fluorescence microscopy showing acridine orange-stained vacuoles induced by 6 Gy radiation alone or with Baf A1 (5 nM) treatment (scale bar = 200 μm). **B. Inhibition of autophagy by Baf A1.** Western blot showing autophagy blockade by Baf A1 (5 nM) based on levels of p62/SQSTMQ (n=2). Cells were pretreated with Baf A1 for 3 h prior to irradiation and protein was isolated after 3 days. **C. Influence of autophagy inhibition on radiation sensitivity.** Cells were pretreated with Baf A1 for 3 h followed by radiation. Cell viability assay indicating that Baf A1 has no effect on radiosensitivity in H460wt cells.

To further confirm these results, short hairpin RNA was used to knock down ATG5, an autophagy-related gene important in proper autophagosome formation (295), in both cell lines. **Figure 2.7A** demonstrates reduced ATG5-ATG12 complex protein levels in both H460wt and H460crp53 cells where ATG5 was knocked down (shATG5 cells) compared to scrambled controls (shControl cells). Moreover, shATG5 cells exposed to radiation in both the H460wt and H460crp53 cells exhibited increased p62 accumulation and reduced LC3 I to II conversion compared to shControl cells treated with 6 Gy IR, confirming that autophagy had been suppressed in both cell lines. Next, radiosensitivity was examined in autophagy-competent and autophagy-deficient H460wt and H460crp53 cell lines. **Figure 2.7B** show autophagy-deficient H460wt and H460crp53 cells did not demonstrate altered radiosensitivity in comparison to autophagy-proficient controls when assessing cell viability over time. Lastly, autophagy inhibition did not interfere with radiation-induced apoptosis in both cell lines **(Figure 2.7C)**. Collectively, pharmacological and genetic inhibition of autophagy did not alter radiosensitivity or radiation-induced cell death in H460wt and H460crp53 cells, indicating that autophagy was nonprotective in function in both of these cell lines. Given that autophagy was nonprotective in both H460wt and H460crp53 cells, these data suggested that differences in autophagic function was unlikely to contribute to the differential radiosensitivity exhibited by the two cell lines.

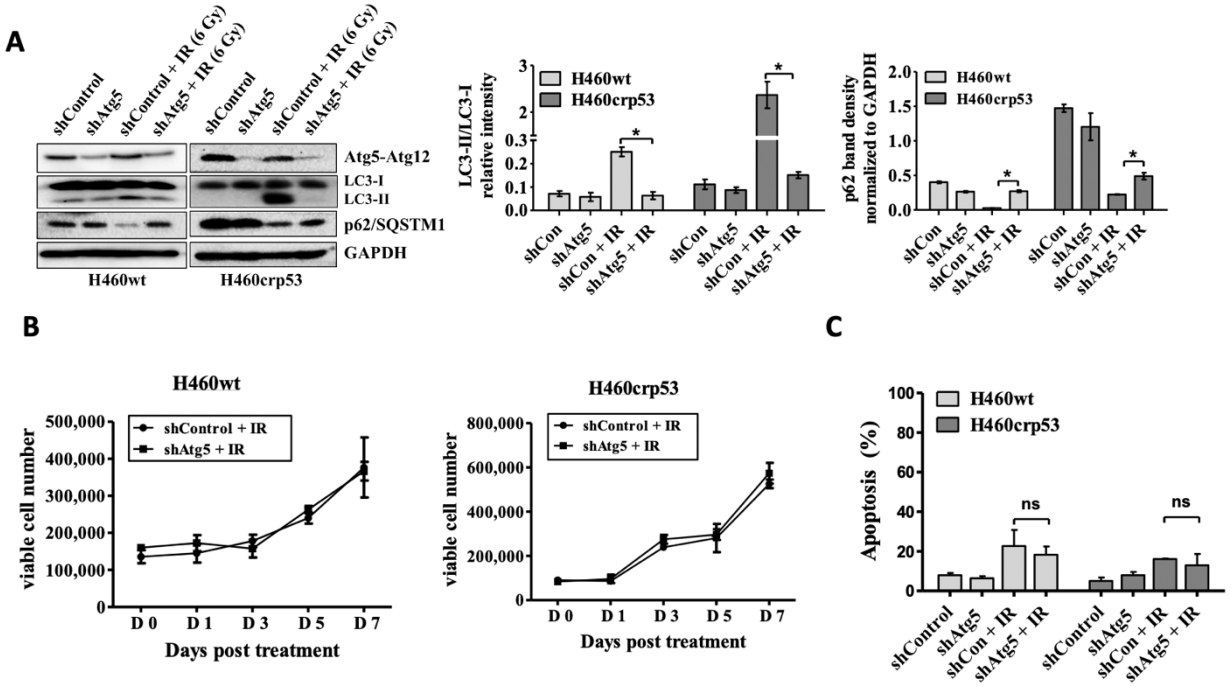

Figure 2.7. Atg5 knockdown fails to alter radiation sensitivity in H460wt and H460crp53 cells.

A. **ATG5 knockdown**. Cells were collected three days after irradiation. Western blot showing ATG5 knockdown in H460wt and H460crp53 cell lines; inhibition of autophagy in shAtg5 H460wt and H460crp53 cell lines is indicated by reduced conversion of LC3I to LC3II and interference with degradation of p62/SQSTM1. The bar graph in each panel indicates the relative band intensity generated from densitometric scans of two independent experiments in arbitrary densitometric units. **B. Lack of radiation sensitization by autophagy inhibition.** Temporal viability assay indicating that Atg5 knockdown has no effect on radiosensitivity in either H460wt or H460crp53 cells. Cells were treated with 6 Gy radiation. **C**. **Autophagy inhibition does not increase the extent of radiation-induced apoptosis** Annexin V/PI staining indicating that apoptosis induced by radiation was unaltered after Atg5 knockdown in both H460wt and H460crp53 cell lines. Unless stated, otherwise data were from three independent experiments. *$p < 0.05$, shControl cells treated with radiation vs shAtg5 cells treated with radiation.

2.3.5. Radiation induced senescence in H460wt and H460crp53 cells

Tumor cells can activate a plethora of cellular processes when exposed to chemotherapy and radiation. p53 plays a central role in modulating various cellular responses under stressed conditions, including autophagy and senescence. Previous literature has demonstrated that autophagy and senescence are primary responses to radiation (91,183,299,300) and often occur in parallel. Thus, we aimed to investigate the role of senescence induction and its contributions to the differential sensitivity seen between H460wt and H460crp53 cells. Cells were exposed to 6 Gy radiation and senescence-associated ß-galactosidase (SA-ß-gal) activity was assayed 72 h post-radiation. **Figure 2.8A** demonstrates ß-galactosidase staining (blue) was qualitatively greater in the H460wt cells compared to H460crp53 cells; furthermore, H460wt cells visually exhibited more pronounced evidence of an enlarged, flatten morphology compared to H460crp53 cells. SA-ß-gal activity was also monitored by flow cytometry for a more quantitative approach. SA-ß-gal was increased in both cell lines; however, SA-ß-gal staining was significantly greater in H460wt cells over time when compared to H460crp53 cells **(Figure 2.8B)**. These data indicate the extent of senescence induction was greater in H460wt cells compared to H460crp53 cells and suggested that the differential extent of senescence induction likely was the critical factor accounting for the differences in radiosensitivity exhibited by the two cell lines. Given limitations in the field, specifically, the lack of availability of a specific senescence inhibitor, the precise contributions of senescence induction towards radiosensitivity remains unclear and will require further inquiry as we gain further insights into the mechanisms underlying therapy-induced senescence.

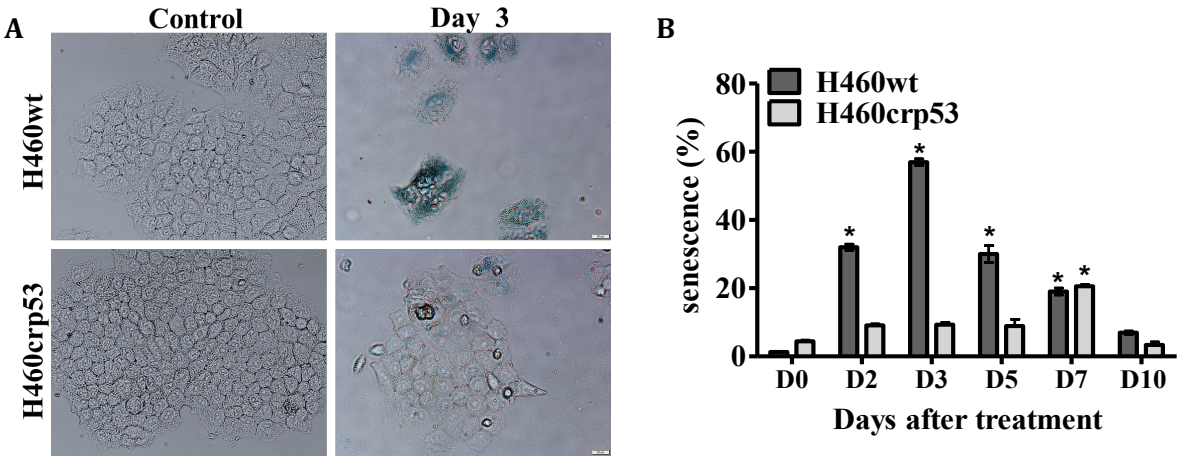

Figure 2.8. Radiation induced senescence in H460wt and H460crp53 cells.
A. β-galactosidase staining and cell morphology. β-galactosidase staining indicating the induction of senescence by radiation (6 Gy) in both cell lines (scale bar = 20 μm). **B. Quantification of senescence.** C_{12}FDG staining and flow cytometry to quantify the extent of senescence in H460wt and H460crp53 cells. H460wt cells exhibited greater induction of senescence than H460crp53 cells. Unless stated, otherwise data were from three independent experiments. *$p < 0.05$, control vs radiation treated group.

2.4 Discussion

Ionizing radiation triggers a spectrum of responses in tumor cells including apoptosis, necrosis, autophagy, and senescence (301–303); however, it is not yet clear what role each of these responses may play in tumor cell radiation sensitivity or resistance and whether the responses are tumor-specific.

While irradiated tumor cells clearly do undergo apoptotic cell death, the extent of apoptosis tends to be relatively low (304–306). Clinically relevant or even significantly higher doses of radiation induced only~ 20-30% apoptosis in several experimental tumor cell lines, including breast cancer, non-small cell lung cancer (NSCLC), and colorectal cancer (141). For instance, Rodel *et al.* demonstrated relative levels of apoptosis between 12% and 27% induced in response to 8 Gy radiation in colorectal cell lines with varying radiosensitivity (306). Similarly, Qu et al reported ~20-25% apoptotic induction in MCF-7 breast cancer cells and A549 lung cancer cells with 8 Gy radiation (307). In agreement with these data, previous work performed in our laboratory demonstrated low levels of apoptosis (~20%) induced in breast, lung, colorectal, and head and neck cell lines when exposed to fractionated radiation (141). It is only when higher levels of radiation are reached (above 10 Gy) that apoptosis becomes a more pronounced response to radiotherapy (308). This is, of course, relevant to stereotactic radiation, wherein patients are delivered multiple precisely focused beams of fractionated radiation to achieve higher effective doses to the tumor while minimizing damage to surrounding tissue (309–311). With regard to cancer treatment modalities, apoptosis or other forms of cell death are, of necessity, the desired outcomes; however, there a number of survival mechanisms cancer cells have employed to evade (apoptotic) cell death. Both autophagy and senescence can allow cancer cells to mitigate or perhaps

delay the damage incurred by clinical therapeutic modalities, escape cell death, and prolong survival.

Resistance to therapy is a primary reason for poor prognosis and treatment failure in cancer patients. Drug resistance in tumor cells can involve multiple mechanisms, including physical barriers, lysosomal trapping of weak bases, and upregulation of several survival pathways. Autophagy is often a "first responder" in times of cellular stress and activated in response to cytotoxic insult. Cytoprotective autophagy has been implicated in tumor cell resistance to therapy, as it prevents the accumulation of toxic damage. Through modulation of ROS levels and removal of radiation-induced damage, cytoprotective autophagy can also contribute to radioresistance. Studies by Chen *et al*. showed that low-dose ionizing radiation after high-dose radiation increased ROS levels, autophagy induction and promoted radioresistance in A549 NSCLC cells (312). Subsequent autophagy inhibition reduced cell viability of A549 cells in response to radiotherapy, suggesting that autophagy was functionally protective in this experimental model. Moreover, administration of N-acetyl-L-cysteine (NAC), a known free radical scavenger, blocked autophagy and was sufficient to suppress the induced radioresistance. These studies indicate that high levels of ROS can promote cytoprotective autophagy in lung cancer cells and thereby contribute to radioresistance. Ko et al also demonstrated that genetic inhibition of autophagy radiosensitizes H460 and A549 cells *in vitro*; however, when moved to an *in vivo* model of immune-competent mice, autophagy inhibition *reduced* responses to radiotherapy, indicating that autophagy may be necessary for immune clearance of tumor cells in response to radiation (176). In contrast, Kuwahara *et al.* utilized radioresistant liver cancer cell lines, which they had previously generated, to better understand the contributions of autophagy towards radioresistance (313). These investigators demonstrated autophagy induction in response to radiotherapy in both the parental

HepG2 cells and in the resistant cells (HepG2-8960-R). Furthermore, exposure to rapamycin, an mTOR inhibitor and autophagy *inducer*, sensitized HepG2-8960-R cells to radiation (10 Gy) but not the parental cell line. Pharmacological and genetic inhibition of autophagy *reduced* rather than increasing sensitivity to acute radiation exposure (2 Gy), suggesting that *suppression* of cytotoxic autophagy could contribute to radioresistance. However, there is relatively limited literature relating to the role of autophagy in acquired radiation resistance. These observations add another layer of intricacy to the overall role and contributions of autophagy to tumor cell growth and host immune cell modulation.

Due to its conventionally cytoprotective function, autophagy has been implicated in tumor cell resistance to therapy (119), making it an attractive target to sensitize tumor cells to anti-cancer therapy. However, in addition to the cytoprotective form of autophagy, there is accumulating evidence suggesting that autophagy can contribute to or mediate drug cytotoxicity (136,313), as well as function in a *non-protective* role, wherein autophagy inhibition would not alter chemosensitivity or radiosensitivity (117). Early studies by the Rodemann group as well as others identified cytoprotective autophagy in response to radiation in a number of experimental tumor cell models (92,183). In comparison, we have previously identified the nonprotective function of autophagy in tumor cells exposed to radiation (118,141) while other laboratories have demonstrated nonprotective autophagy in response to chemotherapy (143).

In the current work, we attempted to address this question by using our models of radiation-induced cytoprotective and non-protective autophagy to compare radiation sensitivity, based on the premise that tumor cells in which autophagy is cytoprotective would be expected to be *significantly less sensitive* to radiation than tumor cells in which the autophagy did not exhibit the cytoprotective function. Unexpectedly, only CQ treatment increased the radiosensitivity of the H460wt cells,

whereas 3-MA, bafilomycin A1 and genetic autophagy inhibition showed no effect on radiosensitivity in either cell line, raising further reservations as to the utilization of chloroquine as an autophagic inhibitor in the experimental discernment of the nature of autophagy. This finding supports the studies by Maycotte *et al.* that suggested chemosensitization by CQ could occur independently of autophagy inhibition (298). Qu *et al.* demonstrated CQ administration, but not 3-MA exposure, in combination with cisplatin increased intracellular hydroxyl radicals in cholangiocarcinoma cells through disruption of lysosomal permeability and mitochondrial bioenergetics (314). Additionally, other laboratories have shown chloroquine may induce apoptosis via p53-dependent pathways leading to increased apoptosis, as well as accentuating mitochondrial fragmentation and dysfunction in already damaged mitochondria, an effect evident in irradiated cells (314–316). Given literature evidence and lack of radiosensitization with added models of autophagy inhibition, we concluded radiosensitivity exhibited by CQ was most likely a result of off-target or autophagy-independent effects. Despite modest sensitization to radiation in p53 wt cells exposed to chloroquine, the autophagy proved to be *nonprotective* in both cell lines in multiple additional pharmacological and genetic inhibition models. Nevertheless, the autophagy was far more extensive in the p53 wild-type cells than in the p53 null cells and this *did not* result in protection from radiation. In fact, the p53 wild-type cells were more radiation-sensitive than the cells lacking functional p53, thus supporting the argument that the promotion of autophagy does not uniformly translate to reduced radiation sensitivity.

The major findings in these studies further complicate the role of p53 in radiation sensitivity. Previous studies demonstrate perturbation of p53 function could allow cancer cells to evade radiation-induced apoptosis, escape cell cycle checkpoints, and continue mitotic proliferation. For example, Hep3B2.1-7 hepatocellular cancer cells that lack p53 expression were less

radiosensitive than HepG2 cells with a functional p53 primarily due to attenuation in apoptosis induction (317). Similarly, Cheng *et al.* reported that induction of p53 expression in H1299 lung cancer cells resulted in enhanced radiosensitivity, again due to increased apoptosis induction (318). In the current work, H460 cells with functional p53 are also more radiosensitive than their p53-null counterparts; however, p53 status had no significant impact on apoptosis levels in response to radiation. Instead, our studies suggest that the difference in radiation sensitivity in the two cell lines may be largely due to their propensity to enter into a state of senescence, as was suggested by earlier work from our group relating to the involvement of p53 in chemotherapy-induced senescence (243).

Accumulating literature has shown that DNA-damaging events and ROS generation associated with radiotherapy can induce a premature senescence in tumor cells (259,319,320). Senescence activation allows tumor cells to undergo an alternative cell fate, aside from cell death, and provides an opportunity for cells to repair and potentially recover from DNA damage while halted in a cell cycle arrest (287). Furthermore, radiation-induced DNA damage can activate p53, resulting in senescence (300). Luo et al showed that 6 Gy radiation did not induce significant apoptosis in A549 and H460 cells, but rather induced a premature senescence indicated by increased SA-β-gal staining. Furthermore, knockdown of p53 inhibited radiation-induced senescence, while restoration of p53 expression sensitized cells to radiation and induced senescence (232). In agreement, Widel et al have shown that induction of senescence differed in p53 $^{+/+}$ and p53 $^{-/-}$ HCT116 cells, where the p53-deficient cells showed a significant reduction in the expression of senescence markers in comparison to the p53-proficients cell (321). However, in these studies p53 $^{-/-}$ HCT116 cells exhibited a higher frequency of apoptosis compared to its counterpart, p53 $^{+/+}$ HCT116 cells, which predominantly underwent senescence in response to irradiation. The data

presented in our findings indicate that H460wt cells undergo senescence induction to a greater extent than H460crp53 cells, resulting in differences in radiosensitivity. The spectrum of responses activated by radiation exposure adds complexity to the effects of p53 status on the toggling of cellular processes induced in response to therapy.

As indicated above, tumor cells almost uniformly undergo autophagy in response to exogenous forms of stress such as chemotherapy and radiation. Although the bulk of the scientific literature tends to consider autophagy as a cytoprotective response to stress and as a mechanism of resistance, this premise is subject to a number of reservations. One is that autophagy is not uniformly cytoprotective; in fact, autophagy can exist in one of four functional forms, only one of which is protective; the other forms are cytotoxic, cytostatic, and nonprotective autophagy (**Figure 2.9**) (117). Consequently, efforts to exploit autophagy inhibition as a therapeutic strategy for radiosensitization (or chemosensitization) are unlikely to be successful unless all autophagic responses to radiation, regardless of the tumor type, actually prove to be cytoprotective, which is highly unlikely based on our preclinical studies.

An abundance of literature, including this study, demonstrates senescence induction in response to radiotherapy. SASP secretion by these senescent cells can result in altered immune infiltration, activate senescence in neighboring cells, and promote changes in the ECM, priming the environment for a migratory phenotype (235). Moreover, promotion and recovery from senescence may be a potential model for tumor dormancy (271,322). Whether senescence is activated in response to radiation in clinical settings is not well understood and begs additional investigation. However, given the array of cellular responses induced in reaction to radiotherapy pre-clinically (**Figure 2.9**), it necessary to determine clinical patient tumor responses to further surmise whether

administration of an autophagy inhibitor or a senolytic, an agent that selectively clears senescent cells, may provide the more advantageous approach to radiosensitize patient tumors.

While these studies provide pre-clinical evidence that tumor cells at least undergo apoptosis, autophagy and senescence in response to radiation, it is also necessary to consider the possibility of additional outcomes to radiotherapy such as mitotic catastrophe, necrosis, and necroptosis. Furthermore, the extent to which of these factors contribute to radiation sensitivity or resistance in a particular malignancy cannot be predicted in clinical settings and is likely to vary depending on the genetic background of the tumor (or tumor cell lines).

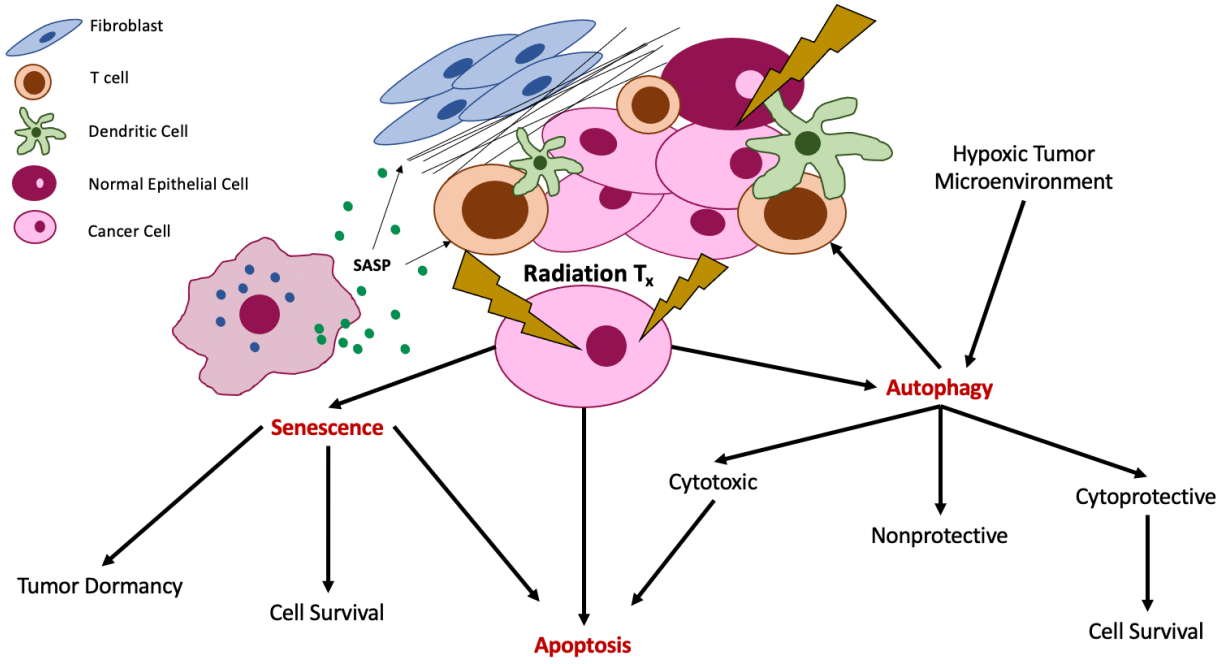

Figure 2.9. Tumor cell responses to radiotherapy.
In response to radiotherapy, tumor cells can upregulate both cell death and cell survival pathways. Whereas apoptotic cell death is the ideal outcome for clinical therapeutic treatment, tumor cells often enter into senescence and autophagy, largely in efforts to evade cell death. However, radiation-induced autophagy can assume different functional roles. Induction of the *cytoprotective* form of autophagy allows cells to evade apoptotic cell death and prolong survival; however, cytotoxic autophagy can facilitate either apoptotic and/or autophagic cell death. Finally, an alternative form of autophagy that does not appear to influence cell sensitivity to radiotherapy can occur, termed *nonprotective* autophagy. Senescence often occurs in parallel with autophagy, sharing a number of mechanistic regulators. Radiation-induced senescence allows cells to transiently arrest in efforts to repair damage. Subsequently, tumor cells may undergo apoptotic cell death if the extent of damage is excessive or may overcome the insult, allowing for continued survival. Senescence may also contribute to tumor dormancy, as a subset of senescent cells endure a prolonged growth arrest and regain proliferative capacity. Senescent cells produce a unique secretory phenotype (SASP), allowing for manipulation of the ECM and influencing surrounding cells in the TME. Through the release of specific cytokines and chemokines, autophagy and senescence can play immune-modulatory effects to create either immune-promoting or immune-suppressive microenvironments, thereby contributing to overall tumor survival or clearance. Both autophagy and senescence have cell-autonomous, as well as cell non-autonomous effects, adding to the complexity of responses and outcomes of clinical radiotherapeutics.

Chapter Three: What is the effect of p53 status on cisplatin sensitivity and the nature of autophagy in NSCLC cells?

3.1 Introduction

Cisplatin is a commonly used anti-cancer therapy. It is prescribed for the treatment of a number of cancer types, including breast cancer, lung cancer, ovarian cancer, head and neck cancers, brain cancer, as well as many others (323). Cisplatin, like most platinum-based drugs, contains a platinum moiety which that forms covalent platinum-DNA interactions, generating both inter-and intrastrand crosslinks in the DNA (324). Formation of these adducts interferes with the proper binding of transcription factors and proteins required for DNA replication and DNA transcription for protein synthesis, ultimately resulting in cell cycle arrest and apoptosis (323). While cisplatin is a highly effective anti-cancer therapeutic, resistance to treatment still remains a prevalent issue. Several mechanisms have been proposed for cisplatin resistance, including drug efflux via the multi-drug resistance pump, DNA damage repair, and inhibition of apoptosis (325). Furthermore, in response to cisplatin treatment, it has been demonstrated that cells upregulate autophagy, a conventionally cytoprotective mechanism, and may contribute to cisplatin resistance in tumor cells (119).

While it is indisputable that inhibition of cytoprotective autophagy proves an advantageous avenue for chemosensitization and radiosensitization (79,123,137), it is essential to acknowledge the multi-functional nature of autophagy in response to therapy (139,141,313). As exemplified from the previous studies on radiation, autophagy was nonprotective in function, regardless of p53 status, indicating autophagy inhibition failed to sensitize cells to radiotherapy (142). Furthermore, previous work from our laboratory demonstrated the existence of an important phenomenon, which

we have termed the “autophagic switch” where the functional form of autophagy can be changed to another form in response to external or biological stressors. In these studies, ZR-75-1 breast cancer cells, expressing wild type p53, exhibited cytoprotective autophagy when treated with radiation alone; however, when given in combination with 1,25-dihydroxy vitamin D3, the functional form of autophagy was “switched” to a cytotoxic form (326). Autophagic function may be regulated by several factors, including p53, oxidative stress, and genotoxic stress (19,51,327). Depending on the spatiotemporal localization of p53, it can both suppress and activate autophagy (328). Whether p53 plays a role in determining the nature of the autophagy is not yet understood; however, in this work, we attempt to further probe this question by utilizing a set of isogenic cell lines, H460wt and H460crp53 NSCLC cell lines, used previously in our studies relating to radiation.

Cisplatin-induced DNA damage can result in the activation of p53 and, depending on the amount of damage, induce a multitude of cellular responses including senescence, autophagy, and apoptosis (104,329). Furthermore, loss of p53 is also associated with increased tolerance to cisplatin-induced DNA adducts and replicative bypass, contributing to cell survival and treatment resistance (325,329). From the studies presented in chapter 2 with radiation, p53 status plays a role in toggling between important cellular responses. In response to radiotherapy, senescence seemed to play a predominant role in mediating the differential radiation sensitivity between H460wt and H460crp53 cells. Similarly, senescence induction in response to cisplatin exposure has been demonstrated in ovarian, NSCLC, and melanoma cell lines (246,330,331). Furthermore, cisplatin-induced SASP secretion in senescent melanoma cells was shown to promote cellular growth in non-senescent counterparts (330). Thus, the current study paralleled the radiation studies to

examine the effects of p53 status on tumor cell responses to cisplatin exposure and whether the type of cytotoxic therapy affects autophagic function.

3.2. Methods

3.2.1. Cell Culture and Treatment

H460wt and H460crp53 NSCLC cells were cultured in DMEM media supplemented with 10% (v/v) fetal bovine serum (Thermo Scientific), 100 U/ml penicillin G sodium (Invitrogen), and 100 μg/ml streptomycin sulfate (Invitrogen). Puromycin (1 μg/ml; Sigma) was used to maintain the selection of shATG5 and shControl transfected cells. Cells were incubated at 37℃ under humidified 5% CO2. Cisplatin was purchased from Cayman Chemical Company (13119) and dissolved in dimethylformamide to a stock solution of 10 mM. Working solutions were further diluted in media.

Cells were seeded on day 0 followed by cisplatin treatment (0, 2.5, 5, 10, 20 μM) on day 1 for 24 h. Media was replenished every other day. For autophagy inhibition studies, cells were treated with pre-treated with 3MA (0 or 1 mM) or CQ (0 or 10 μM) 3 h prior to cisplatin (0 or 10 μM) exposure for 24 h. All assays performed as previously described.

3.3 Results

3.3.1. Cisplatin sensitivity in H460wt and H460crp53 NSCLC cells

In previous work, we reported that radiation-induced autophagy was nonprotective in the H460 non-small cell lung cancer cell line regardless of p53 status (142). In order to investigate whether autophagy is universally nonprotective in this cell line, the current studies utilized the antitumor

drug, cisplatin, as the primary autophagy inducer. Furthermore, utilizing the same set of isogenic cell lines, H460wt and H460crp53 NSCLC cells, we aimed to discern the effects of p53 status on cisplatin sensitivity and autophagic function.

Initially, cells were exposed to 10μM cisplatin for 24 hrs. Temporal analysis of cell viability was assessed to confirm the differential sensitivity to cisplatin. **Figure 3.1A** demonstrates that H460wt cells were more sensitive to 10 μM cisplatin exposure than H460crp53 cells. Temporal response pattern largely showed that the H460wt underwent a rapid growth decline, indicative of cell death, while the H460crp53 cells initially continue to proliferate and only begin to succumb to the drug effects after 3 days.

It is generally thought that lack of p53 function attenuates apoptosis (289,332). An evaluation of the extent of apoptosis by annexin V/PI staining demonstrated that H460wt cells exposed to different doses of cisplatin underwent a much more pronounced degree of apoptosis/necrosis than H460crp53 cells (**Figure 3.1B)**. This did not appear to be a consequence of differential DNA damage since cisplatin promoted equivalent DNA damage in the H460p53 wt and H460crp53 cells, based on γH2AX staining performed over a 24 h time period (**Figure 3.1C-D**).

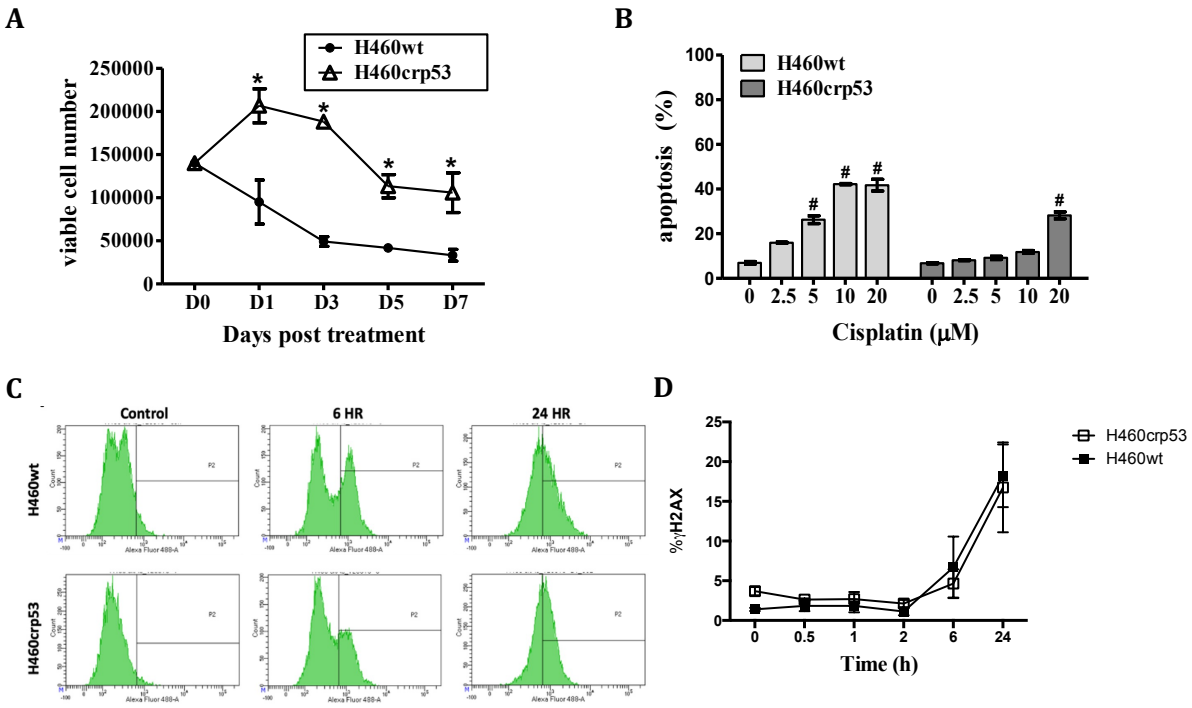

Figure 3.1. Cisplatin sensitivity in H460wt cells and H460crp53 cells.
A. Cell viability. Cells were treated with cisplatin (10 μM) for 24 h, washed free of drug, incubated with fresh medium and stained with trypan blue (n=3). **B. Apoptosis Dose Response.** Cells were treated with cisplatin at the indicated doses for 24 h and apoptosis was assessed by Annexin V-FITC staining. Apoptosis was measured 24 h after cisplatin removal (n=3). **C-D. DNA Damage.** Temporal assessment of DNA damage in response to cisplatin. Extent of DNA damage was measured utilizing flow cytometry to quantify γH2AX staining in cells treated with cisplatin (10 μM). (D) Representative images of flow cytometry data and (E) quantification of fluorescence was graphed (n=3). Unless stated, otherwise data were from three independent experiments, *$p < 0.05$, cisplatin treated H460wt group vs cisplatin treated H460crp53 group. $^{\#}p < 0.05$ cisplatin treated group vs untreated control group in each cell line.

3.3.2. Cisplatin-induced autophagy in p53 wt and p53 knockout cells

Cytoprotective autophagy is generally considered to be a mechanism to ameliorate or evade apoptosis; hence, we assessed the capacity of cisplatin to promote autophagy in both cell lines. **Figure 3.2A** presents images of acridine orange staining of autophagic vacuoles, a rough but generally accurate indication of the extent of autophagy. Assessment of acidic vesicle formation by flow cytometry indicated that the extent of autophagy induced by cisplatin was similar in the two cell lines (**Figure 3.2B**). To further compare the extent of autophagy and whether cisplatin-induced autophagy is going to completion, degradation of p62/SQSTM1 was evaluated by western blotting. **Figure 3.2C** (and quantification of the band densities in **Figure 3.2D**) indicates that autophagic flux is clearly occurring in both cell lines. Moreover, both H460wt and H460crp53 cells demonstrate co-localization of LC3 and the lysosomal marker, LAMP-2, when exposed to cisplatin indicative of autophagic flux (**Figure 3.2E**). Consistent with the previous studies in radiation, untreated H460crp53 exhibited greater LC3/LAMP2 co-localization when compared to H460wt cells, suggesting basal autophagy was higher in H460crp53 cells. These studies indicate that autophagy appears to be induced to a similar extent in cisplatin-treated H460 cell, regardless of the status of p53, although the process is slightly more rapid in the H460crp53 cells.

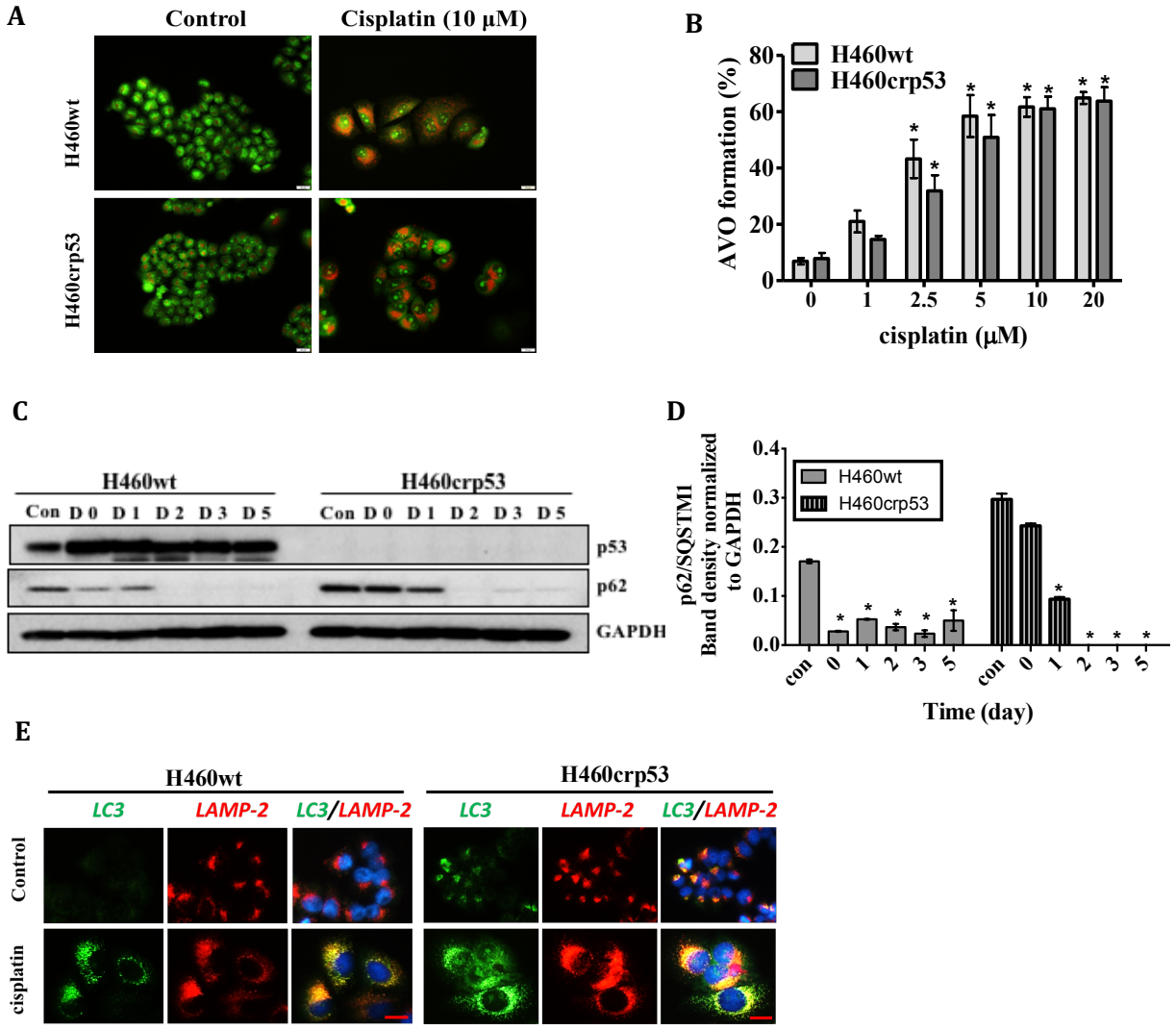

Figure 3.2. Cisplatin induces autophagy in H460wt cells and H460crp53 cells.
A-E. Cells were treated with cisplatin at the indicated doses for 24 h (day 0), after which cells were washed and incubated in fresh medium. **A.** Acridine orange staining was performed after exposure to 10 μM cisplatin. Cells were treated with cisplatin for 24 h (day 0) and stained 48 h post-drug removal (day 3, n=3). **B. Quantification of acridine orange staining.** Autophagy induction was quantified by flow cytometry in response to increasing concentration of cisplatin 2 days after drug exposure (n=3). **C**. **Western blotting.** Levels of p62 were determined by western blotting at the indicated times after 10 μM cisplatin exposure for 24 h (D0). Lysates were collected on indicated days. One of three representative experiments is shown (n=3). **D. Western blot Densitometry.** The bar graph in each panel indicates the relative band intensity generated from densitometric scans of two independent experiments in arbitrary densitometric units (n=3). **E**. **Co-localization of LC3 and LAMP.** Fluorescence microscopy showing LC3 and LAMP2 co-localization in response to 10 μM cisplatin exposure 2 days after cisplatin removal. (20X objective, n=2) Unless stated, otherwise data were from three independent experiments, *p < 0.05, cisplatin treated group vs untreated control group in each cell line.

3.3.3. Evidence for cytoprotective autophagy in the p53 knock-out cells and nonprotective autophagy in the p53 wild-type H460 cells

Next, the nature of the autophagy was evaluated based on sensitization or lack of sensitization when autophagy was inhibited using the pharmacological autophagy inhibitors, CQ or 3-MA. Both H460 cell lines were pre-treated with CQ (10 μM) or 3-MA (1 mM) for 3 h followed by exposure to 10 μM cisplatin for 24 h. Autophagy was measured after 2 days of incubation with fresh medium. The increase in LC3-II puncta formation in the presence of CQ (due to inhibition of autolysosome formation and accumulation of autophagosomes) and decrease in LC3-II puncta with 3-MA (due to interference with autophagosome formation) indicated that CQ and 3-MA inhibited cisplatin-induced autophagy (**Figure 3.3A**). Interference with p62/SQSTM1 degradation further confirmed that CQ and 3-MA inhibited cisplatin-induced autophagy (**Figure 3.3B**). Temporal response studies were then performed to determine the impact of autophagy inhibition on sensitivity to cisplatin. **Figures 3.3D and 3.3E-F** show that administration of CQ and 3-MA increased cell death and apoptosis in response to cisplatin in the H460crp53 cells but failed to influence cisplatin-induced cell death and apoptosis in the H460wt cells (**Figure 3.3C and 3.3E-F**), indicating that autophagy was *cytoprotective* in function in the H460crp53 cells but *nonprotective* in the H460wt cells.

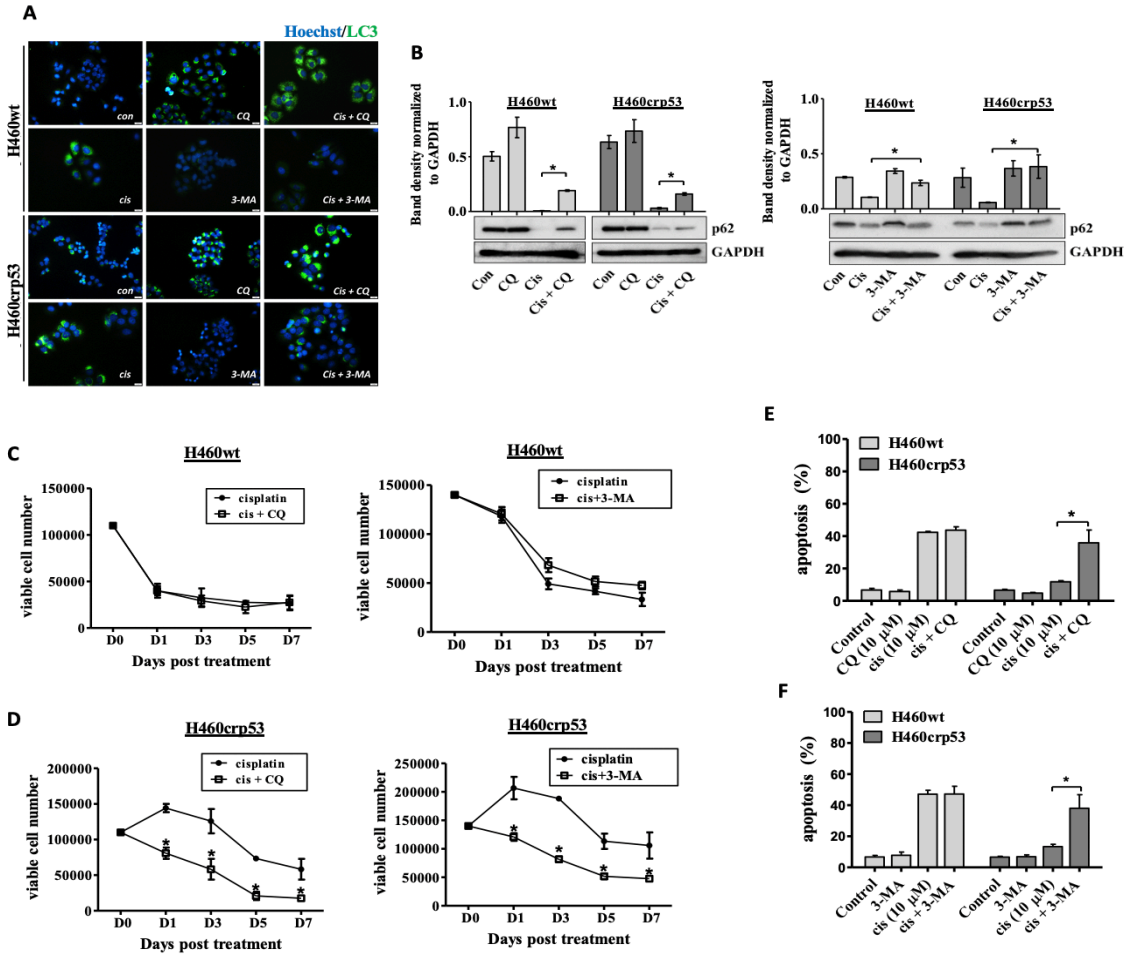

Figure 3.3. Pharmacological autophagy inhibition sensitizes H460crp53 cells, but not H460wt cells to cisplatin exposure.

A. Inhibition of autophagy by CQ and 3-MA. Fluorescence microscopy showing increased LC3 puncta following CQ (10 μM) co-treatment with 10 μM cisplatin, and decreased LC3 puncta following 3-MA (1 mM) co-treatment with 10 μM cisplatin. Cells were pretreated with CQ (10 μM) and 3-MA (1 mM) followed by an additional 24 h with cisplatin. Images were taken 48 h after cisplatin removal. Nuclei were stained with Hoechst 33342 and vacuoles with LC3 antibody (20x objective, n=2). **B. Inhibition of autophagy by CQ and 3-MA.** Western blot showing autophagy blockade by CQ (10 μM) and 3-MA (1 mM) based on levels of p62/SQSTM1 (n=3). The bar graph in each panel indicates the relative band intensity generated from densitometric scans of two independent experiments in arbitrary densitometric units. **C and D. Influence of autophagy inhibition on cisplatin sensitivity.** Viability of H460wt cells and H460crp53 cells was monitored based on trypan blue exclusion at indicated days following 10 μM cisplatin exposure in combination with CQ (10 μM) or 3-MA (1 mM) (n=3). **E-F. Influence of autophagy inhibition on cisplatin induced apoptosis**. Annexin V-PI staining showing influence of CQ (10 μM) and 3-MA (1 mM) on apoptosis of H460 cells exposed to cisplatin (10 μM). Cells were pretreated with CQ or 3-MA for 3 h followed by co-treatment with cisplatin for 24 h. Apoptosis was measured 24 h after cisplatin removal (n=3). Unless stated otherwise, data were from three independent experiments, *p<0.05, cisplatin versus cisplatin + CQ (3-MA).

To confirm the findings generated using pharmacological inhibition, short hairpin RNA (shRNA) was used to knockdown *Atg5*, an autophagy regulatory gene, in both H460 cell lines. **Figure 3.4A** verifies the status of Atg5 by Western blotting in the two cell lines. The decrease in LC3 puncta (**Figure 3.4B**) indicates that shAtg5 effectively inhibited autophagy in both H460 cell lines. Inhibition of autophagy was confirmed by interference with cisplatin-induced degradation of p62/SQSTM1 (**Figure 3.4C**). Here, genetic interfere with autophagy yielded a similar outcome to that observed with the pharmacological autophagy inhibitors; specifically, autophagy inhibition failed to alter growth inhibition and apoptosis in response to cisplatin in H460wt cells (**Figures 3.4D and 3.4E-F**), but increased cell growth inhibition and apoptosis in the H460crp53 cells (**Figures 3.4D and 3.4E-F**).

Taken together, these data demonstrate that in the H460wt cells the autophagy is *nonprotective* since there is no further sensitization with autophagy inhibition and no increase in apoptosis. In contrast, autophagy "switches" to the *cytoprotective* form/function when p53 is knocked out, as autophagy inhibition increases sensitivity to cisplatin and results in enhanced apoptosis in the H460crp53 cells.

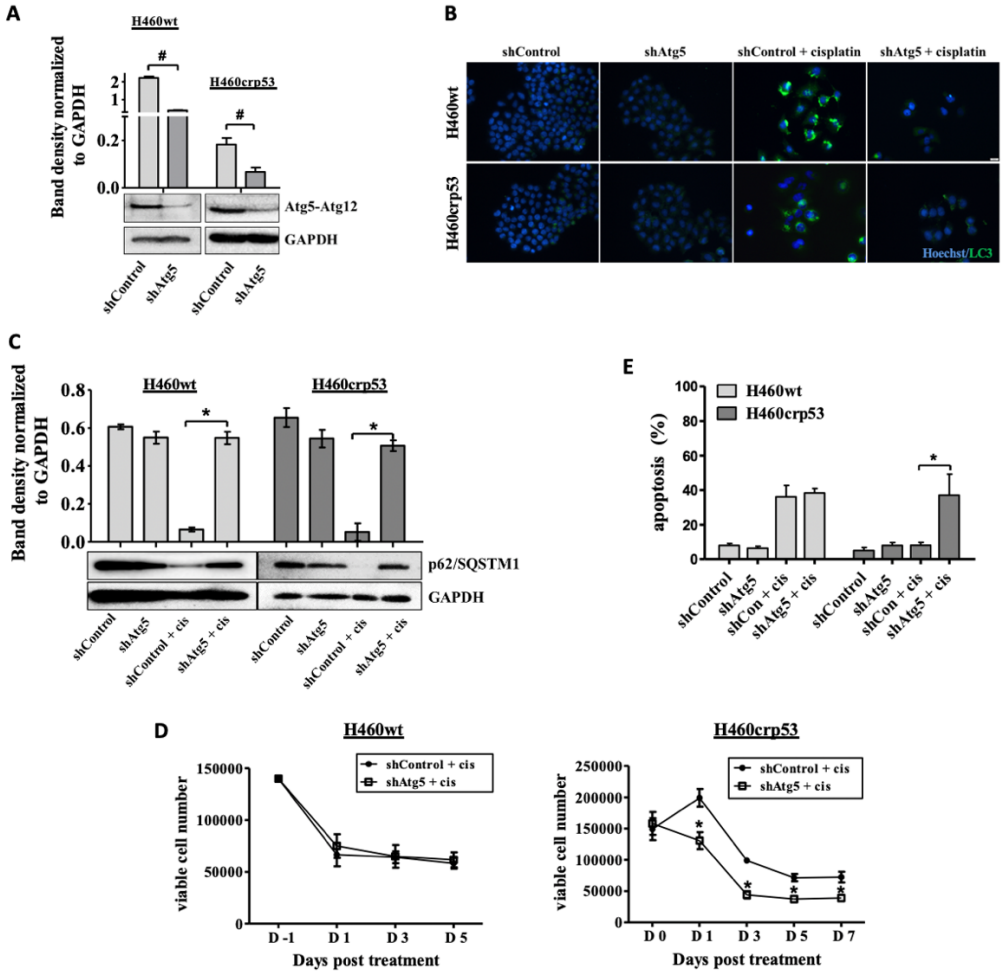

Figure 3.4. Influence of genetic autophagy inhibition on cisplatin sensitivity in H460wt cells and H460crp53 cells.

A. Western blot showing the **silencing of *Atg5*** in H460wt cells and H460crp53 cells. **B and C**. **Autophagy inhibition by *Atg5* silencing.** The bar graph in each panel indicates the relative band intensity generated from densitometric scans of three independent experiments in arbitrary densitometric units (n=3). **B.** Fluorescence microscopy showing decreased LC3 puncta following treatment with 10 μM cisplatin in shAtg5 H460 cells. Images were taken 48 h after cisplatin removal. Nuclei were stained with Hoechst 33324 (20x objective, n=2). **C.** Western blot showing autophagy blockade by Atg5 knockdown based on levels of p62/SQSTM1. Proteins were collected 48 h after cisplatin removal (n=2). The bar graph in each panel indicates the relative band intensity generated from densitometric scans of two independent experiments in arbitrary densitometric units. **D**. **Influence of autophagy inhibition on cisplatin sensitivity.** Viability of H460wt cells and H460crp53 cells was monitored based on trypan blue exclusion at indicated days following cisplatin exposure in shATG5 in H460wt cells and H460crp53 cells (n=3). **E**. **Influence of autophagy inhibition on cisplatin induced apoptosis**. Annexin V-PI staining showing apoptosis in H460 cells exposed to cisplatin (10 μM) with and without ATG5 silencing. Apoptosis was measured 24 h after cisplatin removal. (n=3). Unless stated otherwise, data were from three independent experiments, *$p < 0.05$, shControl + cisplatin versus shAtg5 + cisplatin, #$p < 0.05$, shControl versus shATG5.

3.3.4. Cisplatin induced senescence in H460wt and H460crp53 NSCLC cells

Autophagy and senescence often occur in parallel in response to cytotoxic therapy. Given that senescence contributed to the differential sensitivity to radiotherapy in our previous studies (142); therefore, we investigated the role of senescence induction and its contributions to the differential sensitivity seen between H460wt and H460crp53 cells. Cells were exposed to 10 μM cisplatin and senescence-associated ß-galactosidase (SA-ß-gal) activity was assayed. **Figure 3.5A** demonstrates ß-galactosidase staining (blue) was increased in both H460wt and H460crp53 cells. SA-ß-gal activity was also monitored by flow cytometry for a more quantitative approach. Temporal analysis of SA-ß-gal activity was increased in both cell lines in response to cisplatin exposure, indicating senescence was induced to similar extents in both the H460wt and H460crp53 NSCLC cells **(Figure 3.5B)**.

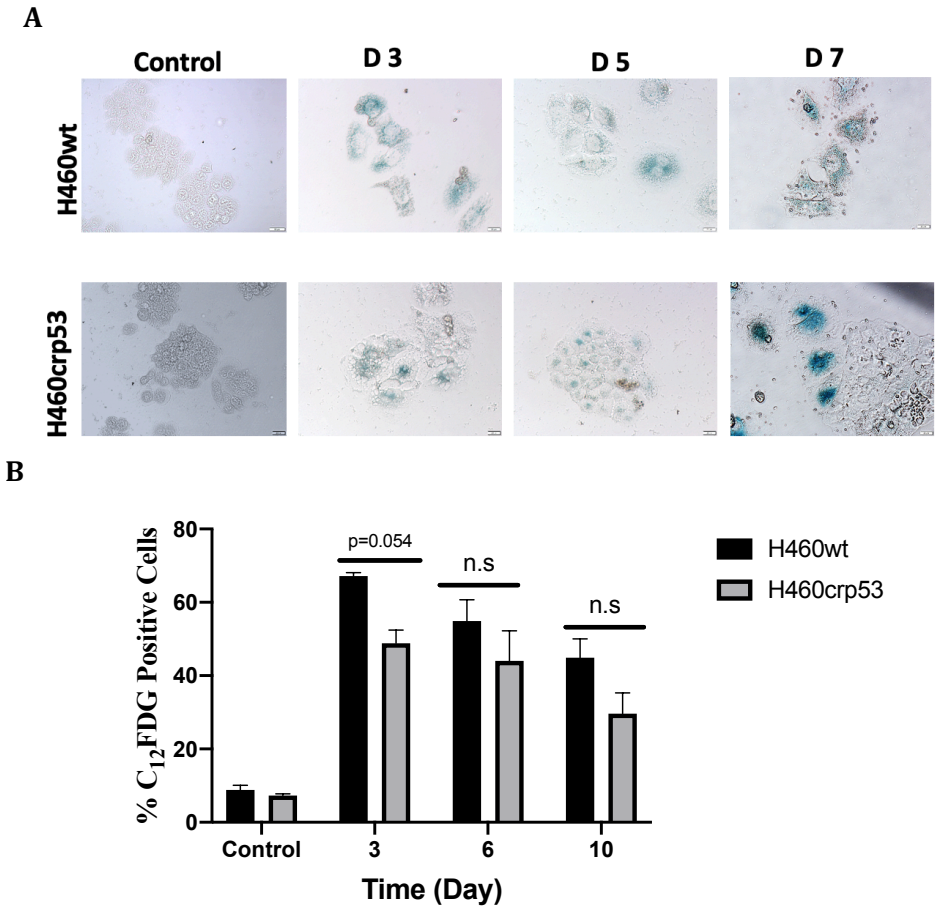

Figure 3.5. Cisplatin induced senescence in H460wt and H460crp53 NSCLC cells.
A. β-galactosidase staining and cell morphology. β-galactosidase staining indicating the induction of senescence by cisplatin (10 μM) in both cell lines (scale bar = 20 μm). **B. Quantification of senescence.** C_{12}FDG staining and flow cytometry to quantify the extent of senescence in H460wt and H460crp53 cells. Cisplatin induced senescence in both H460wt and H460crp53 cells. Unless stated, otherwise data were from three independent experiments. n.s. H460wt versus H460crp53 cells.

3.3.5. Inhibition of cytoprotective autophagy shifts the temporal response to cisplatin in the H460crp53 cells

Given that senescence was induced to similar extents in both cell lines, we wanted to confirm the differential sensitivity to cisplatin exhibited between the two cells lines was partly due to differences in autophagic function. To examine the contributions of cytoprotective autophagy to cisplatin sensitivity, we compared the temporal responses shown in **Fig. 3.3C and 3.3D** with and without pharmacological autophagy inhibition by replotting these time courses in **Figures 3.6A and 3.6B**. The blockade of cytoprotective autophagy in H460crp53 cells exposed to cisplatin resulted in a temporal decline in cell viability that was essentially identical to that in the cisplatin treated H460 p53wt cells. In agreement with these observations, **Figure 3.6C** demonstrates a similar relationship when cell viability data from **Fig. 3.4D** is plotted together to show an overlap of the decline in cell viability in response to cisplatin when autophagy has been genetically inhibited in H460crp53 cells when compared to H460wt shControl cells exposed to cisplatin.

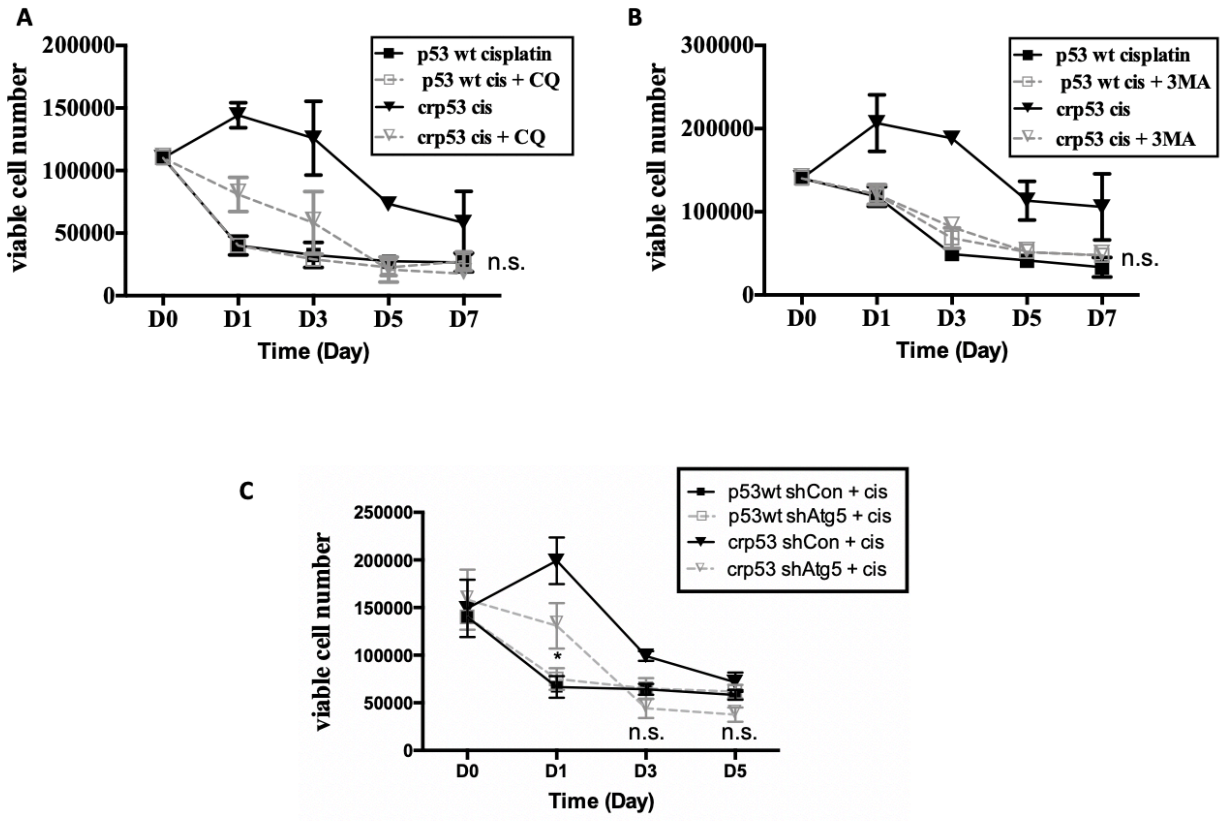

Figure 3.6. Inhibition of cytoprotective autophagy shifts the temporal response to cisplatin in the H460crp53 cells.

A-C. Influence of autophagy inhibition on cisplatin sensitivity in p53 wt and p53 KO cells. Cell viability data from figure 3C and 3D were overlaid to compare the functional role of autophagy in H460crp53 and H460wt cells using the pharmacological inhibitors, CQ **(A)** and 3MA **(B). C.** Cell viability data from figure 4D were overlaid to compare the functional role of autophagy on cisplatin sensitivity via genetic silencing of ATG5. *$p<0.05$ p53wt shControl + cis vs. crp53 shATG5 + cis, n.s. p53wt + cis vs. crp53 + cis + CQ (3-MA or shAtg5).

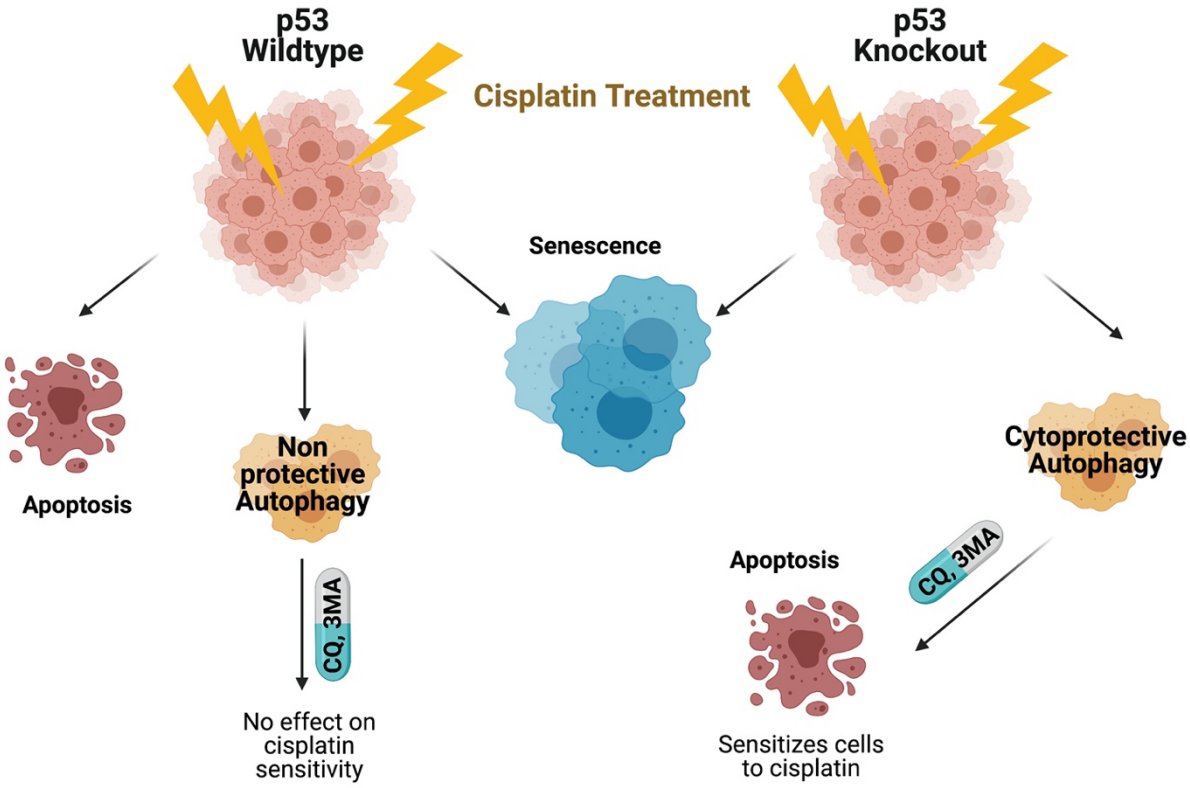

Figure 3.7. Cellular responses to cisplatin exposure in H460wt and H460crp53 NSCLC cells.

In response to cisplatin exposure, H460wt cells were significantly more sensitive than H460crp53 cells. H460wt cells underwent greater apoptosis induction compared to H460crp53 cells; however, the extent of autophagy induction and senescence induction was similar in the two cell lines. Autophagy inhibition revealed H460wt cells underwent nonprotective autophagy, while H460crp53 cells induced cytoprotective autophagy. These data demonstrated nonprotective autophagy induced in p53wt non-small cell lung cancer cells in response to cisplatin can be "switched" to protective autophagy in isogenic crp53 cells, and that inhibition of cytoprotective autophagy is sufficient to restore cisplatin sensitivity in the crp53 cells, through the promotion of apoptosis. Created with BioRender.com.

3.4 Discussion

Autophagy is one mechanism thought to be induced by cancer cells to evade apoptosis (333). Cisplatin exposure resulted in autophagy induction to a similar extent in both the H460wt and H460crp53 cells. Autophagy inhibition (by pharmacological and genetic interventions) increased cisplatin-induced cell death and apoptosis in H460crp53 cells (i.e., evidence of cytoprotective autophagy) but did not alter either outcome in the p53 wt H460 cells (i.e. evidence of nonprotective autophagy). As a result, the temporal decline in cell viability in the H460crp53 cells when autophagy was inhibited essentially paralleled that observed in p53 wt H460 cells, suggesting cytoprotective autophagy was contributing to the differential sensitivity and differing extent of apoptosis observed between the two cell lines when exposed to cisplatin. Consequently, these findings support the premise that cytoprotective autophagy can confer a relative degree of resistance to chemotherapy.

This work further interrogates the relative contributions of p53 status and autophagy to sensitivity and resistance to chemotherapy. Tasdemir et al and colleagues had reported that inhibition of cytoplasmic p53 led to autophagy in enucleated cells and conversely that cytoplasmic p53 was able to repress the enhanced autophagy of p53 null cells, providing evidence of a relationship between p53 and autophagy (170). Topotecan, a topoisomerase I inhibitor, induced *cytoprotective* autophagy in p53wt colon cancer cells *in vitro* and *in vivo*, but induced *cytotoxic* autophagy in p53 null colon cancer cells (334). Tripathi *et al.* demonstrated that cisplatin induced protective autophagy in p53 knockdown embryonal carcinoma cells, which would be consistent with the findings presented in this work (169). However, Maycotte et al reported on nonprotective autophagy in p53 null 4T1 breast tumor cells exposed to cisplatin (298). These differential

outcomes indicate that it cannot be predicted, *a priori*, the nature that drug or radiation-induced autophagy will exhibit, based solely on the status of p53 in the cells.

The Autophagic Switch. Collectively, autophagy can play various functional roles in response to chemotherapy and radiation; moreover, the mechanism(s) determining the functional form induced in response to therapy still remains unclear. To add further complexity to these observations, a unique phenomenon whereby autophagy can "switch" between functions has also been demonstrated (141,142,326). For instance, the "autophagic switch" was also exhibited in studies of estrogen receptor (ER) expression in breast cancer cells exposed to gemcitabine (335). Gemcitabine induced cytoprotective autophagy in ER- BCap37 breast cancer cells, but cytotoxic autophagy in ER+ Bcap37 cells. Further, genetic silencing of the ERα receptor in the ER+ Bcap37 cells was sufficient to switch the cytotoxic form of autophagy to the cytoprotective form. In an osteosarcoma model, drug-resistant cells exhibited cytoprotective autophagy, with greater reliance on autophagy for metabolic maintenance, whereas, drug-sensitive cells exhibited cytotoxic autophagy in response to camptothecin (336). The studies presented with cisplatin and radiotherapy in the same set of isogenic H460 NSCLC cell lines provide further evidence of the existence of an "autophagic switch" in tumor cells in response to anti-cancer therapy.

In the current work, cisplatin induced similar levels of autophagy in both p53 wt and H460crp53 cells, indicating that the capacity to undergo cisplatin-induced autophagy is essentially p53-*independent*. However, as in many of the studies cited above, the nature of the autophagy changed in association with the different p53 statuses of the two cell lines. Inhibition of autophagy increased drug sensitivity and apoptosis in the H460crp53 cell to similar extents as p53wt cells exposed to cisplatin. This critical observation from the current work suggests that *cells lacking*

functional p53 are capable of undergoing apoptosis to the same degree as p53 wild-type cells. As was shown in **Figure 3.6**, autophagy inhibition in H460crp53 cells shifted the temporal response to cisplatin to be virtually identical to that in the H460wt cells, suggesting that *cytoprotective autophagy and not p53 function was largely responsible for the reduced sensitivity to cisplatin of the crp53 cells* **(Figure 3.7)**.

Interestingly, this differs from our recent findings in studies involving ionizing radiation, where radiation sensitivity appeared to be a function primarily of the extent of senescence and appeared to be largely *unrelated* to autophagy (337) **(Table 3.1)**. In these studies, the same set of isogenic cell lines, H460wt and H460crp53 cells, exhibited nonprotective autophagy in both cells when exposed to radiation and autophagy inhibition failed to alter radiation sensitivity or radiation-induced apoptosis in either cell line. However, through these studies, we demonstrate that crp53 cells have the capacity to undergo nonprotective autophagy (in the case of radiation), and this response is "switched" to protective autophagy in the case of cisplatin treatment **(Table 3.1)**. Collectively, these studies suggest the existence of an autophagic switch, not only between isogenic cell lines differing in p53 status but also depending on the therapeutic agent utilized.

While these studies with H460wt and H460crp53 cells isogenic cell lines exposed to radiation or chemotherapy demonstrated functional switches in autophagy, both in terms of cytotoxic agent and p53 status, very little is understood as to the mechanisms regulating autophagic function. DNA damage is the primary mechanism through which both radiation and cisplatin exert their antitumor effects. Furthermore, autophagy is induced in response to both anti-cancer treatments and aids in the turnover of proteins involved in DDR and cell cycle checkpoints (296). Below we consider whether the extent and/or temporal profile of DNA damage may contribute to the differential

functions of autophagy induced in response to cisplatin and radiation in H460wt versus H460crp53 cells. The extent of DNA damage was similar in both cell lines when exposed to radiation or cisplatin; however, there were differences in the temporal profile of DNA damage incurred between the two treatment modalities **(Figure 3.8)**. Radiotherapy induced maximal DNA damage around 30 mins-1 h post-IR exposure which seemed to resolve to basal levels within 24 h post-IR; in contrast, cisplatin demonstrated a delayed and much lower extent of DNA damage, but one that did not exhibit resolution in the timeframe monitored **(Figure 3.8).**

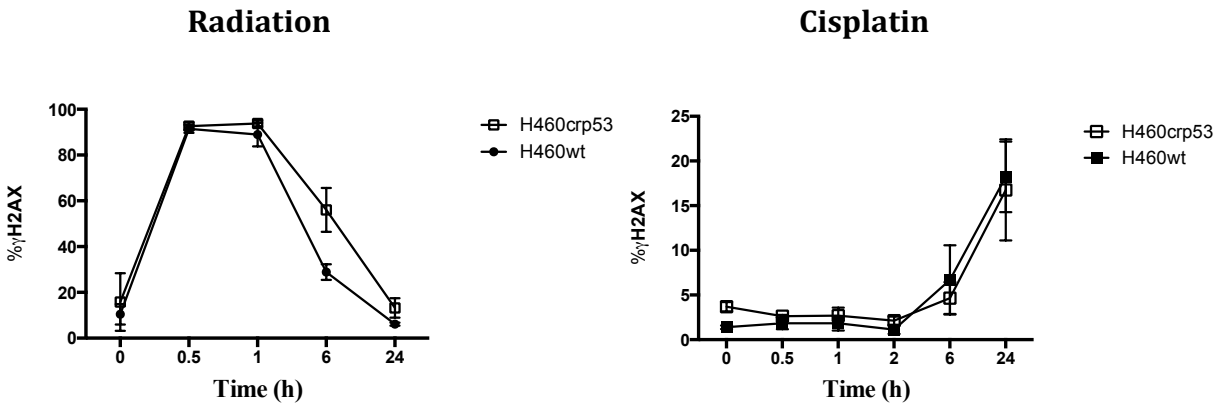

Figure 3.8. Radiation and Cisplatin induced DNA damage in H460wt and H460crp53 cells.

A. Radiation-induced DNA damage. Cells were exposed to 6 Gy radiation and DNA damage was measured by γ-H2AX staining. Fluorescence of γ-H2AX was quantified using flow cytometry. **B. Cisplatin-induced DNA damage.** Cells were exposed to 10M of cisplatin for 24 h and DNA damage was measured by γ-H2AX staining throughout the 24 h exposure time. Fluorescence of γ-H2AX was quantified using flow cytometry.

A potential factor contributing to resistance to therapy is that in tumor cells with higher basal autophagy, autophagy may facilitate the removal of therapy-induced damage more efficiently. Studies by Liang *et al.* examining the role of autophagy in multidrug-resistant ovarian carcinoma observed that radiation induced relatively low levels of apoptosis; inhibition of apoptosis with ZVAD did not significantly alter survival or cell death, confirming that apoptosis is not the primary therapeutic response to radiation, at least in this experimental model (126). These studies also demonstrated higher basal autophagy in the multidrug-resistant phenotype SKVCR cells compared to human SKOV3 ovarian carcinoma cells, suggestive of a cytoprotective function. Moreover, inhibition of autophagy with 3-MA sensitized the multidrug-resistant cells to radiation while having only modest effects on the parental SKOV3 cells. Similarly, when examining LC3/LAMP2 colocalization in untreated control cells in **figures 2.3D** and **3.2E**, H460crp53 cells exhibit greater LC3 fluorescence compared to H460wt NSCLC cells, allowing initial indications that basal autophagy may be higher in H460crp53 cells compared to H460wt cells. While further examination is required, this difference in basal autophagy may play a role in the differences in autophagic function exhibited between the two cell lines in response to cisplatin. The lower and more delayed extent of DNA damage in response to cisplatin exposure may allow H460crp53, with higher basal autophagy, to maintain sufficient clearance of cisplatin-induced damage, sustaining cells below critical cell death thresholds.

Inhibition of *cytoprotective* autophagy in the H460crp53 cells in response to cisplatin restored apoptosis induction and cisplatin sensitivity to similar extents as H460wt cells, where the autophagy was *nonprotective*, suggesting the crosstalk between autophagy and apoptosis may play a key role (73). One possibility is that autophagy may sequester and remove damaged mitochondria through mitophagy, preventing cytochrome C release and activation of intrinsic death pathways

(338). Another potential mechanism through which autophagy modulates apoptosis is through selective cargo shuttling and degradation of pro-apoptotic proteins. Autophagy can mediate the degradation of caspase 8 and evade caspase 8-mediated cell death; furthermore, deficiencies in autophagy can also result in caspase 8 dimerization on the membrane of the autophagosome and promote TRAIL-mediated cell death (339,340). Similarly, Wang *et al.* demonstrated NOXA, a BH3-only member of the Bcl2 family that promotes apoptosis, is targeted by p62 for autophagic degradation, reducing apoptotic induction in NSCLC and colorectal cancer cell lines (341). Autophagy inhibition increased NOXA protein accumulation, suppressed tumor growth, and activated cell death pathways (341). Thus, if autophagy was cytoprotective in nature due to its capabilities of trafficking pro-apoptotic proteins, then it could be hypothesized that autophagy inhibition would relieve this sequestration and switch responses to a pro-apoptotic cell fate. Future studies focusing on in-depth analysis of autophagic cargo may provide promising insights on the role of specific cargo on the nature of autophagic function and maybe a potential rationale for why one functional form of autophagy "switches" to another as exhibited in the H460crp53 cells.

Taken together, these studies provide proof of concept that cytoprotective autophagy can confer intrinsic resistance to chemotherapy, based on a comparison of cisplatin sensitivity in two isogenic cell lines where autophagy demonstrated cytoprotective and nonprotective functions. However, it is necessary to recognize that autophagy induced by chemotherapy or radiation may not always be cytoprotective in the clinic and the therapeutic benefit of autophagy inhibition may only be successful in scenarios where the autophagy is *cytoprotective.* These studies further provide an additional model of the "autophagic switch" in cancer therapy using a set of isogenic cell lines, but the mechanistic basis for the autophagic switch still remains to be determined.

While these studies with cisplatin indicate p53 status may influence autophagic function, collectively taking the findings of both the radiation and cisplatin studies as a whole suggest that it cannot be predicted whether therapy-induced autophagy will be protective or nonprotective based *solely* on functional p53 status. If autophagy inhibition is to be incorporated into therapeutic intervention, it will likely be necessary to identify the functional form(s) of autophagy for each therapeutic intervention in a particular patient (i.e. personalized medicine), reiterating the importance of screening prior to the inclusion of autophagic inhibitors to clinical regimens (285).

Table 3.1. Tumor responses to radiation and cisplatin exposure in H460wt and H460crp53 NSCLC cells.

Cellular Response	Radiation		Cisplatin	
	p53wt	p53 KO	p53wt	p53 KO
Apoptosis	Low	Low	↑↑	↑
Autophagy	nonprotective	nonprotective	nonprotective	cytoprotective
Senescence	↑↑↑	↑	↑↑↑	↑↑↑

Chapter Four: What are the contributions of nonprotective autophagy to senescence?

4.1 Introduction

Autophagy and senescence are both activated in response to radiotherapy and chemotherapy, often concomitantly in tumor cells exposed to anti-cancer therapy as shown in the studies presented within this dissertation (142,342). Given the coexistence of autophagy and senescence in response to therapy and overlapping mechanistic triggers, it is necessary to gain further insights into the relationship between the two processes.

Initiation of autophagy and senescence can be achieved through a number of cellular stress regulators, including p53, ROS, and mTOR (134,168,280,300,343,344) **(Figure 4.1)**. Therapy-induced damage activates p53, an essential tumor suppressor and stress sensor that can modulate the activity of both senescence and autophagy. p53 regulates a vast number of cellular processes, including by not limited to apoptosis, autophagy, and senescence. p53 mediates the transcription of a number of cell cycle inhibitors, including $p21^{waf1}$ and p16, which inhibit the interaction between cyclins and cyclin-dependent kinases and induce cell cycle arrest (345). Luo *et al.* demonstrated that p53 activation using Nutlin-3a radiosensitized H1299 (p53 null) cells by activating p53-$p21^{waf1}$ pathways and inducing cellular senescence (232). Furthermore, depending on the cellular localization of p53, nuclear p53 can activate autophagy through the transcriptional regulation of key ATG proteins or directly stimulate autophagy through DRAM (346). In contrast, cytoplasmic p53 mediates an inhibitory effect on autophagy through ubiquitin-mediated beclin1 degradation and inhibition of AMPK (169,170). Another common meditator of both autophagy and senescence is oxidative stress. Anti-cancer therapies, such as radiation and cisplatin, can result in increased ROS levels due to mitochondrial dysfunction and uncoupling, as well as genotoxic

stress. Autophagy serves to alleviate the cellular burden of the damaged proteins and mitochondria and to remove stressors, such as damaged mitochondrial DNA (mtDNA), which may stimulate senescence induction (347–349). While autophagy is induced in response to oxidative stress, prolonged autophagy impairment in the presence of oxidative stress can result in senescence induction (343,350). Furthermore, excessive ROS accumulation can further exacerbate DNA damage, resulting in p53/p21^{Cip1} activation and promotion of senescence (227,319,351,352). Taken together, pathways activated in response to therapy-induced damage can regulate both autophagy and senescence.

Additionally, mTOR is an important regulator of both senescence and autophagy. mTOR prevents activation of autophagy initialization; thus, mTOR inhibition has been shown to upregulate autophagy (134,353). Studies by Nam et al demonstrated autophagy activation in response to mTOR inhibition in glioma, lung, colorectal, and breast cancer cell lines when exposed to radiotherapy; furthermore, mTOR blockade (which promotes autophagy) resulted in premature senescence and restoration of radiosensitivity (354). Seminal work by Narita *et al.* showed that mTOR and autophagic machinery may be important in SASP processing during senescence (178,355). The authors observed a specialized compartment, which they termed the TOR-autophagy spatial coupling compartment (TSACC), where products of cellular catabolic processes, such as autophagic degradation could feed into cellular anabolic processes, to promote protein synthesis. Disruption of mTOR localization to TSACC was shown to inhibit interleukin-6/8 synthesis in Ras-induced senescence, suggesting that autophagy may play a role in SASP generation, which can reinforce the senescent phenotype.

A plethora of anticancer therapies and concomitant stress pathways activate both autophagy and senescence as alternative cell fates in response to genotoxic and oxidative stress induced by clinically relevant doses of anti-cancer agents; however, whether autophagy and senescence are interdependent still remains elusive and further inquiry is necessary. Given that both autophagy and senescence are currently being examined as attractive means for tumor sensitization to therapy, it is important to understand the relationship between the two processes in order to gauge which pathway could be manipulated to provide a more favorable therapeutic outcome. In these studies, we delved deeper into the role of autophagy in senescence maintenance and recovery from the growth-arrested phenotype in HCT116 cells exposed to radiotherapy. One intrinsic limitation in studies aiming to address the relationship between autophagy and senescence is that in the case of cytoprotective autophagy, autophagy inhibition results in apoptosis, as exemplified both in the literature and studies within this dissertation (142,211); consequently, it becomes difficult to distinguish whether the exhibited responses are due to the impact of autophagy inhibition on cell killing or direct effects of autophagy inhibition on senescence. In an effort to circumvent this limitation, these studies examined the effects of nonprotective autophagy on senescence induction and recovery from the senescent phenotype, since by definition blockade of nonprotective autophagy does not alter sensitivity or apoptotic cell death to anti-cancer therapy.

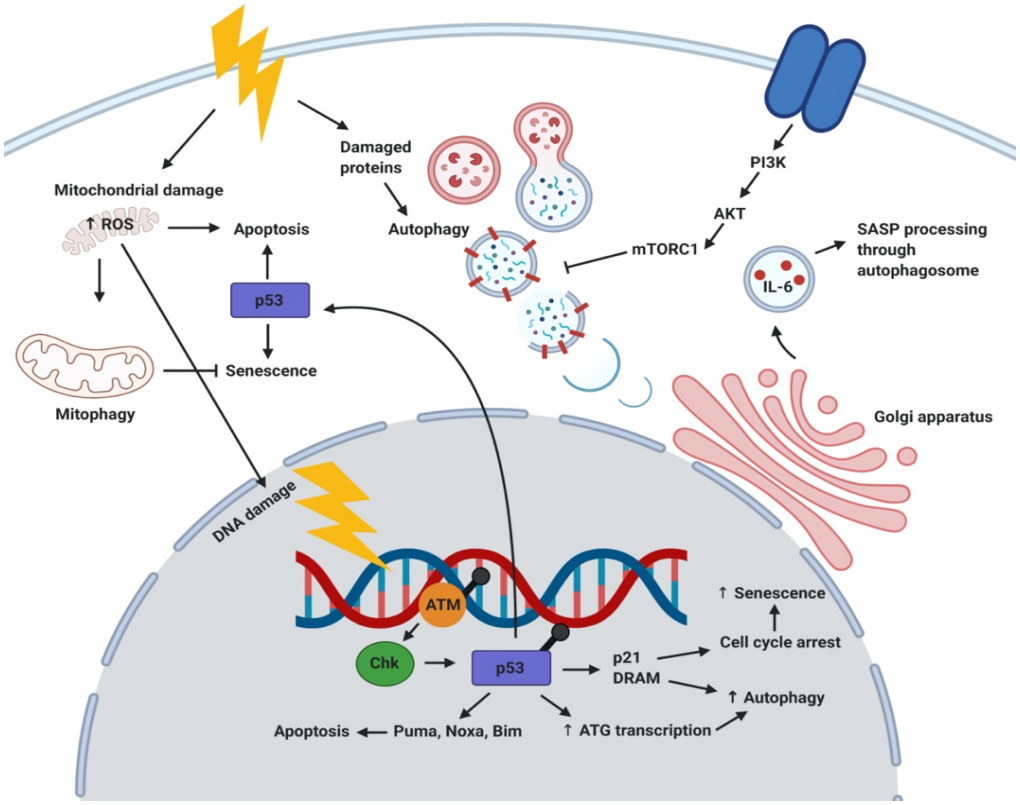

Figure 4.1. Interconnectivity of cellular senescent and autophagic pathways.
Given that both autophagy and senescence are two pathways activated by cellular stress and damage, it is plausible and well-exhibited in pre-clinical models that the two processes often occur in parallel in response to chemotherapy and radiation. Both autophagy and senescence converge on several molecular signaling pathways and share multiple regulators, such as p53, mTOR, and ROS. DNA damage induced by genotoxic therapies, such as etoposide, cisplatin, or radiotherapy, is recognized by ATM and results in its subsequent phosphorylation. Phosphorylated ATM activates DDR pathways, as well as initiates signaling cascades for p53 phosphorylation and activation. Activated p53 regulates a number of responses, including p21 induction resulting in cellular growth arrest and senescence, as well as transcription of proapoptotic proteins. Furthermore, subcellular localization of p53 can also modulate autophagy. Nuclear p53 and DRAM/p53 signaling axis leads to increased transcription of autophagic machinery; whereas cytoplasmic p53 can block autophagy induction and promote apoptosis and growth arrest. Anticancer therapies increasing intracellular ROS levels and inducing mitochondrial dysfunction can result in upregulation of mitophagy. Clearance of dysfunctional and aged mitochondria can prevent senescence, which occurs due to damage accumulation and oxidative stress. Moreover, elevated ROS levels can exacerbate DNA damage activating cellular growth arrest and apoptotic pathways. Lastly, autophagy may contribute to the senescent phenotype through the processing of SASP factors, such as IL-6 and IL-8, via a specialized compartment, TASCC, in which products from the cells catabolic autophagic processes provide raw materials for the anabolic protein synthesis processes. Collectively, while both autophagy and senescence are induced in parallel in response to anti-cancer therapeutics, whether the two processes are interdependent still remains elusive and requires further inquiry. Created with BioRender.com.

4.2. Methods

4.2.1. Cell Culture and Treatment

HCT116 were generously provided by Dr. Sarah Spiegel, at Virginia Commonwealth University. HCT116 cells were cultured in RPMI both supplemented with 10% (*v*/*v*) fetal bovine serum (Thermo Scientific, SH30066.03), 100 U/mL penicillin G sodium (Invitrogen, 15140–122), and 100 μg/mL streptomycin sulfate (Invitrogen, 15140–122). Cells were maintained at 37 °C under a humidified, 5% CO2 atmosphere at sub-confluent densities.

The ATG5-knockdown was generated as follows: Mission shRNA bacterial stocks for ATG5 were purchased from Sigma Aldrich. Lentiviruses were produced in HEK 293T cells co-transfected using EndoFectinTM Lenti Transfection Reagent (GeneCopoeia, 1001-01) with a packaging mixture of psPAX2 and pMD2.G constructs (Addgene). Media containing the viruses was used to infect the HCT116 cells; puromycin (1 μg/ml) was used as a selection marker to enrich for the infected cells.

Cells were seeded on day 0 followed by irradiation (0,2,4,6, 8 or 10 Gy) on day 1 utilizing a ^{137}Cs irradiator. Media was replenished every other day. For autophagy inhibition studies, cells were treated with pre-treated with CQ (0 or 10 μM) 3 h prior to IR (0 or 4 Gy) exposure. All assays performed as previously described.

4.3.Results

4.3.1. Radiation induced senescence and autophagy in HCT116 cells

Cells were exposed to varying doses of radiation, media was replenished, and cells were stained with either X-gal or C_{12}FDG to monitor senescence induction or acridine orange to monitor autophagy induction 72 h post-IR. **Figures 4.2A-B** show the collateral, parallel and dose-dependent induction of autophagy and senescence by ionizing radiation in the HCT116 tumor cell lines.

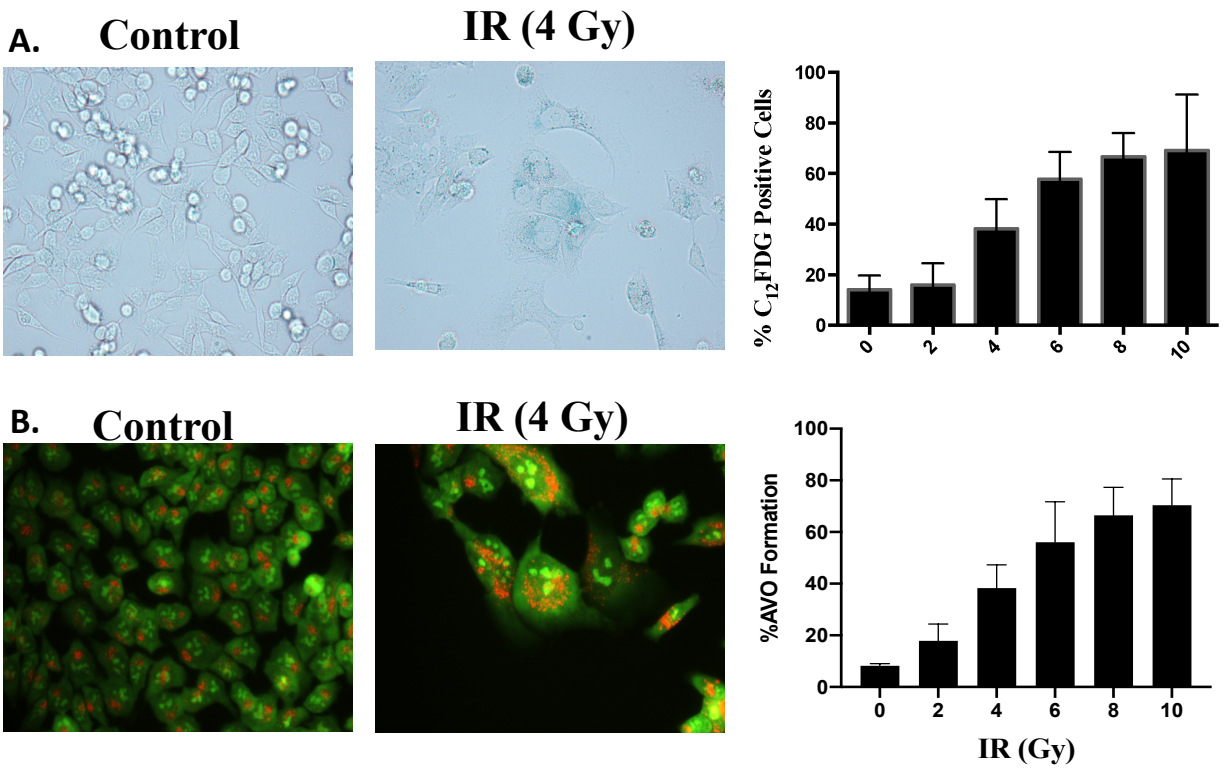

Figure 4.2. The induction of senescence and autophagy in HCT116 cells in response to radiation.

A. SA-β-galactosidase staining of HCT116 cells treated with 4 Gy radiation demonstrating induction of senescence (20x objective). Fluorescence was quantified using flow cytometry 72 h post-irradiation. **B.** Fluorescent microscopy images of acridine orange staining 48 hours post-radiation (4 Gy). Increased acidic vesicle formation is visualized (20x objective). Fluorescence was quantified using flow cytometry 72 h post-irradiation (n=2). Results presented were from three independent experiments, unless otherwise indicated.

4.3.2. Radiation induced nonprotective autophagy in HCT116 colorectal cells

Autophagy was pharmacologically inhibited using CQ to assess the impact of autophagy inhibition on senescence induction and recovery. Specifically, HCT116 cells were pretreated with CQ (5 μM) for 3 hours before being irradiated and then maintained in culture medium for an additional 24 hours. Failure of lysosomal acidification in cells treated with CQ was demonstrated through the yellow staining of autophagic vacuoles **(Figure 4.3A**). Moreover, inhibition of autophagy was confirmed via western blot analysis demonstrating accumulation of the p62 protein levels with prior CQ exposure. **(Figure 4.3B).** Pharmacological inhibition of autophagy by CQ did not alter the sensitivity of HCT116 cells to radiation and did not promote radiation-induced growth arrest (**Figures 4.3C**). Autophagy inhibition also failed to alter radiation-induced apoptosis (**Figure 4.3D**). Collectively, autophagy exhibited a nonprotective function HCT116 colorectal cells in response to radiotherapy; furthermore, these data are consistent with the previous studies with H460 NSCLC cells in which nonprotective autophagy was induced in response to radiation in this experimental model.

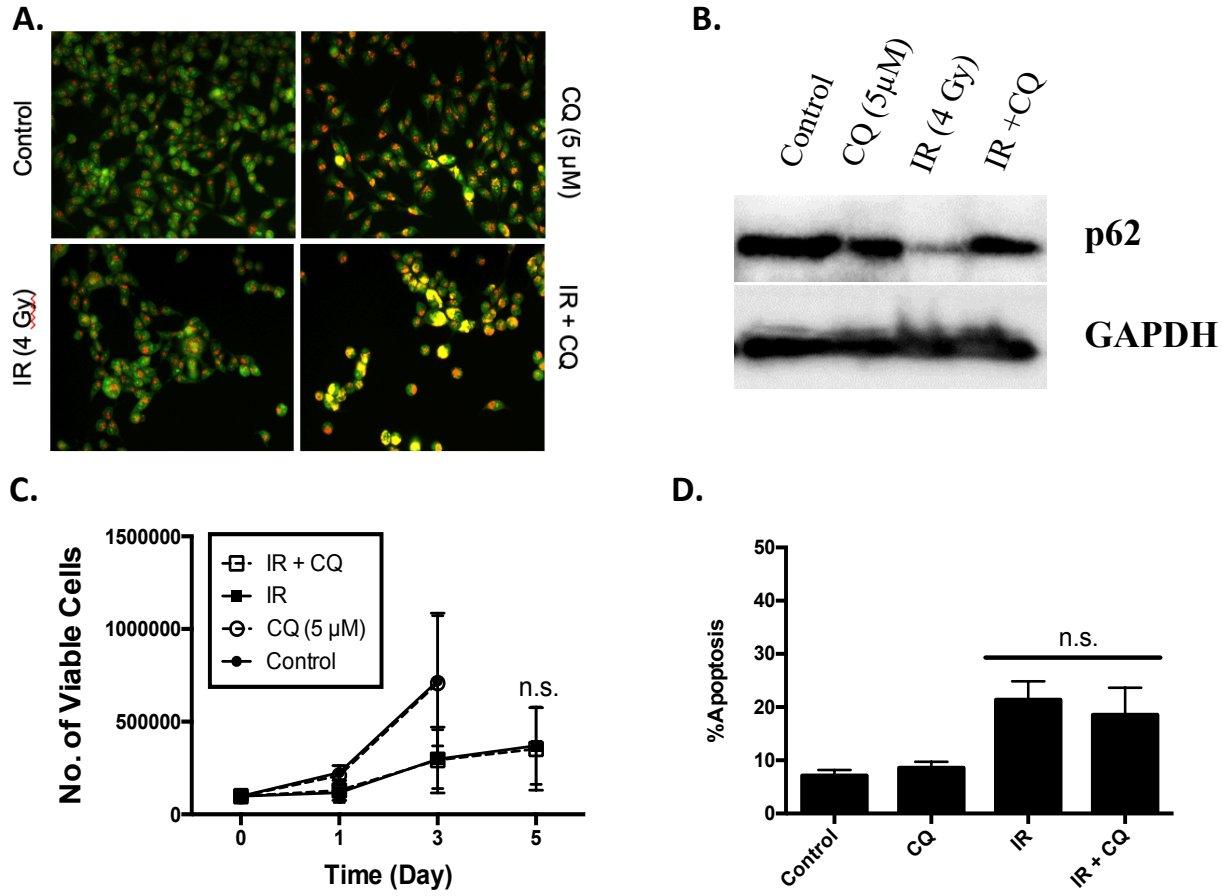

Figure 4.3. Radiation induced nonprotective autophagy in HCT116 colorectal cells.
A-E. Cells were pre-treated with CQ (5 μM) 3 h prior to radiation (4 Gy) exposure. Media was replenished 24 h post-treatment. **A.** Acridine orange staining indicating blockade of lysosomal fusion in cells pre-treated with CQ (20x objective). **B.** Western blot analysis demonstrating autophagy blockade via p62 accumulation in cells pre-treated with CQ (n=2). **C.** Cells were treated with 4 Gy radiation alone or with CQ pre-treatment, and viable cell number was assessed via trypan blue exclusion on the indicated days. **D.** Annexin 5/PI staining was used to assess apoptosis 48 h post-radiation [radiation (4 Gy) alone or with CQ (5 μM) pre-treatment]. Autophagy blockade did not alter radiation-induced apoptosis (n = 2). Results presented were from three independent experiments, unless otherwise indicated. n.s. compared to radiation alone.

4.3.3. Senescence induction and recovery from growth arrest was independent of autophagy in HCT116 cells exposed to radiation

As shown in **Figure 4.4A-B**, senescence induced by radiation in the HCT116 cells was not affected by autophagy inhibition. Figures **4.4C** shows that HCT116 cells underwent growth arrest followed by proliferative recovery upon exposure to radiation, where HCT116 cells proliferative recovery was evident 3 days after radiation exposure. Furthermore, growth arrest and proliferative recovery profiles were virtually identical in HCT116 cells with and without pharmacological autophagy inhibition.

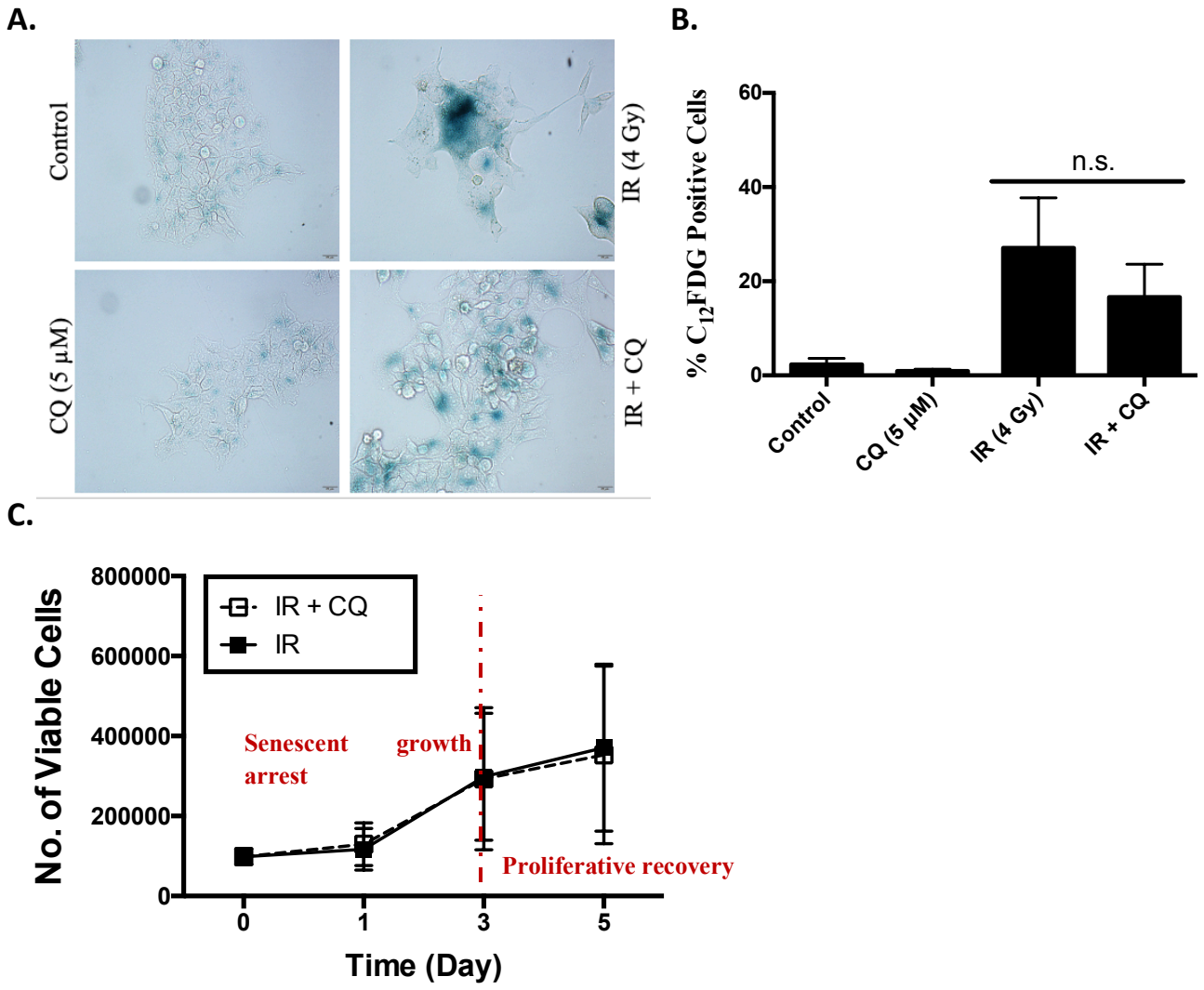

Figure 4.4. Pharmacological autophagy inhibition did not alter senescence induction or recovery in HCT116 cells exposed to radiation.

A. SA-β-galactosidase staining demonstrating increased SA-β-galactosidase activity in both cells exposed to radiation alone or pre-treated with CQ prior to radiation. **B.** SA-β-galactosidase activity was monitored by measuring C_{12}FDG staining using flow cytometry. **C.** Cell viability data from figure 2D. The expanded scale for the lower portion of the graph is shown to visualize proliferative recovery from senescent growth arrest. Results presented were from three independent experiments, unless otherwise indicated. n.s. compared to radiation alone.

Finally, short hairpin RNA was utilized to knockdown ATG5 to assess the effect of genetic autophagy inhibition (silencing of ATG5, **Figure 5A**) on senescence induction and maintenance. In agreement with the pharmacological inhiation data, genetic interference of autophagy did not influence radiation sensitivity or proliferative recovery (**Figure 5B-C**).

While the data is not shown in this dissertation, these studies were also performed with H460 (p53 wt) NSCLC cells and 4T1 (p53 null) breast cancer cells exposed to etoposide and doxorubicin, respectively (251). Consistent with the HCT116 studies, doxorubicin and etoposide exposure induced both senescence and autophagy in H460 NSCLC and 4T1 breast cancer cells; moreover, both of these DNA-damaging chemotherapies induced nonprotective autophagy. Pharmacological and genetic autophagy interference did not alter senescence induction or proliferative recovery from the senescent state. Collectively, H460 NSCLC cells, 4T1 breast cancer cells, and HCT116 colorectal cells exhibited nonprotective autophagy in response to etoposide, doxorubicin, and radiation, respectively. Further, nonprotective autophagy did not significantly contribute to senescence initiation or maintenance (**Figure 6**).

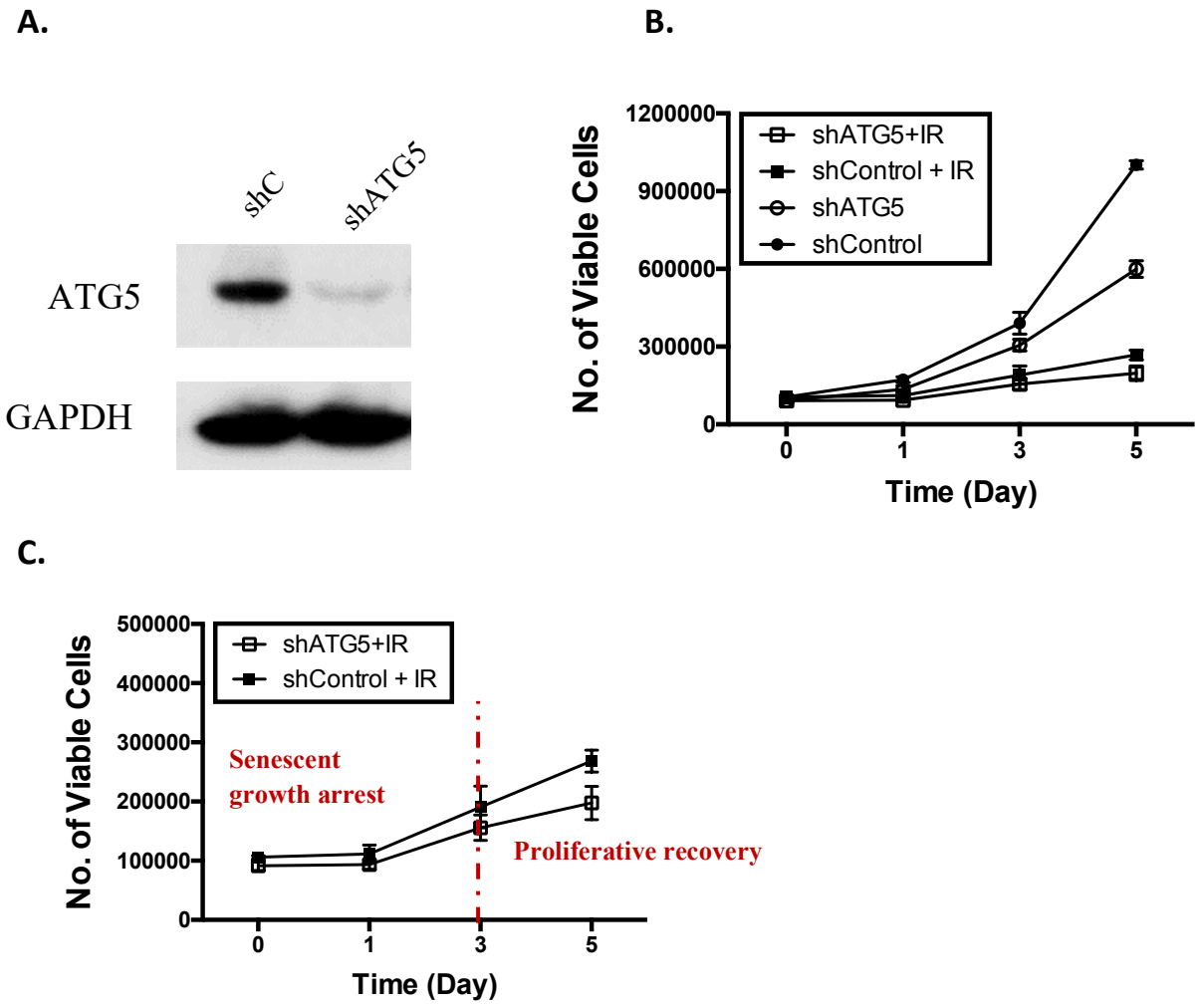

Figure 4.5. Genetic autophagy inhibition did not alter senescence induction or recovery in HCT116 cells exposed to radiation.

A. Western blot demonstrating ATG5 knockdown. **B-C.** Viable cell number was assessed in shControl and shATG5 HCT116 cells exposed to 4 Gy radiation. Representative curves of three independent studies are shown ($n = 3$). Results presented were from three independent experiments, unless otherwise indicated. n.s. compared to radiation alone.

4.4. Discussion

While autophagy and senescence often occur concomitantly in response to chemotherapy and radiotherapy, very little is understood on whether these two processes are interdependent. These studies, utilizing multiple cytotoxic therapies and multiple cell lines, demonstrated senescence induction and proliferative recovery are independent of autophagy when the autophagy is "nonprotective" in function (251). Furthermore, we observed that autophagy inhibition did not alter the extent of senescence induction or recovery in HCT116 colorectal carcinoma cells exposed to 4 Gy radiation. In these studies, at least in the scenario of nonprotective autophagy where senescence is the predominant response, it is feasible to speculate that while autophagy may be induced in a "conventional" effort to maintain survival, the senescence response predominates in conferring a survival advantage to the cells. Whether autophagy contributes to the senescent phenotype or is a relic of senescence is not fully understood; however, these studies indicate that at least in scenarios where the autophagy is *nonprotective*, senescence induction and recovery is not *reliant* on autophagy. Alternatively, Vijayaraghavan *et al.* examined the effects of autophagy inhibition in breast cancer cells exposed to hormonal therapy in combination with CDK 4/6 inhibitors (356). These authors demonstrated that autophagy inhibition significantly reduced cell viability and tumor burden of breast tumors exposed to Palbociclib, as well as the combination of Palbociclib and letrozole, suggesting that autophagy was cytoprotective in function. Furthermore, this sensitization resulting from the administration of an autophagy inhibitor in combination with Palbociclib was mediated through the synergistic induction of senescence. However, the studies by Vijayaraghavan *et al*. suggest autophagy and senescence may share an inverse relationship, complicating the association between the two processes as well as those originally proposed by

Young *et al.* in which blockade of autophagy was shown to suppress senescence in a model of oncogene-induced senescence (357,358).

Building on the previously established literature that autophagy may be a component for the maintenance of the senescent phenotype, autophagy inhibitors have been proposed as potential agents to clear senescent cells. Was *et al*. demonstrated exposure to BafA1 in doxorubicin-induced senescent HCT116 cells reduced cell viability and delayed tumor cell repopulation in the short-term; however, in the long run, single pulse BafA1 exposure resulted in re-activation of autophagy and increased proliferation in the HCT116 recovering subpopulation *in vitro* and increased tumor-burden *in vivo* when compared to chemotherapy alone (359). Therefore, while autophagy inhibitors may be an alluring therapeutic for the clearance of senescent cells, further studies elucidating the nature behind the contributions of autophagy to the senescent phenotype are necessary. In another closely related study, Vera-Ramirez *et al*. showed autophagy inhibition with HCQ decreased survival of dormant breast cancer cells and reduced lung metastasis; however, HCQ administration was minimally effective once dormant cells had regained proliferative capacity, suggesting the role of autophagy in sustaining tumor dormancy in breast cancer cells (360). While the extent of senescence was not assessed in these studies, senescence may a potential tumor dormancy model and further studies are warranted interrogating the contributions of autophagy in senescence-associated tumor dormancy and recovery (209). Alternatively, aberrant activation of autophagy may serve as a potential senolytic. Studies by Wakita *et al.* demonstrated that administration of a BET inhibitor, ARV825, cleared oncogene-induced senescent cells, as well as doxorubicin-induced senescent HCT116 colorectal cells *in vitro* and *in vivo* (361). Furthermore, the authors showed the senolytic capabilities of ARV825 were partially mediated through autophagy modulation. Genetic and pharmacological blockade of autophagy compromised

ARV825-induced senolysis, indicating autophagy was necessary for senescent cell clearance by the BET inhibitor (361).

The tumor suppressor gene, *TP53*, coding for p53, regulates cellular mechanisms modulating both autophagy and senescence, as well as various other cell fates. Whether p53 is important in toggling cellular responses between senescence and autophagy is not well understood; however, our studies, albeit indirectly, provide some insights on the role of p53 status on autophagy and senescence induction. H460 NSCLC cells and HCT116 colorectal cells, expressing wild type p53, and 4T1 breast cancer cells, which are null in p53 status, underwent senescence and autophagy to similar extents in response to etoposide, radiation, and doxorubicin, respectively. Regardless of p53 status and therapeutic exposure, all three cell lines underwent nonprotective autophagy; this concurs with our earlier conclusion that while p53 status may influence the function of autophagy, the specific function induced is inconsistent. Along the same lines of investigation, Sui *et al.* examined the effect of p53 status on autophagy and senescence induction in HCT116 under serum-starved conditions (362). Under serum starvation, HCT116 $p53^{+/+}$ cells exhibited significantly greater autophagic flux than HCT116 $p53^{-/-}$ colorectal cells and protected p53 wild-type cells from starvation-induced cell death. Moreover, HCT116 $p53^{+/+}$ cells underwent autophagy and quiescence in response to serum starvation, while HCT116 $p53^{-/-}$ cells induced senescence to a greater extent than p53 wild-type cells (362). Autophagy inhibition in HCT116 $p53^{+/+}$ cells demonstrated cytoprotective autophagy in response to serum starvation, as expected, and increased ß-gal staining, indicating inhibition of cytoprotective autophagy enhanced senescence induction in HCT116 $p53^{+/+}$ cells. However, autophagy inhibition did not alter senescence induction in HCT116 $p53^{-/-}$ cell (362). Collectively, these studies reiterate the ability of tumor cells to toggle

between cellular responses, the inconsistency of p53 status as a predictive marker for senescence induction and autophagy function, as well as the complexity underlying the relationship between autophagy and senescence.

Summary The premise that autophagy confers resistance to various treatment modalities has been the basis for ongoing clinical trials combining chemotherapy or radiation; however, there are a number of conceptual and experimental reservations relating to these clinical trial strategies. One is that, as we and others have shown in multiple publications, autophagy is not uniformly cytoprotective, often exhibiting cytotoxic and non-protective functions. In scenarios where autophagy inhibition may be beneficial (i.e., when autophagy is cytoprotective in function), from a directly clinical perspective, it is highly uncertain whether chloroquine and hydroxychloroquine can achieve levels in the circulation and the tumor to sufficiently inhibit autophagy to the extent necessary for radiosensitization or chemosensitization. Finally, if and when more efficacious autophagy inhibitors are identified, it is necessary to acknowledge that autophagy also provides homeostatic regulation in normal tissues such as the central nervous system, and therefore autophagy inhibition might represent a double-edged sword that would induce unanticipated and undesirable (intolerable) toxicities.

While autophagy plays a role in initial sensitivity to radiation and chemotherapy in tumor cells, accumulating literature has supported the induction of a prolonged growth arrest, characteristic of senescence, as a response to radiation and chemotherapy. Furthermore, a subset of these senescent cells is capable of regaining proliferative capacity, a possible contributor to tumor dormancy and disease recurrence. Consequently, senescent growth arrest may provide a significant contribution

to chemotherapy and radiation resistance and disease reemergence. Though the implications of senescence cannot be truly resolved due to a lack of effective inhibitors of senescence induction, senolytics may provide a novel class of therapeutics to add to the arsenal of cancer chemotherapy in attempts to eliminate senescent cancer cells. Moreover, given the fact that autophagy and senescence often occur in tandem, it is also important to gain insights as to the predominant responses activated to evade cell death in response to the respective anti-cancer regiment in order to determine whether incorporation of either an autophagy inhibitor or a senolytic with anti-neoplastic therapy may provide a more favorable means to eliminate residual tumor cells.

Disclosure: The work presented in this dissertation has been previously published in Radiation Research (190(5):538-557, Nov 2018; 194(2):103-115, Aug 2020), Biochemical Pharmacology (175:113896, May 2020), International Journal of Medicine (21(4):1427, Feb 2020; 21(23), Nov 2020), and Advances in Cancer Research (2021).

References

1. Siegel R, Miller K, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020;70(1).
2. Duma N, Santana-Davila R, Molina JR. Non-Small Cell Lung Cancer: Epidemiology, Screening, Diagnosis, and Treatment. Mayo Clin Proc. 2019;94(8):1623–40.
3. Zappa C, Mousa SA. Non-small cell lung cancer: current treatment and future advances. Transl lung cancer Res. 2016 Jun;5(3):288–300.
4. Simon GR, Wagner H. Small Cell Lung Cancer. Chest. 2003 Jan 1;123(1):259S-271S.
5. Varghese AM, Zakowski MF, Yu HA, Won HH, Riely GJ, Krug LM, et al. Small-cell lung cancers in patients who never smoked cigarettes. J Thorac Oncol. 2014 Jun;9(6):892–6.
6. Ettinger DS, Aisner J. Changing face of small-cell lung cancer: real and artifact. J Clin Oncol. 2006 Oct 1;24(28):4526–7.
7. Vescio RA, Connors KM, Bordin GM, Robb JA, Youngkin T, Umbreit JN, et al. The Distinction of Small Cell and Non-Small Cell Lung Cancer by Growth in Native-State Histoculture1. Vol. 50, J. Clin. Endocrinol. Metab. 1990.
8. Vollmer RT. The effect of cell size on the pathologic diagnosis of small and large cell carcinomas of the lung. Cancer. 1982 Oct 1;50(7):1380–3.
9. Travis WD. Update on small cell carcinoma and its differentiation from squamous cell carcinoma and other non-small cell carcinomas. Mod Pathol. 2012 Jan 3;25(S1):S18–30.
10. Loo PS, Thomas SC, Nicolson MC, Fyfe MN, Kerr KM. Subtyping of Undifferentiated Non-small Cell Carcinomas in Bronchial Biopsy Specimens. J Thorac Oncol. 2010 Apr 1;5(4):442–7.
11. Bishop JA, Benjamin H, Cholakh H, Chajut A, Clark DP, Westra WH. Accurate classification of non-small cell lung carcinoma using a novel microRNA-based approach.

Clin Cancer Res. 2010 Jan 15;16(2):610–9.

12. Halvorsen AR, Silwal-Pandit L, Meza-Zepeda LA, Vodak D, Vu P, Sagerup C, et al. TP53 Mutation Spectrum in Smokers and Never Smoking Lung Cancer Patients. Front Genet. 2016;7:85.

13. Jeanson A, Tomasini P, Souquet-Bressand M, Brandone N, Boucekine M, Grangeon M, et al. Efficacy of Immune Checkpoint Inhibitors in KRAS-Mutant Non-Small Cell Lung Cancer (NSCLC). 2019;

14. Yang JC-H, Schuler M, Popat S, Miura S, Heeke S, Park K, et al. Afatinib for the Treatment of NSCLC Harboring Uncommon EGFR Mutations: A Database of 693 Cases. J Thorac Oncol. 2020 May 1;15(5):803–15.

15. Midha A, Dearden S, McCormack R. EGFR mutation incidence in non-small-cell lung cancer of adenocarcinoma histology: a systematic review and global map by ethnicity (mutMapII). Am J Cancer Res. 2015;5(9):2892–911.

16. Ahrendt S, Hu Y, Buta M, McDermott M, Benoit N, Yang S, et al. p53 mutations and survival in stage I non-small-cell lung cancer: results of a prospective study. J Natl Cancer Inst. 2003;95(13).

17. Gu J, Zhou Y, Huang L, Ou W, Wu J, Li S, et al. TP53 mutation is associated with a poor clinical outcome for non-small cell lung cancer: Evidence from a meta-analysis. Mol Clin Oncol. 2016;5(6).

18. Gibbons D, Byers L, Kurie J. Smoking, p53 mutation, and lung cancer. Mol Cancer Res. 2014;12(1).

19. White E. Autophagy and p53. Cold Spring Harb Perspect Med. 2016 Apr 1;6(4):a026120.

20. Itahana K, Dimri G, Campisi J. Regulation of cellular senescence by p53. Eur J Biochem.

2001;268(10):2784–91.

21. Zuckerman V, Wolyniec K, Sionov R V, Haupt S, Haupt Y. Tumour suppression by p53: the importance of apoptosis and cellular senescence. J Pathol. 2009 Sep 1;219(1):n/a-n/a.

22. Krawczyk P, Kowalski DM, Ramlau R, Kalinka-Warzocha E, Winiarczyk K, Stencel K, et al. Comparison of the effectiveness of erlotinib, gefitinib, and afatinib for treatment of non-small cell lung cancer in patients with common and rare EGFR gene mutations. Oncol Lett. 2017 Jun;13(6):4433–44.

23. Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, et al. Comparison of Four Chemotherapy Regimens for Advanced Non–Small-Cell Lung Cancer. N Engl J Med. 2002 Jan 10;346(2):92–8.

24. Kosmidis P. Chemotherapy in NSCLC: historical review. Lung Cancer. 2002 Dec 1;38:19–22.

25. Shinomiya N. New concepts in radiation-induced apoptosis: ?premitotic apoptosis? and ?postmitotic apoptosis? J Cell Mol Med. 2001 Jul;5(3):240–53.

26. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007 Jun;35(4):495–516.

27. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat Rev Cancer. 2008 Oct;8(10):782–98.

28. Bhatnagar B, Gilmore S, Goloubeva O, Pelser C, Medeiros M, Chumsri S, et al. Chemotherapy dose reduction due to chemotherapy induced peripheral neuropathy in breast cancer patients receiving chemotherapy in the neoadjuvant or adjuvant settings: a single-center experience. Springerplus. 2014 Jul 16;3(1):366.

29. Bonadonna G, Valagussa P, Moliterni A, Zambetti M, Brambilla C. Adjuvant

Cyclophosphamide, Methotrexate, and Fluorouracil in Node-Positive Breast Cancer — The Results of 20 Years of Follow-up. N Engl J Med. 1995 Apr 6;332(14):901–6.

30. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol. 2010 May;221(1):3–12.

31. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell Res. 2014 Jan;24(1):24–41.

32. Brahimi-Horn MC, Chiche J, Pouysségur J. Hypoxia and cancer. J Mol Med. 2007 Dec 20;85(12):1301–7.

33. Maycotte P, Thorburn A. Autophagy and cancer therapy. Cancer Biol Ther. 2011 Jan 15;11(2):127–37.

34. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol. 2007 Nov;8(11):931–7.

35. Levine B, Packer M, Codogno P. Development of autophagy inducers in clinical medicine. J Clin Invest. 2015 Jan 2;125(1):14–24.

36. Mijaljica D, Prescott M, Devenish RJ. Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. Autophagy. 2011 Jul;7(7):673–82.

37. Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. Antioxid Redox Signal. 2014 Jan 20;20(3):460–73.

38. Deosaran E, Larsen KB, Hua R, Sargent G, Wang Y, Kim S, et al. NBR1 acts as an autophagy receptor for peroxisomes. J Cell Sci. 2013 Feb 15;126(Pt 4):939–52.

39. Stolz A, Ernst A, Dikic I. Cargo recognition and trafficking in selective autophagy. Nat Cell Biol. 2014 Jun 30;16(6):495–501.

40. Ding W-X, Ni H-M, Li M, Liao Y, Chen X, Stolz DB, et al. Nix is critical to two distinct

phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. J Biol Chem. 2010 Sep 3;285(36):27879–90.

41. Kim J, Kundu M, Viollet B, Guan K-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol. 2011 Feb 23;13(2):132–41.

42. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. Nat Rev Mol Cell Biol. 2018 Jun 4;19(6):349–64.

43. Petherick KJ, Conway OJL, Mpamhanga C, Osborne SA, Kamal A, Saxty B, et al. Pharmacological inhibition of ULK1 kinase blocks mammalian target of rapamycin (mTOR)-dependent autophagy. J Biol Chem. 2015 May 1;290(18):11376–83.

44. Ylä-Anttila P, Vihinen H, Jokitalo E, Eskelinen E-L. 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy. 2009 Nov 16;5(8):1180–5.

45. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, et al. Mitochondria Supply Membranes for Autophagosome Biogenesis during Starvation. Cell. 2010 May 14;141(4):656–67.

46. Geng J, Nair U, Yasumura-Yorimitsu K, Klionsky DJ. Post-Golgi Sec proteins are required for autophagy in Saccharomyces cerevisiae. Mol Biol Cell. 2010 Jul 1;21(13):2257–69.

47. Puri C, Vicinanza M, Ashkenazi A, Gratian MJ, Zhang Q, Bento CF, et al. The RAB11A-Positive Compartment Is a Primary Platform for Autophagosome Assembly Mediated by WIPI2 Recognition of PI3P-RAB11A. Dev Cell. 2018;45(1):114-131.e8.

48. Lee Y-K, Lee J-A. Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. BMB Rep. 2016

Aug;49(8):424–30.

49. Gammoh N, Wilkinson S. Autophagy in cancer biology and therapy. Front Biol (Beijing). 2014 Feb 6;9(1):35–50.

50. Mizushima N. Autophagy : process and function. Genes Dev. 2007;21(22):2861–73.

51. Poillet-Perez L, Despouy G, Delage-Mourroux R, Boyer-Guittaut M. Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy. Redox Biol. 2015 Apr 1;4:184–92.

52. Shaw RJ. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. Acta Physiol (Oxf). 2009 May;196(1):65–80.

53. Egan D, Kim J, Shaw RJ, Guan K-L. The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. Autophagy. 2011 Jun 27;7(6):643–4.

54. Jang M, Park R, Kim H, Namkoong S, Jo D, Huh YH, et al. AMPK contributes to autophagosome maturation and lysosomal fusion. Sci Rep. 2018 Dec 23;8(1):12637.

55. Daskalaki I, Gkikas I, Tavernarakis N. Hypoxia and Selective Autophagy in Cancer Development and Therapy. Front cell Dev Biol. 2018;6:104.

56. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008 Jan 11;132(1):27–42.

57. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. 2003.

58. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature. 1999 Dec;402(6762):672–6.

59. Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, et al. Autophagy-

deficient mice develop multiple liver tumors. Genes Dev. 2011 Apr 15;25(8):795–800.

60. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen H-Y, et al. Autophagy suppresses tumorigenesis through elimination of p62. Cell. 2009 Jun 12;137(6):1062–75.

61. Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, et al. Autophagy in malignant transformation and cancer progression. EMBO J. 2015 Apr 23;34(7):856–80.

62. Elgendy M, Sheridan C, Brumatti G, Martin SJ. Oncogenic Ras-Induced Expression of Noxa and Beclin-1 Promotes Autophagic Cell Death and Limits Clonogenic Survival. Mol Cell. 2011 Apr 8;42(1):23–35.

63. Martins I, Wang Y, Michaud M, Ma Y, Sukkurwala AQ, Shen S, et al. Molecular mechanisms of ATP secretion during immunogenic cell death. Cell Death Differ. 2014 Jan;21(1):79–91.

64. Levine B, Deretic V. Unveiling the roles of autophagy in innate and adaptive immunity. Nat Rev Immunol. 2007 Oct;7(10):767–77.

65. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. Nature. 2011 Jan 20;469(7330):323–35.

66. Deng L, Liang H, Xu M, Yang X, Burnette B, Arina A, et al. STING-Dependent Cytosolic DNA Sensing Promotes Radiation-Induced Type I Interferon-Dependent Antitumor Immunity in Immunogenic Tumors. Immunity. 2014 Nov 20;41(5):843–52.

67. Xu Y, Jagannath C, Liu X-D, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like Receptor 4 Is a Sensor for Autophagy Associated with Innate Immunity. Immunity. 2007 Jul;27(1):135–44.

68. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, et al. Autophagy-

dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science. 2011 Dec 16;334(6062):1573–7.

69. Chemali M, Radtke K, Desjardins M, English L. Alternative pathways for MHC class I presentation: a new function for autophagy. Cell Mol Life Sci. 2011 May 10;68(9):1533–41.

70. Li Y, Wang L-X, Yang G, Hao F, Urba WJ, Hu H-M. Efficient Cross-presentation Depends on Autophagy in Tumor Cells. Cancer Res. 2008 Sep 1;68(17):6889–95.

71. Li B, Lei Z, Lichty BD, Li D, Zhang G-M, Feng Z-H, et al. Autophagy facilitates major histocompatibility complex class I expression induced by IFN-γ in B16 melanoma cells. Cancer Immunol Immunother. 2010 Feb 13;59(2):313–21.

72. Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, et al. Pancreatic cancers require autophagy for tumor growth. Genes Dev. 2011 Apr 1;25(7):717–29.

73. Boya P, González-Polo R-A, Casares N, Perfettini J-L, Dessen P, Larochette N, et al. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol. 2005 Feb 1;25(3):1025–40.

74. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes Dev. 2007 Jul 1;21(13):1621–35.

75. Guo JY, Chen H-Y, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev. 2011 Mar 1;25(5):460–70.

76. Rabinowitz JD, White E, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, et al. Autophagy and metabolism. Science. 2010 Dec 3;330(6009):1344–8.

77. Giannoni E, Buricchi F, Grimaldi G, Parri M, Cialdai F, Taddei ML, et al. Redox regulation of anoikis: reactive oxygen species as essential mediators of cell survival. Cell Death Differ. 2008 May 8;15(5):867–78.

78. Gough DR, Cotter TG. Hydrogen peroxide: a Jekyll and Hyde signalling molecule. Cell Death Dis. 2011 Oct 6;2(10):e213.

79. Kaminskyy VO, Piskunova T, Zborovskaya IB, Tchevkina EM, Zhivotovsky B. Suppression of basal autophagy reduces lung cancer cell proliferation and enhances caspase-dependent and -independent apoptosis by stimulating ROS formation. Autophagy. 2012 Jul 1;8(7):1032–44.

80. Harris IS, Treloar AE, Inoue S, Sasaki M, Gorrini C, Lee KC, et al. Glutathione and Thioredoxin Antioxidant Pathways Synergize to Drive Cancer Initiation and Progression. Cancer Cell. 2015 Feb 9;27(2):211–22.

81. Sun Y, Xing X, Liu Q, Wang Z, Xin Y, Zhang P, et al. Hypoxia-induced autophagy reduces radiosensitivity by the HIF-1α/miR-210/Bcl-2 pathway in colon cancer cells. Int J Oncol. 2015 Feb;46(2):750–6.

82. Noman MZ, Janji B, Kaminska B, Van Moer K, Pierson S, Przanowski P, et al. Blocking Hypoxia-Induced Autophagy in Tumors Restores Cytotoxic T-Cell Activity and Promotes Regression. Cancer Res. 2011 Sep 15;71(18):5976–86.

83. Paglin S, Hollister T, Delohery T, Hackett N, Mcmahill M, Sphicas E, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res. 2001;61(2):439–44.

84. Levine B. Autophagy and cancer. Nature. 2007 Apr 11;446(7137):745–7.

85. Hughson LRK, Poon VI, Spowart JE, Lum JJ. Implications of Therapy-Induced Selective

Autophagy on Tumor Metabolism and Survival. Int J Cell Biol. 2012;2012:1–11.

86. Abedin MJ, Wang D, McDonnell MA, Lehmann U, Kelekar A. Autophagy delays apoptotic death in breast cancer cells following DNA damage. Cell Death Differ. 2007 Mar 22;14(3):500–10.

87. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007 Sep;8(9):741–52.

88. Classen F, Kranz P, Riffkin H, Pompsch M, Wolf A, Göpelt K, et al. Autophagy induced by ionizing radiation promotes cell death over survival in human colorectal cancer cells. Exp Cell Res. 2019 Jan 1;374(1):29–37.

89. Rosenfeld MR, Ye X, Supko JG, Desideri S, Grossman SA, Brem S, et al. A phase I/II trial of hydroxychloroquine in conjunction with radiation therapy and concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma multiforme. Autophagy. 2014 Aug;10(8):1359–68.

90. Eriksson D, Stigbrand T. Radiation-induced cell death mechanisms. Tumor Biol. 2010 Aug 20;31(4):363–72.

91. Paglin S, Hollister T, Delohery T, Hackett N, Mcmahill M, Sphicas E, et al. A Novel Response of Cancer Cells to Radiation Involves Autophagy and Formation of Acidic Vesicles 1. Vol. 61, Cancer Research. 2001.

92. Chaachouay H, Ohneseit P, Toulany M, Kehlbach R, Multhoff G, Rodemann HP. Molecular radiobiology Autophagy contributes to resistance of tumor cells to ionizing radiation. 2011;

93. Lomonaco SL, Finniss S, Xiang C, DeCarvalho A, Umansky F, Kalkanis SN, et al. The induction of autophagy by γ-radiation contributes to the radioresistance of glioma stem cells. Int J Cancer. 2009 Aug 1;125(3):717–22.

94. Ren J, Liu T, Han Y, Wang Q, Chen Y, Li G, et al. GSK-3β inhibits autophagy and enhances radiosensitivity in non-small cell lung cancer. Diagn Pathol. 2018 Dec 24;13(1):33.

95. Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y. Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. Int J Oncol. 2005 May 1;26(5):1401–10.

96. Shen S, Kepp O, Michaud M, Martins I, Minoux H, Métivier D, et al. Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. Oncogene. 2011 Nov 10;30(45):4544–56.

97. Köchl R, Hu XW, Chan EYW, Tooze SA. Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes. Traffic. 2006 Feb;7(2):129–45.

98. Bordin DL, Lima M, Lenz G, Saffi J, Meira LB, Mésange P, et al. DNA alkylation damage and autophagy induction. Mutat Res Mutat Res. 2013 Oct 1;753(2):91–9.

99. Saito S, Goodarzi AA, Higashimoto Y, Noda Y, Lees-Miller SP, Appella E, et al. ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. J Biol Chem. 2002 Apr 12;277(15):12491–4.

100. Bitomsky N, Hofmann TG. Apoptosis and autophagy: Regulation of apoptosis by DNA damage signalling - roles of p53, p73 and HIPK2. FEBS J. 2009 Nov;276(21):6074–83.

101. Hirao A, Kong YY, Matsuoka S, Wakeham A, Ruland J, Yoshida H, et al. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science. 2000 Mar 10;287(5459):1824–7.

102. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, et al. DRAM, a p53-Induced Modulator of Autophagy, Is Critical for Apoptosis. Cell. 2006 Jul 14;126(1):121–34.

103. Vessoni AT, Filippi-Chiela EC, Menck CF, Lenz G. Autophagy and genomic integrity. Cell Death Differ. 2013 Nov 9;20(11):1444–54.

104. Lin J, Lin Y, Tsai T, Chen H, Chou K, Hwang T. Cisplatin induces protective autophagy through activation of BECN1 in human bladder cancer cells. Drug Des Devel Ther. 2017;11:1517–33.

105. Li J, Hou N, Faried A, Tsutsumi S, Kuwano H. Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. Eur J Cancer. 2010 Jul 1;46(10):1900–9.

106. Aydinlik S, Erkisa M, Cevatemre B, Sarimahmut M, Dere E, Ari F, et al. Enhanced cytotoxic activity of doxorubicin through the inhibition of autophagy in triple negative breast cancer cell line. Biochim Biophys acta Gen Subj. 2017 Feb;1861(2):49–57.

107. Zhang M, Qiu Q, Li Z, Sachdeva M, Min H, Cardona DM, et al. HIF-1 Alpha Regulates the Response of Primary Sarcomas to Radiation Therapy through a Cell Autonomous Mechanism. Radiat Res. 2015 May 14;183(6):594.

108. Choi Y-M, Kim H-K, Shim W, Anwar MA, Kwon J-W, Kwon H-K, et al. Mechanism of Cisplatin-Induced Cytotoxicity Is Correlated to Impaired Metabolism Due to Mitochondrial ROS Generation. Mukhopadhyay P, editor. PLoS One. 2015 Aug 6;10(8):e0135083.

109. Bursch W, Ellinger A, Kienzl H, Török L, Pandey S, Sikorska M, et al. Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. Carcinogenesis. 1996 Aug;17(8):1595–607.

110. Shao Y, Gao Z, Marks PA, Jiang X. Apoptotic and autophagic cell death induced by histone deacetylase inhibitors. Proc Natl Acad Sci U S A. 2004 Dec 28;101(52):18030–5.

111. Henson E, Chen Y, Gibson S. EGFR Family Members' Regulation of Autophagy Is at a

Crossroads of Cell Survival and Death in Cancer. Cancers (Basel). 2017 Mar 24;9(4).

112. Li X, Fan Z. The epidermal growth factor receptor antibody cetuximab induces autophagy in cancer cells by downregulating HIF-1alpha and Bcl-2 and activating the beclin 1/hVps34 complex. Cancer Res. 2010 Jul 15;70(14):5942–52.

113. Pan H, Wang Z, Jiang L, Sui X, You L, Shou J, et al. Autophagy inhibition sensitizes hepatocellular carcinoma to the multikinase inhibitor linifanib. Sci Rep. 2014 Dec 17;4(1):6683.

114. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012 Mar 22;12(4):252–64.

115. Yamamoto K, Venida A, Yano J, Biancur DE, Kakiuchi M, Gupta S, et al. Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. Nature. 2020 May 22;581(7806):100–5.

116. Liang X, De Vera ME, Buchser WJ, de Vivar Chavez AR, Loughran P, Stolz DB, et al. Inhibiting Systemic Autophagy during Interleukin 2 Immunotherapy Promotes Long-term Tumor Regression. Cancer Res. 2012 Jun 1;72(11):2791–801.

117. Gewirtz DA. The Four Faces of Autophagy: Implications for Cancer Therapy. Cancer Res. 2014 Feb 1;74(3):647–51.

118. Gewirtz DA. Cytoprotective and nonprotective autophagy in cancer therapy. Autophagy. 2013 Sep 29;9(9):1263–5.

119. Mani J, Vallo S, Rakel S, Antonietti P, Gessler F, Blaheta R, et al. Chemoresistance is associated with increased cytoprotective autophagy and diminished apoptosis in bladder cancer cells treated with the BH3 mimetic (−)-Gossypol (AT-101). BMC Cancer. 2015 Dec 7;15(1):224.

120. Cufí S, Vazquez-Martin A, Oliveras-Ferraros C, Corominas-Faja B, Cuyàs E, López-Bonet E, et al. The anti-malarial chloroquine overcomes Primary resistance and restores sensitivity to Trastuzumab in HER2-positive breast cancer. Sci Rep. 2013 Dec 22;3(1):2469.

121. Wang F, Tang J, Li P, Si S, Yu H, Yang X, et al. Chloroquine Enhances the Radiosensitivity of Bladder Cancer Cells by Inhibiting Autophagy and Activating Apoptosis. Cell Physiol Biochem. 2018;45(1):54–66.

122. Qadir MA, Kwok B, Dragowska WH, To KH, Le D, Bally MB, et al. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. Breast Cancer Res Treat. 2008 Dec 3;112(3):389–403.

123. Selvakumaran M, Amaravadi RK, Vasilevskaya IA, O'Dwyer PJ. Autophagy Inhibition Sensitizes Colon Cancer Cells to Antiangiogenic and Cytotoxic Therapy. Clin Cancer Res. 2013 Jun 1;19(11):2995–3007.

124. Sum Y, Xing X, Liu Q, Wang Z, Xin Y, Zhang P, et al. Hypoxia-induced autophagy reduces radiosensitivity by the HIF-1α/miR-210/Bcl-2 pathway in colon cancer cells. Int J Oncol. 2015 Feb 1;46(2):750–6.

125. Zheng B, Zhu H, Gu D, Pan X, Qian L, Xue B, et al. MiRNA-30a-mediated autophagy inhibition sensitizes renal cell carcinoma cells to sorafenib. Biochem Biophys Res Commun. 2015 Apr 3;459(2):234–9.

126. Liang B, Kong D, Liu Y, Liang N, He M, Ma S, et al. Autophagy inhibition plays the synergetic killing roles with radiation in the multi-drug resistant SKVCR ovarian cancer cells. Radiat Oncol. 2012 Dec 17;7(1):213.

127. Sun WL, Lan D, Gan TQ, Cai ZW. Autophagy facilitates multidrug resistance development through inhibition of apoptosis in breast cancer cells. Neoplasma. 2015;62.

128. Chittaranjan S, Bortnik S, Dragowska WH, Xu J, Abeysundara N, Leung A, et al. Autophagy inhibition augments the anticancer effects of epirubicin treatment in anthracycline-sensitive and -resistant triple-negative breast cancer. Clin Cancer Res. 2014 Jun 15;20(12):3159–73.

129. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005 Apr;5(4):275–84.

130. Chang NC. Autophagy and Stem Cells: Self-Eating for Self-Renewal. Front Cell Dev Biol. 2020 Mar 4;8:138.

131. Guo B, Tam A, Santi SA, Parissenti AM. Role of autophagy and lysosomal drug sequestration in acquired resistance to doxorubicin in MCF-7 cells. BMC Cancer. 2016 Dec 29;16(1):762.

132. Thorburn J, Staskiewicz L, Goodall ML, Dimberg L, Frankel AE, Ford HL, et al. Non-cell-autonomous Effects of Autophagy Inhibition in Tumor Cells Promote Growth of Drug-resistant Cells. Mol Pharmacol. 2017 Jan;91(1):58–64.

133. Huang Q, Li F, Liu X, Li W, Shi W, Liu F-F, et al. Caspase 3–mediated stimulation of tumor cell repopulation during cancer radiotherapy. Nat Med. 2011 Jul 3;17(7):860–6.

134. Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, et al. Inhibition of Mammalian Target of Rapamycin or Apoptotic Pathway Induces Autophagy and Radiosensitizes PTEN Null Prostate Cancer Cells. Cancer Res. 2006 Oct 15;66(20):10040–7.

135. Kim EJ, Jeong J-H, Bae S, Kang S, Kim CH, Lim Y-B. mTOR inhibitors radiosensitize PTEN-deficient non-small-cell lung cancer cells harboring an *EGFR* activating mutation by inducing autophagy. J Cell Biochem. 2013 Jun 1;114(6):1248–56.

136. Talarico C, Dattilo V, D'Antona L, Barone A, Amodio N, Belviso S, et al. SI113, a SGK1 inhibitor, potentiates the effects of radiotherapy, modulates the response to oxidative stress and induces cytotoxic autophagy in human glioblastoma multiforme cells. Oncotarget. 2016 Mar 29;7(13):15868–84.

137. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ. 2004 Apr 9;11(4):448–57.

138. Zhang Q, Wang X, Cao S, Sun Y, He X, Jiang B, et al. Berberine represses human gastric cancer cell growth in vitro and in vivo by inducing cytostatic autophagy via inhibition of MAPK/mTOR/p70S6K and Akt signaling pathways. Biomed Pharmacother. 2020 Aug 1;128:110245.

139. Sharma K, Goehe RW, Di X, Hicks MA, Torti S V, Torti FM, et al. A novel cytostatic form of autophagy in sensitization of non-small cell lung cancer cells to radiation by vitamin D and the vitamin D analog, EB 1089. Autophagy. 2014;10(12):2346–61.

140. Dou Q, Chen HN, Wang K, Yuan K, Lei Y, Li K, et al. Ivermectin induces cytostatic autophagy by blocking the PAK1/Akt Axis in breast cancer. Cancer Res. 2016;76(15):4457–69.

141. Chakradeo S, Sharma K, Alhaddad A, Bakhshwin D, Le N, Harada H, et al. Yet another function of p53--the switch that determines whether radiation-induced autophagy will be cytoprotective or nonprotective: implications for autophagy inhibition as a therapeutic strategy. Mol Pharmacol. 2015 May;87(5):803–14.

142. Xu J, Patel NH, Saleh T, Cudjoe EK, Alotaibi M, Wu Y, et al. Differential Radiation Sensitivity in p53 Wild-Type and p53-Deficient Tumor Cells Associated with Senescence

but not Apoptosis or (Nonprotective) Autophagy. Radiat Res. 2018 Nov 10;190(5):538–57.

143. Eng CH, Wang Z, Tkach D, Toral-Barza L, Ugwonali S, Liu S, et al. Macroautophagy is dispensable for growth of KRAS mutant tumors and chloroquine efficacy. Proc Natl Acad Sci U S A. 2016 Jan 5;113(1):182–7.

144. Gallagher LE, Radhi OA, Abdullah MO, McCluskey AG, Boyd M, Chan EYW. Lysosomotropism depends on glucose: a chloroquine resistance mechanism. Cell Death Dis. 2017 Aug 24;8(8):e3014.

145. Schaaf MBE, Jutten B, Keulers TG, Savelkouls KGM, Peeters HJM, van den Beucken T, et al. Canonical autophagy does not contribute to cellular radioresistance. Radiother Oncol. 2015 Mar 1;114(3):406–12.

146. Michaud M, Xie X, Bravo-San Pedro JM, Zitvogel L, White E, Kroemer G. An autophagy-dependent anticancer immune response determines the efficacy of melanoma chemotherapy. Oncoimmunology. 2014 Jul 3;3(7):e944047.

147. Cechakova L, Ondrej M, Pavlik V, Jost P, Cizkova D, Bezrouk A, et al. A Potent Autophagy Inhibitor (Lys05) Enhances the Impact of Ionizing Radiation on Human Lung Cancer Cells H1299. Int J Mol Sci. 2019 Nov 23;20(23):5881.

148. Vogl DT, Stadtmauer EA, Tan K-S, Heitjan DF, Davis LE, Pontiggia L, et al. Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. Autophagy. 2014 Aug 20;10(8):1380–90.

149. Mauthe M, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema K-J, et al. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. Autophagy. 2018 Aug 3;14(8):1435–55.

150. Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y. Bafilomycin A1, a specific

inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. J Biol Chem. 1991 Sep 15;266(26):17707–12.

151. McAfee Q, Zhang Z, Samanta A, Levi SM, Ma X-H, Piao S, et al. Autophagy inhibitor Lys05 has single-agent antitumor activity and reproduces the phenotype of a genetic autophagy deficiency. Proc Natl Acad Sci U S A. 2012 May 22;109(21):8253–8.

152. Amaravadi RK, Winkler JD. Lys05: a new lysosomal autophagy inhibitor. Autophagy. 2012 Sep;8(9):1383–4.

153. Wu Y-T, Tan H-L, Shui G, Bauvy C, Huang Q, Wenk MR, et al. Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. J Biol Chem. 2010 Apr 2;285(14):10850–61.

154. Li J, Hou N, Faried A, Tsutsumi S, Takeuchi T, Kuwano H. Inhibition of Autophagy by 3-MA Enhances the Effect of 5-FU-Induced Apoptosis in Colon Cancer Cells. Ann Surg Oncol. 2009 Mar 31;16(3):761–71.

155. Ronan B, Flamand O, Vescovi L, Dureuil C, Durand L, Fassy F, et al. A highly potent and selective Vps34 inhibitor alters vesicle trafficking and autophagy. Nat Chem Biol. 2014 Dec 19;10(12):1013–9.

156. Pasquier B. SAR405, a PIK3C3/Vps34 inhibitor that prevents autophagy and synergizes with MTOR inhibition in tumor cells. Autophagy. 2015 Apr 3;11(4):725–6.

157. Schlütermann D, Skowron MA, Berleth N, Böhler P, Deitersen J, Stuhldreier F, et al. Targeting urothelial carcinoma cells by combining cisplatin with a specific inhibitor of the autophagy-inducing class III PtdIns3K complex. Urol Oncol Semin Orig Investig. 2018 Apr 1;36(4):160.e1-160.e13.

158. Shao S, Li S, Qin Y, Wang X, Yang Y, Bai H, et al. Spautin-1, a novel autophagy inhibitor,

enhances imatinib-induced apoptosis in chronic myeloid leukemia. Int J Oncol. 2014 May 1;44(5):1661–8.

159. Liu J, Xia H, Kim M, Xu L, Li Y, Zhang L, et al. Beclin1 Controls the Levels of p53 by Regulating the Deubiquitination Activity of USP10 and USP13. Cell. 2011 Sep 30;147(1):223–34.

160. Blommaart EFC, Krause U, Schellens JPM, Vreeling-Sindelarova H, Meijer AJ. The Phosphatidylinositol 3-Kinase Inhibitors Wortmannin and LY294002 Inhibit Autophagy in Isolated Rat Hepatocytes. Eur J Biochem. 1997 Jan 1;243(1–2):240–6.

161. Feng Y, Gao Y, Wang D, Xu Z, Sun W, Ren P. Autophagy Inhibitor (LY294002) and 5-fluorouracil (5-FU) Combination-Based Nanoliposome for Enhanced Efficacy Against Esophageal Squamous Cell Carcinoma. Nanoscale Res Lett. 2018 Dec 17;13(1):325.

162. Hansen SH, Olsson A, Casanova JE. Wortmannin, an inhibitor of phosphoinositide 3-kinase, inhibits transcytosis in polarized epithelial cells. J Biol Chem. 1995 Nov 24;270(47):28425–32.

163. Qiu L, Zhou G, Cao S. Targeted inhibition of ULK1 enhances daunorubicin sensitivity in acute myeloid leukemia. Life Sci. 2020 Feb 15;243:117234.

164. Egan DF, Chun MGH, Vamos M, Zou H, Rong J, Miller CJ, et al. Small Molecule Inhibition of the Autophagy Kinase ULK1 and Identification of ULK1 Substrates. Mol Cell. 2015 Jul 16;59(2):285–97.

165. Akin D, Wang SK, Habibzadegah-Tari P, Law B, Ostrov D, Li M, et al. A novel ATG4B antagonist inhibits autophagy and has a negative impact on osteosarcoma tumors. Autophagy. 2014;10(11):2021–35.

166. Givvimani S, Munjal C, Tyagi N, Sen U, Metreveli N, Tyagi SC. Mitochondrial

division/mitophagy inhibitor (Mdivi) Ameliorates Pressure Overload Induced Heart Failure. Tjwa M, editor. PLoS One. 2012 Mar 27;7(3):e32388.

167. Mizumura K, Cloonan SM, Nakahira K, Bhashyam AR, Cervo M, Kitada T, et al. Mitophagy-dependent necroptosis contributes to the pathogenesis of COPD. J Clin Invest. 2014 Sep 2;124(9):3987–4003.

168. Mrakovcic M, Fröhlich LF, Mrakovcic M, Fröhlich LF. p53-Mediated Molecular Control of Autophagy in Tumor Cells. Biomolecules. 2018 Mar 21;8(2):14.

169. Tripathi R, Ash D, Shaha C. Beclin-1-p53 interaction is crucial for cell fate determination in embryonal carcinoma cells. J Cell Mol Med. 2014 Nov 1;18(11):2275–86.

170. Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, et al. Regulation of autophagy by cytoplasmic p53. Nat Cell Biol. 2008 Jun;10(6):676–87.

171. Liu J, Xia H, Kim M, Xu L, Li Y, Zhang L, et al. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell. 2011 Sep 30;147(1):223–34.

172. Li Y, Lam S, Mak J, Zheng C, Ho J. Erlotinib-induced autophagy in epidermal growth factor receptor mutated non-small cell lung cancer. Lung Cancer. 2013;81(3).

173. Tung M, Lin P, Wang Y, He T, Lee M, Yeh S, et al. Mutant p53 confers chemoresistance in non-small cell lung cancer by upregulating Nrf2. Oncotarget. 2015;6(39).

174. Saini H, Hakeem I, Mukherjee S, Chowdhury S, Chowdhury R. Autophagy Regulated by Gain of Function Mutant p53 Enhances Proteasomal Inhibitor-Mediated Cell Death through Induction of ROS and ERK in Lung Cancer Cells. J Oncol. 2019;2019.

175. Wu T, Wang M, Jing L, Liu Z, Guo H, Liu Y, et al. Autophagy facilitates lung adenocarcinoma resistance to cisplatin treatment by activation of AMPK/mTOR signaling

pathway. Drug Des Devel Ther. 2015;9.

176. Ko A, Kanehisa A, Martins I, Senovilla L, Chargari C, Dugue D, et al. Autophagy inhibition radiosensitizes in vitro, yet reduces radioresponses in vivo due to deficient immunogenic signalling. Cell Death Differ. 2014 Jan 13;21(1):92–9.

177. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1β–dependent adaptive immunity against tumors. Nat Med. 2009 Oct 20;15(10):1170–8.

178. Narita M, Young ARJ, Arakawa S, Samarajiwa SA, Nakashima T, Yoshida S, et al. Spatial coupling of mTOR and autophagy augments secretory phenotypes. Science. 2011 May 20;332(6032):966–70.

179. Ramakrishnan R, Huang C, Cho H-I, Lloyd M, Johnson J, Ren X, et al. Autophagy Induced by Conventional Chemotherapy Mediates Tumor Cell Sensitivity to Immunotherapy. Cancer Res. 2012 Nov 1;72(21):5483–93.

180. Chude CI, Amaravadi RK, Chude CI, Amaravadi RK. Targeting Autophagy in Cancer: Update on Clinical Trials and Novel Inhibitors. Int J Mol Sci. 2017 Jun 16;18(6):1279.

181. Al-Bari MAA. Chloroquine analogues in drug discovery: new directions of uses, mechanisms of actions and toxic manifestations from malaria to multifarious diseases. J Antimicrob Chemother. 2015;70(6):1608–21.

182. Lee H-O, Mustafa A, Hudes GR, Kruger WD. Hydroxychloroquine Destabilizes Phospho-S6 in Human Renal Carcinoma Cells. PLoS One. 2015;10(7):e0131464.

183. Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. Blocked Autophagy Sensitizes Resistant Carcinoma Cells to Radiation Therapy. 2008;

184. Cook KL, Clarke R. Estrogen receptor-α signaling and localization regulates autophagy and

unfolded protein response activation in ER+ breast cancer. Recept Clin Investig. 2014;1(6).

185. Barnard RA, Wittenburg LA, Amaravadi RK, Gustafson DL, Thorburn A, Thamm DH. Phase I clinical trial and pharmacodynamic evaluation of combination hydroxychloroquine and doxorubicin treatment in pet dogs treated for spontaneously occurring lymphoma. Autophagy. 2014 Aug;10(8):1415–25.

186. Mulcahy Levy JM, Zahedi S, Griesinger AM, Morin A, Davies KD, Aisner DL, et al. Autophagy inhibition overcomes multiple mechanisms of resistance to BRAF inhibition in brain tumors. Elife. 2017 Jan 17;6.

187. Levy JMM, Thompson JC, Griesinger AM, Amani V, Donson AM, Birks DK, et al. Autophagy Inhibition Improves Chemosensitivity in BRAFV600E Brain Tumors. Cancer Discov. 2014 Jul 1;4(7):773–80.

188. Wolpin BM, Rubinson DA, Wang X, Chan JA, Cleary JM, Enzinger PC, et al. Phase II and pharmacodynamic study of autophagy inhibition using hydroxychloroquine in patients with metastatic pancreatic adenocarcinoma. Oncologist. 2014 Jun 1;19(6):637–8.

189. Brazil L, Swampillai A, Mak K, Hackshaw A, Edwards D, Mesiri P, et al. P01.072 Hydroxychloroquine and short course radiotherapy for elderly patients with glioma: a randomised study. Neuro Oncol. 2018 Sep 19;20(suppl_3):iii246–iii246.

190. Wolpin BM, Rubinson DA, Wang X, Chan JA, Cleary JM, Enzinger PC, et al. Phase II and Pharmacodynamic Study of Autophagy Inhibition Using Hydroxychloroquine in Patients With Metastatic Pancreatic Adenocarcinoma. Oncologist. 2014 Jun 1;19(6):637–8.

191. Rangwala R, Chang YC, Hu J, Algazy KM, Evans TL, Fecher LA, et al. Combined MTOR and autophagy inhibition: phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma. Autophagy. 2014 Aug;10(8):1391–402.

192. Rangwala R, Leone R, Chang YC, Fecher LA, Schuchter LM, Kramer A, et al. Phase I trial of hydroxychloroquine with dose-intense temozolomide in patients with advanced solid tumors and melanoma. Autophagy. 2014 Aug;10(8):1369–79.

193. Zeh HJ, Bahary N, Boone BA, Singhi AD, Miller-Ocuin JL, Normolle DP, et al. A Randomized Phase II Preoperative Study of Autophagy Inhibition with High-Dose Hydroxychloroquine and Gemcitabine/Nab-Paclitaxel in Pancreatic Cancer Patients. Clin Cancer Res. 2020 Jul 1;26(13):3126–34.

194. Malhotra J, Jabbour S, Orlick M, Riedlinger G, Joshi S, Guo JY, et al. Modulation of autophagy with hydroxychloroquine in patients with advanced non-small cell lung cancer (NSCLC): A phase Ib study. J Clin Oncol. 2018 May 20;36(15_suppl):e21138–e21138.

195. Neal JW, Wakelee HA, Feliciano JL, Goldberg SB, Morgensztern D, Das MS, et al. A multicenter randomized phase II trial of erlotinib with and without hydroxychloroquine (HCQ) in TKI-naive patients (pts) with epidermal growth factor receptor (EGFR) mutant advanced non-small cell lung cancer (NSCLC). J Clin Oncol. 2014 May 20;32(15_suppl):8088–8088.

196. Hayflick L, Moorhead PS. The Serial Cultivation of Human Diploid Cell Strains. Exp Cell Res. 1961;25:585–621.

197. Hayflick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res. 1965;636:614–36.

198. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes and Development. 2010.

199. Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, et al. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. Dev Cell.

2014;31(6):722–33.

200. Storer M, Mas A, Robert-Moreno A, Pecoraro M, Ortells MC, Di Giacomo V, et al. Senescence is a developmental mechanism that contributes to embryonic growth and patterning. Cell. 2013;155(5):1119–30.

201. Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell. 2005;120(4).

202. Campisi J. Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol. 2001 Nov;11(11):S27-31.

203. Hoenicke L, Zender L. Immune surveillance of senescent cells--biological significance in cancer- and non-cancer pathologies. Carcinogenesis. 2012 Jun 1;33(6):1123–6.

204. Hassona Y, Cirillo N, Heesom K, Parkinson EK, Prime SS. Senescent cancer-associated fibroblasts secrete active MMP-2 that promotes keratinocyte dis-cohesion and invasion. Br J Cancer. 2014 Sep 12;111(6):1230–7.

205. Krtolica A, Parrinello S, Lockett S, Desprez P, Desprez J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A. 2001;98(21).

206. Coppé J-P, Desprez P-Y, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol. 2010;5:99–118.

207. Ghosh D, Pena CM, Quach N, Xuan B, Lee AH, Dawson MR. Senescent mesenchymal stem cells remodel extracellular matrix driving breast cancer cells to a more-invasive phenotype. J Cell Sci. 2020 Jan 15;133(2).

208. Laberge R-M, Awad P, Campisi J, Desprez P-Y. Epithelial-Mesenchymal Transition Induced by Senescent Fibroblasts. Cancer Microenviron. 2012;5(1):39.

209. Saleh T, Tyutyunyk-Massey L, Gewirtz DA. Tumor Cell Escape from Therapy-Induced Senescence as a Model of Disease Recurrence after Dormancy. Cancer Res. 2019 Mar 15;79(6):1044–6.

210. Roberson RS, Kussick SJ, Vallieres E, Chen S-YJ, Wu DY. Escape from Therapy-Induced Accelerated Cellular Senescence in p53-Null Lung Cancer Cells and in Human Lung Cancers. Cancer Res. 2005 Apr 1;65(7):2795–803.

211. Alotaibi M, Sharma K, Saleh T, Povirk LF, Hendrickson EA, Gewirtz DA. Radiosensitization by PARP Inhibition in DNA Repair Proficient and Deficient Tumor Cells: Proliferative Recovery in Senescent Cells. Radiat Res. 2016 Mar;185(3):229–45.

212. Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM, et al. A senescence program controlled by p53 and p16INK4acontributes to the outcome of cancer therapy. Cell. 2002;109(3):335–46.

213. Hayward RL, Macpherson JS, Cummings J, Monia BP, Smyth JF, Jodrell DI. Antisense Bcl-xl Down-Regulation Switches the Response to Topoisomerase I Inhibition from Senescence to Apoptosis in Colorectal Cancer Cells, Enhancing Global Cytotoxicity. Clin Cancer Res. 2003 Jul 1;9(7):2856–65.

214. Yosef R, Pilpel N, Tokarsky-Amiel R, Biran A, Ovadya Y, Cohen S, et al. Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. Nat Commun. 2016 Sep 6;7(1):11190.

215. Saleh T, Carpenter V, Tyutyunyk-Massey L, Murray G, Leverson J, Souers A, et al. Clearance of therapy-induced senescent tumor cells by the senolytic ABT-263 via interference with BCL-X L -BAX Interaction. Mol Oncol. 2020;1–16.

216. Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of Cellular Senescence. 2018;

217. Narita M, Nuñez S, Heard E, Narita M, Lin AW, Hearn SA, et al. Rb-Mediated Heterochromatin Formation and Silencing of E2F Target Genes during Cellular Senescence. Cell. 2003 Jun 13;113(6):703–16.

218. Pare R, Soon PS, Shah A, Lee CS. Differential expression of senescence tumour markers and its implications on survival outcomes of breast cancer patients. Ahmad A, editor. PLoS One. 2019 Apr 18;14(4):e0214604.

219. Kurz DJ, Decary S, Hong Y, Erusalimsky JD. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. J Cell Sci. 2000;113(20).

220. Carmona-Gutierrez D, Hughes AL, Madeo F, Ruckenstuhl C. The crucial impact of lysosomes in aging and longevity. Ageing Res Rev. 2016;32:2–12.

221. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and in vivo. Nat Protoc. 2009 Dec 19;4(12):1798–806.

222. Bent EH, Gilbert LA, Hemann MT. A senescence secretory switch mediated by PI3K/AKT/mTOR activation controls chemoprotective endothelial secretory responses. Genes Dev. 2016;30(16):1811–21.

223. Druelle C, Drullion C, Deslé J, Martin N, Saas L, Cormenier J, et al. ATF6a regulates morphological changes associated with senescence in human fibroblasts. Oncotarget. 2016 Oct;7(42):67699–715.

224. Martínez J, Tarallo D, Martínez-Palma L, Victoria S, Bresque M, Rodríguez-Bottero S, et al. Mitofusins modulate the increase in mitochondrial length, bioenergetics and secretory phenotype in therapy-induced senescent melanoma cells. Biochem J. 2019;476(17):2463–

86.

225. Zhang R, Chen W, Adams PD. Molecular Dissection of Formation of Senescence-Associated Heterochromatin Foci. Mol Cell Biol. 2007;

226. Aird KM, Zhang R. Detection of senescence-associated heterochromatin foci (SAHF). Methods Mol Biol. 2013;

227. Macip S, Igarashi M, Fang L, Chen A, Pan Z, Lee SW, et al. Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. EMBO J. 2002 May 1;21(9):2180–8.

228. Chang B-D, Swift ME, Shen M, Fang J, Broude E V., Roninson IB. Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent. Proc Natl Acad Sci. 2002 Jan 8;99(1):389–94.

229. Yosef R, Pilpel N, Papismadov N, Gal H, Ovadya Y, Vadai E, et al. p21 maintains senescent cell viability under persistent DNA damage response by restraining JNK and caspase signaling. EMBO J. 2017 Aug;36(15):2280–95.

230. Zindy F, Quelle DE, Roussel MF, Sherr CJ. Expression of the p16(INK4a) tumor suppressor versus other INK4 family members during mouse development and aging. Oncogene. 1997;15(2):203–11.

231. Cerella C, Grandjenette C, Dicato M, Diederich M. Roles of Apoptosis and Cellular Senescence in Cancer and Aging. Curr Drug Targets. 2016 Feb 12;17(4):405–15.

232. Luo H, Yount C, Lang H, Yang A, Riemer EC, Lyons K, et al. Activation of p53 with Nutlin-3a radiosensitizes lung cancer cells via enhancing radiation-induced premature senescence. Lung Cancer. 2013 Aug 1;81(2):167–73.

233. Ritschka B, Storer M, Mas A, Heinzmann F, Ortells MC, Morton JP, et al. The senescence-

associated secretory phenotype induces cellular plasticity and tissue regeneration. Genes Dev. 2017 Jan 15;31(2):172–83.

234. Webb AH, Gao BT, Goldsmith ZK, Irvine AS, Saleh N, Lee RP, et al. Inhibition of MMP-2 and MMP-9 decreases cellular migration, and angiogenesis in in vitro models of retinoblastoma. BMC Cancer. 2017 Dec 20;17(1):434.

235. Ortiz-Montero P, Londoño-Vallejo A, Vernot J-P. Senescence-associated IL-6 and IL-8 cytokines induce a self- and cross-reinforced senescence/inflammatory milieu strengthening tumorigenic capabilities in the MCF-7 breast cancer cell line. Cell Commun Signal. 2017 Dec 4;15(1):17.

236. Korkaya H, Liu S, Wicha MS. Regulation of Cancer Stem Cells by Cytokine Networks: Attacking Cancer's Inflammatory Roots. Clin Cancer Res. 17.

237. Meng Y, Efimova E V, Hamzeh KW, Darga TE, Mauceri HJ, Fu Y-X, et al. Radiation-inducible immunotherapy for cancer: senescent tumor cells as a cancer vaccine. Mol Ther. 2012 May 1;20(5):1046–55.

238. Schosserer M, Grillari J, Breitenbach M. The Dual Role of Cellular Senescence in Developing Tumors and Their Response to Cancer Therapy. Front Oncol. 2017;7:278.

239. Tareq Saleh, Sarah Bloukh, Valerie J. Carpenter , Enas Alwohoush JB, Sarah Darwish , Belal Azab and DAG. Therapy-Induced Senescence: An "Old" Friend Becomes the Enemy. :1–38.

240. Liao E-C, Hsu Y-T, Chuah Q-Y, Lee Y-J, Hu J-Y, Huang T-C, et al. Radiation induces senescence and a bystander effect through metabolic alterations. Cell Death Dis. 2014 May 22;5(5):e1255–e1255.

241. He X, Yang A, McDonald DG, Riemer EC, Vanek KN, Schulte BA, et al. MiR-34a

modulates ionizing radiation-induced senescence in lung cancer cells. Oncotarget. 2017 Sep 19;8(41):69797–807.

242. Luo H, Wang L, Schulte BA, Yang A, Tang S, Wang GY. Resveratrol enhances ionizing radiation-induced premature senescence in lung cancer cells. Int J Oncol. 2013 Dec 1;43(6):1999–2006.

243. Jones KR, Elmore LW, Jackson-Cook C, Demasters G, Povirk LF, Holt SE, et al. p53-Dependent accelerated senescence induced by ionizing radiation in breast tumour cells. Int J Radiat Biol. 2005 Jun;81(6):445–58.

244. Mirzayans R, Scott A, Cameron M, Murray D. Induction of Accelerated Senescence by γ Radiation in Human Solid Tumor-Derived Cell Lines Expressing Wild-Type TP53. Radiat Res. 2005 Jan 1;163(1):53–62.

245. Cui F, Hou J, Huang C, Sun X, Zeng Y, Cheng H, et al. C-Myc regulates radiation-induced G2/M cell cycle arrest and cell death in human cervical cancer cells. J Obstet Gynaecol Res. 2017 Apr;43(4):729–35.

246. Li W, Wang W, Dong H, Li Y, Li LI, Han L, et al. Cisplatin-induced senescence in ovarian cancer cells is mediated by GRP78. Oncol Rep. 2014;31(6):2525–34.

247. Wang X, Wong S, Pan J, Tsao S, Fung K, Kwong D, et al. Evidence of Cisplatin-induced Senescent-like Growth Arrest in Nasopharyngeal Carcinoma Cells. Cancer Res. 1998;58(22):5019–22.

248. Nagano T, Nakano M, Nakashima A, Onishi K, Yamao S, Enari M, et al. Identification of cellular senescence-specific genes by comparative transcriptomics. Sci Rep. 2016;22(6):1–13.

249. Taymaz-Nikerel H, Karabekmez ME, Eraslan S, Kırdar B. Doxorubicin induces an

extensive transcriptional and metabolic rewiring in yeast cells. Sci Rep. 2018 Dec 12;8(1):13672.

250. Pommier Y, Leo E, Zhang H, Marchand C. DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. Chem Biol. 2010 May 28;17(5):421–33.

251. Saleh T, Tyutyunyk-Massey L, H. Patel N, K. Cudjoe E, Alotaibi M, A. Gewirtz D. Studies of Non-Protective Autophagy Provide Evidence that Recovery from Therapy-Induced Senescence is Independent of Early Autophagy. Int J Mol Sci. 2020 Feb 20;21(4):1427.

252. Bojko A, Czarnecka-Herok J, Charzynska A, Dabrowski M, Sikora E. Diversity of the Senescence Phenotype of Cancer Cells Treated with Chemotherapeutic Agents. Cells. 2019;8(12):1501.

253. Elmore LW, Rehder CW, Di X, McChesney PA, Jackson-cook CK, Gewirtz DA, et al. Adriamycin-induced senescence in breast tumor cells involves functional p53 and telomere dysfunction. J Biol Chem. 2002 Sep;277(38):35509–15.

254. Su D, Zhu S, Han X, Feng Y, Huang H, Ren G, et al. BMP4-Smad signaling pathway mediates adriamycin-induced premature senescence in lung cancer cells. J Biol Chem. 2009;284(18):12153–64.

255. Dabrowska M, Skoneczny M, Uram L, Rode W. Methotrexate-induced senescence of human colon cancer cells depends on p53 acetylation, but not genomic aberrations. Anticancer Drugs. 2019 Apr;30(4):374–82.

256. Pazdur R, Kudelka AP, Kavanagh JJ, Cohen PR, Raber MN. The taxoids: paclitaxel (Taxol®) and docetaxel (Taxotere®). Cancer Treat Rev. 1993;

257. Khongkow P, Gomes AR, Gong C, Man EPS, Tsang JWH, Zhao F, et al. Paclitaxel targets FOXM1 to regulate KIF20A in mitotic catastrophe and breast cancer paclitaxel resistance.

Oncogene. 2016;35(8):990–1002.

258. Jia M, Su B, Mo L, Qiu W, Ying J, Lin P, et al. Circadian clock protein CRY1 prevents paclitaxel-induced senescence of bladder cancer cells by promoting p53 degradation. Oncol Rep. 2020 Dec 30;45(3):1033–43.

259. Lee YH, Kang BS, Bae YS. Premature senescence in human breast cancer and colon cancer cells by tamoxifen-mediated reactive oxygen species generation. Life Sci. 2014;97(2):116–22.

260. Dolfi SC, Jäger A V., Medina DJ, Haffty BG, Yang JM, Hirshfield KM. Fulvestrant treatment alters MDM2 protein turnover and sensitivity of human breast carcinoma cells to chemotherapeutic drugs. Cancer Lett. 2014;350(1–2):52–60.

261. Burton DGA, Giribaldi MG, Munoz A, Halvorsen K, Patel A, Jorda M, et al. Androgen Deprivation-Induced Senescence Promotes Outgrowth of Androgen-Refractory Prostate Cancer Cells. PLoS One. 2013;8(6):e68003.

262. Bollard J, Miguela V, Ruiz De Galarreta M, Venkatesh A, Bian CB, Roberto MP, et al. Palbociclib (PD-0332991), a selective CDK4/6 inhibitor, restricts tumour growth in preclinical models of hepatocellular carcinoma. Gut. 2017;66(7):1286–96.

263. Liu M, Liu H, Chen J. Mechanisms of the CDK4/6 inhibitor palbociclib (PD 0332991) and its future application in cancer treatment (Review). Oncol Rep. 2018 Mar;39(3):901–11.

264. Vijayaraghavan S, Karakas C, Doostan I, Chen X, Bui T, Yi M, et al. CDK4/6 and autophagy inhibitors synergistically induce senescence in Rb positive cytoplasmic cyclin E negative cancers. Nat Commun. 2017 Aug 27;8(1):15916.

265. Valenzuela CA, Vargas L, Martinez V, Bravo S, Brown NE. Palbociclib-induced autophagy and senescence in gastric cancer cells. Exp Cell Res. 2017;360(2):390–6.

266. Morelli MB, Amantini C, Nabissi M, Cardinali C, Santoni M, Bernardini G, et al. Axitinib induces senescence-associated cell death and necrosis in glioma cell lines: The proteasome inhibitor, bortezomib, potentiates axitinib-induced cytotoxicity in a p21(Waf/Cip1) dependent manner. Oncotarget. 2017;8(2):3380–95.

267. Hasan MR, Ho SHY, Owen DA, Tai IT. Inhibition of VEGF induces cellular senescence in colorectal cancer cells. Int J Cancer. 2011;129(9):2115–23.

268. Wang LX, Wang JD, Chen JJ, Long B, Liu LL, Tu XX, et al. Aurora A Kinase Inhibitor AKI603 Induces Cellular Senescence in Chronic Myeloid Leukemia Cells Harboring T315I Mutation. Sci Rep. 2016;6(35533):1–13.

269. Cells MH, Meng A, Wang Y, Zant G Van, Zhou D. Ionizing Radiation and Busulfan Induce Premature Senescence in Murine Bone Marrow Hematopoietic Cells. Cancer Res. 2003;63(17):5414–9.

270. Seignez C, Martin A, Rollet CE, Racoeur C, Scagliarini A, Jeannin JF, et al. Senescence of tumor cells induced by oxaliplatin increases the efficiency of a lipid A immunotherapy via the recruitment of neutrophils. Oncotarget. 2014;5(22):11442–51.

271. Saleh T, Tyutyunyk-Massey L, Gewirtz DA. Tumor cell escape from therapy-induced senescence as a model of disease recurrence after dormancy. Cancer Res. 2019;79(6).

272. Saleh T, Tyutynuk-Massey L, Cudjoe EK, Idowu MO, Landry JW, Gewirtz DA. Non-Cell Autonomous Effects of the Senescence-Associated Secretory Phenotype in Cancer Therapy. Front Oncol. 2018 May 18;8:164.

273. Short S, Fielder E, Miwa S, von Zglinicki T. Senolytics and senostatics as adjuvant tumour therapy. EBioMedicine. 2019 Mar 1;41:683–92.

274. Samaraweera L, Adomako A, Rodriguez-Gabin A, McDaid HM. A Novel Indication for

Panobinostat as a Senolytic Drug in NSCLC and HNSCC. Sci Rep. 2017 Dec 15;7(1):1900.

275. Malavolta M, Bracci M, Santarelli L, Sayeed MA, Pierpaoli E, Giacconi R, et al. Inducers of Senescence, Toxic Compounds, and Senolytics: The Multiple Faces of Nrf2-Activating Phytochemicals in Cancer Adjuvant Therapy. Mediators Inflamm. 2018;2018:1–32.

276. Baskar R, Dai J, Wenlong N, Yeo R, Yeoh K-W. Biological response of cancer cells to radiation treatment. Front Mol Biosci. 2014 Nov 17;1:24.

277. Colliez F, Gallez B, Jordan BF. Assessing Tumor Oxygenation for Predicting Outcome in Radiation Oncology: A Review of Studies Correlating Tumor Hypoxic Status and Outcome in the Preclinical and Clinical Settings. Front Oncol. 2017;7:10.

278. Zhang Y, Martin SG. Redox Proteins and Radiotherapy. Clin Oncol. 2014 May 1;26(5):289–300.

279. Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Updat. 2004 Apr 1;7(2):97–110.

280. Azad MB, Chen Y, Gibson SB. Regulation of Autophagy by Reactive Oxygen Species (ROS): Implications for Cancer Progression and Treatment. Antioxid Redox Signal. 2009 Apr 24;11(4):777–90.

281. Mazure NM, Pouysségur J. Hypoxia-induced autophagy: cell death or cell survival? Curr Opin Cell Biol. 2010 Apr 1;22(2):177–80.

282. He L, Zhang J, Zhao J, Ma N, Kim SW, Qiao S, et al. Autophagy: The Last Defense against Cellular Nutritional Stress. Adv Nutr. 2018 Jul 1;9(4):493–504.

283. Saleh T, Cuttino L, Gewirtz DA. Autophagy is not uniformly cytoprotective: a personalized medicine approach for autophagy inhibition as a therapeutic strategy in non-small cell lung cancer. Biochim Biophys Acta. 2016;1860(10):2130–6.

284. Bristol ML, Emery SM, Maycotte P, Thorburn A, Chakradeo S, Gewirtz DA. Autophagy inhibition for chemosensitization and radiosensitization in cancer: do the preclinical data support this therapeutic strategy? J Pharmacol Exp Ther. 2013 Mar;344(3):544–52.

285. Gewirtz DA. The Switch between Protective and Nonprotective Autophagy; Implications for Autophagy Inhibition as a Therapeutic Strategy in Cancer. Biology (Basel). 2020;9(12):1–6.

286. Marcotte R, Lacelle C, Wang E. Senescent fibroblasts resist apoptosis by downregulating caspase-3. Mech Ageing Dev. 2004;125(10–11):777–83.

287. d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer. 2008 Jul;8(7):512–22.

288. Saleh T, Tyutyunyk-Massey L, Murray GF, Alotaibi MR, Kawale AS, Elsayed Z, et al. Tumor cell escape from therapy-induced senescence. Biochem Pharmacol. 2019;162:202–12.

289. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell. 1993 Sep 24;74(6):957–67.

290. Stewart-Ornstein J, Iwamoto Y, Miller MA, Prytyskach MA, Ferretti S, Holzer P, et al. p53 dynamics vary between tissues and are linked with radiation sensitivity. Nat Commun. 2021 Dec 9;12(1):898.

291. Lee JM, Bernstein A. p53 mutations increase resistance to ionizing radiation. Proc Natl Acad Sci U S A. 1993 Jun 15;90(12):5742–6.

292. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A. 1995 Sep 26;92(20):9363–7.

293. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hosteller R, Cleary K, et al. Mutations in the p53 gene occur in diverse human tumour types. Nature. 1989 Dec;342(6250):705–8.

294. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature. 1993 Apr;362(6423):847–9.

295. Klionsky D, Abdelmohsen K, Abe A, Abedin M, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy. 2016;12(1).

296. Gomes LR, Menck CFM, Leandro GS. Autophagy Roles in the Modulation of DNA Repair Pathways. Int J Mol Sci. 2017 Nov 7;18(11).

297. Lin W, Yuan N, Wang Z, Cao Y, Fang Y, Li X, et al. Autophagy confers DNA damage repair pathways to protect the hematopoietic system from nuclear radiation injury. Sci Rep. 2015 Dec 18;5(1):12362.

298. Maycotte P, Aryal S, Cummings CT, Thorburn J, Morgan MJ, Thorburn A. Chloroquine sensitizes breast cancer cells to chemotherapy independent of autophagy. Autophagy. 2012 Feb;8(2):200–12.

299. Huang Y-H, Yang P-M, Chuah Q-Y, Lee Y-J, Hsieh Y-F, Peng C-W, et al. Autophagy promotes radiation-induced senescence but inhibits bystander effects in human breast cancer cells. Autophagy. 2014 Jul 29;10(7):1212–28.

300. Lehmann BD, McCubrey JA, Jefferson HS, Paine MS, Chappell WH, Terrian DM. A Dominant Role for p53-Dependent Cellular Senescence in Radiosensitization of Human Prostate Cancer Cells. Cell Cycle. 2007 Mar 28;6(5):595–605.

301. Okada H, Mak TW. Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer. 2004 Aug;4(8):592–603.

302. Maier P, Hartmann L, Wenz F, Herskind C. Cellular Pathways in Response to Ionizing Radiation and Their Targetability for Tumor Radiosensitization. Int J Mol Sci. 2016 Jan 14;17(1).

303. Patel NH, Sohal SS, Manjili MH, Harrell JC, Gewirtz DA. The Roles of Autophagy and Senescence in the Tumor Cell Response to Radiation. Radiat Res. 2020;194(2):103–15.

304. Roninson IB, Broude E V., Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Updat. 2001;4(5):303–13.

305. Gewirtz DA, Alotaibi M, Yakovlev VA, Povirk LF. Tumor Cell Recovery from Senescence Induced by Radiation with PARP Inhibition. Radiat Res. 2016 Oct;186(4):327–32.

306. Rödel C, Haas J, Groth A, Grabenbauer GG, Sauer R, Rödel F. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: Survivin as a radioresistance factor. Int J Radiat Oncol. 2003 Apr;55(5):1341–7.

307. Qu Y, Hu S, Xu X, Wang R, Yu H, Xu J, et al. Nimotuzumab Enhances the Radiosensitivity of Cancer Cells In Vitro by Inhibiting Radiation-Induced DNA Damage Repair. Shi X, editor. PLoS One. 2013 Aug 16;8(8):e70727.

308. Lee J-J, Kim BC, Park M-J, Lee Y-S, Kim Y-N, Lee BL, et al. PTEN status switches cell fate between premature senescence and apoptosis in glioma exposed to ionizing radiation. Cell Death Differ. 2011 Apr 12;18(4):666–77.

309. Lu X-Q. Stereotactic Body Radiation Therapy Systems. In: Gaya A, Mahadevan A, editors. Stereotactic Body Radiotherapy: A Practical Guide. London: Springer London; 2015. p. 27–43.

310. Andrews DW, Scott CB, Sperduto PW, Flanders AE, Gaspar LE, Schell MC, et al. Whole brain radiation therapy with or without stereotactic radiosurgery boost for patients with one

to three brain metastases: phase III results of the RTOG 9508 randomised trial. Lancet (London, England). 2004 May 22;363(9422):1665–72.

311. Doyen J, Poudenx M, Gal J, Otto J, Guerder C, Naghavi AO, et al. Stereotactic ablative radiotherapy after concomitant chemoradiotherapy in non-small cell lung cancer: A TITE-CRM phase 1 trial. Radiother Oncol. 2018 May;127(2):239–45.

312. Chen N, Wu L, Yuan H, Wang J. ROS/Autophagy/Nrf2 Pathway Mediated Low-Dose Radiation Induced Radio-Resistance in Human Lung Adenocarcinoma A549 Cell. Int J Biol Sci. 2015;11(7):833–44.

313. Kuwahara Y, Oikawa T, Ochiai Y, Roudkenar MH, Fukumoto M, Shimura T, et al. Enhancement of autophagy is a potential modality for tumors refractory to radiotherapy. Cell Death Dis. 2011 Jun 30;2(6):e177–e177.

314. Qu X, Sheng J, Shen L, Su J, Xu Y, Xie Q, et al. Autophagy inhibitor chloroquine increases sensitivity to cisplatin in QBC939 cholangiocarcinoma cells by mitochondrial ROS. PLoS One. 2017;12(3):e0173712.

315. Kim EL, Wüstenberg R, Rübsam A, Schmitz-Salue C, Warnecke G, Bücker E-M, et al. Chloroquine activates the p53 pathway and induces apoptosis in human glioma cells. Neuro Oncol. 2010 Apr;12(4):389–400.

316. Espinoza JA, Zisi A, Kanellis DC, Carreras-Puigvert J, Henriksson M, Hühn D, et al. The antimalarial drug amodiaquine stabilizes p53 through ribosome biogenesis stress, independently of its autophagy-inhibitory activity. Cell Death Differ. 2020 Feb 8;27(2):773–89.

317. Gomes AR, Abrantes AM, Brito AF, Laranjo M, Casalta-Lopes JE, Gonçalves AC, et al. Influence of P53 on the radiotherapy response of hepatocellular carcinoma. Clin Mol

Hepatol. 2015 Sep;21(3):257–67.

318. Cheng G, Kong D, Hou X, Liang B, He M, Liang N, et al. The tumor suppressor, p53, contributes to radiosensitivity of lung cancer cells by regulating autophagy and apoptosis. Cancer Biother Radiopharm. 2013 Mar;28(2):153–9.

319. Luo H, Yang A, Schulte BA, Wargovich MJ, Wang GY. Resveratrol induces premature senescence in lung cancer cells via ROS-mediated DNA damage. PLoS One. 2013;8(3):e60065.

320. te Poele RH, Okorokov AL, Jardine L, Cummings J, Joel SP. DNA damage is able to induce senescence in tumor cells in vitro and in vivo. Cancer Res. 2002 Mar;62(6):1876–83.

321. Widel M, Lalik A, Krzywon A, Poleszczuk J, Fujarewicz K, Rzeszowska-Wolny J. The different radiation response and radiation-induced bystander effects in colorectal carcinoma cells differing in p53 status. Mutat Res Mol Mech Mutagen. 2015 Aug 1;778:61–70.

322. Saleh T, Tyutynuk-Massey L, Cudjoe EK, Idowu MO, Landry JW, Gewirtz DA. Non-Cell Autonomous Effects of the Senescence-Associated Secretory Phenotype in Cancer Therapy. Front Oncol. 2018;8(164):1–14.

323. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol. 2014 Oct 5;740:364–78.

324. Basu A, Krishnamurthy S. Cellular Responses to Cisplatin-Induced DNA Damage. J Nucleic Acids. 2010;2010:1–16.

325. Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, et al. Molecular mechanisms of cisplatin resistance. Oncogene. 2012 Apr 5;31(15):1869–83.

326. Wilson EN, Bristol ML, Di X, Maltese WA, Koterba K, Beckman MJ, et al. A switch between cytoprotective and cytotoxic autophagy in the radiosensitization of breast tumor

cells by chloroquine and vitamin D. Horm Cancer. 2011 Oct;2(5):272–85.

327. Vessoni AT, Filippi-Chiela EC, Menck CF, Lenz G. Autophagy and genomic integrity. Cell Death Differ. 2013 Nov 9;20(11):1444–54.

328. Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, et al. Regulation of autophagy by cytoplasmic p53. Nat Cell Biol. 2008 Jun 4;10(6):676–87.

329. Wang Y, Blandino G, Oren M, Givol D. Induced p53 expression in lung cancer cell line promotes cell senescence and differentially modifies the cytotoxicity of anti-cancer drugs. Oncogene. 1998 Oct 14;17(15):1923–30.

330. Sun X, Shi B, Zheng H, Min L, Yang J, Li X, et al. Senescence-associated secretory factors induced by cisplatin in melanoma cells promote non-senescent melanoma cell growth through activation of the ERK1/2-RSK1 pathway. Cell Death Dis. 2018;9(3):260.

331. Fang K, Chiu C-C, Li C-H, Chang Y, Hwang H. Cisplatin-induced senescence and growth inhibition in human non-small cell lung cancer cells with ectopic transfer of p16INK4a. Oncol Res. 2007;16(10):479–88.

332. Kuribayashi K, El-Deiry WS. Regulation of Programmed Cell Death by the P53 Pathway. In Springer, Dordrecht; 2008. p. 201–21.

333. Harhaji-Trajkovic L, Vilimanovich U, Kravic-Stevovic T, Bumbasirevic V, Trajkovic V. AMPK-mediated autophagy inhibits apoptosis in cisplatin-treated tumour cells. J Cell Mol Med. 2009 Sep;13(9B):3644–54.

334. Li D-D, Sun T, Wu X-Q, Chen S-P, Deng R, Jiang S, et al. The inhibition of autophagy sensitises colon cancer cells with wild-type p53 but not mutant p53 to topotecan treatment. PLoS One. 2012;7(9):e45058.

335. Shen P, Chen M, He M, Chen L, Song Y, Xiao P, et al. Inhibition of ERα/ERK/P62 cascades

induces "autophagic switch" in the estrogen receptor-positive breast cancer cells exposed to gemcitabine. Oncotarget. 2016 Jul 26;7(30):48501–16.

336. Hollomon MG, Gordon N, Santiago-O'Farrill JM, Kleinerman ES. Knockdown of autophagy-related protein 5, ATG5, decreases oxidative stress and has an opposing effect on camptothecin-induced cytotoxicity in osteosarcoma cells. BMC Cancer. 2013 Oct 26;13:500.

337. Xu J, Patel NH, Saleh T, Cudjoe EK, Alotaibi M, Wu Y, et al. Differential Radiation Sensitivity in p53 Wild-Type and p53-Deficient Tumor Cells Associated with Senescence but not Apoptosis or (Nonprotective) Autophagy. Radiat Res. 2018 Aug 10;190(5):538.

338. Yee KS, Wilkinson S, James J, Ryan KM, Vousden KH. PUMA- and Bax-induced autophagy contributes to apoptosis. Cell Death Differ. 2009 Aug 20;16(8):1135–45.

339. Hou W, Han J, Lu C, Goldstein LA, Rabinowich H. Autophagic degradation of active caspase-8. Autophagy. 2010 Oct 27;6(7):891–900.

340. Tang Z, Takahashi Y, Chen C, Liu Y, He H, Tsotakos N, et al. Atg2A/B deficiency switches cytoprotective autophagy to non-canonical caspase-8 activation and apoptosis. Cell Death Differ. 2017;24(12):2127–38.

341. Wang J, Cui D, Gu S, Chen X, Bi Y, Xiong X, et al. Autophagy regulates apoptosis by targeting NOXA for degradation. Biochim Biophys Acta - Mol Cell Res. 2018 Aug 1;1865(8):1105–13.

342. Patel NH, Xu J, Saleh T, Wu Y, Lima S, Gewirtz DA. Influence of nonprotective autophagy and the autophagic switch on sensitivity to cisplatin in non-small cell lung cancer cells. Biochem Pharmacol. 2020 May 1;175:113896.

343. Tai H, Wang Z, Gong H, Han X, Zhou J, Wang X, et al. Autophagy impairment with

lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced senescence. Autophagy. 2017 Jan 2;13(1):99–113.

344. Qu K, Lin T, Wang Z, Liu S, Chang H, Xu X, et al. Reactive oxygen species generation is essential for cisplatin induced accelerated senescence in hepatocellular carcinoma. Front Med China. 2014;8(2):227–35.

345. Artuso M, EsteveA, Brésil H, Vuillaume M, Hall J. The role of the Ataxia telangiectasia gene in the p53, WAF1/CIP1(p21)- and GADD45-mediated response to DNA damage produced by ionising radiation. Oncogene. 1995 Oct 1;11(8):1427–35.

346. Cui L, Song Z, Liang B, Jia L, Ma S, Liu X. Radiation induces autophagic cell death via the p53/DRAM signaling pathway in breast cancer cells. Oncol Rep. 2016 Jun 1;35(6):3639–47.

347. Rubinsztein DC, Mariño G, Kroemer G. Autophagy and Aging. Cell. 2011 Sep 2;146(5):682–95.

348. Kang HT, Lee KB, Kim SY, Choi HR, Park SC. Autophagy impairment induces premature senescence in primary human fibroblasts. PLoS One. 2011;6(8):e23367.

349. Han X, Tai H, Wang X, Wang Z, Zhou J, Wei X, et al. AMPK activation protects cells from oxidative stress-induced senescence via autophagic flux restoration and intracellular NAD(+) elevation. Aging Cell. 2016;15(3):416–27.

350. Kwon Y, Kim JW, Jeoung JA, Kim M-S, Kang C. Autophagy Is Pro-Senescence When Seen in Close-Up, but Anti-Senescence in Long-Shot. Mol Cells. 2017 Sep 30;40(9):607–12.

351. Fitzgerald AL, Osman AA, Xie T-X, Patel A, Skinner H, Sandulache V, et al. Reactive oxygen species and p21Waf1/Cip1 are both essential for p53-mediated senescence of head

and neck cancer cells. Cell Death Dis. 2015 Mar 12;6(3):e1678–e1678.

352. Passos JF, Nelson G, Wang C, Richter T, Simillion C, Proctor CJ, et al. Feedback between p21 and reactive oxygen production is necessary for cell senescence. Mol Syst Biol. 2010 Jan 16;6(1):347.

353. Paquette M, El-Houjeiri L, Pause A. mTOR Pathways in Cancer and Autophagy. Cancers (Basel). 2018;10(1).

354. Nam HY, Han MW, Chang HW, Lee YS, Lee M, Lee HJ, et al. Radioresistant Cancer Cells Can Be Conditioned to Enter Senescence by mTOR Inhibition. Cancer Res. 2013 Jul 15;73(14):4267–77.

355. Young ARJ, Narita M, Narita M. Spatio-temporal association between mTOR and autophagy during cellular senescence. Autophagy. 2011 Nov 27;7(11):1387–8.

356. Vijayaraghavan S, Karakas C, Doostan I, Chen X, Bui T, Yi M, et al. CDK4/6 and autophagy inhibitors synergistically induce senescence in Rb positive cytoplasmic cyclin e negative cancers. Nat Commun. 2017;8:1–17.

357. Narita M, Young ARJ, Narita M. Autophagy facilitates oncogene-induced senescence. Autophagy. 2009 Oct 27;5(7):1046–7.

358. Young ARJ, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JFJ, et al. Autophagy mediates the mitotic senescence transition. Genes Dev. 2009 Apr 1;23(7):798–803.

359. Was H, Barszcz K, Czarnecka J, Kowalczyk A, Uzarowska E, Koza P, et al. Bafilomycin A1 triggers proliferative potential of senescent cancer cells in vitro and in NOD / SCID mice. Oncotarget. 2017;8(6):9303–22.

360. Vera-Ramirez L, Vodnala SK, Nini R, Hunter KW, Green JE. Autophagy promotes the survival of dormant breast cancer cells and metastatic tumour recurrence. Nat Commun.

2018 Dec 22;9(1):1944.

361. Wakita M, Takahashi A, Sano O, Loo TM, Imai Y, Narukawa M, et al. A BET family protein degrader provokes senolysis by targeting NHEJ and autophagy in senescent cells. Nat Commun. 2020 Dec;11(1).

362. Sui X, Fang Y, Lou H, Wang K, Zheng Y, Lou F, et al. p53 suppresses stress-induced cellular senescence via regulation of autophagy under the deprivation of serum. Mol Med Rep. 2015 Feb 1;11(2):1214–20.