ELUCIDATION OF THE ROLE OF AUTOPHAGY AND MITOPHAGY IN RADIATION RESPONSE

THESIS

Submitted to Delhi Technological University
for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

MADHURI CHAURASIA

DEPARTMENT OF BIOTECHNOLOGY
DELHI TECHNOLOGICAL UNIVERSITY
DELHI, 110042

JUNE 2019

Dedicated

to

My Family

DECLARATION

I, Madhuri Chaurasia, certify that the work embodied in this thesis entitled **"Elucidation of the role of Autophagy and Mitophagy in Radiation response"** to be submitted for the Degree of Doctor of Philosophy is my own bonafied work carried out under the joint supervision of **Dr. Kulbhushan Sharma** (Scientist' D', Institute of Nuclear Medicine and Allied Sciences, DRDO, Delhi) and **Dr. Asmita Das** (Assistant Professor, Department of Biotechnology, Delhi Technological University) for a period of July 2014 to June 2019 at INMAS and DTU. The matter embodied in this thesis has not been submitted for the award of any degree/ diploma.

I declare that I have faithfully acknowledged, given credit and referred to the research workers wherever their works have been cited in the text and the body of the thesis.

Date:

Place:

Madhuri Chaurasia
(Reg. No: 2K14/Ph.D/BT/07)

DELHI TECHNOLOGICAL UNIVERSITY

CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"Elucidation of the role of Autophagy and Mitophagy in Radiation response"** submitted by **Madhuri Chaurasia (**Reg. No: 2K14/PhD/BT/07) to the Delhi Technological University, Delhi for the award of the degree of **Doctor of philosophy** is based on the original work carried out under our supervision. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other University or Institution for the award of any degree or diploma.

Dr. Kulbhushan Sharma
(Supervisor)
Scientist 'D'
INMAS
DRDO, Delhi

Dr. Asmita Das
(Supervisor)
Asst. Professor
Department of Biotechnology
Delhi Technological University

Prof. Jai Gopal Sharma
Head of Department
Department of Biotechnology
Delhi Technological University

ACKNOWLEDGEMENT

First of all I will to thank Almighty God who bestowed on me the determination and dedication over the years for successfully completing the research work, my enduring dream. The work presented in this thesis would not have been possible without my close association with many people. I take this great opportunity to thank all those people who have been in some or the other way being part of my struggle, success and failure of this long journey. I will like to extend my extreme gratitude to my PhD supervisors, ***Dr. Kulbhushan Sharma (Sci. D, INMAS)*** *and* ***Dr. Asmita Das (Assistant Prof. Dept. Of Biotechnology, DTU)*** *for their constant and impeccable encouragement and guidance throughout the course of my research work. I will be forever indebted to Kulbhushan Sir for believing me and providing me his round the clock help, guidance and freedom he gave to me as a researcher to fulfill my principal aspiration i.e. PhD. I have no words to express my gratefulness to him. I thank him for although being a guide and acting as a true guardian. His parental nature to his students and assistance even at the dead line stand him apart as a guide. Throughout my life, I will cherish the time and effort I spend with him and in his lab.*

I am truly indebted to ***Dr. B.S. Dwarakanath*** *who gave me constant encouragement and invaluable suggestions throughout, his motivating and enthusiastic approach will always be a source of inspiration to me.*

I am extremely grateful to ***Dr. Ajay Kumar Singh ex- Director INMAS****, For providing me the platform to accomplish my research work and taking keen interest in the ongoing programmmes at the institutr and giving deadlines to supervisors of research scholars that every resraech scholars work shouls be justified and to be full fillled well before the completion of fellows research tenure. I would also like to aapreciate* ***Dr Tarun Sekhari, Director INMAS*** *for providing me the opportunity to work as a researcher in such an esteemed institute. I feel privileged to be part of INMAS and express my thankfulness to him for getting the best infrastructure for efficient working of research facility.*

I want to expresse my sincere gratutude to ***Prof. Yogesh Singh, Vice Chancellor DTU*** *for providing me a privilege to be part of this exteemed university as a research scholar.*

I take this opportunity to acknowledge ***Prof. Jai Gopal sharma, Head, Dept. of Biotechnology DTU*** *for his kind support.*

My gratitude will also extend to ***Dr. Kailash Manda, Sci. F*** *for being always a helping hand when I was in a crunch situation. He always extended his support to me whenever I needed it. I thank him for being a temporary supervisor for me during my guide's study leave. I will also be grateful to him encouraging me, providing an insight and steering me to right direction.*

I will also like to express my gratitude to ***Dr. Anant Narayan Bhatt, Sci E*** *for providing his constant technical support and enchouragement in the absence of my Mentor. I express my sincere gratitudt to* ***Dr. Ravi Soni, Sci E*** *for his constant help in providing research materials, enthusisasm and encouragements and* ***Dr N K Chaudhary , Sci G*** *and* ***Dr. Paban Kumar Agarawala Sci. F*** *for free access to their lab, for their kindness and constant enchoragements and always happening tea or lunch parties.*

I also feel great to express my thankfulness to my closes't friends ***Mrinalini Tiwari Akanchha, Aastha, Ankit Chauhan, Sandeep, Teena Haritwal, Noopur Gupta, Neeraj Kumari, Bincy Babu*** *and* ***Anuranjini*** *for their constant help to me either scientifically or personally. They have been a solid force behind my determination and dedication during my tough and easy days as a researcher. I specially express my gratitude to* ***Attinder Chaddha*** *and* ***Ayushi Singhal from ACBR, DU*** *who were always been there for me.*

I also extend my thanks to my lab colleagues ***Shashank Misra, Simran Kaur, Late Mr. Abdullah, Suchit from INMAS*** *and* ***Richa Sharma and Divya Joshi from DTU*** *for their support for fulfillment of my research endeavours and making lab atmosphere joyous and congenial.*

I am also indebted to the technical staff from DTU ***Mr. C. B. Singh, Mr. Jitendra*** *and* ***Soumya Maurice*** *fro smooth running of the technical fecilities and infomations well before their deadlines.*

I appreciate the technical staff of INMAS ***Mrs Namita Kalra, Mrs Anjali, Dinesh Singh*** *and* ***Mr Nitin Kumar*** *for smooth running of the machineries in research facility and helping me in achieving my research objectives.*

I gratefully acknowledge ***Indian Council of Medical Research*** *for providing financial support to enable me to carry out research in this esteemed institute.*

I also want to express my deepest gratitude to ***Mr. Rahul singh*** *for his unconditional support motivation , great patience and inspiration. I am indebted to him for being caring and helping in every good or bad experiences of my life. He has been a strong pillar of strength to me, a sheer and constant force behind my thesis writing. I thank him for being there in my life.*

My utmost gratitude and respect goes towards my ***parents*** *who have always being my strength. Their constant endeavors and encouragement has kept me motivated throughout my journey as researcher. I have no words in expressing my gratitude to them what they mean in my life. I feel blessed and honored to be their child.*

I appreciate all whom I mentioned here or forgot to mention here for their support.

Madhuri Chaurasia

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAA	:	ATP associated with diverse cellular activities
ACTB	:	actin, beta
ALFY	:	autophagy-linked FYVE protein
AMBRA-1	:	activating molecule in Beclin 1-regulated autophagy
AMPK	:	5′-AMP-activated protein kinase
ATM	:	ataxia-telangiectasia mutated protein
ATF6	:	activating transcription factor 6
Atg	:	autophagy related proteins
BafA1	:	bafilomycin A1.
Bag1	:	BCL-2-associated athanogene1
BECN1	:	beclin1
CASP3	:	caspase 3
$[Ca^{2+}]$	:	Calcium ion
$[Ca^{2+}]I$	:	intracellular calcium ion
CHIP	:	heat-shock cognate70-interacting protein
CQ	:	chloroquine
DBSA	:	3,5-dibromosalicylaldehyde
DDR	:	DNA damage response
DRAM	:	damage-regulated autophagy modulator
2-DG	:	2-deoxy-D-glucose
EIF2AK3/PERK	:	eukaryotic translation initiation factor 2 alpha kinase 3
EIF2AK3-EIF2S1/eIF2alpha	:	eukaryotic translation initiation factor 2, subunit 1 alpha
ERN1/IRE1	:	endoplasmic reticulum [ER] to nucleus signaling 1
GI	:	Gastrointestinal

HCQ	:	hydroxychloroquine
HI	:	Hematopoietic
HR	:	Homologous recombination
HSC70	:	heat-shock cognate70
HSPA5/GP78	:	heat shock protein 5
IR	:	Ionizing radiation
iNOS	:	inducible nitric oxide synthase gene
IP3	:	Inositol 1,4,5-trisphosphate
JNK	:	c-Jun N-terminal kinase
KLF6	:	Kruppel like factor 6
LC3	:	Microtubule-associated protein light chain 3
LIR	:	LC3 Interacting Region
MFN1	:	mitofusin1
MFN2	:	mitofusin2
3-MA	:	3-methyladenine
NAC	:	N-acetyl-L-cysteine
NHEJ	:	Non-homologous end joining
PARL	:	Presenselin associated, rhomboid-like protease
PARP1	:	PolyADP-ribose polymerase 1
4-PBA	:	4-phenylbutyrate
PDT	,	photodynamic therapy
PINK1	:	PTEN induced putative kinase 1
PMN	:	Piecemeal microautophagy
PtdIns3K	:	class III phosphatidylinositol 3-kinase
Rap	:	rapamycin
RIP1	:	receptor interacting protein 1
RNS	:	reactive nitrogen species
ROS	:	reactive oxygen species

RyR	:	Ryanodine receptor
SASP	:	senescence associated secretory phenotype
TORC1	:	Target of rapamycin complex 1
TSC1/2	:	Tuberous Sclerosis Complex1/2
ULK1	:	Unc-51-like kinase
UPR	:	Unfolded protein response
UPS	:	Ubiquitin proteasomal system
VDAC	:	Voltage-dependent anion channel
Vps34	:	Vacuolar protein sorting 34
XBP1	:	x-box binding protein 1

LIST OF PUBLICATIONS

Publications

1. **Madhuri Chaurasia**, Swapnil Gupta, Asmita Das, B.S. Dwarakanath, Anne Simonsen, Kulbhushan Sharma. "Radiation induces EIF2AK3 (PERK) and ERN1 (IRE1) mediated pro-survival autophagy". *Autophagy 2019 (Impact factor- 11.1)*

2. **Madhuri Chaurasia,** Anant Narayan Bhatt, Asmita Das, Bilikere Dwarakanath and Kulbhushan Sharma. Radiation induced Autophagy: Mechanisms and Consequences. *Free Radical Research 2016 (Impact factor 3.1).*

3. **Madhuri Chaurasia**, Asmita Das, Kulbhushan Sharma. Role of Autophagy in recovery of Ionizing radiation induced intestinal damage in C57BL6 mice. *(Manuscript in preparation).*

4. **Madhuri Chaurasia**, Shashank Misra, Anant N Bhatt, Asmita Das, Bilikere Dwarakanath and Kulbhushan Sharma. "Metabolic imbalance associated mitophagy in tumor cells: genesis and implications". *Journal of Cancer Research Updates 2015.*

List of Conferences

1. International conference on radiation research **(ICRR-HHE 2018**). 'Role of autophagy in recovery of ionizing radiation induced intestinal damage in C57BL6 mice'. **Madhuri Chaurasia**, Asmita Das, Kulbhushan Sharma.

2. **Keystone Symposia conference on Autophagy Network integration in health and disease** (B1), 2017, Copper mountain, Colorado, USA. 'Radiation induces ROS dependent prosurvival autophagy in macrophages by activation of UPR pathways'. **Madhuri Chaurasia**, Asmita Das, B.S. Dwarakanath, Kulbhushan Sharma.

3. International conference on radiation biology **(ICRB 2016**). 'Radiation induces ROS dependent prosurvival autophagy'. **Madhuri Chaurasia**, Asmita Das, B.S. Dwarakanath, Kulbhushan Sharma.

4. International conference on radiation biology **(ICRB 2014**). 'Radiation induces ROS dependent and UPR mediated prosurvival autophagy in Raw264.7 macrophages'. **Madhuri Chaurasia**, Asmita Das, B.S. Dwarakanath, Kulbhushan Sharma

ABSTRACT

Autophagy is well preserved cellular recycling machinery which elicits upon numerous cellular insults including hypoxia, starvation, pathogenic infection etc. for salvaging damaged cellular cargos, thus helping in maintaining cellular homeostasis. Autophagic pathway starts with formation of a double membrane organelle i.e. autophagosome, which engulfs cellular cargos destined to recycle. Major categories of autophagy includes macroautophagy, microautophagy and chaperon mediated autophagy. Among the other forms of autophagy, Macroautophagy is most widely studied.

Autophagy has become an emerging scientific filed of research in the context of cancer resistance/ sensitivity, hypoxic signalling, nutrient starvation and in numerous harmful pathogenic infections including TB etc. or other harmful stress conditions.

Due to advancements in technologies, incidents and accidents of radiation exposure (due to natural calamities) leading to leakage of radioactive material in the environment imposes a great threat to mankind. Accidental/ incidental radioactivity leakage demands trained personnel (first responders) to go to the accident site for the help of individuals' stuck in these sites, who are in need of immediate medical help. The first responders going to those sites impose themselves to radioactivity exposure. Under these conditions, radioprotection strategy may be helpful. In order to attain better radioprotective response, a deep knowledge of cell survival responses and their modulation under radiation exposure in order to attain improved protection strategies is required.

Since radiation-induced macromolecular damage is associated with ROS generation and UPR induction, we hypothesised that autophagy may get induced to recycle damaged macromolecules (cargos), thereby protecting the cell against the radiation stress. The present study was started with the specific aim to understand the link between autophagy, ER stress and ROS generation, and the impact of this link on cell survival during radiation induced stress conditions. Macrophages serve as an important line of defense under most of the stress conditions in our body. Therefore, in this study, we investigated the induction of autophagy following irradiation in murine macrophage cells both *in-vitro* and *ex vivo*. We found that radiation induced autophagy is ROS-dependent and proceeded by UPR, specifically through the activation of EIF2AK3/Eukaryotic translation initiation factor 2 alpha kinase 3 (PERK) and ERN1/Endoplasmic reticulum to nucleus signaling 1

(IRE1) UPR pathways. Further we confirmed our *in-vitro* findings at systemic levels as well. In next part of our study, we extended our work in murine model system using pharmacological modulators of autophagy; Rapamycin and Chloroquine. We found enhancement in mice survival and better intestinal recovery in autophagy inducer treated mice following radiation exposure.

Nucleus is a vital cell organelle, containing genetic information in the form of DNA. Exposure to ionizing radiation can lead to direct as well as indirect DNA damage depending on the dose of exposure. Ionizing radiation generates excessive ROS/RNS leading to an indirect DNA damage and genomic instability. Therefore, there are indirect evidences available which suggests the possible role of radiation induced autophagy in genome integrity maintenance. We further performed study to comprehend the differential response of tumorigenic colon carcinoma and normal intestinal cells towards radiation exposure induced autophagy and its association with radiation induced DNA damage response. We found that cells (normal/tumorigenic) were showing differential response in autophagy levels as well as final cell fate. Furthermore, distinct DNA damage repair pathway induction was found in different cells in a cell type specific manner.

After investigating the differential role of autophagy, we also investigated the specific induction of mitochondrial recycling through autophagy (mitophagy) following radiation exposure and its role in cellular providence if any. Under conditions of extensive mitochondrial damage, the cell adapts mitophagy in order to exterminate the damaged and dysfunctional mitochondria. In this way, mitophagy results in cell survival after harmful stress condition injury. Most of the radiation exposure induced ROS/RNS is largely produced in the mitochondria. Mitochondria are known to play an important role in radiation-induced cellular response, but the underlying mechanisms are largely unknown. Therefore, after establishing the role of radiation induced autophagy in deciding cellular fate decision under radiation exposed conditions,

We next elucidated the role of mitophagy during radiation exposure (if at all its induction occurs specifically or not after radiation exposure). We found cell survival improvement following mitophagy induction in radiation exposed cells further suggesting prosurvival role of mitophagy post radiation exposure by recycling of damaged mitochondria.

Chapter 1

Introduction

CHAPTER 1

INTRODUCTION

Autophagy is a process where the cell starts recycling its damaged cellular cargos; organelles and proteins during cellular trauma (like nutrient deficiency, hypoxia, pathogenic infection etc.) by delivering damaged cytoplasmic constituents, destined to recycle to the lysosome (Mizushima N, et al. 2004, Levine B, et al, 2007), thus, helping in maintaining cellular homeostasis. The process has been shown to promote cells towards survival under various stress conditions but excessive autophagy may also lead to autophagic cell death. Various steps involved in autophagy include sequestration, transport of damaged cargos to lysosomes, their degradation and recycling (Levine B. et al, 2007, Levine B. and Kroemer G., 2008). In autophagy a unique double membrane organelle, autophagosome is formed, which engulfs cellular cargos (either damaged or destined to recycle) of cytoplasm. In addition to recycling of cargos, autophagy also plays other different roles including organelle and protein quality control. As autophagy is involved in cell growth, survival, development and death, its levels must be regulated cautiously. Dysregulated autophagy has been linked to many human pathophysiologies such as cancer, myopathies, neurodegeneration, heart and liver diseases, gastrointestinal disorders etc (Levine B. and Kroemer G., 2007; Mizushima N. et al., 2008).

There are mainly three types of autophagy namely macroautophagy, microautophagy and chaperon mediated autophagy. Among all these forms, macroautophagy is the most extensively studied (Mizushima N. et al., 2004). In addition, specific terms have been given for the forms of autophagy involved in the selective removal of damaged

organalles; pexophagy (for peroxisome recycling, occurs when cells adapt to glucose metabolism), ribophagy (autophagic removal of ribosomes) (Nair U, et al 2005, Mao K, et al., 2013), xenophagy (autophagic degradation of pathogenic microbes) and mitophagy (for recycling damaged or older mitochondria) (Nair U, et al 2005, Zhang et al., 2007, Mao K, et al., 2013).

Autophagy has a provocative role as it promotes the cells towards cell survival but at elevated levels it may also lead to cell death through apoptosis (Marino G, et al., 2014). Cancerous cells in comparison to their normal counterparts are more competent to chemotherapy, as they are highly efficient to escape any kind of stress. It has been reported in several studies that cancerous cells may use enhanced levels of autophagy for their survival during chemotherapeutic stress. Thus, combination of chemotherapy with autophagic inhibitors has been suggested be a better strategy in certain cancer types and their stage (Li T, et al., 2013).

Autophagy is a very well conserved process and is observed in various organisms including yeast as well as mammals. Signalling pathway linked with autophagy induction include PI3K-Akt pathway and TORC1 and 2 (target of rapamycin complex 1 and 2) (Mizushima N. et al., 2007, Marino G, et al., 2014). Various specific stress sensors like IRE1 (inositol requiring ER to nucleus signal kinase 1), PERK (RNA dependent protein kinase like ER kinase), ATF6 (activating transcription factor 6) present over the endoplasmic reticulum membrane gets activated due to stress, leading to the activation of unfolded protein response (UPR) within a cell which is commonly known as ER stress. Certain studies have shown the association between ER stress and autophagy (Li T, et al., 2013). The specific ER stress markers include HSPA5 (GRP78),

ERN1 (IRE1), EIF2AK3 (PERK), ATF6. The ER stress in-turn induces autophagy by negatively regulating the levels of AKT/TSC/MTOR pathway (Ding WX, et al., 2007, Li T, et al., 2013).

Rationale

Radiation exposure results in the damage of exposed organs and cells, leading to both acute radiation syndrome and delayed effects. After exposure, besides the cutaneous syndrome, three types of acute radiation syndromes may arise in a dose dependent manner, namely Hematopoietic (HI), Gastrointestinal (GI) and central nervous system (CNS) syndrome. Hematopoietic, Gastrointestinal, skin and vascular endothelium are amongst the most radio-sensitive organs (Fliedner TM, et al., 2007, Gamerdinger M, et al., 2009, MacNaughton WK., 2000). Cellular effects caused by IR exposure include mutation and transformation that arise from oxidative damage to macromolecules (DNA, protein and lipids), alterations in cell and nuclear membrane permeability, chromosome aberrations and metabolic imbalances. At the systemic level, decrease in lymphocytes, macrophages, neutrophils, stem cells and disturbance in tissue integrity takes place, which may (depending on the level of exposure) finally lead to multiple organ failure, resulting in mortality and morbidity. A number of intracellular events are initiated/activated including generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), activation of p53/Bax pathway, increase in DNA double strand breaks (DSB), single strand breaks (SSB) and activation of different signaling pathways involved in apoptosis, growth and autophagic induction (Waselenko JK, et al., 2004, Kiang JG, et al., 2013). Radiation induced oxidative stress can also lead to compromised mitochondrial functioning, protein misfolding and ER stress, besides DNA damage. Most of these factors have been shown to induce autophagy (Buytaert E,

et al., 2007, Farrukh MR, et al., 2014). However, detailed mechanisms underlying the induction of autophagy after radiation exposure has not been completely elucidated.

Despite concerted efforts over the last few decades, the exact role of autophagy in cellular radiation response has remained controversial. Two schools of thought exist; one suggests that it is a cell survival phenomena while the other nurtures the notion that autophagy is a type II programmed cell death helping the removal of affected cells. Current understanding suggests that the type, extent and time of stress are important determinants of the fate of a cell following autophagy induction (Li J, et al., 2009, Pang XL, et al., 2013, Zhang X, et al., 2014).

The process of autophagy is stimulated during various cellular insults e.g. oxidative stress, endoplasmic reticulum stress, imbalances in calcium homeostasis and altered mitochondrial potential. Radiation-induced damage involves ROS generation leading to oxidative stress. In turn, oxidative stress may lead to various imbalances in the cell, including DNA damage, compromised mitochondrial functioning, protein misfolding etc. In contrast to other stresses, autophagy induction following exposure of cells to radiation has received little attention (Nakai A, et al., 2007, Chen Y, et al., 2009, Fulda S, et al., 2010, Yang Z, et al., 2013). Although, various studies have shown the induction of autophagy during radiation exposure, an in-depth analysis of the relationship has not been explored (Chen Y, et al., 2015, Hu JL, et al., 2018, Wang F, et al., 2018). Autophagy has been shown to affect the survival of various cancer types when exposed to radiation (Scriven P, et al., 2007, Black HS. 2004. Hu JL, et al., 2018). The endoplasmic reticulum (ER) is a crucial intracellular Ca^{2+} reservoir that serves as a platform for numerous cellular processes including translation, post-translational modification and proper folding. ER is also the starting point for sorting and trafficking

of proteins and lipids to various organelles and the cell surface. During ER stress, newly synthesized proteins are unable to fold properly, leading to a process collectively known as the unfolded protein response (UPR) (Scriven P, et al., 2007). During UPR, protein synthesis shuts down until removal of all unfolded proteins from the cell system. It has been well established that stress-induced ROS formation causes indirect macromolecular damage (to DNA, proteins and lipids) (Black HS. 2004, Briganti S, et al., 2003). It also elicits an activation signal to boost the cytosolic calcium load released from ER (Farrukh MR, et al., 2014). ROS generation thus causes activation of ER stress leading to the induction of UPR (Ding W, et al., 2012, Ron D, et al., 2007, Malhotra JD, et al., 2007). Although studies have shown a correlation between radiation, UPR and autophagy, the mechanisms are not very clear (Mikkelsen RB, et al., 2003, Buytaert E, et al., 2007, Kim KW, et al., 2010, Mac Naughton WK. 2000). Therefore, it is considered worthwhile to study the possible association between ROS, ER stress and autophagy following irradiation.

Since radiation-induced macromolecular damage is associated with ROS generation, we hypothesised that autophagy is induced to recycle damaged macromolecules (cargos), thereby protecting the cell against the radiation stress. This study focuses on the current understanding of the mechanisms underlying radiation induced autophagy and its association with macromolecular damage, oxidative stress and ER stress. Furthermore, it also explores the systemic response of radiation induced autophagy in murine model system and the final fate of irradiated mice model. Overall, we propose autophagy and mitophagic phenomena as an approach for radiomodification.

Accidential/ incidential clinical exposure to UV, X-rays, gamma rays etc. can lead three different types of acute radiation syndromes depending on the dose of exposure namely;

hematopoietic (HI), gastrointestinal (GI) and central nervous system (CNS) syndrome. Ionizing radiation exposure from 1-7 Gy dose results in hematopoietic syndrome while GI syndrome occurs after a whole body exposure of more than 8 Gy in humans. Acute effects of radiation exposure causing gastrointestinal syndrome in murine system are well studied, however we are yet to acquire a complete knowledge about the functional contribution of radiation induced autophagy in intestinal damage recovery in radiation exposed mice.

Nucleus, being foremost crucial cell organelle, it stores genetic information in the form of DNA. Within a cell DNA being the most critical target to be hampered upon exposure to ionizing radiation in both direct damage (due to high radiation dose) as well as by indirect DNA damage at low dose of exposure, thus leading to genomic instability (Wu LJ, et al., 1999). Nuclear-cytosolic shuttling of numerous autophagy related proteins e.g. p62 and ALFY (autophagy-linked FYVE protein) has been shown indicating the role of this phenomena in nucleus (Simonsen A, et al., 2004). Following stress, ALFY is extruded from the nucleus to cytoplasm and interacts with p62 bodies. Collectively, these circumstantial evidences suggests direct or indirect role of autophagy in the DDR and ROS/ RNS-mediated genotoxic stress. However, precise mechanisms underlying DDR mediated autophagy are still not very clear. Autophagy has also been shown to influence the dynamics of DNA repair wherein it helps in recycling of key proteins involved in the processing of lesions; besides aiding the metabolic precursors for the generation of ATP, as well as regulating the supply of dNTPs required for repair (Dyavaiah M, et al., 2011). Autophagy shows a pleomorphic role in the context of DNA damage response. Majority of the studies indicate that autophagy inhibition in cells treated with DNA damaging agents leads to enhanced cell death, supporting a pro-survival role for autophagy.

In addition to canonical autophagy, other similar processes which are involved in the removal of specific damaged organelles do exist. Mitophagy (specific removal of mitochondria) is one of them (Chaurasia M, et al., 2015). Mitochondrial oxidative phosphorylation leads to the generation of toxic by-products involving ROS, which cause oxidative damage to mitochondrial lipids, DNA and proteins, making mitochondria further prone to the production of excessive ROS. The damaged mitochondria in turn, release huge amount of calcium ions (Ca^{2+}) and cytochrome-c to the cytosol thereby triggering apoptosis (Saraste M., 1999, Wallace DC., 2005). On contrary to this, it is well established that radiation exposure leads to extensive mitochondrial biogenesis. However, under conditions of extensive mitochondrial damage, the cell can also adopt mitophagy in order to exterminate the damaged and dysfunctional mitochondria. The exact role of mitophagy in radiation exposed conditions has not been explored in greater details (Srivastava S, 2017). Keeping these facts in mind, the specific aim of our study was to understand the link between autophagy, mitophagy, ER stress and ROS generation, and the impact of this link on cell survival during radiation induced stress conditions. Finally, we tested the potential of using autophagy as a radio-modifier during radiation exposed circumstances.

Objectives

The research work was devised with the following objectives:-

1. To study the autophagic and mitophagic induction in cells exposed to radiation.
2. To study the effect of radiation induced autophagy on ER stress and DNA damage.
3. To understand the influence of autophagic modifications on programmed cell death (apoptosis) under radiation induced stress.

4. To compare the levels of radiation induced autophagy in malignantly transformed and untransformed cells.
5. To understand the effects of autophagy and mitophagy modifiers at systemic levels using animal model system.

Chapter 2

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

2.1 Radiation exposure causes and consequences

Radiation is a form of energy that comes from a radioactive material (such kind of materials have ability of self-decay) those can be both naturally occurring and synthetic substances. Radiation can be introduced in body by clinical exposure, radiation accident and incidents. Based on their ionization potential, radiations can be classified in two categories viz. ionizing (these radiations have the capacity to ionize matter exposed to it) and non-ionizing (these radiations do not ionize the substances exposed under it) radiations (Hall EJ, Giaccia AJ. eds. Radiobiology for the Radiologist, 6th ed. Philadelphia, PA, USA: Lippincott Williams & Wilkins, 2006). Light, radio, and microwaves are few examples of non-ionizing radiation. Risks associated with ionising radiation are more. When an ionizing particle travels through a material it loses its energy per unit distance which is known as Linear energy transfer (LET). Based on their LET, ionizing radiations can be High LET (such type of radiations deposit huge amount of energy in a short distance) for e.g. alpha particles and neutrons and low LET (such type of radiations deposit less amount of energy in their path) e.g. X-rays and gamma rays. If we talk about damage manifestations caused due to high LET is an easy task as these radiations are less penetrating therefore we can stop these radiations with a piece of paper in its path. While if we talk about low LET radiations, these are more penetrating and have more penetration potential and in-order to stop these radiations through strong concrete walls and protective lead sheets are required (Binks W, 1955).

High LET radiations causes cell damage by direct ionization of macromolecules including DNA, RNA, lipids, and proteins. On contrary to this, low LET radiations cause indirect macromolecular damage by production of reactive oxygen and nitorogen species (ROS/ RNS) (Hall EJ, Giaccia AJ. eds. Radiobiology for the Radiologist, 6th ed. Philadelphia, PA, USA: Lippincott Williams & Wilkins, 2006:16-180, Zhao W, et al., 2007, Lomax ME, et al., 2013).

2.1.1 Radiation induced effects; deterministic and stochastic

Deterministic effects are those effects which are certain to occur after radiation exposure. These effects are directly related to a known dose of radiation and have a dose threshold; their severity is also dose related (Choudhary S, 2018). Deterministic effects can also be described as radiation induced tissue toxicities as these effects are not predetermined at the time of irradiation (Stewart F.A, et al., 2012). Deterministic effects include- acute and chronic radiation syndrome.

Radiation exposure causes different types of effects which are in the form of acute and chronic radiation syndrome. *Acute radiation syndrome*- these effects arise within 24 to 48 h after radiation exposure. These are seen in tissues with rapid turnover e.g. gastrointestinal tissue, bone marrow, skin and esophageal mucosa. Acute radiation damage leads to induction of various cell signalling responses within an exposed tissue which ultimately has effects in exposed person's health. *Chronic radiation injury* occurs on exposure due to low or mild radiation doses due to repeated exposures. The symptoms may take months to years to get developed to traceable extent (Grammaticos P, et al., 2013, Reeves GI, et al., 1995). *Chronic radiation injury* - include atrophy, necrosis, ulceration, metaplasia, dysplasia or neoplasia which can occur in epithelial

and parenchymal cells (Fajardo LG LF. et al., Principles & Practice of Radiation Oncology, 3rd ed. Philadelphia, New York : Lippincott-Raven, 1998;143-154).

Hematopoietic, gastrointestinal tract, lymphoid organs are the most radiosensitive organs. Therefore, acute radiation syndrome includes; hematopoietic syndrome (HI), gastrointestinal syndrome, and central nervous system syndrome (Macia IGM, et al., 2011). Hematopoietic syndrome sometimes referred as bone marrow syndrome; also it occurs usually at low dose range (1-7 Gy in humans). During hematopoietic syndrome there is leucopenia, thrombocytopenia and reduction in lymphocytes with anaemia and delay in wound healing. Gastrointestinal syndrome occurs usually with dose range >7 Gy and characterized by infection, dehydration, loss of electrolytes and diarrhoea. GI syndrome may occur along with hematopoietic syndrome. Central nervous system syndrome occur usually with the exposure of 10 Gy or more and show symptoms like perplexing situation, delusion, cerebral edema, hypotension etc (Dainiak N, 2018).

2.2 Radiation induced cellular fate mechanisms

The induction of cell fate mechanisms upon exposure to radiation depends upon radiation dose, type of radiation, cell susceptibility and magnitude of radiation exposure (Eriksson D, et al., 2010). Main signalling pathway activated following irradiation mainly pushes the cell towards death response which may be induced via diverse modes including apoptosis (programmed cell death), necrosis, senescence, autophagy and mitotic catastrophe (Figure 2.1). These pathways can be described as follows:

2.2.1 Apoptosis

Programmed cell death, is a highly regulated cell death mechanism. The cells undergoing apoptosis can be identified by distinct morphologic changes in cytoplasmic and nuclear

constituents. Characteristic features of a cell undergoing apoptosis are- cell shrinkage, membrane blebbing, nuclear condensation, DNA fragmentation etc. (Fuchs Y, et al., 2011). Two key pathways of apoptosis induction are intrinsic and extrinsic pathways. The intrinsic pathway is triggered by internal cell signaling, regulating mitochondrial integrity, mitochondrial Cytochrome-c release and *via* apoptosome complex formation. On the other hand, the extrinsic pathway is activated by extracellular signals transduced by transmembrane "Death Receptors" mainly Fas with Fas Ligand, which belong to the tumor necrosis factor (TNF) receptor superfamily (Sinha K, et al.,2013, Riedl SJ, et al., 2007). Both intrinsic and extrinsic apoptotic pathways may occur in radiation exposed cells, depending upon delivered doses and cell type (Panganiban RA, et al., 2013).

2.2.2 Necrosis

Radiation exposure can also induce cell death in exposed cells *via* induction of non-programmed cell death which is also known as necrosis. Necrotic cells can be characterised by distinct morphological features, such as plasma membranes permeabilization with loss of intracellular contents, organelle swelling, mitochondrial dysfunction etc. (Golden EB, et al. 2012). In addition to necrosis, a special mechanism of regulated necrosis or programed necrosis is defined as necroptosis (Degterev A, et al., 2005, Zhou W, et al., 2014). It may be induced during inefficient apoptotic conditions *via* death receptor ligands. Necroptosis induction within a cell requires kinase activity of RIP1 (receptor interacting protein 1), which facilitates the activation of RIP3 (kinase) and MLKL (critical downstream mediators of necroptosis). Necroptosis can be inhibited by necrostatins which blocks the kinase activity of RIP1 (Degterev A, et al., 2008, Cho YS, et al., 2009). Nowadays, scientists are targeting necroptosis inducers for achieving better radiosensitization of tumor cells by induction of RIP1 or its downstream effectors (Nehs MA, et al., 2011).

2.2.3 Mitotic catastrophe

It is a type of cell death that occurs during mitosis, hence the name, mitotic catastrophe (Vakifahmetoglu H, et al., 2008). Exposure to chemical or physical stresses including radiation exposure leads to premature entry of mitotic cells into mitosis finally inducing death of those cells. It can be initiated by many chemical agents like anticancer drugs, agents influencing microtubule stability, and mitotic failure caused due to defective cell cycle checkpoints. During mitotic catastrophe, multinucleated giant cells can be seen, therefore it can also be described as delayed form of reproductive death (Hall LL, et al., 1996, Waldman T, et al., 1996). The process may result in death that requires both caspase-dependent and caspase-independent mechanisms; therefore it may be considered as ('prestage') necrosis or apoptosis, (Castedo M, et al., 2004, Mansilla S, et al., 2006).

2.2.4 Senescence

Senescence is a stage during which cells stop dividing and enter into a stage of mitotic inactivity. It is a known strategy during aging or in irradiated tissues. Senescent cells show alterations in gene expression, deregulated expression of cell cycle regulatory proteins involved in cell cycle arrest, upregulation of anti-apoptotic factors, high expression of inflammatory cytokines, growth factors and proteases. All of these characteristics shown by senescent cells are known as senescence associated secretory phenotype (SASP) (Muller M. et al., 2009, Tchkonia T, et al., 2013). Increased expression of senescence-associated β-galactosidase (SA-β-gal has been correlated with senescence in many cell types. Lower doses of radiation may induce senescence in exposed cells which despite being vital are no longer competent for proliferation. It has been demonstrated that senescence is the principal response of some cell types at lower doses of radiation.

2.2.5 Autophagy

Autophagy is a cellular recycling mechanism involved in removal and recycling of damaged cellular components, thus helps in maintaining cellular homeostasis (Mizushima N, et al., 2004). Autophagy is considered as programmed cell survival phenomena in context of cancer cells. A detailed relationship between autophagy induction and radiation exposure has not been explored in greater details. Further details about autophagy have been explained in section 2.4.

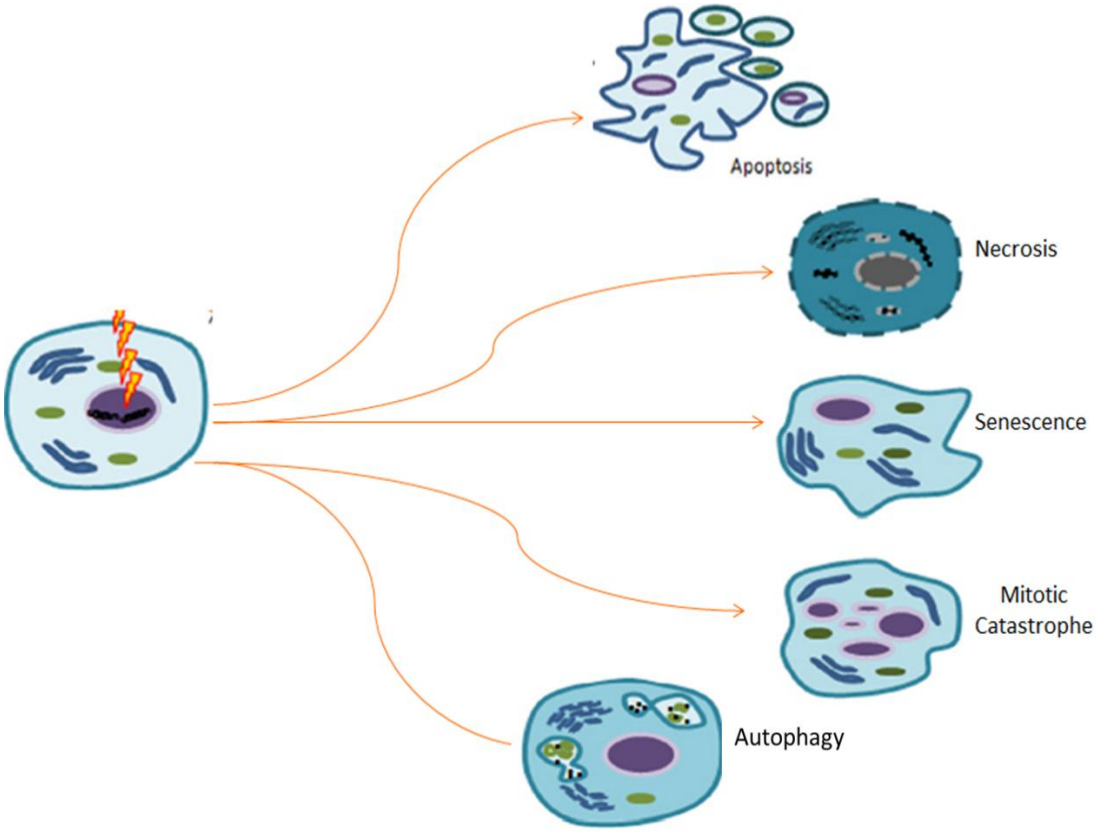


Figure 2.1: Radiation induced cellular fates. (Minafra L, et al., 2014)

2.3 Radiotherapy- Radio sensitization and radioprotection

Use of ionizing radiation to kill/inhibit tumor growth is known as radiotherapy. Radiotherapy, evolved over the years and become the key cancer treatment modality

worldwide (Johnke RM, et al., 2014). Over the past decade, radiotherapy has successfully been employed in cases of colon, stomach, cervix, head and neck, lung and brain tumor (Macdonald JS, et al., 2001, Pearcey R, et al., 2002, Stupp R, et al., 2005). Radiotherapy alone or in combination with chemotherapy, target cancer cells by inducing DNA damage, reducing rate of DNA damage repair and by induction of various signalling cascades including apoptosis, autophagy etc (Kolesnick R, et al., 2003). During radiotherapy, in addition to tumor cell killing, normal cells present in the vicinity of tumor microenvironment also get damaged (Johnke RM, et al., 2014). It has been identified that alterations in response to radiation leading to cancer cell killing can be classified as radio sensitization, whereas, alternation in response which helps in preventing normal tissue damage against radiation is classified as radioprotection (Spalding AC, et al., 2006).

2.3.1 Radio sensitization

Use of specific agents alongwith radiation to kill tumor cells is known as raiosensitization (Raviraj J, et al., 2014). Radio sensitization is achievable either alone or in combination with additional treatments. Radiotherapy in combination with chemotherapy causes more sensitization of cancer cells at the cellular and tissue levels (Blanco R, et al., 2008). Radisensitizers work on the principle by enhancing oxygen sensitivity to hypoxic tumor cells (Hyperbaric Oxygen agents), agents which alters biochemical synthesis pathways in tumor cells (Radiosensitizing Nucleosides; Fluoropyrimidines, Thymidine analogs, Hydroxyurea, Gemcitabine etc.) and some Novel Radiosensitizers include; Taxanes (Paclitaxel) and Irinotecan, Tunicamycin and Rapamycin etc. Some clinically approved Radiosensitisers is shown below in Table 2.1 (Raviraj J, et al., 2014).

Table 1: List of various Radiosensitizers (Raviraj J, et al., 2014).

Hyperbaric oxygen
Carbogen
Nicotinamide
Metronidazole and its analogs (misonidzole, etanidazole, nimorazole)
Hypoxic cell cytotoxic agents (Mitomycin-C, Tirapazamine)
Membrane active agents (procaine, lidocaine, chlorpromazine)
Radiosensitizing nucleosides (5-Fluorouracil, Fluorodeoxyuridine, Bromodeoxyuridine, Iododeoxyuridine, Hydroxyurea, Gemcitabine, Fludarabine)
Texaphyrins (motexafin gadolinium)
Supressors of sulfhydral groups (N- Ethylmalemide, Diamide and Diethylmaleate)
Hyperthermia
Novel radiosensitizers (paclitaxel, docetaxel, irinotecan)

2.3.2 Radioprotection

The increase of radiation applications in our daily life has made us susceptible to accidental radiation injury (Mishra KN, et al., 2018). There is urgent need of radiation countermeasure agents to counteract any radiation incidents and accidents. Accidental radiation exposure leads to radiation injury to normal tissue, there is a need to identify measures to dodge normal tissue toxicity (Hosseinimehr SJ, 2007). During past decade, immense progress has been made in the field of radiotherapy. However, not much success has been achieved to reduce normal tissue toxicity in vicinity of tumor, owing to no clear demarcation between tumor and normal tissue (Bourgier C, et al., 2012, Citrin D, et al., 2010). A new school of thought has been the dose optimization for radiotherapy, which would either increase radioresistance of normal cells and the same dose leads to enhanced radio sensitization of tumor cells (Kumar S., et al., 2016) Another school of thought is working in the direction of development of radiation countermeasure agents (synthetic or natural) to avoid toxicity to normal tissues. These agents may work as radioprotectors/mitigators (Singh VK, et al., 2015b).Agents or

molecules that are given prior to the exposure to radiation are classified as radiprotectors; those agents that can be administered immediately to get protection after radiation exposure before emergence of symptoms are known as radiomitigators (Mishra KN, et al., 2018). Most of the radioprotectors have been stated to act through free radical scavenging mechanism; on the other hand mitigators employ strategies targeting the DNA repair mechanism, signal transduction pathways and inflammation (Johnke RM, et al., 2014).

2.4 Autophagy and radiation exposure

Autophagy is an evolutionary conserved, indispensable, lysosome mediated degradation process, which helps in maintaining homeostasis during various cellular traumas. During stress, a context dependent role of autophagy has been observed which drives the cell towards survival or death depending upon the type, time and extent of the damage. The process of autophagy is stimulated during various cellular insults e.g. oxidative stress, endoplasmic reticulum stress, imbalances in calcium homeostasis and altered mitochondrial potential. Ionizing radiation causes ROS dependent as well as ROS independent damage in cells that involve macromolecular (mainly DNA) damage, as well as ER stress induction, both capable of inducing autophagy. A basal level of autophagy is maintained in a healthy cell to sustain cellular homeostasis, which gets modulated under stress conditions (like starvation, hypoxia etc.). The process has been shown to promote cells towards survival but excessive autophagy may also lead to autophagic cell death. Various steps involved in autophagy include sequestration, transport of cargo to lysosomes, degradation and utilization of the degraded products (Levine B, et al., 2007, Levine B, Kroemer G. et al., 2008). In autophagy, a unique double membrane organelle, autophagosome is formed, which engulfs the cellular

cargos (either damaged or destined to recycle) (Levine B, et al., 2007). As autophagy is involved in cell growth, survival, development and death; its levels must be regulated properly. Ionizing radiation causes macromolecular (DNA, protein and lipid) damage and imbalances in metabolism eliciting several intracellular responses that collectively determine the fate of the irradiated cell.

2.4.1 Induction of autophagy

Autophagy is a well conserved process observed in various organisms including yeast as well as mammals. The proteins involved in autophagy are known as autophagy related proteins (Atg). These include a series of proteins from Atg1 to Atg32. Under experimental conditions, autophagy initiation is marked by taking into account few parameters which include (i) LC3-II (Atg 8) to LC3-I ratio, (ii) increased levels of Atg5-Atg12 complex, (iii) increased levels of Beclin1 and (iv) decreased levels of p62 (Mizushima N. et al., 2007, Marino G, et al., 2014).

Various signaling pathways are associated with autophagy induction which include PI3K-Akt pathway and TORC1 and 2 pathways (target of rapamycin complex 1 and 2) (Mizushima N. et al., 2007). The TORC1 is rapamycin sensitive and gets inhibited in its presence, leading to the stimulation of autophagy. Under normal conditions, TORC1 remains active and keep a check on autophagy induction (Noda T, et al., 1998). During starvation on the other hand, Atg1 is dephosphorylated to take part in autophagosome formation. After activation, the binding affinity of Atg1 (ULK1) to Atg13 and Atg17 gets enhanced by several folds, leading to the stimulation of Atg1-Atg13-Atg17 scaffold, which further helps in the recruitment of numerous Atg proteins thus initiating the autophagosome formation (Noda T, et al., 1998, He C, et al., 2009, Gozuacik D, et

al., 2008, Kabeya Y, et al., 2005). AMPK (5′-AMP-activated protein kinase) has also been known to play a role in autophagy induction. During metabolic stress, reduced cellular ATP concentration is detected by AMPK. In mammals, cellular AMPK is activated by a reduced ATP to AMP ratio through the upstream molecules. Activated AMPK causes phosphorylation and activation of the Tuberous Sclerosis Complex1/2 (TSC1/2), which inhibits MTOR activity (Inoki K, et al., 2003, Qin L, et al., 2010). Further, LKB1-AMPK may also phosphorylate and activate p27 kip1, a cdk inhibitor leading to the cell cycle arrest, which prevents apoptosis and induces autophagy for cell survival (Liang J, et al., 2007).

One of the key components required for autophagy induction is a specific complex called class III phosphatidylinositol 3-kinase (PtdIns3K) complex. This complex composed of PtdIns3K, Vps34 (vacuolar protein sorting 34), Vps15 in myristoylated form (p150), Atg14 (Barkor) and Atg6/Vps30 (Beclin 1 in mammals) is required for the assembly and initiation of phagophore membrane (Kihara A, et al., 2001, Itakura E, et al., 2008, He C, et al., 2009). PtdIns3K complex along with Atg proteins further recruits two ubiquitin-like conjugation complexes, Atg12-Atg5-Atg16 and Atg8-PE. Initially Atg12 is activated by Atg7, which is then transferred to Atg10 and finally covalently attaches with Atg5 protein. The Atg12-Atg5 further interacts with Atg16 to form Atg12-Atg5-Atg16 complex which finally gets attached to the phagophore (He C, et al., 2009). Ultimately Atg8 in nonlipidated form (LC3-I) attaches to Atg12-Atg5-Atg16 complex and gets converted into its lipidated form (LC3-II), leading to the elongation of autophagosome. After complete autophagosome formation, its outer membrane fuses with the lysosome to form autophagolysosome, where lysosomal hydrolases degrade damaged cargos (Kirisako T, et al., 2000, Mizushima N, et al., 2004). In mammalian

cells, the autophagosome and lysosome fusion requires a lysosomal membrane protein namely LAMP-2 and a small GTPase Rab7 (Jager S, et al., 2004, Tanaka Y, et al., 2000). Processes involved in the induction of autophagy following exposure of cells to stress have been briefly summarized in (Figure 2.2).

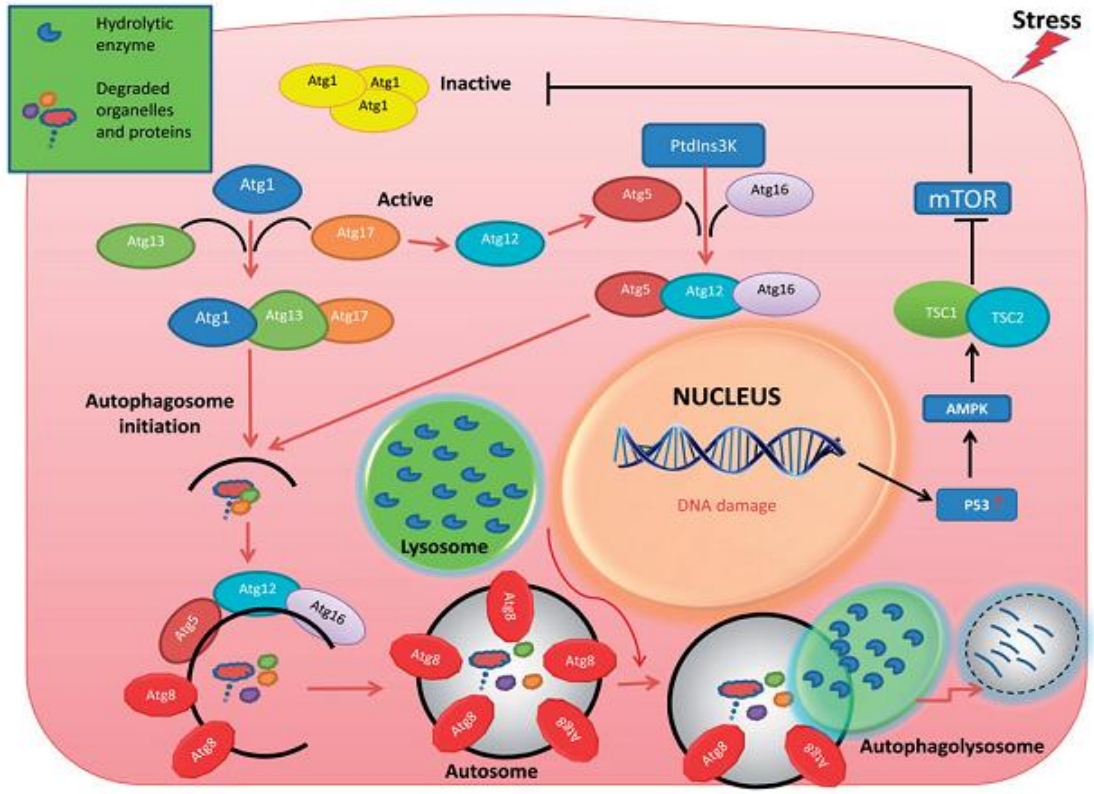


Figure 2.2: Schematic mechanism of autophagy induction. (Chaurasia M, et al., 2016).

Deregulated autophagy has been associated with various human pathophysiological conditions such as cancer, myopathies, neurodegeneration, heart, liver, and gastrointestinal disorders (Valente EM, et al., 2004, Kitada T, et al., 1998, Aita VM, et al., 1999). In most of these pathogenic conditions, autophagy has been shown to play a provocative role as indicated by the presence of mutated autophagy related genes like Beclin1, PARKIN and PINK1 in various cancers and neurodegenerative disorders (Valente EM, et al., 2004, Kitada T, et al., 1998). Literature suggests a close association between

cancer development and autophagy. Malignant cells with enhanced autophagy appear to be highly resistant to a variety of stress and chemotherapy in comparison to their normal counterparts. Thus, combination of chemotherapy with inhibitors of autophagy has been suggested to be a better strategy in these cases (Ding WX, et al., 2007, Li T, et al., 2013). In contrast, elevated levels of autophagy have also been shown to promote cell death through apoptosis (Marino G, et al., 2014, Maiuri MC, et al., 2007). Thus, in these cases autophagy acts as a barrier in cancer induction as well as progression.

2.4.2 Autophagy vs Proteasomal machinery

Autophagy and ubiquitin proteasomal system (UPS) are the two major machineries involved in the removal of misfolded or unfolded proteins and their aggregates. Due to its additional involvement in the recycling of damaged organelles, autophagic machinery is considered relatively more advanced in cargo recycling as compared to proteasomal machinery. Ubiquitination is the common step between proteasomal and autophagic degradation pathway. During this step, the ubiquitin moiety gets covalently attached with the protein to be recycled. The enzymatic cascade involved in ubiquitination are termed E1 (activation), E2 (conjugation) and E3 (ligation) (Hershko A, et al., 1998). Based on the conjugation of ubiquitin moiety, ubiquitination can be mono, bi and polyubiquitination. There are at least seven lysine residues on which ubiquitination can take place; these include K6, K11, K27, K29, K33, K48 or K63 (Ikeda F, et al., 2008).

The decision for the mode of degradation of a misfolded or damaged protein depends on several factors. One of these factors is the position of lysine residue to be ubiquitinated. For instance, K48 ubiquitin chains are considered as the classical signal

to target proteins for proteasomal degradation (Hershko A, et al., 1998). On the other hand, non-classical linkage type such as K63 ubiquitination signals for autophagic pathway (Haglund K, et al., 2005, Tan JM, et al., 2008). During autophagy, p62 and PARKIN are the two important E3 ubiquitin ligases which help in the removal of aggregated proteins. Among these, p62 have been found to be involved in K63-linked ubiquitination followed by the removal of cargos *via* both macro as well as specific autophagy. On the other hand, PARKIN can form K48 linked ubiquitin chains and cause removal of damaged protein *via* proteasomal machinery (Hattori N, et al., 2004). Conversely, when PARKIN forms K63-linked polyubiquitin chains on misfolded protein, it leads to the recruitment of ubiquitinated protein into aggresome finally helping in the removal of proteins *via* autophagic machinery (Olzmann JA, et al., 2007, Narendra D, et al., 2008, Kubota H, et al., 2009). Similarly, if the protein refolding is not successful, E3 ubiquitin ligase co-chaperone carboxyl terminus of heat-shock cognate70 (HSC70)-interacting protein (CHIP) may cause protein ubiquitination thereby selecting unfolded proteins for degradation preferentially through the proteasomal system. However, when chaperone mediated refolding and proteasomal system is overloaded, protein aggregation ensues; thus formed protein aggregates under UPS burdened condition are destined to autophagic machinery for removal (Kubota H. et al., 2009, Shaid S, et al, 2013, Gamerdinger M, et al., 2009). The possibility of coexistence of UPS and autophagy can't be ruled out.

The autophagic targeting of protein aggregates are determined by the 'LC3 Interacting Region' (LIR) motif of p62 and NBR1. Co-chaperones such as BCL-2-associated athanogene 1 (Bag1) and Bag3 also play crucial regulatory role in determining protein degradation pathway. Bag1 helps in the removal of ubiquitinated proteins *via* UPS,

whereas Bag3 helps in autophagic removal of degraded proteins (Fliedner TM, et al., 2007). In young cells, Bag1 co-chaperone expression is relatively higher as compared to Bag3, whereas in aged cells, protein aggregation gets enhanced leading to enhanced Bag3 expression. In fact, Bag1/Bag3 ratio plays key role in determining the predominant pathway for the removal of misfolded protein (Gamerdinger M, et al., 2009, Fliedner TM, et al., 2007).

The decision for removal of the complete organelles (mitochondria, peroxisomes etc.) *via* proteasomal or autophagic machinery is also signalled through ubiqutinated proteins present over these organelles. For instance, degradation of damaged mitochondria can take place either through removal of misfolded mitochondrial proteins or *via* complete and specific removal of mitochondria (mitophagy). Interestingly, in both conditions, misfolded proteins serve as the main initiating signals.

2.4.3 Radiation induced autophagy

Radiation exposure results in the damage of exposed organs and cells, leading to both acute radiation syndrome and delayed effects. After exposure, three different types of acute radiation syndromes may arise in a dose dependent manner namely Hematopoietic, Gastrointestinal) and central nervous system syndrome, besides the cutaneous syndrome (skin damage) independent of these three syndromes. Hematopoietic, Gastrointestinal, skin and vascular endothelium are amongst the most radio-sensitive organs (MacNaughton WK, 2000, Meistrich ML, et al., 1997, Coleman CN, et al., 2004). Doses in the range of 1 to 7 Gy results in hematopoietic syndrome in humans, which is associated with overall decline in blood cells, increased susceptibility of radiation exposed persons to several infections and haemorrhage. GI syndrome occurs

after a whole body exposure of more than 8 Gy (Waselenko JK, et al., 2004, Kiang JG, et al., 2010).

Cellular effects caused by IR exposure include death, mutation and transformation that arise from oxidative damage to macromolecules (DNA, protein and lipids), alterations in cell and nuclear membrane permeability, chromosome aberrations and metabolic imbalances. At the systemic level, decrease in lymphocytes, macrophages, neutrophils, stem cells and disturbance in tissue integrity takes place finally leading to multiple organ failure, resulting in mortality and morbidity depending on the level of exposure. A number of intracellular events are initiated/activated including generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), activation of p53/ Bax pathway, increase in DNA double strand breaks (DSB), single strand breaks (SSB) and activation of different signaling pathways involved in apoptosis, growth and autophagic induction (Gorbunov NV, et al., 2009, Kiang JG, et al., 2009, Mikkelsen RB, et al., 2003, Buytaert E, et al., 2007). Amongst the key molecules activated during radiation exposure, inducible nitric oxide synthase gene (iNOS) and nitric oxide (NO) have been shown to be involved in radiation induced apoptosis and autophagy (Kiang JG, et al., 2009, Mikkelsen RB, et al., 2003). As iNOS gene promotor region contains motifs of many transcription factors such as nuclear factor κB (NF-κB) and kruppel like factor 6 (KLF6), it results in increased NO production that causes caspase mediated apoptosis and protein nitration mediated autophagic induction (Kiang JG, et al., 2009).

Radiation induced oxidative stress can cause compromised mitochondrial functioning, protein misfolding and ER stress, besides DNA damage. Most of these factors have been shown to induce autophagy (Farrukh MR, et al., 2014, Nakai A, et al., 2007, Chen

Y, et al., 2009, Fulda S, et al., 2010, Zois CE, et al., 2009). However, detailed mechanisms underlying the induction of autophagy after radiation exposure has not been completely elucidated. Formation of acidic vacuoles has been found with increasing doses of radiation indicating an increased autophagic activity within these cells (Yi H, et al., 2013). Autophagy regulation during various stress condition e.g. hypoxia, nutrient starvation or ionizing radiation has also been linked to various micro-RNAs. More recent studies suggest the role of miR-199a-5p in autophagic regulation following irradiation (Codogno P, et al., 2013). Interestingly, autophagy has been reported to control miRNA biogenesis and activity, suggesting a feedback loop between miRNAs and autophagy (Pawlik TM, et al., 2004). Over expression of this miRNA has been shown to suppress radiation-induced autophagy in MCF7 breast cancer cell line (Yao KC, et al., 2003, Pang XL, et al., 2013).

Despite concerted efforts over the last few decades, the exact role of autophagy in cellular radiation response has remained controversial. Two schools of thought exist; one suggests that it is a cell survival phenomena while the other nurtures the notion that autophagy is a type II programmed cell death helping the removal of affected cells. Cumulative understanding suggest that the type, extent and time of stress are important determinants of the fate of a cell following autophagy induction (Schmukler E, et al., 2013, Zhang X, et al., 2014, Li J, et al., 2009, Sui X, et al., 2014, Chaurasia M, et al., 2015).

2.4.4 Mitochondrial association of autophagy

Mitochondrion is the energy currency for a cell and is indispensable for critical metabolic functions. The damaged, dysfunctional mitochondria have been linked with a series of patho-physiological conditions and neurodegenerative diseases (Valente EM, et al., 2004,

Kitada T, et al., 1998, Aita VM, et al., 1999). In addition to canonical autophagy, other similar processes which are involved in the removal of specific damaged organelles do exist. Mitophagy (specific removal of mitochondria) is one of them (Schweers RL, et al., 2007). Mitophagy is an important process involved in the development of reticulocytes to mature erythrocytes. Mitochondrial oxidative phosphorylation leads to the generation of toxic by-products involving ROS particularly superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) which cause oxidative damage to mitochondrial lipids, DNA and proteins, making mitochondria further prone to production of excessive ROS. The damaged mitochondria in turn, release huge amount of calcium ions (Ca^{2+}) and cytochrome-c to the cytosol and thereby trigger apoptosis (Wallace DC, 2005, Parsons MJ, et al., 2010, Langer T, et al., 2001).

Although, the consequences of mitophagy and detailed pathways have been poorly understood, accumulating evidences reveal that there are three major pathways by which mitochondrial quality control can be regulated. The first two are mitochondrial proteolytic systems. In the first one, AAA (ATP associated with diverse cellular activities) protease complexes present in the inner mitochondrial membrane degrades misfolded membrane proteins; while in the other pathway, vesicular transport of degraded mitochondrial protein for removal to lysosomes takes place (Chen Y, et al., 2013). The third pathway, known as mitophagy involves sequestration of damaged mitochondrion within a double-membrane vesicle, the autophagosome, followed by fusion with a lysosome (Schweers RL, et al., 2007, Youle RJ, e al., 2011).

There are two major pathways that results in the induction of mitophagy. One of these depends on the interaction between PTEN induced putative kinase 1 (PINK1), a

mitochondria specific kinase and PARKIN, an E3- ubiquitin ligase (Figure 2.3) (Matsuda N, et al., 2010).

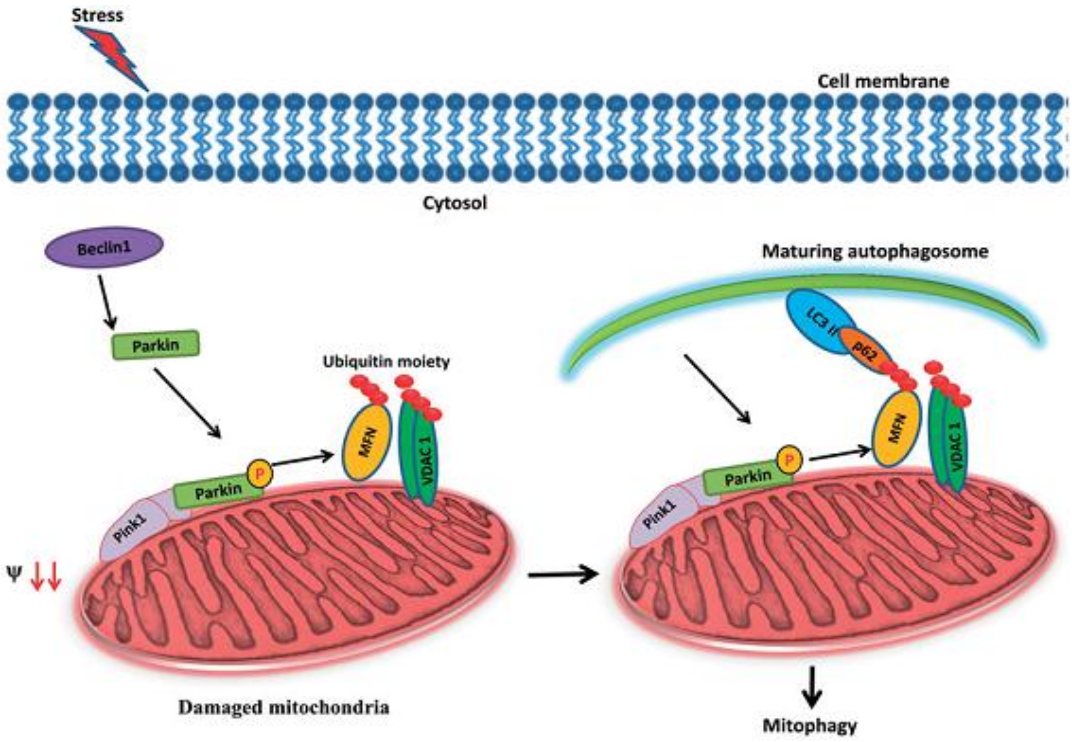


Figure 2.3: Mechanism of mitophagy induction. (Chaurasia M, et al., 2016).

Under normal conditions, PINK1 binds with the mitochondrial outer membrane and gets translocated to inner mitochondrial membrane where PARL (presenselin associated, rhomboid-like) protease causes its proteolytic degradation (Wang SH, et al., 2008). Under reduced mitochondrial potential, PINK1 accumulates over mitochondria where it interacts with PARKIN and causes its phosphorylation (Fu M, et al., 2013). Activated PARKIN causes ubiquitination of mitochondrial proteins. These ubiquitinated mitochondrial surface proteins acts as a landing platform for p62/SQSTM1 which finally forms a functional link between ubiquitinated proteins, including MFN1/2 (Mitofusin1/2) and LC3, leading to the initiation of autophagosome with the help of Atg32. Additionally, the outer mitochondrial membrane voltage-

dependent anion channel (VDAC), acts as signal for the removal of damaged mitochondria (Bartoletti-Stella A, et al., 2013). Other pathways of mitophagy induction are independent of PINK1-PARKIN and are mainly mediated *via* ER associated E3 ubiquitin ligase GP78 (Glycoprotein 78) and NIX/BNIP3L in a context dependent manner (Schweers RL, et al., 2007, Matsuda N, et al., 2010, Yamamori T, et al.,2012) .

It is well established that radiation exposure leads to extensive mitochondrial biogenesis providing additional advantage for the cell survival (Wu LJ, et al., 1999, Zhang B, et al., 2013). However, under conditions of extensive mitochondrial damage, the cell adapts mitophagy in order to exterminate the damaged and dysfunctional mitochondria. In this way, mitophagy results in cell survival after radiation injury. As discussed above, ionizing radiation (IR) can generate excessive ROS/RNS leading to DNA damage and genomic instability (Kim I, et al., 2011). Most of this IR-induced ROS/RNS is largely produced in the mitochondria (Zhang B, et al., 2013, Kim I, et al., 2011). Mitochondria are known to play an important role in radiation-induced cellular response, but the underlying mechanisms by which cytoplasmic stimuli modulate mitochondrial dynamics and functions are largely unknown. Numerous studies have pointed out the effect of radiation on mitochondrial dysfunction. Targeted cytoplasmic irradiation has been shown to cause mitochondrial fragmentation and a reduction in cytochrome-c oxidase followed by succinate dehydrogenase activity and a diminished respiratory chain function (Nishioka T, et al., 2014). Gamma-rays also induce a p53-independent mitochondrial biogenesis in human colorectal carcinoma cells (Wu LJ, et al., 1999). This radiation induced mitochondrial dysfunction and biogenesis has been shown to be associated with mitophagy induction (Black HS, 2004). Photo-irradiation of individual mitochondria from primary hepatocytes cause altered mitochondrial potential, inner membrane

permeablization, excessive ROS generation and mitophagy induction in a dose dependent manner and phosphatidylinositol 3-kinase independent manner (Black HS, 2004).

2.4.4.1 Metabolic reprogramming and mitochondrial alterations during tumorigenesis

Tumor cells are heterogeneous in terms of metabolism and morphology. Metabolic heterogeneity includes variations in the levels of oxidative phosphorylation and Warburg effect due to fluctuations in the oxygen and nutrient supply. Besides the tumor cells, heterogeneity has also been shown in the stromal cells present in the tumor micro milieu consisting cells of hematopoietic (T cells, B cells, NK cells, macrophages and MDSC) and mesenchymal origin (fibroblasts, myofibroblasts, mesenchymal stem cells (MSCs), adipocytes and endothelial cells) (Chen X, et al., 2015). However, the role of these cells in metabolic reprogramming of tumor cells has remained elusive. Recently, a new concept of "Reverse Warburg effect" or "Battery-operated tumor growth" (hereafter called as non-Warburgian phenotyope) has been proposed where the stromal cells appear to influence the metabolic reprogramming of tumor cells through a host-parasite relationship, with stromal cells acting as host and cancer cells as parasites (Pavlides S, et al., 2012). The stromal cells surrounding tumor cells have also been shown to display efficient mechanism for recycling dysfunctional mitochondria acting as a nutrient supplier (Pavlides S, et al., 2012). However, the implications of efficient recycling of mitochondria in the tumor cells and micro milieu on the resistance against chemo and radiotherapies have not been well understood.

2.4.4.2 Role of calcium in mitochondrial dysfunction

Hypoxia and/or altered metabolism are the major source of oxidative stress in cancer cells. This persistent oxidative stress leads to the chain reaction of cellular lipid

oxidation. Oxidized lipid metabolites (by-products) either alter the membrane fluidity or gets released inside the cytoplasm or the respective organelles. The oxidized lipid alter the permeability of membrane to calcium or directly acts as a calcium ionophore, leading to increased cytosolic calcium (Mishra PJ, et al., 2008, Dwarakanath B, et al., 2009). Mitochondria buffer this overloaded cytosolic calcium by acting as a sink thus preventing cell death. However, calcium accumulation in mitochondria leads to hormesis effect, called mitohormesis (Griffiths EJ, et al., 2009, Ristow M, et al., 2014). At low concentration, calcium enhances the oxidative phosphorylation capacity by activating many mitochondrial dehydrogenases (Griffiths EJ, et al., 2009) leading to aggressive metabolic phenotype. Majority of the cancer cells show mitochondrial accumulation in the close proximity of ER, creating the microdomain of high calcium for mitochondrial calcium uniporter leading to regulated increase in mitochondrial calcium, thus assisting in the development of the aggressive metabolic phenotype for enhanced growth and survival (Rimessi A, et al., 2013). On the other hand, calcium overload in the mitochondria leads to mitochondrial damage (Verma A, et al., 2011).

Since the accumulation of damaged mitochondria is detrimental and one of the major causes of cancers, mitochondrial quality control is essential for maintaining the cellular function, (Gogvadze V, et al., 2008). Therefore, damaged and functionally compromised mitochondria undergo to the process of degradation and regeneration of newer mitochondria called mitophagy and mitochondrial biogenesis respectively. As oxidative stress induced and calcium induced mitochondrial damage is continuous process in cancer cells, damaged mitochondria can be observed in them at any given time in the form of mitochondria derived vesicles (MDVs) and “I-Bodies” (Verma A, et al., 2011, Gogvadze V, et al., 2008, McLelland GL, et al., 2014). Taken together, all

these events appear to be inter-dependent and work in a cyclic manner in the cancer cell. Oxidative stress leads to disturbance in cellular calcium homeostasis causing mitochondrial damage and altered metabolism, further resulting in to enhanced ROS production in cancer cells (Figure 2.4).

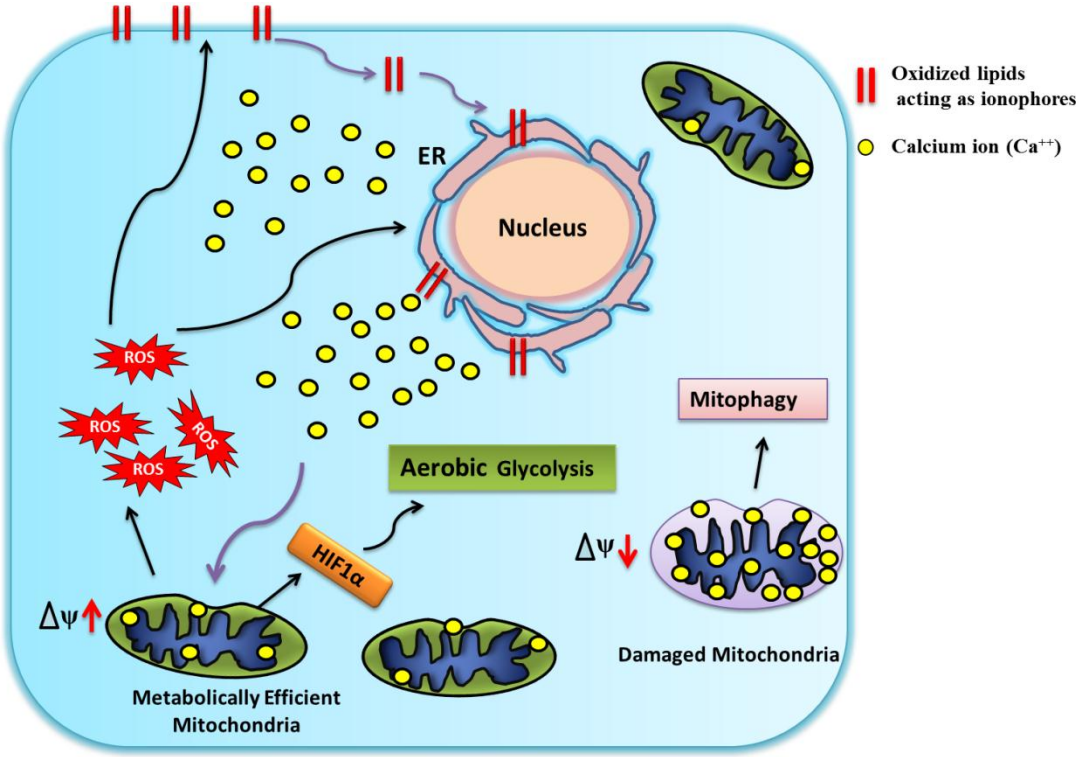


Figure 2.4: Schematic diagram showing oxidative stress induced alterations in calcium homeostasis during metabolic reprogramming of cancer cells. (Chaurasia M, et al., 2015)

2.4.4.3 Mitophagy and Cancer

During trauma, mitophagy supports tumor cell survival by providing substrates for mitochondrial metabolism (Guo JY, et al., 2011). Aggressive tumor cells appear to harbour robust mitochondria, although due to severe mutations in tumor suppressor as well as TCA cycle genes, they rely more on aerobic glycolysis to meet their energy demands. Such mitochondria show 'Warburg phenomenon'. In addition to the mutations in metabolic regulatory genes, several mitophagy related genes have also

been found to be mutated in many types of cancers during initial stages of tumor development (Fujiwara M, et al., 2008, Zhang C, et al., 2011). This results in the induction of defective mitophagy in these cells, leading to a higher accumulation of dysfunctional mitochondria ultimately leading to enhanced ROS generation and tumor induction (Kongara S, et al., 2012). Such mitochondria are more robust, having high antioxidant defence mechanism and can survive in highly hypoxic environment.

Cellular compositions of tumors are highly heterogeneous, with clonal variations of tumor cells and other tumor-associated cell types including fibroblasts, endothelial and immune cells. These cells constitutes the tumor stroma and have also been shown to display efficient dysfunctional mitochondria recycling which acts as nutrient supplier thus fertilizing the tumor niche and thereby helping in tumor progression and resistance (Valente EM, et al., 2004, Kitada T, et al., 1993). However, the effects of the efficient recycling of mitochondria (mitophagy) in tumor cells/micro milieu on their resistance against chemo and radiotherapies have not been clearly understood.

PARKIN (mitophagy related protein) has been identified as a p53 target gene and has been reported to prevent the Warburg effect by encouraging oxidative metabolism (Zhang C, et al., 2011). PARKIN has also been found to be deleted in numerous cancers conditions namely; ovarian, lung, and breast cancer (Picchio MC, et al., 2004, Cesari R, et al., 2003). Further, mice with severe PARKIN mutations have been found to be more vulnerable to spontaneous liver tumors (Fujiwara M, et al., 2008, Zhang C, et al., 2011). Mutations in other mitophagy related adaptor proteins like BNIP3 and NIX enhances tumor invasiveness and malignancies (Koop EA, et al., 2009, Okami J, et al., 2004, Sowter HM, et al., 2003, Tan EY, et al., 2007, Abe T, et al., 2005, Castro M, et al.,

2010) in lung, colorectal, hematologic, liver, and pancreatic cancers (Okami J, et al., 2004, Sowter HM, et al., 2003, Tan EY, et al., 2007, Abe T, et al., 2005, Castro M, et al., 2010). Thus there appears to be a inverse relationship between initiation, progression and resistance the therapies vis-a-vis the mitophagy potential of tumors. In contrast, mitophagy has also been shown to be a tumor-promoting process which is supported by its ability to maintain a healthy mitochondrial pool required to fulfil the enhanced energy need of tumor cells (Guo JY, et al., 2013).

2.4.4.4 Tumor associated mitophagy and aerobic glycolysis

Although not well established, circumstantial evidences indicate a direct relationship between tumorigenesis and mitophagy (Fujiwara M, et al., 2008, Lu H, et al., 2013). Similar to autophagy, mitophagy is also involved in maintaining functional (and thus energy generating) mitochondrion pool as well as nutrients for better cancer cell survival. A direct relationship between mitophagy and glycolysis are still lacking. Available evidences suggest that as functional mitochondria are a prerequisite for energy generation through glycolysis in a tumor cell (Warburg effect), mitophagy may add on to the survival and progression of tumorigenesis even during therapeutic stress (Chatterjee A, et al., 2006, Rosenfeldt MT, et al., 2016). For instance, Ras oncogene positive tumors have been shown to activate mitophagy which is associated with enhanced glycolysis (Kim JH, et al., 2013).

The impact of alterations in metabolic reprogramming and mitophagy of cells in the tumor micro milieu has been recently explored. Many tumor cells appear to maintain their mitochondrial function of enhanced glycolysis *via* a complex mechanism wherein tumor cells indirectly derive energy from the neighbouring cells in the tumor

microenvironment; the tumor stromal cells which exhibit a higher glycolytic phenotype i.e. Warburg Effect (Pavlides S, et al., 2012). As a messenger, tumor cells generate enormous amounts of reactive oxygen species (ROS), which gets released into the tumor micro milieu. Tumor stromal cells gets influenced by this huge ROS supply thus initiating the onset of stromal oxidative stress, autophagy, and mitophagy due to the activation of key transcription factors, namely HIF1-alpha (aerobic glycolysis) and NFkB (inflammation) (Casey TM, et al., 2008, Desmouliere A, et al., 1993, Kojima Y, et al., 2010, Martinez-Outschoorn UE, et al., 2010). Two types of mitochondria may exist in these stromal cells, those which are less robust and signal mitophagy initiation on sensing the ROS released into the micro environment followed by their altered membrane potential (Non-Warburgian); and those which are more robust and start L-lactate production after sensing oxidative stress (Warburgian). Mitophagic degradation of non-Warburgian mitochondria provides recycled products as well as raw materials for the Warburgian mitochondria to facilitate aerobic glycolysis and enhanced tumor stromal lactate production. This lactate produced by Warburgian mitochondria is released into the tumor microenvironment with the help of MCT4 and MCT1 (Whitaker-Menezes D, et al., 2011, Pinheiro C, et al., 2008). In response to the nutrient (in form of lactate) released into the micro milieu, cancer cells exhibit 'reverse Warburg phenomena' where L-lactate functions as an onco-metabolite, stimulating mitochondrial biogenesis, glutaminolysis and oxphos in them; thereby directly providing energy for their growth and mitochondrial biogenesis (Sharma K L, et al., 2015).

Cancer associated fibroblasts have also been shown to over express mitochondrial fission factor (MFF) which is considered as the prerequisite for mitophagy (Otera H, et al., 2010, Guido C, et al., 2010). The MFF over-expressing fibroblasts undergo oxidative

stress, with augmented ROS production, and NF-kB activation, thus driving the onset of mitophagy and, ultimately, glycolytic metabolism (Guido C, et al., 2010). Similarly, MFF has been shown to promote a glycolytic phenotype *in vivo*, under conditions of hypoxia, where cancer associated fibroblasts (MFF fibroblasts) become more glycolytic and display an efflux of high-energy mitochondrial fuels into the extracellular microenvironment which help drive mitochondrial biogenesis in cancer cells.

Mitophagy and glycolysis show strong interrelationship in stromal cells as well as cancer cells thereby promoting tumor cell survival even under adverse conditions of therapy (Kubli DA, et al., 2012). Therefore, mitophagy appears to be a key quality control deciding the response of cancer cells to therapy and may thus be a potential target for adjuvant therapy.

2.4.4.5 Therapeutic implications of targeting mitophagy

Association of Glut-4 and over-expression of MCT as well as deletion in Caveolin-1 have been shown in resistant and aggressive tumors (Sotgi F, et al., 2011, Witkiewicz AK, et al., 2012). Since these are associated with reverse Warburgian phenotype as well as enhanced mitophagy, they may serve as markers for identifying tumors where mitophagy inhibitors could be useful in combination with other therapeutic agents. Combinations of antioxidants like N- acetyl cystein and quercetin which can inhibit mitophagy as well as lactate production leading to the accumulation of more dysfunctional mitochondria ultimately driving the cell towards apoptosis (Ma Q, et al., 2015) could also be a potential strategy that requires systematic investigations. Furthermore, inhibitors of mitochondrial fission that inhibit mitophagy in stromal as well as tumor cells could also be potential adjuvants.

Mitophagy exhibits a double faceted role in tumorigenesis i.e. either survival-supporting or death-promoting (Gao P, et al., 2007, Ma Q, et al., 2015). Therefore, inducing prolonged or robust mitophagy using mitophagy modifiers along with the conventional anti-cancer therapies could also be explored as an anti-cancer strategy. Prolonged mitophagy in tumor cells would exhaust the metabolites required for sustaining the tumor growth ultimately leading to cell death. Induction of robust mitophagy using linamarase/linamarin/glucose oxidase (lis/lin/GO) system leading to the loss of mitochondrial membrane potential and irreversible cell death of tumor cells has been reported recently (Narendra D, et al., 2008, Gargini R, et al., 2011, Saddoughi SA, et al., 2013). Similarly, induction of mitophagy by ceramide; and enhanced cell death of nasopharyngeal carcinoma (CNE2) during low-intensity ultrasound therapy in the presence of curcumin on induction of mitophagy further substantiate the potential of targeting robust or treatment induced prolonged mitophagy (Narendra D, et al., 2008, Gargini R, et al., 2011, Saddoughi SA, et al., 2013). Even though induction of prolonged or robust mitophagy appears reasonable, care must be taken as robust induction would depend upon the type and degree of stress. The lack of specific biomarkers and understanding of the mitophagy associated tumor cell death is another hurdle that needs to be considered in order to make this strategy feasible in the clinics.

Inhibitors of glycolysis like 2-DG and 3-bromopyruvate have been shown to selectively induce tumor cell death as well as enhance death induced by anticancer therapies like ionizing radiation and chemotherapeutic drugs (Jain V, 1996, Dwarakanath B, et al., 2009). However, a great deal of heterogeneity has been observed in both these effects among well-established tumor cell lines *in vitro*, animal tumors *in vivo* and clinical response (Jain V, 1996, Dwarakanath B, et al., 2009). This heterogeneity may be partly

attributed to the presence of both Warburgian as well as non-Warburgian mitochondria in resistant tumors. Mitophagy as well as enhanced glycolysis in these non-Warburgian mitochondria assists in providing nutrients to the Warburgian phenotype, thereby augmenting the tumor resistance. Thus, inhibitors of mitophagy in combination with metabolic modifiers (like 2-deoxy-glucose, metformin etc.) can be a potential approach for improving the efficacy of radio- and chemotherapies (Figure 2.5).

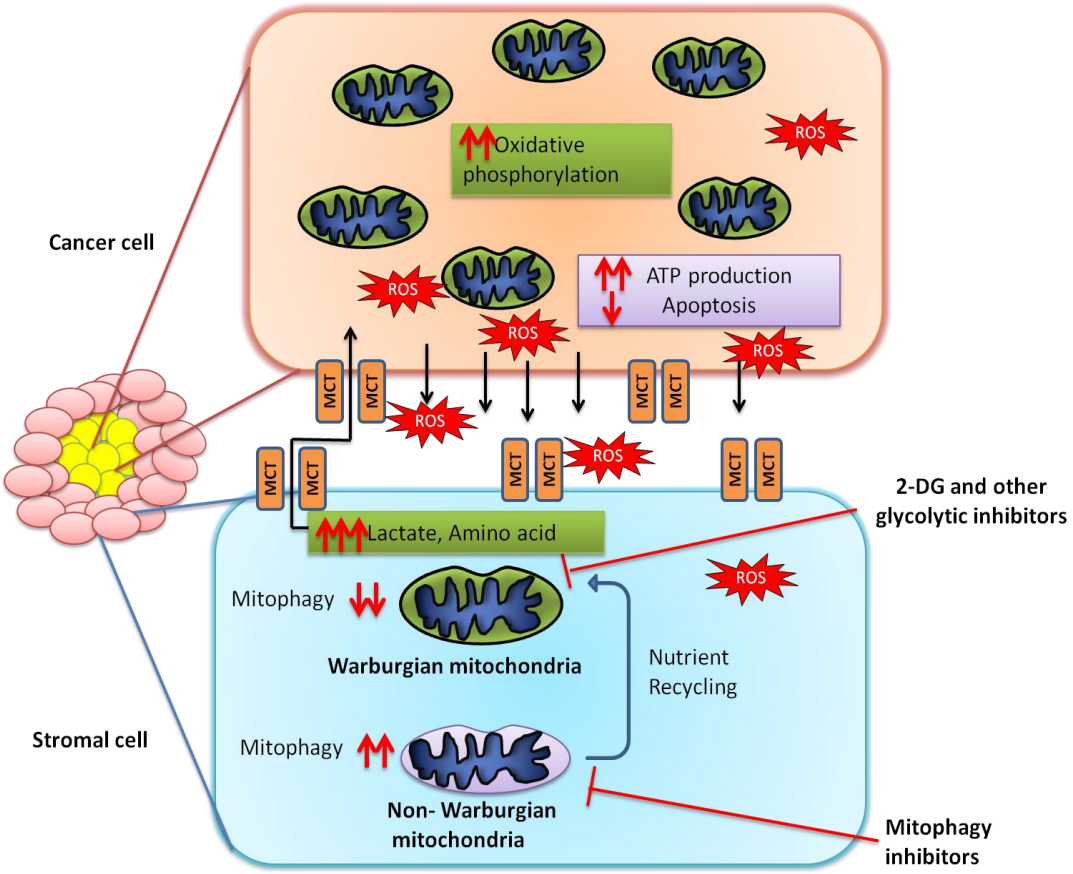


Figure 2.5: Host parasite relationship between stromal and cancer cells and the influence of metabolic reprogramming in tumorigenesis. (Chaurasia M, et al., 2015)

To what an extent variations in the treatment induced mitophagy (or autophagy) contributes to the heterogeneous responses observed in pre-clinical and clinical studies needs further investigations using genetically modified malignant cell systems. Since host

factors also contribute to the responses of tumors under *in vivo* conditions, identification (establishment) of appropriate surrogate markers will be helpful in individualizing therapies targeting mitophagy/autophagy for improving therapeutic gain.

2.4.5 ER stress and radiation induced autophagy

The endoplasmic reticulum is a crucial intracellular Ca^{2+} reservoir which provides executive machinery for numerous cellular processes including translation, post translational modification and proper folding. ER is also involved in the initiation of several pathways of the vesicular movement of membrane and proteins to various organelles as well as the cell surface. It has been well established that ROS generated following stress conditions including radiation exposure causes indirect macromolecular damage to DNA, proteins and lipids etc. (Scriven P, et al., 2007, Ding W, et al., 2012). In response to excessive unfolding of proteins (due to damage caused by radiation induced ROS), a process collectively known as unfolded protein response (UPR) gets induced in ER (Malhotra JD, et al., 2007). Additionally, it also elicits an activation signal to boost the cytosolic calcium load released from ER (a store house of Ca^{2+}) (Nakai A, et al., 2007). ROS generation thus causes activation of ER stress which is mediated by UPR response (Lin JH, et al., 2008, Ma Y, et al., 2004). UPR in-turn has been shown to have a strong correlation with autophagy (Li T, et al., 2013, Malhotra JD, et al., 2007). These couplings indicate a possible association between ROS, ER stress, $[Ca^{2+}]i$ and autophagy.

Accumulating evidences suggest a strong association between ER stress and autophagy in various organisms including yeast and mammals (Li T, et al., 2013). In healthy conditions, Grp78 (the main marker of UPR activation) remains bound with IRE1,

PERK (eukaryotic translation initiation factor 2-alpha kinase 3) and ATF6 (activating transcription factor 6) present over the endoplasmic reticulum membrane. In contrast, affinity of Grp78 for unfolded proteins increases several folds during UPR. It dissociates from its ER-sensing transducers and binds to unfolded proteins in the ER lumen leading to the activation of all three distinct ER stress sensors. PERK and IRE1 get activated by phosphorylation whereas ATF6 gets activated by its fragmentation and translocation from ER to golgi and finally to the nucleus. These three sensors initiate transcription of different target genes (Figure 2.6).

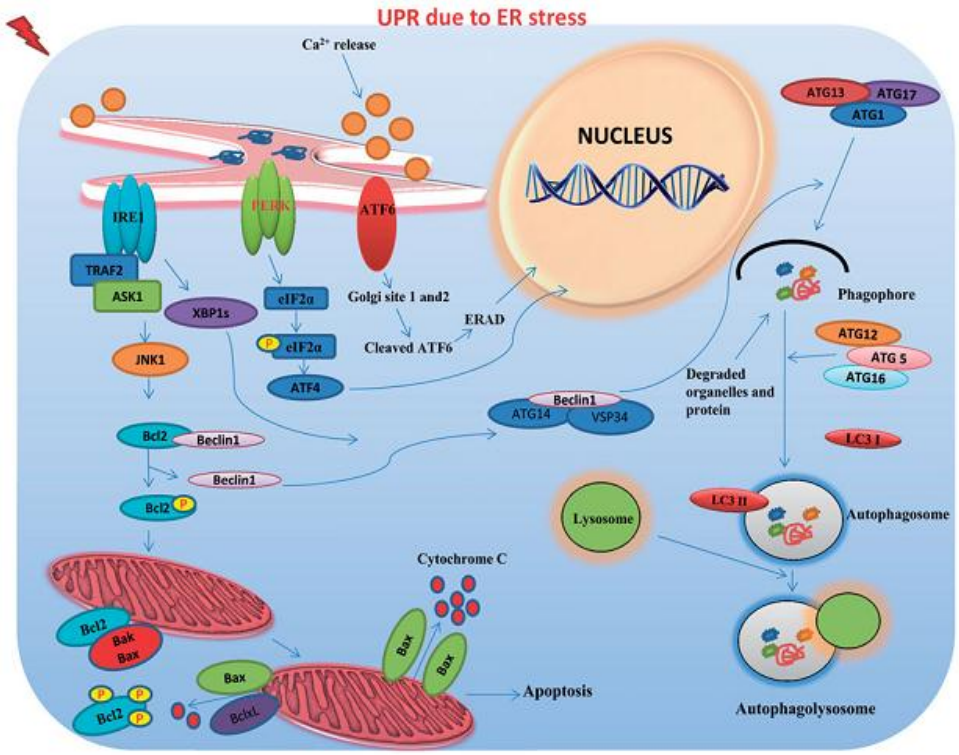


Figure 2.6: Schematic diagram showing signaling activated during UPR to promote ER stress, autophagy and apoptosis induction in a stressed cell. (Chaurasia M, et al., 2016)

One of the target genes is c-Jun N-terminal kinase (JNK) which is essential for lipid conjugation of LC3 and may act as a connecting link between ER stress and autophagy induction (Kubota H, 2009). All these events prime the activation of ER stress. The

specific ER stress markers like ERM1, XBP1, eIF2α, ATF4, DDIT3 or Chop II have been found to be up-regulated during UPR. Studies also suggest that in response to ER stress, the phosphorylation of eIF2α (eukaryotic initiation factor 2α) by PERK (an eIF2α kinase) causes global shutdown of protein synthesis except Atg4 which is required for mediating LC3I to LC3II conversion thus ultimately leading to autophagy induction (Malhotra JD, et al., 2007, Kouroku Y, et al., 2007, Yang Z, et al., 2013).

ER stress has emerged as a novel traumatic condition to the cells which is involved in induction of autophagy by negatively regulating the levels of AKT/TSC/MTOR pathway (Ding WX, et al., 2007). Numerous ER stress inducers like tunicamycin (inhibitor of N-linked gycosylation), DTT (causes intervention in disulphide bond formation), MG132 (intrusion in proteasome function), cisplatin, thapsigargin (inhibitor of the sarcoplasmic calcium ATPase, namely SERCA2) etc. are proposed to induce autophagy. ER stress-induced autophagy has been predominantly shown to have a prosurvival role; but in parallel, there are studies suggesting that excessive ER stress may cause autophagic cell death followed by apoptosis (Gozuacik D, et al., 2008, Ding WX, et al., 2007, Li T, et al., 2013). Although it is known that ER stress is one of the autophagy inducing pathway but the exact mechanism is still under elucidation. Moreover, only few reports exist on radiation exposure induced autophagy through ER stress.

Exposure of cells to ionizing radiation causes oxidative stress which in turn may initiate unfolded protein response. Available evidences suggest an association between radiation exposure and ER stress, which finally results in the commencement of efficient autophagic machinery in the exposed cells (Kim KW, et al., 2010, Kim EJ, et al., 2014).

Although the main signaling pathway of ER stress getting activated following irradiation is still a debatable one; some recent evidences suggest that PERK-eIF2α and/or IRE1α may serve as the main executing pathways of ER stress in irradiated scenarios (Kim EJ, et al., 2014, Saglar E, et al., 2014). Furthermore, recent studies have also indicated the importance of further downstream molecules (i.e. eIF2α/ATF4) of PERK mediated UPR pathway in irradiated endothelial cells (HUVEC and HCAEC). Significant alterations have not been observed in IRE1 and ATF6 branches in these cells (Chen X, et al., 2012). Recently published data from our lab also suggest predominant activation of PERK and IRE1 pathway in radiation exposed conditions. Treatment of spinal metastasis with Iodine-125 has been shown to activate ER stress through the activation of PERK-eIF2α which finally causes induction of autophagy (Kim EJ, et al., 2014). In line with this, IR induced ER stress has also shown autophagy induction in a dose dependent manner in the blood samples of human cancer patients (Brookes PS, et al., 2004).

The autophagic process induced in response to ER stress is found to be involved in providing survival advantage to the cells. However, if the exposure burden is too large to handle, the same PERK-eIF2α pathway can activate several cell death pathways like apoptosis and necrosis for the removal of damaged cells (Kim EJ, et al., 2014). Further studies are required to understand the relationship between radiation-induced ER stress and autophagy.

2.4.6 Role of calcium signaling in radiation induced autophagy

Intracellular calcium is distributed between several sub-domains like ER lumen and mitochondria. During stress conditions, the sub-cellular distribution of unbound $[Ca^{2+}]i$ gets altered and it gets released into the cytoplasm, promoting either cell proliferation or

cell death (Decuypere JP, et al., 2011, Cardenas C, et al, 2012). Altered $[Ca^{2+}]i$ is indeed shown to regulate autophagy, mainly macroautophagy (Gordon PB, et al., 1993, Pfisterer SG, et al., 2011). At a first glance, evidences in the literature seems mystifying and suggest that elevations of $[Ca^{2+}]i$ can both activate and inhibit autophagy (Gordon PB, et al., 1993, Buytaert E, et al., 2006). However, emerging evidences and in-depth analysis suggest that the distribution of $[Ca^{2+}]i$ in different sub-domains and extent of Ca^{2+} release from the ER lumen leads to the activation of different signaling pathways causing either activation or inhibition of autophagy.

Evidences showing increased $[Ca^{2+}]i$ as an activator of autophagy have mainly used stress inducing agents like anti-cancer drugs, radiation, photodynamic therapy (PDT), Ca^{2+} ionophore and SERCA inhibitor, thapsigargin etc., which mobilize Ca^{2+} from one sub-domain to the other and also lead to elevated $[Ca^{2+}]i$ (Hoyer-Hansen M, et al., 2007, Sakaki K, et al., 2008, Brady NR, et al., 2007). However, Ca^{2+} chelators *viz.* BAPTA-AM inhibit the induction of autophagy, confirming the involvement of cytosolic Ca^{2+} (Bartoletti-Stella A, et al., 2013, Brady NR, et a., 2007, Gao W, et al., 2008, Szabadkai G, et al., 2008). Autophagy induced by starvation and inhibition of MTOR using rapamycin is also reversed by BAPTA-AM, suggesting the indirect role of Ca^{2+} signaling in starvation induced autophagy (Szabadkai G, et al., 2008). Autophagy induced by extracellular calcium is countered by extracellular and intracellular buffering, suggesting that ER is probably the main, but not the only source of free Ca^{2+} during Ca^{2+} induced autophagy (Verma A, et al., 2011).

It is well established that stress induced elevated cytoplasmic Ca^{2+} influx, originating either from ER or extracellular environment is first buffered by mitochondria

(Vingtdeux V, et al., 2010). However, sustained elevation exceeding the buffering capacity of the mitochondria leads to accumulation in the cytoplasm, suppressing the MTOR activity in a CaMKK- and AMPK-dependent manner (Cardenas C, et al, 2012, Gordon PB, et al., 1993, Brady NR, et a., 2007, Vingtdeux V, et al., 2010, Zalckvar E, et al., 2009, Kim HJ, et al., 2009). The Ca^{2+} overloaded mitochondria also become non-functional, which increases AMP/ATP ratio and activation of AMPK signaling (Gordon PB, et al., 1993). Moreover, the elevated [Ca^{2+}]i also activates calmodulin-dependent DAPK which phosphorylates Beclin1, thereby promoting its dissociation from Bcl-2 leading to the induction of autophagy (Ionescu L, et al., 2006). Besides activation of these signaling cascades, the excess Ca^{2+} overloading in to mitochondria also irreversibly damages them by precipitating all the inorganic phosphates (Pi) in to calcium phosphate, insoluble form (Cardenas C, et al, 2012, Zalckvar E, et al., 2009). These damaged mitochondria get cleared from cells by mitophagy or macroautopahgy (Black HS, 2004, Zalckvar E, et al., 2009). TRPML3, a Ca^{2+}-permeable channel, recruited to autophagosomes, has been shown to be important in autophagy (Taylor CW, et al., 2002). Overexpression of TRPML3 positively correlates with induction of autophagy (Gordon PB, et al., 1993). Further the role of Ca^{2+} signaling is not only limited to the induction of autophagy, but may be important for its progression as well.

Low levels of Ca^{2+} oscillations for shorter period of time have been suggested to inhibit autophagy, that appears to act through the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R, 3 isoforms), a ubiquitously expressed intracellular Ca^{2+} release channel, located mainly in the ER (Gordon PB, et al., 1993, Nadif Kasri N, et al., 2002, Vicencio JM, et al., 2009, Criollo A, et al., 2007, Khan MT, et al., 2010, Cardenas C, et al., 2010). IP3R forms channel in the mitochondria with the help of Beclin 1 and Bcl-2 thus, decreasing

the level of free Beclin 1 to induce autophagy (Cardenas C, et al., 2010). Therefore, the absence of IP3Rs would lead to the dissociation of Beclin 1 from Bcl-2, followed by autophagy stimulation. This hypothesis has indeed been verified with the IP3Rs triple knock out (TKO) chicken DT40 B lymphocytes, which exhibit enhanced autophagy levels (Williams A, et al., 2008, Rong YP, et al., 2008). Interestingly, the expression of other ER Ca^{2+} channels like the ryanodine receptor (RyR) do not restore the elevated autophagy levels (Rong YP, et al., 2008), confirming the involvement of IP3Rs. Release of low levels of Ca^{2+} mainly from ER is directly buffered by mitochondria through IP3Rs channel. Low levels of Ca^{2+} accumulation in mitochondria induces majority of TCA cycle enzymes leading to enhanced production of ATP (Cardenas C, et al, 2012). This reduces the ratio of AMP/ATP, which inhibits AMPK and therefore autophagy in cells (Rong YP, et al., 2008). There are also evidences to suggest that activation of calpain by intracellular Ca^{2+} can lead to increased IP3 production through cAMP and activate IP3R-mediated Ca^{2+} release, thereby inhibiting autophagy (Bhandary B, et al., 2012). The anti-apoptotic Bcl-2 protein appears to be a critical regulator of Ca^{2+} induced autophagy at the ER level as its over-expression in an ER targeted manner is most effective in reducing Ca^{2+}-induced autophagy (Brady NR, et al., 2007, Szabadkai G, et al., 2008). Bcl-2 inhibits autophagy by facilitating the buffering of Ca^{2+} by mitochondria through IP3Rs-Bcl-2-Beclin-1 channel, besides sequestering Beclin-1 and thus Ca^{2+} seems to regulate autophagy in both positive and negative manner depending on the degree of disturbance in Ca^{2+} homeostasis and cellular status (normal cells and stressed cells) (Brady NR, et al., 2007, Moretti L, et al., 2007).

Radiation induced oxidative stress leads to ER stress thereby causing enhancement in $[Ca^{2+}]i$ apart from extracellular Ca^{2+} intake (Vessoni AT, et al., 2013, Shoji JY, et al.,

2010). Keeping both radiation induced calcium imbalance and Ca^{2+} imbalance induced autophagy in consideration, it seems that calcium induced autophagy must also be elicited during radiation exposed conditions and may be linked with ROS and ER stress, where increased cytosolic Ca^{2+} may cause suppression of MTOR activity in a CaMKK- and AMPK-dependent manner (Brady NR, et al., 2007, Kim HJ, et al., 2009). Moreover, the elevated [Ca^{2+}]i may cause activation of calmodulin-dependent DAPK which by phosphorylation of Beclin1, helps in induction of autophagy. However, studies confirming triangular relationship between radiation exposure, autophagy and calcium imbalance are need of the hour. The possible effect of this triangular relationship on cellular fate has been depicted in (Figure 2.7).

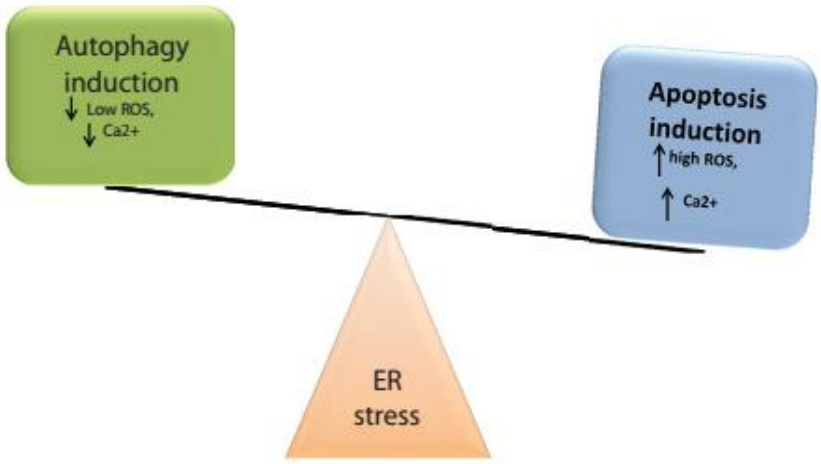


Figure 2.7: Triangular association between ER stress, ROS generation and cytosolic calcium, and their role in the induction of autophagy/ apoptotic cascade in a cell. (Chaurasia M, et al., 2016)

2.4.7 Radiation induced DNA damage response and autophagy

Nucleus is an important part of a cell which contains genetic information in the form of DNA and therefore autophagic degradation of the entire nucleus appears to be intriguing. A novel form of nuclear specific autophagy called Nucleophagy has been

recently reported wherein the elimination of damaged DNA occurs *via* autophagic vacuoles (McGee MD, et al., 2014). However, in certain multinucleated fungi and nematodes like filamentous fungus *Aspergillus oryzae* and *Caernohabiditis elegans*, nuclear DNA degradation by a highly selective form of autophagy called piecemeal microautophagy (PMN) has been reported under exceptional circumstances (Krick R, et al., 2009, Rello-Varona S, et al., 2012). Specific removal of damaged nuclear DNA has also been reported in certain mammalian cells (Park YE, et al., 2009, Filimonenko M, et al., 2010, Pankiv S, et al., 2010).

Interestingly, while autophagy is a strictly cytoplasmic process, several autophagy-related proteins e.g. p62 are enriched in the nucleus or undergo fast nuclear-cytosolic shuttling (Simonsen A, et al., 2004, Clausen TH, et al., 2010). Another protein, ALFY (autophagy-linked FYVE protein) has been shown to be involved in autophagy and localize predominantly in the nucleus (Simonsen A, et al., 2004, Isakson P, et al., 2013, Rodriguez-Rocha H, et al., 2011). However, following stress, ALFY is extruded from the nucleus to cytoplasm and interacts with p62 bodies in a similar manner certain nuclear proteins exit out of the nucleus for their removal *via* autophagic machinery. Recent reports suggest that compromised autophagy leads to delayed degradation of damaged nuclear components (DNA, RNA and nucleoproteins) (Filimonenko M, et al., 2010). In line with this, mutated lamins have been shown to induce deformations in the nuclear envelope that induces nucleophagy (Pankiv S, et al., 2010). Micronuclei containing whole chromosomes or parts of the chromosomes are also suggested to be removed by autophagy; thus facilitating the maintenance of genomic stability (Filimonenko M, et al., 2010). The nature and functional significance of this nuclear sequestration of autophagy-related proteins is not clear, although the nuclear-cytosolic

shuttling of Beclin1 has been shown to be important for its autophagic and tumor suppressor functions (Bae H, et al., 2009).

Autophagy appears to play a crucial role in regulating cellular fate following the induction of DNA damage (McGee MD, et al., 2014, Mao Z, et al., 2008). For instance, in cells with DNA damage and defective in apoptosis, autophagy facilitates cell death; thereby acting as a tumor suppressor (Maiuri MC, et al., 2007). In line with this, the suppression of ULK1-interacting protein FIP200 has been reported to impair DDR, thus triggering cell death upon ionizing radiation-induced oxidative stress (Brandsma I, et al., 2012). Collectively, these circumstantial evidences suggests direct or indirect role of autophagy in the DDR and ROS/ RNS-mediated genotoxic stress. However, precise mechanisms underlying DDR mediated autophagy are still not very clear.

Autophagy also takes care of the micronuclei as shown by a recent study where co-localisation of micronuclei, autophagic vacuole with p62 and γ-H2AX foci (a DNA damage marker) has been reported (Filimonenko M, et al., 2010). Non-autophagic micronuclei were p62-negative suggesting that the presence of DNA damage directly or indirectly signals for autophagic engulfment. Accumulating evidences suggest that radiation induced DNA damage induces autophagy. In response to DNA double strand breaks (generally considered lethal), two repair pathways are mainly activated. Homologous recombination (HR) which depends on sequence homology and restricted to the S and G2 phases of the cell cycle is associated with high fidelity while Non-homologous end joining (NHEJ), independent of the sister chromatid is relatively error prone (Mathew R, et al., 2007, Liu EY, et al., 2015). Cells deficient in autophagy have been shown to accumulate higher levels of mutated DNA suggesting deficiency in the

HR repair (Park C, et al., 2015, Robert T, et al., 2011). The role of chaperone mediated autophagy has been recently implicated in maintaining the genome stability. Chaperone mediated autophagy plays an essential role in the degradation of Chk1 following exposure to DNA damaging agents (etoposide and gamma radiation). Furthermore, inactivation of chaperone mediated autophagy under these conditions results in the accumulation of DNA damage (Dyavaiah M, et al., 2011).

Inhibition of NHEJ in these irradiated cells (thus lacking HR machinery and thus completely disabled in terms of DNA repair) results in enhanced apoptosis (Robert T, et al., 2011). Autophagy has also been shown to influence the dynamics of DNA repair wherein it helps in recycling of key proteins involved in the processing of lesions; besides aiding the metabolic precursors for the generation of ATP as well as regulating the supply of dNTPs required for repair (Dotiwala F, et al., 2013, Ryan KM., 2011). Studies carried out in yeast have shown that activation of autophagy following the induction of DSBs results in anaphase arrest, which persists till autophagy is blocked or vacuolar proteolysis is inhibited suggesting that DDR induced autophagic process may also contribute to cytotoxicity (Mao Z, et al., 2008, Gao W, et al., 2011) .

Two essential proteins, p53 and ATM serve as connecting links between radiation-induced DDR and autophagy. Following DNA damage, p53 provokes autophagy by transcriptionally inducing several genes including damage-regulated autophagy modulator (DRAM), ULK1/2, sestrin1/2 and bnip3 (Budanov AV, et al., 2008). These genes can directly regulate autophagy e.g. the lysosomal proteins DRAM1 and ULK1/2 interact with Atg13 and FIP200 to induce autophagy (Alexander A, et al., 2010). Similarly, Sestrin1 and 2 activate AMPK and the TSC1/2 complex, leading to the

inactivation of MTORC1 and thus autophagy induction (Figure 2.6) (Morselli E, et al., 2008). ATM can activate autophagy by both p53 dependent as well as independent mechanism. Cytosolic ATM can activate TSC2 tumor suppressor to inhibit MTORC1 through the LKB1/AMPK pathway and induce autophagy during ROS-mediated cellular damage (Tasdemir E, et al., 2008). On the other hand, nuclear ATM can initiate autophagy *via* AMPK by direct activation of LKB1 (the AMP kinase) (Tasdemir E, et al., 2008). Thus, these new findings integrate different stress response pathways taking place in different cellular compartments. From this perspective, ATM would be required for both initiation (nucleus) and mediation (cytosol) of DDR. Interestingly, induction of autophagy during starvation requires destruction of cytosolic p53 revealing a multifaceted role of p53 in autophagy regulation (Munoz-Gamez JA, et al., 2009, Rodriguez-Vargas JM, et al., 2012). PolyADP-ribose polymerase 1 (PARP1) is another protein directly linking DDR and autophagy (Batista LF, et al., 2009, Sui X, et al., 2013). PARP1 is hyperactivated upon radiation-induced DNA damage that consumes NAD^+ resulting in ATP depletion. Such energetic imbalance can activate autophagy *via* AMPK pathway (Batista LF, et al., 2009, Sui X, et al., 2013).

Autophagy shows a pleomorphic role in the context of DNA damage response. Majority of the studies indicate that autophagy inhibition in cells treated with DNA damaging agents leads to enhanced cell death, supporting a prosurvival role for autophagy. In this scenario, transcription factors such as p53, p73 and E2F1; which not only promote DNA repair, cell cycle arrest or apoptosis in response to different degrees of DNA damage but also control autophagy would have pivotal roles (Li J, et al., 2010). On the other hand, autophagy has also been shown to promote degradation of acetylated Sae2 in valproic acid treated yeast cells in an intricate manner, wherein autophagy

activation could contribute to perseverance of DNA damage and further enhancement of apoptotic signaling in mammalian cells by controlling turnover of certain DNA repair-related enzymes (Dotiwala F, et al., 2013). Taken together, these studies indicate that repair of radiation induced DNA damage may be linked with autophagy, which may either enable the cell to overcome the radiation stress or may activate cell death in a context dependent manner (Figure 2.8) .

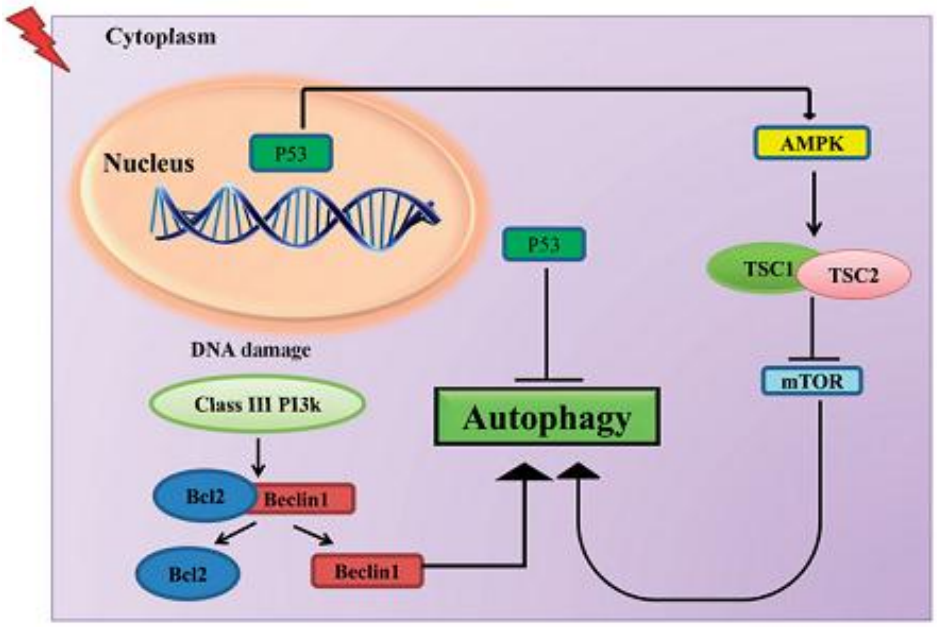


Figure 2.8: Schematic model illustrating the possible signaling pathways induced following irradiation and their involvement in the regulation of radiation-induced autophagy in cells. (Chaurasia M, et al., 2016)

2.5 Targeting autophagy for altering radiosensitivity

Most of the studies linking radiation with autophagy have been performed on cancer patients undergoing radiotherapy. Elevated levels of autophagy have been found to be associated with chemo as well as radio-resistance of various cancerous types (Wu LJ, et al., 1999, Zhang B, et al., 2013, Scriven P, et al., 2007, Ding W, et al., 2012, Kouroku Y, et al., 2007, Yang Z, et al., 2013). Clinical trials combining chemotherapeutic agents with

autophagy inhibitors such as chloroquine (CQ), hydroxychloroquine (HCQ), 3-Methyladenine (3-MA) etc. provide survival benefits and increased life span in patients with breast cancer, myeloma, prostate cancer and several other advanced tumors (Table 2.2) (Selvakumaran M, et al., 2013, Mahalingam D, et al., 2014, Rangwala R, et al., 2014, Wang K, et al., 2011, Vogl DT, et al., 2014, Bommareddy A, et al., 2009, Claerhout S, et al., 2010, Levy JM, et al., 2011, Lomonaco SL, et al., 2009). Similar finding are also reported with radiotherapy (Table 2.3) (Schmukler E, et al., 2013, Lomonaco SL, et al., 2009, Kim KW, et al., 2009, Fujiwara K, et al., 2007, Mammucari C, et al., 2007). Reduced expression of Beclin1 protein has been associated with decreased cell survival in radio-resistant cancer cell lines exposed to low dose irradiation during radiotherapy (Polager S, et al., 2008). These studies support the role of autophagy in cell survival under radiation stress. In addition of its role in carcinogenesis, autophagy has also been reported to play a role in angiogenesis. Ionizing radiation induce contrasting effects on vascularisation by enhancing autophagic levels in cells, which in turn enhances production of pro-angiogenic factors e.g. VEGF finally leading to enhanced radioresistance (Yue Z, et al., 2013, Li M, et al., 2008, Amaravadi RK, et al., 2007, Thorburn J, et al., 2009).

Table 2.2: Pre clinical studies using modifiers of autophagy for enhancing the efficacy of anticancer therapeutic (Chaurasia M, et al.,2016).

Cancer type	Modifiers of autophagy	Primary therapeutic
Colorectal cancer	3-MA	5-FU
	CQ, siRNA against Beclin1	Bevacizumab, Oxaliplatin
	HCQ	HDAC inhibition
	HCQ	Temozolomide
Gastric cancer	CQ, siRNA against ATG5, Beclin1	Quercetin
Gastrointestinal stromal tumors (GISTs)	CQ, Quinacrine, siRNA against ATG7, ATG12	Imatinib
Multiple myeloma (MM)	HCQ	Bortezomib
Lymphoma	CQ, siRNA against ATG5	Tamoxifen
Prostate cancer	3-MA, CQ, siRNA against ATG7	Saracatinib
	3-MA, siRNA against ATG5	Phenethyl isothiocyanate
Skin cancer	3-MA, siRNA against ATG5	Cisplatin
Glioma	3-MA, HCQ, E64 + Pepstatin A	Cannabinoid

Table 2.3: Modifiers of autophagy as potential radiosensitizers in various cancers (Chaurasia M, et al, 2016).

Cancer type	Modifier of autophagy	Type of ionizing radiation
Breast cancer	siRNA against Beclin 1, ATG3, ATG4b, ATG5, ATG12	γ-Radiation
Glioma	3-MA, BFA, siRNA Beclin 1, ATG5	γ-Radiation
Colorectal cancer	Tunicamycin, siRNA against Beclin 1	γ-Radiation
	siRNA against Beclin 1, ATG5, ATG7, UVRAG	γ-Radiation
Lung cancer	ABT-737/rapamycin	X-Rays

A dose dependent correlation has been observed between radiation-induced autophagy and cell cycle arrest (Schmukler E, et al., 2013). A lower dose of ionizing radiation mainly induces G2/M arrest. Also, co-treatment of cells with ER stress and autophagy activators along with radiation further enhances the G2/M block extent (Schmukler E, et al., 2013, Thorburn J, et al., 2009). This associated cell cycle arrest plays a key role in overall radiation resistance in various cancer conditions.

In contrast to the role of autophagy in radio-resistance, there are evidences suggesting that autophagy can also promote cell death (Maiuri MC, et al., 2009, White E, et al., 2010). Various tumor suppressors have been shown to induce high levels of autophagy (Kim I, et al., 2011). For instance, loss of Beclin1 gene function has been associated with various solid tumors including breast, ovarian and prostate tumors (Roberts DJ, et al., 2014, Dwarakanath B, et al., 2009a, Dwarakanath B, et al., 2009b). Similarly, combined treatment of Akt inhibitors along with radiation has been shown to induce autophagy in numerous carcinoma conditions, thus enhancing radiosensitization of the cancer cells (Maiuri MC, et al., 2009). Molecular mechanisms through which autophagy helps tumor suppression are poorly understood. The best-determined mechanism is autophagy's ability to degrade damaged and mutated components of a cell which may otherwise gain oncogenic properties (Dwarkanath BS, et al., 2011). Metabolic inhibitors like 2-deoxy-D-glucose (2-DG) have been shown to induce autophagy under conditions of starvation like

in hypoxic tumor cells (Dwarakanath BS. et al., 2009). Whether variations in the extent of autophagy induction is partly responsible for the heterogeneity in the response of tumor cells *in vitro* and *in vivo* to 2-DG alone and in combination with ionizing radiation or anticancer drugs needs to be investigated, so as to individualize the therapy using 2-DG as adjuvant (Gupta S, et al., 2005, Dwarakanath BS. et al., 2009).

Chapter 3

Material and Methods

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell culture

3.1.1 Experimental cell lines

Murine macrophage, *RAW 264.7* cells were obtained from the American Type Culture Collection. Whereas Human colon carcinoma, HCT 116 and INT 407 cells used during this work, were purchased from National Centre for Cell Science (NCCS) Pune. *RAW 264.7* cells were maintained in high glucose DMEM medium (Sigma-Aldrich, D5648) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco) whereas HCT 116 and INT407 cells were maintained in DMEM and MEM (Sigma-Aldrich, M0275) medium respectively supplemented with 10% (v/v) fetal bovine serum. All cells were kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were treated with various chemicals including 3-Methyladenine (Sigma-Aldrich, M9281), Chloroquine (Sigma-Aldrich, C6628), Rapamycin (Sigma-Aldrich, PZ0020), Sulphorhodamine-B (Sigma-Aldrich, S1402), Trichloroacetic acid (MP Biomedical, 0215259290), DCFDA (Sigma-Aldrich, D6883), BafA1 (Sigma-Aldrich, B1793), 4-phenylbutyrate (Sigma-Aldrich, P21005), N-Acetyl-L-Cysteine (Sigma-Aldrich, A9165), GSK2606414(Cayman chemicals, 17376) and 3,5-Dibromosalicylaldehyde (Sigma-Aldrich, 122130). *Atg7* and *Ulk1* specific siRNA were purchased from Dharmacon (L-020112-00-0005). The GFP-LC3 plasmid was purchased from Addgene (plasmid no. 21073).

3.1.2 Sub culture and maintenance of cells

RAW 264.7 cells were obtained from the American Type Culture Collection. The cells were maintained in high glucose DMEM medium supplemented with 10% (v/v) heat-

inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) as well as 100 units/ml penicillin G and 100 mg/ml streptomycin. On the other hand, INT 407 cells were cultured in MEM containing 10% FBS as well as 100 units/ml penicillin G, 100 mg/ml streptomycin, 2mM glutamine and colon carcinoma cells HCT 116 were cultured in MEM and high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS as well as 100 units/ml penicillin G, 100 mg/ml streptomycin. All cells were maintained as a monolayer culture in 25 cm^2 tissue culture flasks (T-25; Corning, USA). Cells on reaching 80-85% confluency were passaged by using Cell scraper (for *Raw 264.7* cells) and via trypsinization (HCT 116 and INT 407 cells). The cells were trypsinized by 0.25% trypsin solution in PBS by washing cells with trypsin twice and then incubating for 2 min in 5% CO2 incubator maintained at 37 °C. Over trypsinization was prevented by addition of growth medium containing serum. Cells in growth medium were made into a single cell suspension by pipetting and later seeded into new flasks in split ratio specific to each cell line. The cells were used between 20 passages for experimentation to prevent growth and behaviour characteristics of each cell line used. Thus, the cells were preserved in liquid nitrogen at a lower passage number by generating cell stocks. Cell stocks were prepared by centrifugation (at 1000 rpm for 10 min) of cells resuspended in growth medium after trypsinization/ scraping. The pellet obtained was resuspended in a freezing solution containing 10% Dimethyl Sulphoxide (DMSO), 70% FBS and 20% serum media. Resuspended cells were transferred as 1ml aliquots in 1.5ml cryovials (NUNC, UK). Cells in cryovials were first stored at-20 °C for 1-2 h, then at -80 °C for overnight and finally were stored in liquid nitrogen until required. The frozen stocks were thawed at 37 °C, thawed cells were resuspended in

8 ml of growth media and then subjected to centrifugation at 1200 rpm for 10 min. The pelleted cells were resuspended in 5ml growth medium and transferred to a new T-25 flask.

3.2 Cell treatments

3.2.1 Autophagy modifier drugs

3.2.1.1 3-MA (early autophagy inhibitor) treatment

A stock solution of 3-MA (Sigma-Aldrich, M9281) was prepared at 100 mM concentration in water, the drug was dissolved by incubation in water bath at 60 ^{o}C. The required dilutions were prepared in the growth medium. Cells were treated with 3-MA for time points depending on the experiment.

3.2.1.2 BafA1 (late autophagy inhibitor) treatment

A stock solution of BafA1 was prepared (100 μM) in filtered DMSO and stored in aliquots for subsequent use. The required dilutions were prepared in growth medium. Cells were treated with desired BafA1 conc. for 1h followed by treatment with desired IR dose for further time until completion of desired time point in BafA1 containing media respectively for BafA1 and BafA1+IR plates.

3.2.1.3 Chloroquine (late autophagy inhibitor) treatment

(a) For *in-vitro* studies

A stock solution of chloroquine was prepared at 5 mM in PBS. The required dilutions were prepared in growth medium. Cells were treated with desired CQ conc. for 1 h followed by treatment with desired IR dose for further time until completion of desired time point in CQ containing media respectively for CQ and CQ+IR plates. Since CQ is light sensitive, necessary precautions were taken to avoid direct light exposure.

(b) For *in-vivo* studies

CQ was reconstituted in PBS and administered 10 mg/kg (prepared in PBS) dose *via* intraperitoneal (i.p.) route in *C57BL/6* female mice (10-12 weeks old) 1 h prior radiation exposure.

3.2.1.4 Rapamycin (MTOR inhibitor, autophagy inducer) treatment

(a) For *in-vitro* studies

A stock solution of rapamycin (Sigma-Aldrich, PZ0020) was prepared (19 mM) in filtered DMSO and stored in aliquots for subsequent use. Further, a diluted stock (200 μM) was prepared in PBS. The required dilutions were prepared in growth medium. Cells were treated with desired Rap conc. for 1 h followed by treatment with desired IR dose for further time until completion of desired time point in Rap containing media respectively for Rap and Rap+IR plates.

(b) For *in-vivo* studies

Rapamycin was reconstituted in DMSO at 20 mg/ml and further diluted in PBS containing 5% DMSO to get the desired 2 mg/kg body weight dose, which was administered through intraperitoneal (i.p.) route. The autophagy modifiers were administered 1 h prior to irradiation until otherwise mentioned.

3.2.2 ER stress inhibitors

3.2.2.1 EIF2AK3 (PERK) inhibitor

Stock solution (100 μM) of GSK2606414 (Cayman chemicals, 17376) was prepared in DMSO. The required dilutions were prepared in growth medium. *Raw 264.7* Cells were treated with desired GSK2606414 conc. for 1 h followed by treatment with desired IR

dose for further time until completion of desired time point in GSK containing media respectively for GSK and GSK+IR plates.

3.2.2.2 ERN1 (IRE1) inhibitor

Stock solution (50 *m*M) of 3,5-Dibromosalicylaldehyde, DBSA (Sigma-Aldrich, 122130) was prepared in methanol. The required dilutions were prepared in growth medium. *Raw 264.7* Cells were treated with desired DBSA conc. for 1h followed by treatment with desired IR dose for further time until completion of desired time point in DBSA containing media respectively for DBSA and DBSA +IR plates.

3.2.3 ROS scavenger

A fresh solution of N-Acetyl-L-Cysteine (NAC) (Sigma-Aldrich, A7250) was prepared at 30 mM conc. in media without serum, pH was maintained to 7.4 every time. Cells were treated with desired NAC conc. for 1 h followed by treatment with desired IR dose for further time until completion of desired time point in NAC containing media respectively for NAC and NAC +IR plates.

3.2.4 Mitophagy modifier drugs

3.2.4.1 CCCP (mitophagy inducer) treatment

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a Protonophore (H^+ ionophore) and uncoupler of mitochondrial oxidative phosphorylation. Due to uncoupling action, it is a potent inducer of mitophagy in living cells. For our mitophagy related studies it was taken as positive control. A 1 mM stock solution of CCCP (Sigma-Aldrich, C2759) was prepared in strile DMSO in dark. Cells were treated with desired CCCP conc. (20 µM) for 1-2 h followed by treatment with desired IR dose for further time until completion of desired time point.

3.2.4.2 Mdivi1 (mitophagy inhibitor) treatment

Mdivi-1 is a cell-permeable selective inhibitor of mitochondrial division DRP (dynamin-related GTPase) and inhibitor of the mitochondrial division dynamin (Dnm1). Hence is a potent mitophagy inhibitor. For our mitophagy related studies, 50 mM stock solution of Mdivi1 (Sigma-Aldrich, M0199) was prepared in strile DMSO. Cells were treated with desired Mdivi1 conc. (20 µM) 1 h prior treatment with desired IR dose. Cell were harvested after completion of desired incubation period and used for various experiments.

3.3 Cell growth and death analysis

3.3.1 Cell viability assays

3.3.1.1 MTT assay

The effects of autophagy modulators (both chemical and genomic), ER stress modifiers and ROS scavengers on the metabolic viability of respective cells were evaluated by the MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl-2H tetrazolium bromide] (Sigma-Aldrich, Missouri, USA) assay. Briefly, approx. $3x10^3$ cells per well were seeded in 96-well microplates and were cultured for overnight. The cells were then treated with indicated concentrations of specific modulator drugs for completion of desired time point. Next, the medium in each well was replaced with 200 µl of fresh medium containing 0.5 mg/ml MTT. The cells were then incubated at 37 °C for next two hour, following which the medium was discarded, and 150 µl of DMSO was added to each well in order to dissolve formazan crystals. The optical density was read at 570 nm using an automated microplate reader (Bio-Tek, Winooski, USA).

3.3.1.2 Sulphorhodamine (SRB) assay

A similar protocol was followed for studying cell viability using Sulphorhodamine-B stain. After completion of indicated time points, cells were fixed in 10% (w/v) trichloroacetic acid for 45 min at 4 ^{o}C followed by incubation with SRB for 30 min at 37 ^{o}C. After completion of the desired incubation period,wells were washed to remove the excess stain by using 1% (v/v) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution. The optical density was read at 510 nm using an automated microplate reader.

3.3.1.3 Growth inhibition kinetics

In order to study relative growth inhibition kinetics, 0.1 x10^6 cells were seeded in 35 mm dish in triplicates and allowed to grow at 37 ^{o}C in CO_2 incubator. Next day, media was changed, and cells were irradiated with the desired dose. Cells were processed for counting using hemocytometer. Relative cell number (N_t:N_0) was calculated with respect to the unirradiated control cells.

3.3.2 Colony formation assay

Macro colony formation assay was performed to assess the effect of radiation-induced cell death in the presence of autophagy, UPR and ROS inhibitors/ activator drugs. Cell in which survival study needs to be performed were seeded in triplicates. Cells were treated with the drugs, one hour prior to radiation. Media was replaced 24 h post-irradiation,and cells were incubated at 37 ^{o}C to form colonies. After 10 days (*Raw 264.7* cells) and 15 days (for HCT 116 and INT 407 cells) colonies were washed with PBS, fixed in methanol and stained with 1% crystal violet for 10 min. Excess stain was removed with PBS. Stained colonies made up of more than 50 cells were scored and manually counted.

$$\text{Plating efficiency} = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100$$

$$\text{Surviving fraction} = \frac{\text{Number of colony formed}}{\text{Number of cells inoculated}} \times \text{PE}$$

3.3.3 ANXA5 and PI staining

Apoptosis was studied using flow cytometry, after completion of desired time point post-irradiation using ANXA5-PI staining assay kit according to manufacturer's instructions (Sigma Aldrich, APOAF-Annexin V-FITC Apoptosis Detection Kit). Briefly, cells ($1x10^6$) were resuspended in 200 µl of binding buffer containing 5 µl ANXA5-FITC and 10 µl propidium iodide (PI). After 15 min of incubation at RT in the dark, samples were acquired using BD FacsCalibur flow cytometer. A minimum of 10,000 cells per sample wereacquired and analyzed using BD FACS Diva software (Becton and Dickinson, San Jose, CA, USA). The percentage of ANXA5-positive and negative cells were estimated by applying appropriate gates and using regional statistical analysis (Flow Jo software). Both early apoptotic (ANXA5 positive, PI-negative) and late apoptotic (ANXA5 positive, PI-positive) cells were considered positive.

3.3.4 DNA ladder assay

Apoptosis was also studied using DNA fragmentation assay as given by Yalda Rahbar Saadat, et al., 2015. In order to study relative DNA fragmentation, 0.25 $x10^6$ cells were seeded in 35 mm dish in triplicates and allowed to grow at 37 ^oC in CO_2 incubator. Next day, media was changed, and cells were irradiated with the desired dose. Cells were harvested by using PBS and stored at -80 ^oC untill further use. Cell lysis buffer for DNA isolation contains (proteinase K, KCl, and SDS).

3.3.5 PI uptake

Apoptosis and necroptosis was studied using flow cytometry, after completion of desired time point post-irradiation using propidium iodide (PI) staining. Briefly, cells ($0.2x10^6$) were seeded in PD 35, followed by deisred drug and IR treatments. After completion of incubation periods cells were harvested and resuspended resuspended in 300 µl of PBS containing 5µg/mL PI (prepared by diluting 1mg/mL stock solution). After 15 min of incubation at RT in the dark, samples were acquired using BD Facs Calibur flow cytometer. A minimum of 10,000 cells per sample wereacquired and analyzed using BD FACS Diva software (Becton and Dickinson, San Jose, CA, USA).

3.3.6 AO staining

Acridine Orange is a lipophilic fluorochrome stain that is permeable through the cell membrane in the neutral state and emits green light (525 nm). Through this stain we can detect the amount of acidic lysosomes present in different conditions (autophagy, apoptosis and after radiation exposure) provided to cells. Autophagy at later stages can be detected using AO stain.

In order to detect acidic vacuole formation which may be an indicative of late autophagic phenomena, $0.1x10^6$ HCT 116 cells were plated in PD-35 containing coverslips and incubated overnight at 37 ºC. Next day, radiation treatment was given and 10 µg/mL of acridine orange (Sigma Aldrich, A923) working solution and 5 µg/mL ethidium bromide (Sigma Aldrich, E7637) was added for 10 minutes. The cells were then washed with PBS. The cells were taken in a glass slide and images were taken using a fluorescent microscope.

3.4 Cell transfections

3.4.1 DNA transfections and confocal microscopy

Constructs used for transient transfections were pEGFP-LC3 (Addgene, plasmid no. 21073), pEGFP-parkin (Addgene, plasmid no. W403A). Cells that have reached 50-60% confluency were used for the transfection of DNA. 2.5μg of DNA was transfected per 35mm petri dishes (PD 35). *Raw 264.7* and HCT 116 cells were seeded at 0.2 x 10^6 per PD 35 containing coverslips, and on reaching 50% confluency was transfected with DNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in OptiMEM (Invitrogen), according to the manufacturer's instructions. Two mixes lipofectamine-optimem and DNA-optimem were prepared simultaneously and incubated for 15 minutes at room temperature. Then, these mixes were combined and left for 20 minutes for lipofectamin-DNA-optimem complexes to form. After 20 minutes, complexes were added to cells washed once with optimem. Six hours post-transfection, optimem was replaced with fresh medium containing serum and antibiotics and harvested at the completion of desired treatment time. Cells transfected with GFP-tagged proteins were observed at different time points, and photomicrographs were captured using a fluorescent microscope (Olympus, Center Valley, PA, USA) or confocal microscope (Zeiss LSM 710 ELYRA, Oberkochen, Germany).

3.4.2 Reverse siRNA transfections

For siRNA transfection, cells were transfected with either *Atg7* or *Ulk1* siRNA (50 nM) using reverse transfection with RNAi max transfection reagent (Invitrogen, 13778075) in non-antibiotic 5% serum supplemented opti-MEM media for 24 h, next day transfection media was replaced with high glucose DMEM containing antibiotic and heat inactivated serum and was processed for various assays including microscopy, Western blotting or viability.

3.5 Transmission electron microscopy (TEM)

RAW 264.7 Cells ($2x10^6$) were seeded in 90 mm dishes and allowed to attach overnight. Next day, cells were exposed to 2.5 Gy radiation dose. After 12 h, cells were washed twice with ice-cold PBS and fixed overnight in ice-cold Karnovsky's fixative [1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4] at 4 ^oC. Cells were then rinsed twice with ice-cold PBS, post-fixed in 1% osmium tetraoxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30-90%) and embedded in Epon. Semi-thin sections (300 nm) were cut using a Reichart Ultracut (Leica Microsystems Inc., Chicago, IL, USA), stained with 0.5% toluidine blue, and examined under a light microscope. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and Reynold's lead citrate and examined using FEI transmission electron microscope (Holland). The electron microscopy was done at All India Institute of Medical Sciences, Delhi, India.

3.6 Analysis of ROS and mitochondrial changes

3.6.1 DCFDA assay

DCFDA is a fluorogenic dye that measures ROS (hydroxyl, peroxyl) activity within the cells. After diffusion into the cells, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound. This compound is later oxidized by ROS into DCF, which is a highly fluorescent form. The murine macrophage cells *RAW 264.7* and HCT 116 were irradiated with desired radiation dose at the indicated time point. Media was removed, and fresh media without serum was added. Next, 10 µM DCFDA was added to each well. The cells were incubated for 30 min at 37 ^oC in dark and processed using BD FACS LSR-II flow cytometer. For 0 h reading, cells were pretreated with 10 µM

DCFDA for 20 min and irradiated; immediately after irradiation, cells were washed, scraped in PBS and used for flow cytometry.

3.6.2 Analysis of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was checked using CMXROS Mitotracker Red (Invitrogen, M7512). MitoTracker red (MTR) reacts by linking to thiol groups in the mitochondria and thus remains after the cell dies or is fixed (Chazotte B, 2011). Exponentially growing cells were seeded for cell mitochondrial potential analysis at a density of approx. $1x10^5$ cells per well of a 6-well plate. After drug/ radiation/ combined radiation treatment depending on the experiment, cells were incubated in growth medium. Following completion of anticipated time point, cells were stained with 100 nM MTR solution from prepared by diluting stock (1µM stock solution in DMSO) solution in media without FBS for 10-15min. Cell were washed with PBS and observed under fluorescence microscope using TRITC laser filter. In order to acquire cells via FACS, following incubation with MTR, cells were harvested via gentle scraping/ trypsinization and 10,000 cells were acquired in BD FACS calibur in FL2 (PE) region.

3.6.3 Analysis of mitochondrial mass

Mitochondrial content was checked using MitoTracker Green FM (Invitrogen, M7514). MitoTracker Green (MTG) is a mitochondrial-selective fluorescent dye. This dye selectively covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues (Presley AD, et al., 2003). Exponentially growing cells were seeded for cell cycle analysis at a density of approx. $1x10^5$cells per well of a 6-well plate. After compound/ radiation/ combined radiation treatment depending on the experiment, cells

were incubated in growth medium. Following completion of anticipated time point, cells were stained with 50 nM MTG solution from prepared by diluting stock (1 µM stock solution in DMSO) solution in media without FBS for 10-15 min. Cell were washed with PBS and observed under fluorescence microscope using FITC laser filter. In order to acquire cells via FACS, following incubation with MTG, cells were harvested via gentle scraping/ trypsinization and 10,000 cells were acquired in BD FACS calibur in FL1 (FITC) region.

3.6.4 MitoSOX red staining for mitochondrial ROS

Mitochondrial superoxide levels were detected using MitoSOX Red (Invitrogen, M36008). MitoSOX Red reacts by specifically targeting mitochondrial superoxide radicals (but not other ROS or RNS). Reaction of MitoSOX Red with mitochondrial superoxide causes Oxidation of MitoSOX, which can be detected via enhancement in red fluorescence. The oxidized product is highly fluorescent upon binding to nucleic acid. Exponentially growing cells were seeded for MitoSOX Red staining at a density of approx. $1x10^5$ cells per well of a 6-well plate. After drug/ radiation/ combined radiation treatment depending on the experiment, cells were incubated in growth medium. Following completion of anticipated time point, cells were stained with 5 µM MitoSOX Red solution prepared by diluting stock (5 mM stock solution in DMSO) solution in media without FBS for 10-15 min. Cell were washed with PBS and observed under fluorescence microscope using TRITC laser filter. In order to acquire cells via FACS, following incubation with MitoSOX Re, cells were harvested via gentle scraping/ trypsinization and 10,000 cells were acquired in BD FACS calibur in FL2 (PE) region.

3.6.5 Mitochondrial colocalization studies using U_2OS-double tag MLS cells

U_2OS-double tag MLS cells were a kind gift from the lab of Professor Anne Simonsen (Institute of Basic Medical Sciences, University of Oslo, Norway). These U_2OS-double tag MLS cells are tagged with mitochondria localization signal expressing specific signals for GFP (green fluorescence protein) and RFP (red fluorescence protein), both of these proteins localizes to mitochondria as tagged with MLS. But GFP is pH sensitive while RFP is insensitive to acidic pH of lysosomes. Therefore, during mitophagy as mitochondria gets engulfed in autophagolysosomal membrane, thus forming mitophagic vacuole. Here due to acidic lysosomal pH GFP gets degraded while RFP remains stable. Therefore enhancement in red puncta gives clear cut signal of ongoing mitophagic activity in those cells. We seeded $1x10^5$cells on sterile coverslips paced in 35 mm petri dishes. After overnight incubation cells were given 2 ug/mL tetracyclin treatment followed by CCCP or radiation treatment till completion of desired time point. Media was discarded on completion of treatment module and cells were washed twice with PBS. Cells were fixed in 1:1 chilled acetone methanol solution for 10 minutes at -20 °C. Following fixation, cells were washed twice with PBS and coverslips were mounted on glass slides using prolong gold antifade mountant (Invitrogen, P36930) containing DAPI. Slides were visualized in FITC, TRITC and UV filter using fluorescence miscroscope, colocalized images from each sample were acquired at same settings and used for scoring and analysis of slides.

3.7 Animals

C57BL/6 female mice (10-12 weeks old) were injected with FDA approved autophagy modulators; Chloroquine or Rapamycin. Chloroquine was reconstituted in PBS and administered 10 mg/kg dose *via* intraperitoneal (i.p.) route. Rapamycin was reconstituted

in DMSO at 20 mg/ml and further diluted in PBS containing 5% DMSO to get the desired 2 mg/kg body weight dose, which was administered through intraperitoneal (i.p.) route. The autophagy modifiers were administered 1 h prior to irradiation until otherwise mentioned.

3.7.1 Macrophage isolation, culture and polarization

Peritoneal macrophages were attracted to mice peritoneal cavity by injecting 4% thioglycollate (chemoattractant, Sigma, B2551) solution in mice peritoneal cavity. After 72 h of thioglycollate stimulus, mice were euthanized, and peritoneal cavity macrophages were isolated by flushing the peritoneal cavity with PBS with the help of a 25G needle. Peritoneal cavity cells were given one wash with PBS, and $0.2x10^6$ cells per group were used to quantitate macrophage population in peritoneal fluid cells using an F4/80-PE-Cy5.5 conjugated antibody.

3.7.2 Histology and immunohistochemical (IHC) staining for Ki-67

GI tissues (ileal and jejuna sections) were fixed in 10% neutral-buffered formalin (SRL) and embedded in paraffin. GI samples were sectioned at 5 μm thickness using Leica microtome, stained with H & E (haematoxylin and eosin). The sections were scored for Surviving Crypt Number, Villi Number, Villi Height etc. Only complete sections, which included the opening of crypt and full length of villi from base to the tip, were considered for scoring. Villus height was determined by measuring the distance from the tip of the villus up to the crypt in pixels. A surviving crypt was defined as containing 10 or more adjacent, healthy-looking, non Paneth cells, some Paneth cells, and a lumen (Chen N, et al, 2010). All counts and measurements from each tissue specimen were obtained "blind" from a minimum of 3 coded sections.

3.7.3 Fluorescence based Immunohistochemistry (IHC-F) in intestinal sections

In order to check intestinal crypt proliferation fluorescence based IHC with Ki-67 antibody detection was performed, sections were processed according to the manufacturer's protocol (ImmunoCruz staining kit; Santacruz, CA). Ki-67-FITC monoclonal primary antibody (Biolegend) was used at 1:800 dilution; overnight at 4°C. Corresponding tissue sections without primary antibody served as negative controls. The sections were examined by fluorescence microscopy to capture FITC and DAPI stained images using Olympus (IX51) microscope were captured at 10 and 40X magnification for quantification and are presented in results. For Microtubule associated protein 1 light chain (LC3-II) staining, sections were depraffinized, antigen retrieved in pH 6.0 citrate buffer (Dako, Carpinteria, CA), and endogenous peroxidase quenched. Sections were incubated in blocking buffer (0.1% bovine serum albumin in PBS) before exposing to LC3-II antibody. Tissue sections were incubated overnight with anti-LC3A/B antibody (CST-4108; dilution 1:100) at 4 °C. After necessary washing steps sections were incubated with goat anti rabbit HRP conjugated secondary antibody SuperPicture TM 3rd Gen IHC detection kit (87-9673; Invitrogen) was used for signal detection and color development. All the IHC slides were mounted and visualized under a bright field microscope and images were captured at microscopic magnification (20X magnification). To determine specificity of the staining, appropriate controls were run in parallel with the experimental sections.

3.7.4 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed on intestinal sections using In situ death detection kit (Sigma Aldrich, 11684795910-roche) as per manufacturer's instruction. Briefly, sections were depraffinized, pretreated with proteinase K, and endogenous peroxidase

was quenched using H_2O_2. The sections were incubated with FITC tagged terminal deoxynucleotidayl transferase (TdT), and counterstained with DAPI. Stained sections were visualized under Zeiss Axio vision microscope and images were captured at 10 and 20X microscopic magnifications for quantification. Representative images captured at 20X magnification are presented in results. Apoptotic cells were scored within the crypts of jejunum.

3.7.5 Lipid peroxidation assay

Lipid peroxidation was estimated spectrophotometrically by modified thiobarbituric acid-reactive substance (TBARS) method (as described by Varshney and Kale (1990). Cell lysate / GI tissue lysate (80 µl) was mixed with 580 µl LP Buffer (0.15M KCl + 10 mM Tris-HCl, pH 7.4) buffer to which of 30% TCA (166 µl) was added and vortex. Then 52 mM (166 µl) TBA was added. The tubes placed in a water bath in dark for 45 min at 80 ^{o}C, cooled in ice and centrifuged at room temperature for 10 min at 3,000 rpm. The absorbance of clear supernatant was measured against reference blank at 532 nm in spectro-photometer. The amount of MDA formed in a sample was estimated according to the equation

$$\text{N moles of MDA} = V \times OD/E \times v \times \text{protein (mg/ml)}$$

where, V= final volume of test solution (ml), OD = optical density, E= extinction coefficient and v=sample volume.

3.7.6 Biochemical measurements

(a) SOD assay

Activities of the anti-oxidant enzyme, Superoxide dismutase (SOD), were measured in various cell lines and GI tissues harvested at 3 days post treatment. The SOD activity

assay is based on the auto-oxidation of pyrogallol, a process highly dependent on superoxide, which is the substrate for SOD. The auto-oxidation of this compound is inhibited in the presence of SOD, whose activity was then indirectly assayed at 420 nm according to the method of Marklund and Marklund (1974). The results were represented as SOD units/mg.

(b) Peroxidase assay

This assay is based on principle of conversion hydrogen donor pyragallol to purpurogallin (yellow coloured product) in the presence of cellular peroxidases which converts H_2O_2 to H_2O and O_2. Followed by measurement of coloured product at 430 nm. Activities of the anti-oxidant enzyme, peroxidase, were measured in various cell lines and GI tissues harvested at 3 days post treatment. In this assay 220 µL pyragallol (0.05M prepared in 0.1M phosphate buffer pH 6.5), 30 µL sample, 50 µL H_2O_2 (1% in phosphate buffer pH 6.5), kinetic reads were recorded after every 30 sec for 5 min.

$$\text{Units /ml enzyme} = \frac{\text{(Change in OD of test sample- change in OD of blank) x dilition factor}}{\text{Extinction coefficient of Purpurogallin x volume of sample in µL}}$$

$$\text{Units / mg protein} = \frac{\text{units / ml enzyme}}{\text{mg protein / ml enzyme}}$$

(c) GSH assay

The level of the non-enzymatic cellular anti-oxidant, Glutathione, was measured according to the method of Moron et al., 1979. The estimation was performed by measuring GSH activity in various cell lines and GI tissues harvested at 3 days post treatment. GSH stock solution (1 mM in 5% TCA) was prepared for generation of GSH standard curve by serial dilution method in triplicates. The sulfhydryl group of GSH

reacts with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) and produces a yellow colored product. The protocol is a colorimetry based assay. Samples in which GSH levels to be analysed were assayed by taking 30 µL of sample and 200 µLof DTNB followed by incubation at 37°C in dark. Activity was measured by taking absorbance at 415 nm using automated microplate reader (Bio-Tek, Winooski, USA).

3.8 Protein studies

3.8.1 Western blotting analysis of cell protein expression

Western blotting is used to assess the relative expression of proteins in between samples. Proteins were resolved on polyacrylamide gel using SDS- polyacrylamide gel electrophoresis (SDS-PAGE) with following the detection of proteins via western blotting.

3.8.1.1 Cell lysis

Media was aspirated from PD 60 and PD 90 and collected in 15 ml falcons kept on ice. Cells washed once in ice-cold PBS were harvested with the help of scrapper in PBS and collected in same 15 ml falcon. Cells plus media was then centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in PBS. Resuspended cells were transferred to chilled microcentrifuge tubes (MCTs) and again centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were discarded and cell pellets were stored at -80 °C until used further. Pellets were lysed with the help of lysis buffer on ice. 100 µl of lysis buffer was added to the pellet. MCTs kept on the ice were vortexed for 10 seconds after every 10 min for an hour and then centrifuged at 14000 rpm for 20 minutes at 4 ºC, the supernatant containing whole cell lysate were stored at -80 °C until used further used.

Composition of Lysis buffer (RIPA buffer):

- 50 mM Tris-Cl (pH 8)
- 150 mM NaCl
- 1% Triton X-100
- 0.5 % Sodium deoxycholate
- 0.1% Sodium dodecyl sulphate (SDS)
- Protease inhibitor cocktail

3.8.1.2 Protein estimation

Estimation of protein concentration in lysed cells was conducted by Binchonic acid (BCA) assay using BCA protein assay kit (Thermo Scientific, USA). BSA (1mg/ml) standard curve was plotted by preparing a two-fold dilution series, and protein concentration of unknown protein samples was determined using the standard curve.

3.8.1.3 Sample preparation

Protein samples were prepared by addition of 2x Lamelli buffer. Prepared samples were incubated in a boiling water bath for 10 min and loaded to SDS-PAGE gel.

Composition of 2x Lamelli buffer:

- 0.6 M Tris-HCl (pH 6.8)
- Glycerol 10%
- β-mercaptoethanol 5%
- SDS 2%
- Bromophenol blue 0.01%

3.8.1.4 SDS-PAGE

SDS-PAGE resolves proteins based on their size. Various gels of various percentages were prepared owing to the protein of interest to be determined. Equal amount of protein along with protein marker (Thermo Scientific, USA) were loaded in the wells of SDS-PAGE gel and was electrophoresed at 80 and 120 volts respectively for stacking and resolving using a Mini-Protean 3 Electrophoresis Cell (Bio-Rad) in running buffer for approximately 1.5-2 hours, to ensure that the bromophenol blue dye-front completely crosses through the gel of interest or on the amount of resolution required.

Composition of Running buffer:

- 192 mM glycine
- 25 mM Tris
- 0.1% w/v SDS

3.8.1.5 Western blotting

Following separation of proteins, the gel was placed in transfer buffer for 5 min to remove traces of SDS. The polyvinylidine difluoride (PVDF) membrane on which gels were to be blotted were first soaked in methanol for 5 minutes and finally placed in transfer buffer. The gels were blotted to PVDF membrane (0.2 µm and 0.45 µm) according to their molecular weight in transfer buffer at 50V for 2.5 hours at room temperature using a Transblot cell (Bio-Rad).

Composition of Transfer buffer:

- 192 mM glycine
- 25 mM Tris
- 20% v/v methanol

The membrane was then washed with PBS and incubated in blocking solution (either PBS containing 5% non-fat, dried milk or 3% BSA in PBST) for 45 minutes at room temperature on a shaker to block non-specific binding of antibodies. Following blocking, membranes were overnight incubated with desired primary antibodies diluted in blocking buffer at 4˚C. The probed membranes on the subsequent day, was washed twice with PBS containing 0.1% tween-20 on a rocker and then incubated in horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 90 min at room temperature. The membrane was then similarly washed as after probing with primary antibody. Protein of interest was then detected using an enhanced chemiluminescence procedure. Detection of the blots was performed using ECL reagents (Amersham Pharmacia Biotechnology, Buckinghamshire, UK). For loading controls, membranes were stripped using stripping buffer (composition) and re-probed with GAPDH/ ACTB (actin beta) antibodies.

3.9 Irradiation

RAW 264.7, HCT 116 and INT 407 cells were irradiated with Tele-Cobalt Facility, Bhabhatron II (Panacea Medical) at 2.5 Gy (a dose rate of 1.62 Gy/min) over an appropriate field size of 35 cm x 35 cm and at 80 SSD in the irradiation centre. 3-MA (0.5 mM), BafA1 (2.5 nM), NAC (30 mM) or 4-PBA (3.5 mM) were added into culture medium 1 h before irradiation. After irradiation, cells were incubated at atmospheric conditions of 5% CO_2 for the desired time points. For the 0 h time point, cells were processed immediately after radiation for the assay of interest.

For *in-vivo* experiments, a group of at least ten mice were given whole body irradiation with Tele-Cobalt Facility, Bhabhatron II (Panacea Medical) over an appropriate field

size of 35 cm x 35 cm and at 80 SSD in the Irradiation Centre. Mice in each group were given 1 h prior treatment of autophagy modulators and exposed to whole body radiation of 8 Gy from ^{60}Co γ -ray irradiator having a dose rate of 1.25 Gy/min followed up for survival till 30 days, animals were monitored daily for any clinical signs of distress such as weight loss, hunched posture, diarrhoea, inability to stand and reduced activity etc. For western blotting and other assays, three mice from each group were sacrificed on the third, eighth and thirtieth day after irradiation. All experiments were complied with the Institutional regulations on animal welfare protocols and were approved by the Institute's ethics committee of laboratory animals.

3.10 DNA damage repair assays

3.10.1 γ-H2AX assay

The γ-H2AX assay is a sensitive assay that has been widely reported or studied for induction of DNA double stranded breaks (DSBs) on various treatments to cells. The formation of γ-H2AX or phosphorylated H2AX in response to irradiation treatment Signifies the introduction of DNA damage in cancer cells. The assay has been extensively applied in basic research to have a sound understanding of DNA damage repair pathways or the mechanisms involved. The γ-H2AX assay was performed to detect the induction of DNA damage and the delay in the induction of DNA damage repair pathways. $1x10^5$cells were seeded on sterile coverslips paced in 35 mm petri dishes. After overnight incubation cells were given the required treatment and cultured in 5% CO_2 incubator maintained at 37 °C. Media was discarded on completion of treatment module and cells were washed twice with PBST. Cells were fixed in 1:1 chilled acetone methanol solution for 10 minutes at -20 °C. Following fixation, cells were washed twice with PBST and blocked in 5% goat serum

prepared in PBST for 30 minutes at room temperature. Cells were then treated with the γ-H2AX antibody (Merck Millipore, USA) in 1:1000 dilution prepared in 1% BSA in PBST for 1 h over parafilms in upside down (cell side in contact with Ab) position at room temperature. After treating with the antibody, cells were washed four times with PBST for 5 minutes each on a rocker and then probed with FITC-tagged secondary antibody (Santacruz, USA) in 1:3000 dilution for 1 h in dark at room temperature. Cells were again washed four times with PBST for 5 minutes each on a rocker. Finally, one drop of DAPI was added in the mounting solution on the slide and the coverslips were placed over the drop with cells facing the drop and visualized in FITC and UV filter.

3.10.2 Micronuclei assay

Micronucleus assay is a comprehensive and sensitive method for measuring DNA damage. Exponentially growing cells were seeded for cell cycle analysis at a density of approx. $1x10^5$ cells per well of a 6-well plate. After compound/ radiation/ combined radiation treatment depending on the experiment, cells were incubated in growth medium, harvested and counted at different time points. Cells were fixed in acetic acid: methanol (3:1) solution at 4 ^oC for overnight. Next day, the cell suspension was dropped over chilled side from a particular height so that cells get adhered on them, slides were dried, stained in 10 µM DAPI (Sigma Aldrich, D9542) solution (from 1.7 mM stock solution) for 5 min. slides were observed under fluorescence microscope using UV filter.

3.10.3 Cell cycle assay

Exponentially growing cells were seeded for cell cycle analysis at a density of approx. $1.5\ x10^5$ cells per well of a 6-well plate. After compound/ radiation/ combined radiation

and compound treatment depending on the experiment, cells were incubated in growth medium, harvested and counted at different time points. DNA content of cells fixed in 70% ethanol was measured by flow cytometry using the intercalating DNA fluorochrome PI (50 µg/ml). Cells untreated and treated were harvested, washed in PBS and then fixed in 70% chilled ethanol overnight at -20 °C. Following day, cells were washed in PBS after removing ethanol and were treated with ribonuclease-A (200 µg/ml) for 30 minutes at 37 °C. Subsequently, cells were stained with PI (50 µg/ml) for 10-15 minutes at room temperature. For each sample, about 10,000 events were acquired with an argon laser-based flow cytometer (FACS-Calibur, Becton Dickinson, San Jose, CA, USA) using the blue line (488 nm) for excitation. Distribution of cells in distinct phases of cell cycle was calculated from the frequency distribution of DNA content by using the Flow Jo software (Tree Star Inc., USA).

Chapter 4

Radiation Induces EIF2AK3/PERK and ERN1/IRE1 Mediated Prosurvival Autophagy

CHAPTER 4

RADIATION INDUCES EIF2AK3/PERK AND ERN1/IRE1 MEDIATED PRO-SURVIVAL AUTOPHAGY

4.1 Introduction

Accumulating evidence suggests that acute exposure to ionising radiation, mainly low-LET (linear energy transfer) causes macromolecular damage as well as reduced mitochondrial potential, leading to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These events finally lead to activation of different signaling pathways including apoptosis, cell-growth and autophagic induction (Gorbunov NV, et al., 2009, Kiang JG, et al., 2009, Kiang JG, et al., 2010, Mikkelsen RB, et al., 2003).

Radiation exposure causes macromolecular damage both by direct interaction and indirectly through the generation of reactive oxygen/nitrogen species (Buytaert E, et al., 2007). Radiation-induced damage involves ROS generation leading to oxidative stress. In turn, oxidative stress may lead to various imbalances in the cell, including DNA damage, compromised mitochondrial functioning, protein misfolding etc. In contrast to other stresses, autophagy induction following exposure of cells to radiation has received little attention (Buytaert E, et al., 2007, Nakai A, et al., 2007, Chen Y, et al., 2009, Fulda S, et al., 2010, Farrukh MR, et al., 2014,). Although, various studies have shown the induction of autophagy during radiation exposure, an in-depth analysis of the relationship has not been explored (Yang Z,et al., 2013, Kim KW, et al. 2010, Kong EY, et al., 2018, Wang F, et al.,2018, Chen Y, et al., 2015, Hu JL, et al., 2018). Autophagy has been shown to affect the survival of various cancer types when exposed to radiation (Wang F, et al., 2018, Chen Y, et al., 2015, Hu JL, et al., 2018, Sailaja GS,

et al., 2013). The endoplasmic reticulum (ER) is a crucial intracellular Ca^{2+} reservoir that serves as a platform for numerous cellular processes including translation, post-translational modification and proper folding. ER is also the starting point for sorting and trafficking of proteins and lipids to various organelles and the cell surface. During ER stress, newly synthesized proteins are unable to fold properly, leading to a process collectively known as the unfolded protein response (UPR) (Scriven P,et al., 2007). During UPR, protein synthesis shuts down until removal of all unfolded proteins from the cell system. It has been well established that stress-induced ROS formation causes indirect macromolecular damage (to DNA, proteins and lipids) (Black HS., 2004, Briganti S,et al., 2003). It also elicits an activation signal to boost the cytosolic calcium load released from ER (Farrukh MR,et al., 2014). ROS generation thus causes activation of ER stress leading to the induction of UPR (Ding W, et al., 2012, Ron D, Walter P., et al., 2007, Malhotra JD, et al., 2007). Although studies have shown a correlation between radiation, UPR and autophagy, the mechanisms are not very clear (Gorbunov NV, et al., 2009, Kiang JG, et al.,2009, Yang Z, et al. 2013, Kim KW, et al., 2010, Moretti L, et al., 2007). Therefore, it is considered worthwhile to study the possible association between ROS, ER stress and autophagy following irradiation.

4.2 Aim

In this chapter, we addressed the influence of radiation induced autophagy on cell survival and the signaling mechanism involved following radiation exposure in murine macrophages. Since radiation-induced macromolecular damage is associated with ROS generation, we hypothesised that autophagy is induced to recycle damaged macromolecules (cargos) thereby protecting the cell against the radiation stress. Macrophages serve as an important line of defense under most of the stress conditions in our body. Therefore, in this study, we have investigated the induction of autophagy

following irradiation in murine macrophage cell line (*RAW 264.7*) as well as peritoneal macrophages *ex vivo*.

The main aims of the current chapter were:

1. To study kinetics of autophagy induction following radiation exposure.
2. To explore the relationship between radiation induced ROS, UPR and autophagy and the signaling pathways involved.
3. To investigate relation ship between radiation induced autophagy and apoptosis.
4. To investigate the influence of radiation induced autophagy as radioprotection strategy in normal mice following radiation exposure.

4.3 Results

4.3.1 Radiation induces autophagy in *RAW 264.7* cells

Autophagy has been suggested to play a pro-survival role under various stress conditions (Castino R, et al., 2005, Alnasser HA, et al., 2016, Hsieh CH, 2011, Li J,et al., 2009, Dalby KN, 2010). To investigate the role of autophagy in radiation-induced cellular stress and cell death; we first determined dose-dependent lethality of *RAW 264.7* cells exposed to IR (0 to 10 Gy) by analyzing growth inhibition. The LD_{50} was found to be approx. 2.5 Gy in these cells (Figure 4.1A). Unless specified otherwise, all further investigations to understand the relationship between radiation-induced cell death and autophagy were carried at an absorbed radiation dose of 2.5 Gy, 12 or 24 h post-irradiation. A time-dependent growth inhibition (relative cell number at 24 h after irradiation) was accompanied by loss of cell as well as metabolic viability, and a significant loss of clonogenic survival at 2.5 Gy (Figure 4.1B), clearly suggesting cell death. Next, we examined the induction of cell death by analyzing phosphatidyl externalization using multi-parametric flow cytometry with ANXA5/Annexin A5 and

PI (apoptosis), as well as uptake of PI (necroptosis) (Figure 4.1C, upper left and right panel respectively) and found 30% to 40% increase in ANXA5 & PI-positive cells (apoptosis and necroptosis) at 12 and 24 h post-irradiation. Radiation-induced apoptosis was confirmed by the enhanced CASP3/Caspase 3 cleavage (Figure 4.1C, lower panel). To understand the role of autophagy under radiation stress, we examined its status and functional relevance in irradiated *RAW 264.7* cells by comparing the level of the autophagosomal membrane-bound form of MAP1LC3A/Microtubule associated protein 1 light chain 3 alpha (also known as LC3-II) relative to ACTB/Actin beta (Klionsky DJ, et al., 2012). A dose-dependent increase in autophagy levels was observed in irradiated cells, which correlated well with a significant decrease in the autophagy substrate SQSTM1/Sequestosome 1 (also known as p62) with increasing dose of radiation suggesting the induction of autophagy (Figure 4.1D). Further, the kinetics of autophagy induction was studied by harvesting irradiated *RAW 264.7* cells at different times post-exposure. The levels of LC3-II peaked at approximately 12 h post-irradiation and stabilizedafter that (Figure 4.1E). The autophagic flux was studied using the lysosomal proton-pump inhibitor BafA1, which further confirmed radiation-induced autophagy in these cells (Figure 4.1F). Cells of human origin (U2OS, human osteosarcoma) also exhibited a similar response after radiation exposure. Furthermore, we quantified the number of LC3 puncta post-irradiation in *RAW 264.7* cells transiently transfected with a pEGFP-LC3 plasmid, 12 h post-irradiation, and observed a nearly 4-5 fold increase in EGFP-LC3 puncta formation as compared to non-irradiated control cells (Figure 4.1G). This observation was complemented by transmission electron microscopy quantifications, where significantly more autophagic vacuoles (autophagosomes) were observed 12 h post-irradiation (Figure 4.1H). Taken together, these results indicate the induction of autophagy as well as apoptosis in irradiated cells.

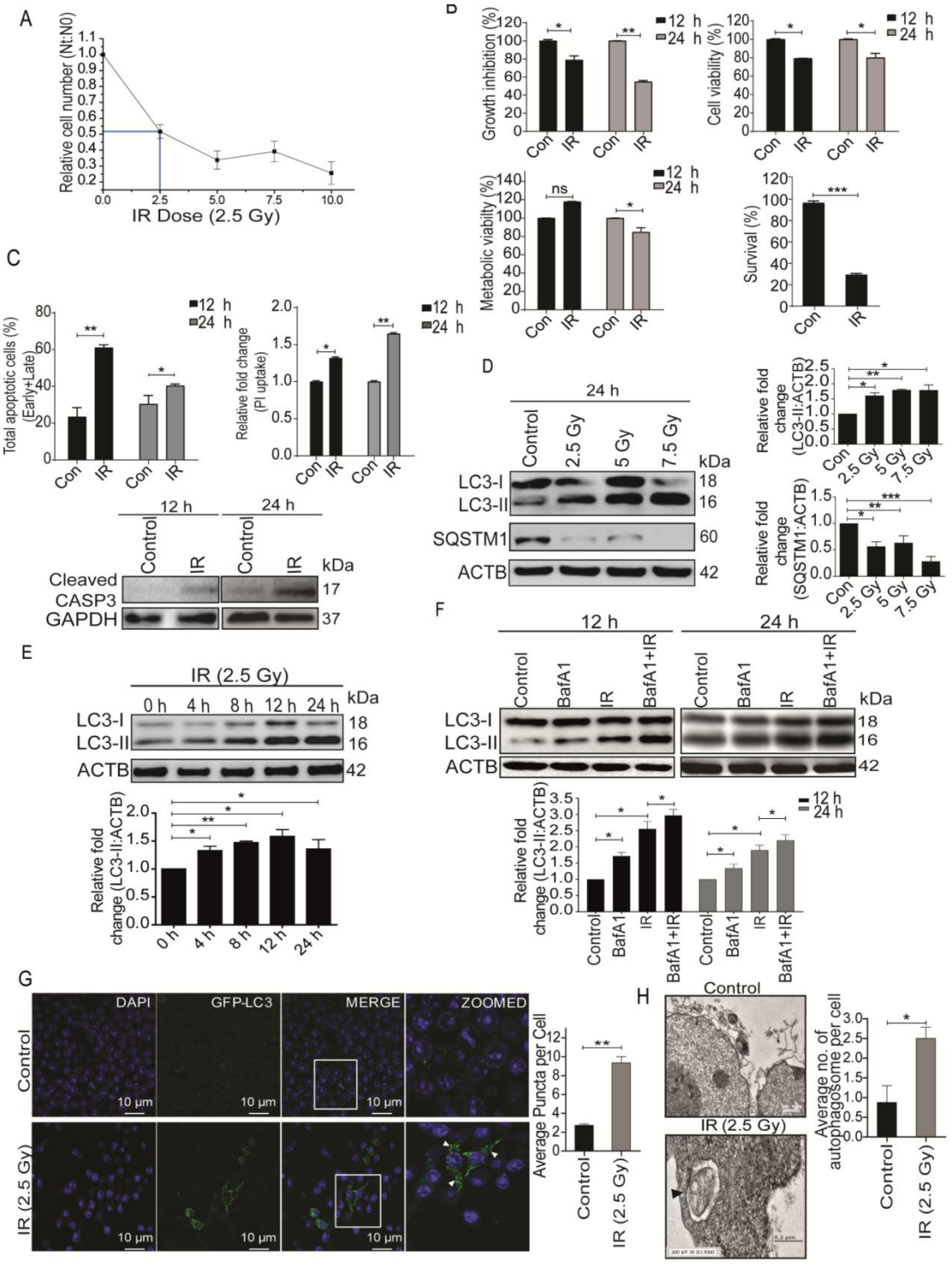


Figure 4.1: Radiation induces autophagy in *RAW 264.7* cells. **(A)** *RAW 264.7* cells were exposed to different doses of IR ranging from 0 to 10 Gy, 24 h post-irradiation cells were counted, and relative growth was calculatedwith respect to unirradiated control cells. The graph represents growth of irradiated cells relative to unirradiated control. LD_{50} of these cells was found to be 2.5 Gy. Data is representative of three independent experiments. **(B)** *RAW 264.7* cells were exposed to 2.5 Gy IR and processed for growth inhibition kinetics, cell viability and metabolic viability. Cells were counted for growth inhibition or processed by Sulphorhodamine B (SRB) or MTT for cell and

metabolic viability respectively after 12 and 24 h. For the clonogenic assay, cells were exposed to 2.5 Gy radiation and were incubated at 37 ^{o}C to form colonies. Upper left and right panel represents growth inhibition and cell viability (by SRB assay) whereas lower left and right panel represent metabolic viability and clonogenicity. **(C)** Upper left panel: A bar graph showing results from ANXA5-PI assay in irradiated vs normal cells. Upper right panel: A bar graph showing mean fluorescence intensity of propidium iodide uptake in irradiated samples as compared to unirradiated control cells using flow cytometry at 12 and 24 h post-IR exposure. Lower Panel: Western blot analysis of cell lysate obtained at 12 and 24 h post-irradiation. Blots were probed with intrinsic apoptosis marker, cleaved CASP3, GAPDH was used as loading control. **(D)** Lysates from *RAW 264.7* cells exposed to increasing IR doses ranging from 0-7.5 Gy were resolved by SDS-PAGE and blotted onto PVDF membranes, followed by immunoblotting with LC3 and SQSTM1 specific antibodies. Each blot is representative of three independent experiments. The graphs show quantitation of band intensities (relative to ACTB) from three independent experiments. **(E)** The kinetics of autophagy induction in *RAW 264.7* cells was explored by Western blot analysis of LC3-II levels (relative to ACTB) in whole cell lysates. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments. **(F)** In order to study autophagic flux, cells were irradiated, and BafA1 (100 nM) was added 2 h before harvesting. Samples were analyzed after 12 and 24 h. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments. **(G)** EGFP-LC3 puncta were analyzed through confocal microscopy (63X magnification) in irradiated *RAW 264.7* cells. Briefly, after 24 h post-transfection with pEGFP-LC3, *RAW 264.7* cells were treated with 2.5 Gy ionizing radiation and images were captured 12 h post-irradiation. Puncta were counted using Image J from at least three fields per experiment. Arrows indicate puncta post-irradiation. Also, the difference in morphology of irradiated cells can be seen in the represented image. The graph on the right represents EGFP-LC3 positive puncta per cell after radiation exposure as compared to control (**P<0.01, IR vs control). **(H)** Electron microscopy based detection of autophagosome in *RAW 264.7* cells. Electron micrographs of control and radiation-exposed cells were taken at 12 h post-irradiation. Arrowhead in the representative micrograph shows the autophagosome. Autophagosomes were counted manually from at least three fields per experiment. The graph on the right represents an average number of autophagosome/cell after radiation exposure as compared to control (*P<0.05, IR vs control).

4.3.2 Radiation-induced autophagy is ROS dependent

Generation of reactive oxygen species (ROS) has been linked with the autophagy regulation (Kiang JG, et al., 2010, Gorbunov NV, 2009, Lin JH, et al., 2008) and we, therefore, investigated whether radiation-induced autophagy in *RAW 264.7* cells is ROS dependent. The kinetics of radiation-induced ROS was studied using flow cytometric analysis of DCFH-DA fluorescence andshowed an initial burst immediately after irradiation (marked as 0 h) followed by a delayed ROS at approximately 12 h (Figure 4.2A). To scavenge radiation-induced ROS production, we determined the optimum dose of the antioxidant N-acetylcysteine (NAC) in *RAW 264.7* cells. ROS production was significantly abolished by

incubation with 30 mM freshly prepared NAC (pH=7.4) for one hour prior to irradiation (Figure 4.2B). NAC treatment also attenuated irradiation-induced autophagy, as suggested by a decrease in the levels of lipidated LC3, indicating oxidative stress to be upstream of autophagy induction (Figure 4.2C). Interestingly, we also observed a reduction in the levels of LC3-I along with a decline in LC3-II in NAC treated cells post exposure. The drug alone group have also shown some reduction in LC3 lipidation probably due to the effect of these drugs on the basal levels of autophagy. Taken together, these results suggest that radiation-induced ROS is involved in the induction of autophagy in *RAW 264.7* cells.

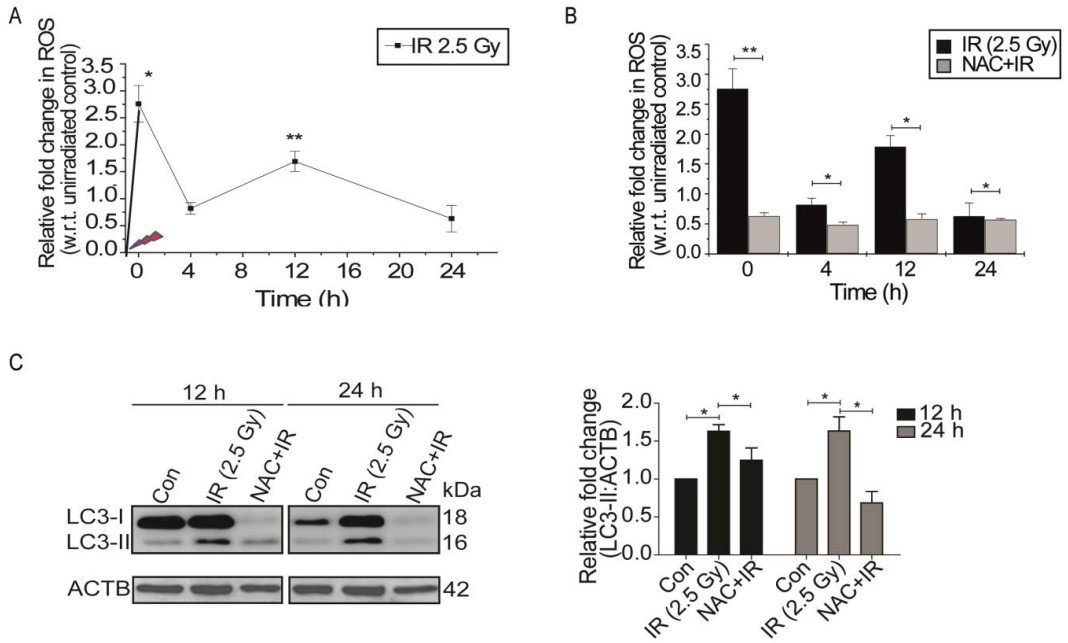


Figure 4.2: Radiation induces oxidative stress in *RAW 264.7* cells. **(A)** Kinetics of radiation-induced oxidative stress in *RAW 264.7* cells. The line graph shows relative fold change in fluorescence intensity for intracellular ROS production with respect to non-irradiated control cells after DCFH-DA staining (10 µM) in response to radiation exposure (2.5 Gy). *P<0.05, at 0 h and **P< 0.01, at the 12 h post-IR respectively. **(B)** Effect of ROS scavenger NAC (freshly prepared, given 1 h prior irradiation) on the IR-induced oxidative burst**.** The values shown are relative to unirradiated control (considered as zero). The 0 h time point represents samples processed immediately after radiation. **(C)** Radiation-induced changes in autophagy levels are ROS-dependent. Immunoblots of total protein lysates from control, IR and NAC+IR samples harvested at 12 h or 24 h post-irradiation and probed with the indicated specific antibodies. For LC3 blotting, Sigma-Aldrich, L7543 was used. Each blot is representative of three independent experiments. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments.

4.3.3 Radiation-induced autophagy induction is mediated through ER stress (the UPR)

Since ER signaling is one of the major processes involved in regulation of autophagy, we hypothesized that ER stress might play an important role in radiation-induced autophagy (Ogata M, et al., 2006, Yorimitsu T, et al., 2006, Li T,et al., 2013). We explored the induction profile of the classical UPR marker, HSPA5/Heat shock protein family A (Hsp70) member 5 (also known as GRP78/Glucose regulatory protein 78), post-irradiation and observed a time-dependent induction (Figure 4.3A), which resembled the pattern of LC3 lipidation (Figure 4.1E) and oxidative burst (Figure 4.2A). Available evidence suggests a link between oxidative stress and ER stress (Malhotra JD, et al., 2007, Cao SS, et al., 2014, Xue X, et al., 2005, Yen YP, et al., 2012). To examine the relationship between radiation-induced ROS and UPR, we suppressed ROS with freshly prepared NAC and monitored the levels of HSPA5. NAC significantly reduced the levels of HSPA5 suggesting that radiation-induced UPR is ROS-dependent (Figure 4.3B).

In order to find out whether radiation-induced autophagy is UPR dependent, we suppressed UPR with its inhibitor 4-phenylbutyrate (4-PBA) and found a significant reduction in HSPA5 (Figure 4.3C). Reduction in the levels of radiation-induced LC3-II (more significant 12 h post-irradiation) in PBA-treated cells (Figure 4.3D), strongly suggests that the irradiation-induced autophagy is indeed UPR dependent and is linked to ROS. Decreased LC3 lipidation in cells treated with drug alone indicates the effect of these drugs on the basal levels of autophagy.

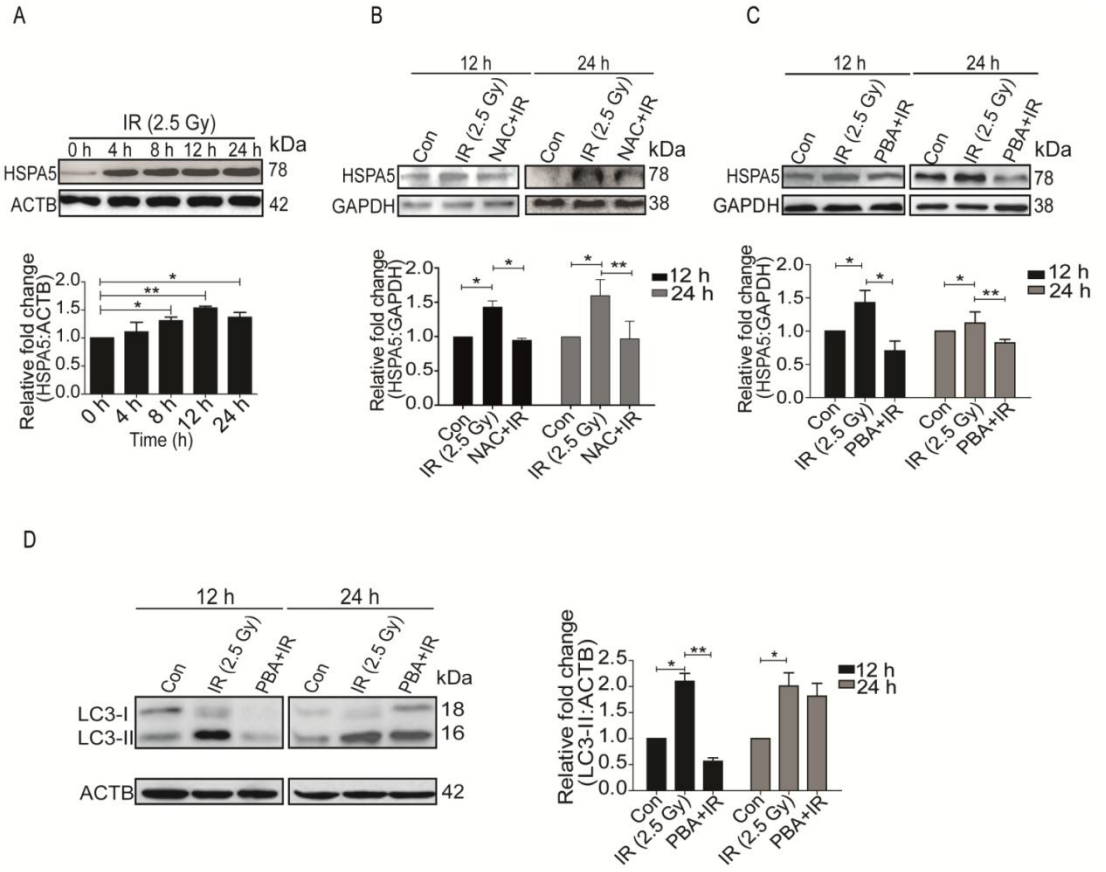


Figure 4.3: Radiation induces ER stress in *RAW 264.7* cells. **(A)** Kinetics of UPR induction after radiation exposure was studied through the expression profile of the UPR marker, HSPA5. The experiment was performed at least three independent times. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments. **(B)** Immunoblots of total protein isolated from control, IR and NAC+IR samples at 12 or 24 h post-irradiation for analysing HSPA5 expression. NAC treatment was given 1 h prior to radiation followed by IR exposure. Blots were probed with the indicated specific antibodies. Each blot is representative of three independent experiments. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments. **(C)** Western blot analysis of UPR marker HSPA5 in cell lysate obtained from *RAW 264.7* cells irradiated in the presence of UPR inhibitor 4-PBA (3.5 mM). The data shown are representative of at least three separate experiments. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments. **(D)** LC3 levels were analyzed in the samples treated with 4-PBA. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments.

4.3.4 EIF2AK3 and ERN1 gets activated after radiation exposure and lead to autophagy induction

UPR is mediated by three major signaling pathways, namely ERN1, EIF2AK3 and ATF6 (Scriven P, et al., 2007, Malhotra JD, et al., 2007, Ding W, et al., 2012, Lin JH, et al., 2008). All these pathways have also been found to play a role in the induction of

autophagy during diverse stress conditions (Ogata M,et al., 2006, Kouroku Y, et al., 2007). However, specific UPR pathways activated in radiation-exposed condition are not well known. The UPR levels (HSPA5) started building post-radiation quickly, with p-EIF2AK3 being specifically activated very rapidly after irradiation (0 h) (Figure 4.4A). Furthermore, proteins like SQSTM1 and LC3 were also altered immediately after irradiation suggesting the possibility of p-EIF2AK3 mediated UPR linked to autophagy induction. As compared to early time points, the levels of both phosphorylated EIF2AK3 and ERN1 were elevated at 12 and 24 h post-IR exposure, while ATF6 remained unaltered, suggesting that EIF2AK3 and ERN1 are the major ER stress pathways involved in the activation of radiation-induced autophagy (Figure 4.4B).

To examine the role of EIF2AK3 pathway in radiation-induced autophagy in *RAW 264.7* cells, we used GSK2606414, a specific pharmacological inhibitor of EIF2AK3 phosphorylation (Sun WT, et al., 2017), and investigated the levels of autophagy. Reduction in the levels of p-EIF2AK3 in irradiated cells treated with GSK2606414 was accompanied by a significant decrease in the levels of LC3-II, indicating the involvement of EIF2AK3 signaling in radiation-induced autophagy (Figure 4.4C). To investigate the role of ERN1 in radiation-induced autophagy, we used 3,5-Dibromosalicylaldehyde (an inhibitor of ERN1 endoribonuclease activity) (Huo Y,et al., 2013, Volkmann K, et al., 2011). A significant decrease in spliced XBP1 protein coupled with a reduction in the levels of LC3-II in irradiated cells strongly suggested that ERN1 was also involved in radiation-induced autophagy (Figure 4.4D). Taken together, these observations advocate that the radiation-induced autophagy is mediated through ER stress and is specifically dependent upon EIF2AK3 and ERN1 pathways.

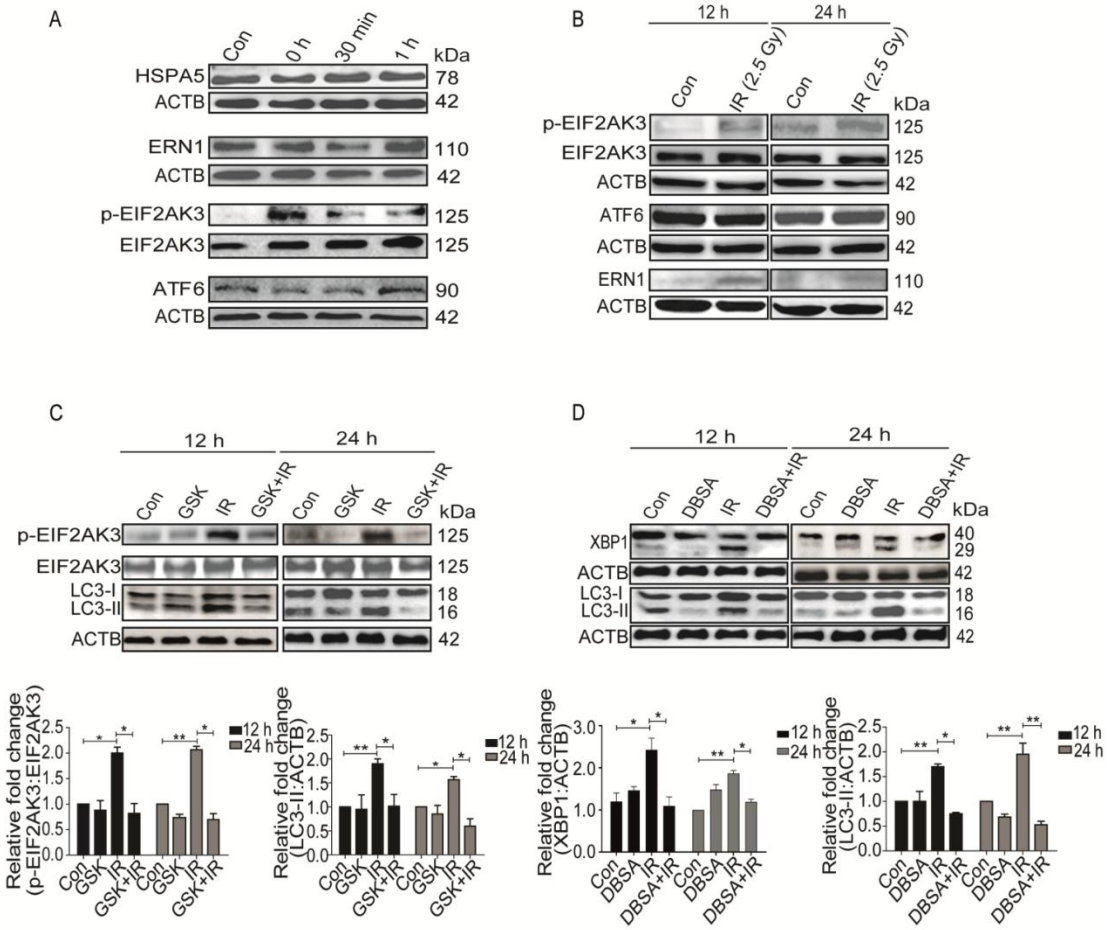


Figure 4.4: Radiation-induced activation profile of specific UPR pathways. **(A)** Immunoblot analysis of radiation-induced changes in specific UPR branch proteins at the early time point (0-1 h post-irradiation); blots were probed with HSPA5, ERN1, ATF6, p-EIF2AK3 and total EIF2AK3 specific antibodies. The time point of 0 h was taken immediately after radiation. **(B)** Western blot analysis of ERN1, ATF6 and p-EIF2AK3 levels at 12 and 24 h post-irradiation. **(C)** Immunoblot analysis of radiation-induced changes in EIF2AK3 phosphorylation and autophagy levels (LC3-II), in the presence of EIF2AK3 inhibitor GSK2606414 at 12 and 24 h post-irradiation. The graph shows quantitation of band intensities (relative to ACTB). **(D)** Immunoblot analysis of radiation-induced changes in the cleavage of XBP1 and autophagy levels (LC3-II), in the presence of ERN1 inhibitor DBSA at 12 and 24 h post-irradiation. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments.

4.3.5 Radiation-induced UPR mediated autophagy is pro-survival and anti-apoptotic

After establishing the induction of autophagy post-irradiation, we investigated its role in determining cell fate. The cellular and metabolic viability of irradiated *RAW 264.7* cells were analyzed with the SRB and MTT assays respectively, in the presence of autophagy inhibitors 3-MA (phosphatidylinositol 3-kinase (PtdIns3K) inhibitor, blocks

the early steps of autophagy) and BafA1, late autophagy inhibitor (vacuolar H^+ ATPase inhibitor; blocks lysosomal degradation) (Klionsky DJ, et al., 2012). Both, cell and metabolic viability of irradiated cells (examined *via* SRB and MTT assay respectively) were significantly reduced in the presence of these autophagy inhibitors (Figure 4.5A and B). As expected, both the inhibitors significantly reduced the clonogenic survival of irradiated cells (Figure 4.5C), while Rapamycin (autophagy inducer) enhanced the clonogenic survival (Figure 4.5D). Cell death induced by some of the drugs used may be attributed to the blockage of the basal levels of autophagy.

Next, we sought to clarify if the pro-survival role of autophagy is a consequence of the inhibition of radiation-induced apoptosis and necroptosis. Increase in PI uptake (suggestive of necroptosis, Figure 4.5E, left panel), as well as the levels of cleaved PARP1/PolyADP-ribose polymerase 1 and cleaved CASP3 (apoptosis; Figure 4.5E, right panel), indicate that loss of clonogenic survival following irradiation is indeed linked to apoptosis and necroptosis. Importantly, suppression of autophagy using siRNA against *Atg7* and *Ulk1*(both important for autophagosome biogenesis (Komatsu M, et al. 2005; Kuma A, et al. 2004; Mizushima N, et al.1998; Komatsu M, et al., 2007; Fan XY, et al., 2015) (Figure 4.5F), compromised the cell viability (Figure 4.5G), showing that autophagy can rescue the cells from radiation-induced lethality. The effectiveness of siRNA was confirmed by LC3B lipidation analysis under both starvation and radiation exposure conditions (Figure 4.5F). Furthermore, the autophagic flux in the presence of *Atg7* siRNA was confirmed using BafA1 in irradiated cells. A significant increase in LC3-II was observed in the presence of BafA1 confirming that autophagic flux is induced during irradiation. This is not the case in cells depleted of ATG7, showing the autophagy-specific effect of irradiation (Figure 4.5F, right panel). These observations lend further support to our proposition that radiation-induced autophagy is pro-survival and anti-apoptotic in nature.

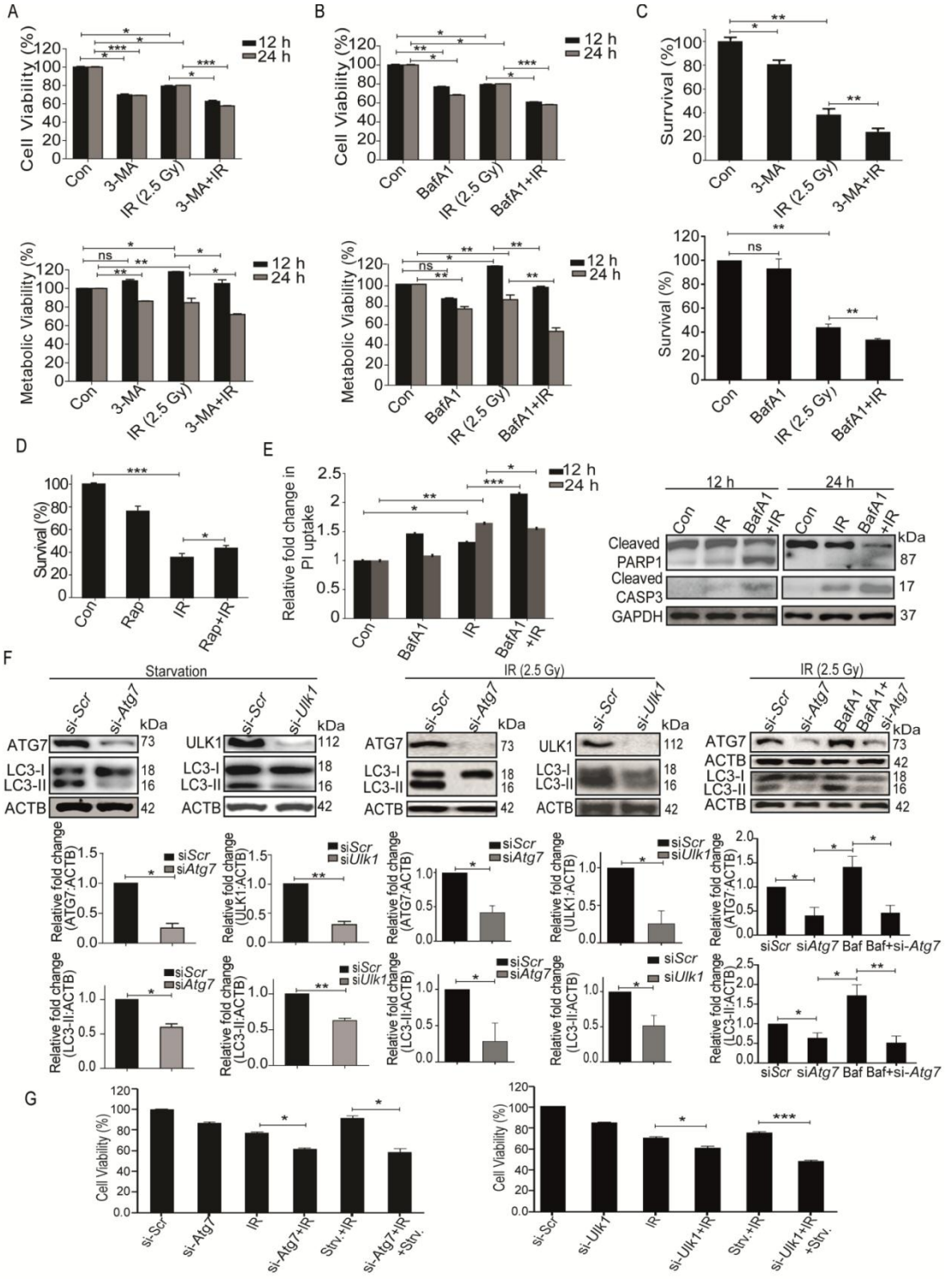


Figure 4.5: Radiation-induced autophagy is pro-survival and anti-apoptotic. **(A)** Cells were irradiated in the presence of autophagy inhibitor, 3-MA (0.5 mM). SRB data (cell viability, shown in upper graph, *P<0.05, ***P<0.001, at 12 and 24 h post-irradiation respectively) and MTT data (metabolic viability, shown in lower graph, *P<0.05, at 12 and 24 h post-irradiation respectively) at 12 and 24 h post-irradiation shows the reduced viability of *RAW 264.7* cells. **(B)** Cells were irradiated in the presence of autophagy inhibitor, BafA1 (2.5 nM). Shown in the upper graph is cell viability (SRB) in the presence of BafA1 (*P<0.05, ***P<0.001, at 12 and

24 h post-irradiation respectively) whereas in the lower graph, metabolic viability in the presence of BafA1 is shown (**P<0.01, at 12 and 24 h post-irradiation respectively). **(C)** The clonogenic assay was performed to study the cell survival in the presence of 3-MA and BafA1. The bar graph shows the survival fraction of cells irradiated in the presence of 3-MA (upper panel) or BafA1 (lower panel). (**P<0.01, *P<0.05, for 3-MA+IR vs IR and BafA1+IR vs IR respectively). **(D)** Cell survival studies in the presence of autophagy inducer, Rapamycin. The clonogenic assay was performed in the presence of 25 nM Rapamycin. The bar graph shows the survival percentage of cells treated with Rapamycin in the absence or presence of radiation. **(E)** Left panel: A bar graph showing mean fluorescence intensity of propidium iodide uptake in irradiated samples in the presence of BafA1 as compared to unirradiated control cells using flow cytometry at 12 and 24 h post-IR exposure. ***P<0.001, *P<0.05, at 12 and 24 h post-irradiation respectively. Right panel:Western blot analysis of cell lysate obtained at 12 and 24 h post-irradiation from BafA1+IR treated cells. Blots were probed with intrinsic apoptosis markers, cleaved PARP1 and cleaved CASP3; GAPDH was used as loading control. **(F)** Effect of *Atg7* and *Ulk1* siRNA on the levels of autophagy. Cells were reverse transfected with *Atg7* and *Ulk1* specific siRNAs (50 nM) and incubated for 24 h. Next,cells were either starved for 3 h or exposed to radiation, harvested after 24 h and immunoblotted with specific antibodies against ATG7, ULK1 and LC3. The effect of si-*Atg7* on autophagic flux was further studied in the presence of BafA1. **(G)** Effect of genetic downregulation of autophagy on cell viability. Cells were reverse transfected with si-*Atg7* and *Ulk1* and incubated for 24 h. Next, cells were either starved for 3 h or kept in complete medium and exposed to 2.5 Gy radiation. After 24 h post-irradiation, SRB assay was performed to study cell viability. *P<0.05, for IR vs si-*Atg7*, Strv.+IR vs si-*Atg7*+IR+Strv., *P<0.05 IR vs si-*Ulk1*+IR and ***P<0.001 Strv.+IR vs si-*Ulk1*+IR+Strv. respectively. Strv. represents starvation.

4.3.6 Pro-survival nature of radiation-induced autophagy is ROS and UPR dependent

To further investigate whether the radiation-induced pro-survival role of autophagy is ROS-dependent and UPR mediated, irradiated *RAW 264.7* cells pre-treated with freshly prepared NAC or 4-PBA were analyzed for clonogenic survival. Both NAC and PBA significantly reduced the clonogenicity post-irradiation (Figure 4.6A and 4.6B respectively). Also, the levels of cleaved PARP1 were enhanced in the presence of these drugs after radiation (Figure 4.6C and 4.6D) indicating that blocking ROS or ER stress which reduces autophagy induction (Figure 4.2C and 4.3D), lead to enhanced apoptosis, which was supported by ANXA5-PI assay as well as the DNA ladder observed under these conditions (Figure 4.6E).

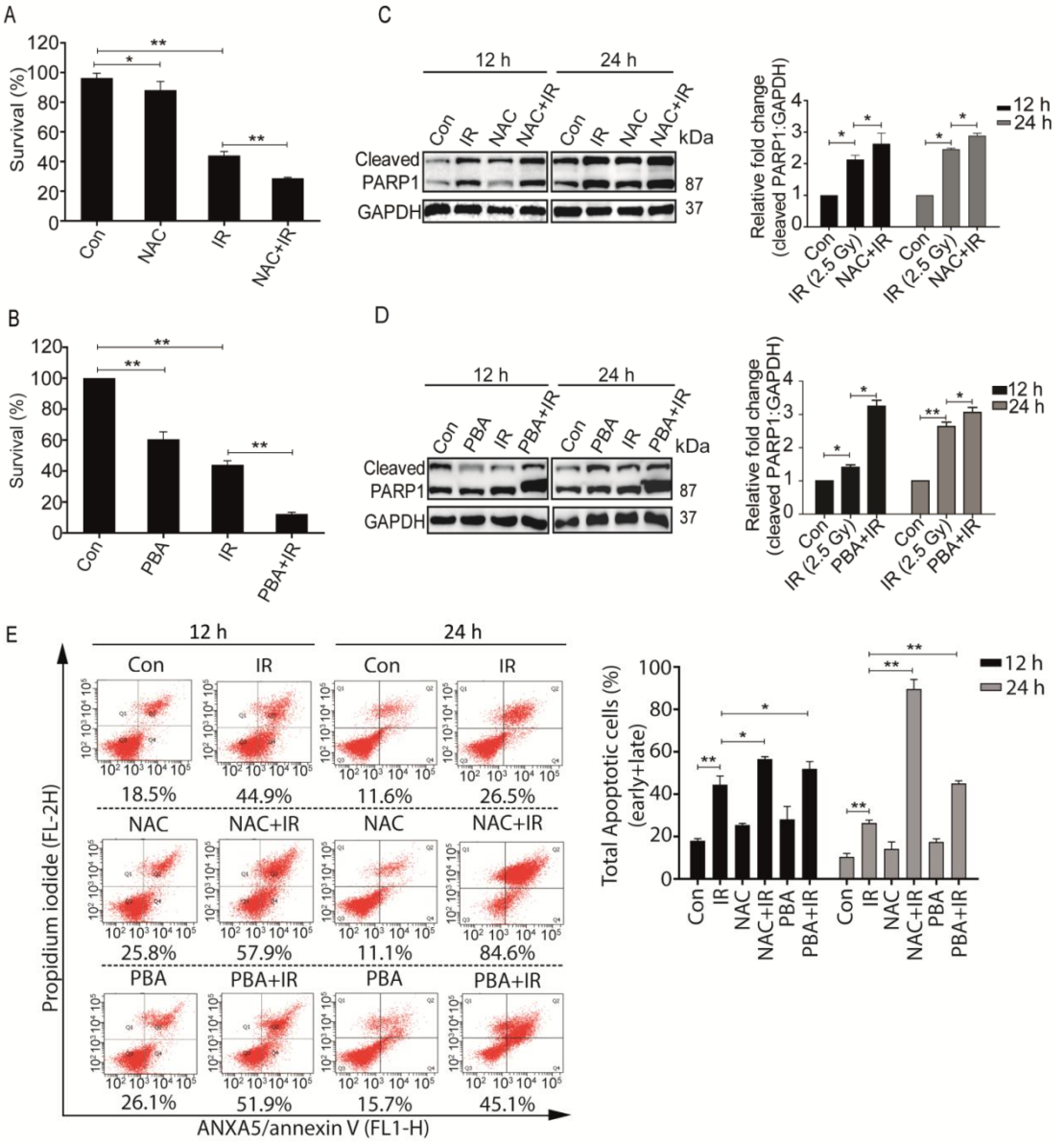


Figure 4.6: The pro-survival nature of radiation-induced autophagy is ROS and UPR dependent. **(A)** The clonogenic assay was performed after exposing cells to radiation in the presence of the ROS quencher NAC. The graph represents percent survival after irradiation compared to control. **P<0.01, for IR and NAC-treated radiation control. **(B)** The clonogenic assay was performed after exposing cells to radiation in the presence of 4-PBA. The graph represents percent survival after irradiation compared to control. **P<0.01, for IR and PBA-treated radiation control. **(C)** Western blot of cell lysates obtained 12 and 24 h post-irradiation from NAC alone, and NAC+IR treated cells was performed for the apoptotic marker, cleaved PARP1.Each blot is representative of three independent experiments. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments. **(D)** Immunoblotting of PBA and PBA+IR treated samples at 12 and 24 h post-irradiation was performed to study apoptosis after ER stress inhibition. Each blot is representative of three independent experiments. The graph shows quantitation of band intensities (relative to ACTB) from three independent experiments. **(E)** Apoptosis was analysed using ANXA5-PI staining followed by flow cytometry in *RAW 264.7* cells treated with ROS and UPR inhibitors, NAC and PBA respectively. Numbers under each cytogram represents total apoptosis (early+late). The same has been represented by bar graph in the right panel. The experiment was performed in triplicates, and the values are represented with SD.

The possibility of cell death induced due to the other off-target effects of these drugs cant be excluded. Taken together, these results further strengthen the notion that radiation-induced autophagy is ROS and UPR dependent. In addition, our data show that blocking either ROS or UPR may not be sufficient to reduce the radiation-induced cell death in *RAW* cells indicating autophagic induction as obligatory for cell survival in these conditions.

4.3.7 Autophagy activation provides a survival advantage to the irradiated animals

In order to investigate the *in-vivo* relevance of our *in-vitro* findings, we studied the effects of modulators of autophagy on the survival of total body irradiated (TBI) *C57BL/6* female mice at an absorbed dose of 8 Gy, which is the LD_{50} dose for the animals used. The MTOR inhibitor and autophagy inducer Rapamycin (Rap), provided the survival advantage in radiation-exposed animals (Figure 4.7A), while the autophagy inhibitor, Chloroquine (CQ) reduced animal survival (Figure 4.7B). Changes in the body weight, also complemented the observations on animal survival under these conditions. The induction of autophagy in these animals was confirmed at the cellular level by reduced expression of SQSTM1 in peritoneal macrophages of mice irradiated in the presence of Rapamycin (Figure 4.7C). Further, we also noted reduced levels of apoptosis (Cleaved CASP3) in Rapamycin-treated animals (Figure 4.7C, upper panel). In contrast, there was accumulation in the levels of SQSTM1 in animals irradiated in the presence of Chloroquine as compared to those exposed to radiation alone. As expected, this was accompaniedby an increase in the levels of cleaved CASP3 (Figure 4.7C, lower panel). Taken together, these results indicate a pro-survival role of radiation-induced autophagy both *in-vitro* and *in-vivo*.

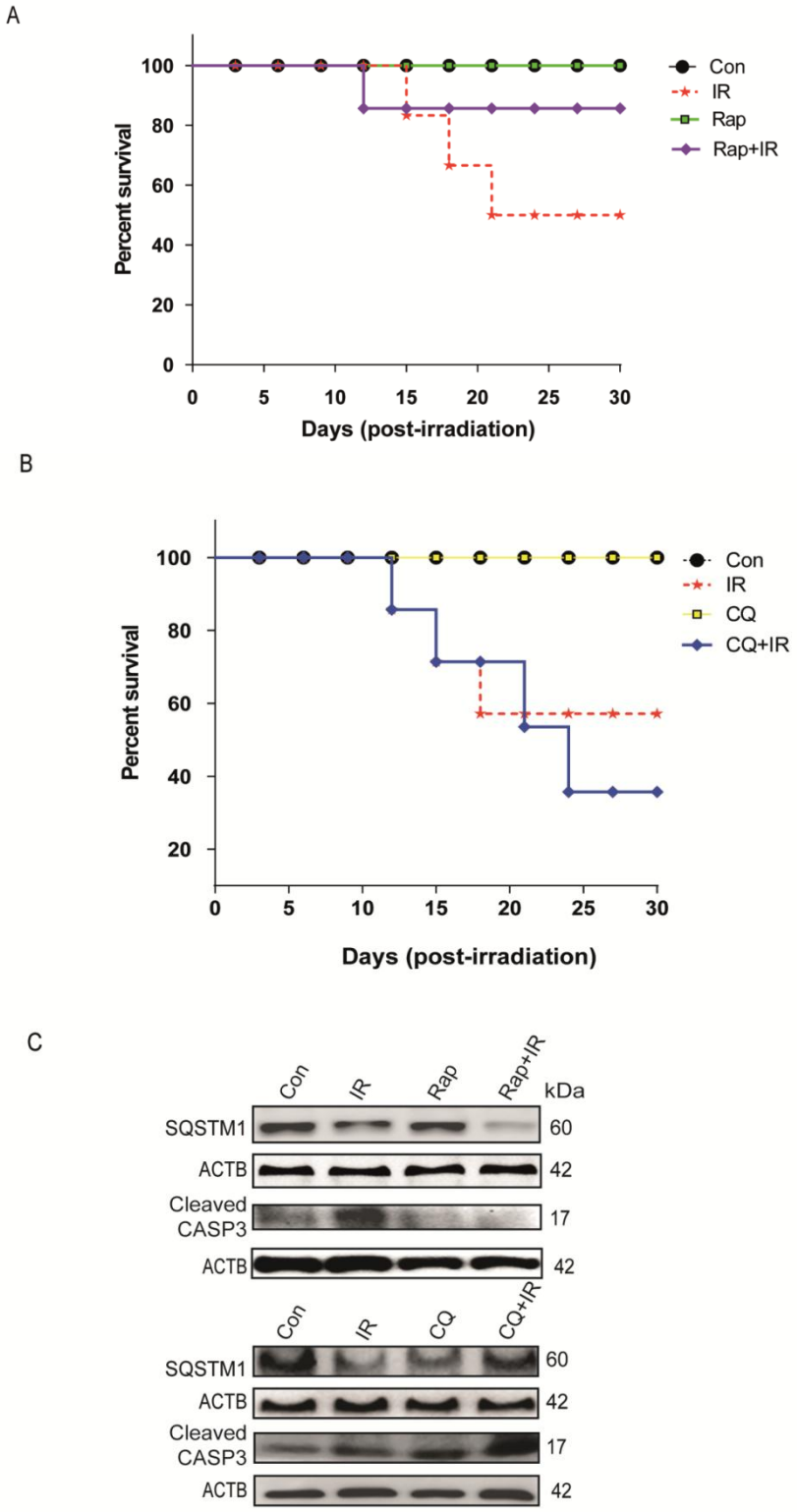


Figure 4.7: Radiation-induced autophagy is pro-survival under *in-vivo* conditions. **(A)** The effects of autophagy inducer Rapamycin (2 mg/kg body weight) on survival during the first 30 days after 8 Gy irradiation in mice. *C57BL/6* mice were randomized into four groups: control, IR, Rap, Rap+IR. Rapamycin was administered *via* intraperitoneal (i.p.) route in a single dose, 1

h prior to irradiation. Mice were observed for their body weight and lethality was scored daily for the first 30 days. Kaplan-Meier analysis was performed for mice receiving 8 Gy of total body irradiation. Each treatment group contained at least six animals. **(B)** The effect of autophagy inhibitor Chloroquine (10 mg/kg body weight) on animal survival was studied for the first 30 days after 8 Gy irradiation in mice. *C57BL/6* mice were randomized into four groups: control, IR, CQ alone and CQ+IR. CQ was administered *via* intraperitoneal (i.p.) injection in a single dose, 1 h prior to irradiation. Mice were observed for their body weight, and lethality was scored daily for the first 30 days. Kaplan-Meier analysis was performed for mice receiving 8 Gy of total body irradiation. Each treatment group contained at least 6 animals. **(C)** Immunoblotting of isolated mice peritoneal cavity cells from Rapamycin and Chloroquine (CQ) treated mice was performed after day 8 of irradiation. Each mouse was given 4% thioglycolate treatment 72 h prior to peritoneal cavity cell isolation. Cells were lysed, and blots were probed with SQSTM1 (as a marker of autophagy) and cleaved CASP3 (as a marker of apoptosis). A total of three animals were sacrificed from each group for Western blotting. Each blot is representative of two independent experiments.

4.4 Discussion

In this study, we have shown that radiation induces ROS dependent autophagy in macrophages through UPR activation. Specific inhibition of EIF2AK3 and ERN1 pathways blocked autophagy, suggesting them as the key players for radiation-induced autophagy activation. Furthermore, the induction of autophagy resulted in a decline in cell death in both cellular as well as animal model system.

Ionizing radiation generates ROS and RNS (causing oxidative stress) causing macromolecular damage in the form of protein nitration, carbonylation and lipid peroxidation, besides many oxidative products of DNA (Kiang JG, et al., 2010, Gorbunov NV,et al., 2009, Kiang JG,et al., 2009, Lin JH,et al., 2008, Li T, et al., 2013). Accumulation of these macromolecular lesions results in cell death, while proper recycling is essential for cell survival. It has been well established that radiation-induced ROS generation causes activation of unfolded protein response (UPR) and ER stress (Chaurasia M,et al., 2016, Farrukh MR, et al., 2014, Briganti S, et al., 2003, Ding W, et al., 2012). Autophagy is activatedduring oxidative stress as well as endoplasmic reticulum stress and may be both protective and detrimental following radiation exposure

(Chaurasia M, et al., 2016, Yang Z, et al., 2013, Kim KW, et al., 2010, Sailaja GS, 2013, Moretti L, et al., 2007). Cancer cells are known to activate pro-survival autophagy to develop resistance against chemo or radiotherapy (Yang ZJ, et al., 2011).In line with this, the radiation-induced autophagy in macrophages was found to be pro-survival in nature (MTT, SRB and clonogenicity data). Apoptosis is one of the major cell death pathways activated post-irradiation, initiated by the accumulation of various types of macromolecular as well as organelle damages caused mainly by oxidative stress (Yang ZJ, et al., 2011, Zong Y, et al., 2017). The survival advantage provided by radiation-induced autophagy may stem from the efficient recycling of damaged mitochondria preventing cytochrome c release (mitophagy), or due to the degradation of pro-apoptotic protein complexes (Wang K,et al., 2011). It will be interesting to understand the role of selective autophagy, e.g. mitophagy, lipophagy, ribophagy, aggrephagy etc. after radiation-induced stress conditions (Reggiori F,et al., 2012).

ROS and ER stress are associated events induced by many cytotoxic agents including ionizing radiation. During stress, unfolded proteins accumulate in the ER, leading to the activation of distinct ER stress sensors and elevation in the levels of HSPA5. Endoplasmic reticulum stress has been shown to mediate radiation-induced autophagy by EIF2AK3-EIF2S1 (eukaryotic translation initiation factor 2 subunit alpha, also known as eIF2alpha) in CASP3/7-deficient MCF-7 breast cancer cells (Kim KW, et al., 2010). Notably, the time-dependent UPR induction (HSPA5 levels) correlated well with the secondary ROS as well as autophagy, which was attenuated by NAC, suggesting that ROS generation is an earlier event to ER stress and the radiation-induced UPR is ROS dependent. Reduction in the levels of LC3-II in the presence of 4-PBA (4-phenylbutyrate, a chemical chaperone and ER stress inhibitor) lent further support to the notion that

radiation-induced autophagy is UPR dependent. Interestingly, NAC also reduced the levels of LC3-I, suggesting a possible effect of NAC on LC3 transcription and requires further investigations to understand its impact on the sustenance of autophagy. The activation of EIF2AK3 pathway is crucial for autophagic flux either through upregulation of ATG12 resulting in more LC3-II formation or through PRKAA1/Protein kinase AMP-activated catalytic subunit alpha 1 (also known as AMPK) upregulation (Kouroku Y,et al., 2007, Avivar-Valderas A,et al., 2013). Results of the present study highlight the importance of EIF2AK3 and ERN pathways in the activation of radiation-induced autophagy. Our results are in line with the earlier studies suggesting the importance of EIF2AK3 and ERN1 during the induction of autophagy (Kim KW, et al., 2010, Sailaja GS, et al., 2013). Interestingly, specific inhibition of ERN1 resulted in reduced lipidation of LC3, not only in the presence of radiation but also in control conditions indicating the importance of this pathway during basal autophagy. However, this is in contrast to the reports showing ERN1 signaling mediated impairment of autophagy flux in Huntington model (Lee H, et al., 2012). The difference in the roles of the ERN1 pathway may be due to the difference in stress conditions. The early phosphorylation of EIF2AK3 (0 h sample, immediately after irradiation) and activation of both EIF2AK3, as well as ERN1 later (12 h and 24 h), are suggestive of a tight regulation of the activationof specific UPR signaling pathways in radiation-induced autophagy. The precise role of these pathways in radiation-induced autophagy needs to be explored further. The possibility of HSPA5 independent EIF2AK3 activation pathways responsible for its activation immediately after radiation may not be excluded.

Various signaling pathways are associated with autophagy induction including PtdIns3K-AKT1 and PRKAA1 (Mizushima N, 2007). During starvation, PtdIns3K-

AKT1 is unable to activate MTOR thus making ATG1 ready to initiate the autophagic process (Chaurasia M. et al., 2016). Similarly, cellular PRKAA1 activation by a reduced ATP to AMP ratio inhibits MTOR activity and results in autophagy induction (Inoki K, et al., 2003, Qin L, et al., 2010). A fragile balance exists between autophagy and apoptosis. The anti-apoptotic protein BCL2 gets released from BECN1/Beclin 1 thus inhibiting apoptosis on autophagy induction (Marquez RT, et al., 2012). Further, STK11/Serine/threonine kinase 11 (LKB1)-PRKAA1 may activate CDKN1B/Cyclin dependent kinase inhibitor 1B (P27KIP1), a CDK inhibitor leading to the cell cycle arrest, which prevents apoptosis and induces autophagy for cell survival (Liang J, et al., 2007). Our study shows that autophagy counteracts necroptosis in radiation-exposed conditions. It will be interesting to explore the molecular mechanism responsible for the balance between necroptosis and autophagy under radiation stress.

Although autophagy and apoptosis have been recognized as important components of cellular responses to oxidative and other stress, the association between radiation-induced autophagy and animal survival has not been studied thoroughly (Stansborough RL, et al., 2017). Induction of autophagy (SQSTM1 clearance) with a concomitant decrease in apoptosis (reduced cleaved CASP3) in macrophages isolated from irradiated mice that were administered Rapamycin, lent support to the proposition that the induction of autophagy in critical cell components contributes to the survival of irradiated mice. It will be interesting to explore the effect of these autophagy modifiers in other cell types of the irradiated mice. Improved radio-protection after Rapamycin and enhanced radio-sensitization after Chloroquine treatment indicate that autophagy is a potential target for the modification of systemic response to radiation that may be utilized for developing radiation countermeasure as well as improved tumor radiotherapy.

4.5 Conclusion

In conclusion, our results suggest that radiation-induced autophagy is a pro-survival response initiated by oxidative stress and mediated by UPR via specific involvement of mediated by EIF2AK3 and ERN1pathways, and emphasize that autophagy is a protection strategy deployed by the irradiated cells for survival (Figure 4.8).

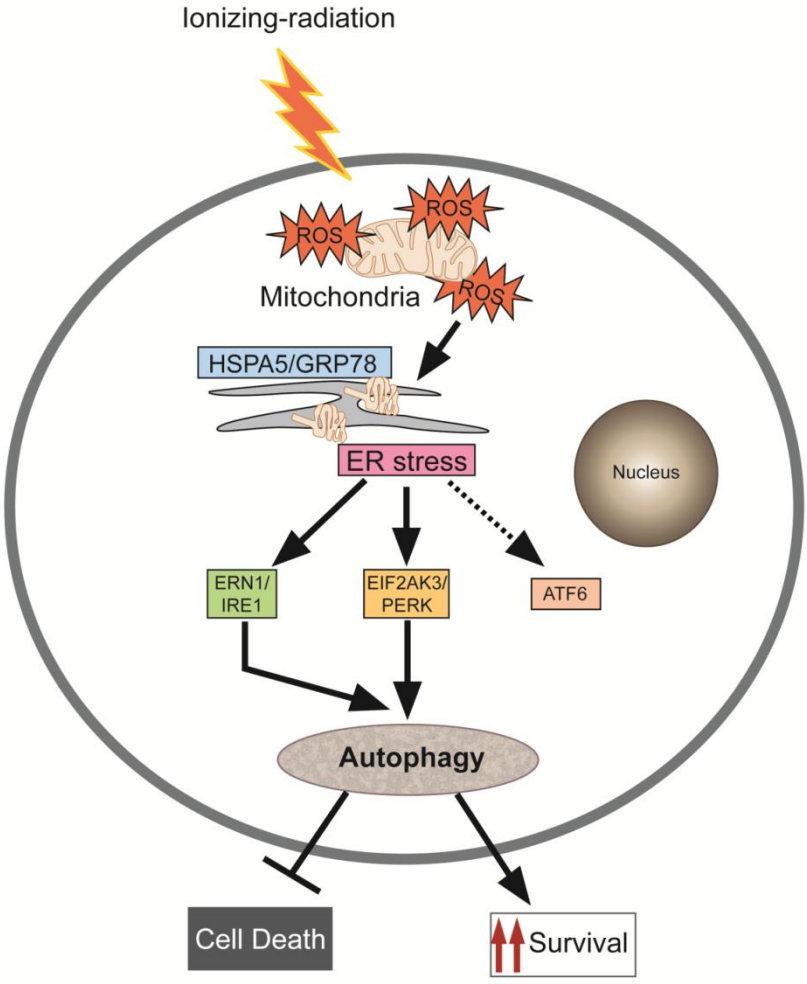


Figure 4.8 Proposed model for molecular signaling involved in radiation-induced autophagy. Radiation exposure results in the generation of numerous reactive oxygen species (ROS) mainly *via* mitochondrial potential disturbance. The formed ROS may cause damage to the macromolecules (primarily DNA, proteins and lipids) leading to protein misfolding and unfolding, resulting in ER stress. This stress is sensed through the UPR sensor HSPA5 (which binds to the unfolded proteins) causing instigation of UPR through predominant activation of EIF2AK3 and ERN1 branches of UPR. UPR results in the induction of autophagy in radiation-exposed conditions.

Chapter 5

Role of Autophagy in Recovery of Ionizing Radiation Induced Intestinal Damage in C57bl6 Mice

CHAPTER 5

ROLE OF AUTOPHAGY IN THE RECOVERY OF IONIZING RADIATION INDUCED INTESTINAL DAMAGE IN *C57BL6* MICE

5.1 Introduction

In the environment, people get exposed to a variety of radiations including UV rays, X-rays gamma rays etc. either via clinical exposures or radiation incidents and accidents. Radiation exposure results in cellular instabilities due to the generation of ROS and RNS leading to macromolecular damage at the cellular level which ultimately gets magnified and causes organ damage, leading to both acute radiation syndrome and delayed effects. Hematopoietic, gastrointestinal, skin and vascular endothelium are among the most radio-sensitive organs (Fliedner TM, et al., 2007, Mac Naughton WK. 2000, Meistrich ML, et al., 1997). Based on the dose of radiation exposure, acute radiation syndromes can be hematopoietic (HI) or Bone marrow syndrome, gastrointestinal (GI) and central nervous system (CNS) syndrome. Doses in the range of less than 7 Gy results in hematopoietic syndrome in humans, which can be identified with an overall decline in blood cells (pancytopenia), increased susceptibility of radiation exposed persons to several infections and haemorrhage. GI syndrome occurs after a whole body exposure of more than 8 Gy (Coleman CN, et al., 2004, Waselenko, et al., 2004). Ionizing radiation causes macromolecular damage and imbalances in metabolism eliciting several intracellular responses that collectively determine the fate of the irradiated cell (Chaurasia M, et al., 2016). Autophagy is one such process which can elicit under numerous stress conditions like hypoxia, nutrient deficiency, pathogenic infections (Levine B, et al., 2007, Levine B, et al., 2008). In case of radiation, just like various traumatic situations, basal levels of autophagy are found constitutive within cells to maintain homeostasis. During stress,

autophagy gets modulated manifold for recycling the damaged constituents. Numerous efforts have been done in the field of cancer biology to influence tumor survival by altering autophagy staus in them. Only a few studies present in the literature suggested the role of autophagy in case of radiation exposure to normal cells or normal healthy individuals with no previous history of ailments (Kim H, et al., 2011, Chaurasia M. et al., 2019) . The question is the strategy to be employed by the clinicians after radiation exposure in these people or other responders to rescue these individuals including army personnels. Specifically, what kind of strategy they have to follow to modulate the levels of autophagy (induction or inhibition) in order to attain better patient survival. Thus, in order to test the effect of autophagy modulation at systemic levels, pharmacological modulators of autophagy; Rapamycin and Chloroquine were used in mouse model. Rapamycin is an FDA approved drug and has been used for several years in the treatment of renal cell carcinoma and mantle cell lymphoma (Nousheen Samad, et al., 2010, Jing Li, et al., 2015). It is an immunosuppressant that was originally used in transplant patients (Gera J, et al., 2011). Rapamycin induces macroautophagy through the inhibition of MTOR/ mechanistic target of rapamycin kinase complex 1 (MTORC1) which is the complex of MTOR with Raptor (Laplante M, et al., 2013). On the other hand, Chloroquine is an endosomal acidification inhibitor which inhibits autophagy at later stages by increasing the pH of lysosomes, thus inhibiting the fusion of autophagosome with lysosomes for cargo clearance. It is traditionally used as an anti-malarial drug, and has recently emerged as an anti-cancer agent as well as a chemosensitizer when used in combination with other anticancer drugs (Klionsky D.J., et al., 2012, Homewood C.A., et al., 1972). It has been shown to help in inhibiting cell growth and inducing cell death in various types of cancer.

In the present study, we have shown the role of autophagy in radiation exposure induced mice intestinal damage recovery in whole body irradiated *C57BL6* mice.

5.2 Aim

In this chapter, we addressed the influence of radiation induced autophagy in mice intestinal damage recovery in whole body irradiated *C57BL6* mice pre- treated with autophagy modifier drugs. The main objectives of the current chapter were:

1. To study the survival profile of *C57BL6* mice treated with autophagy modifiers drugs and radiation.
2. To study the changes in the histological profile of mice GI tissue in presence of autophagy modifier drugs and radiation.
3. To explore the levels of autophagy and apoptotic markers in mice GI tissue following combination therapy and to study the proliferation ability of GI of these animals.

5.3 Results

5.3.1 Effect of autophagy modifiers on the survival of whole body irradiated *C57BL6* mice

To explore the *in vivo* radio modulative potential of pharmacological modifiers of autophagy; Rapamycin (Rap, 2mg/kg body weight) and Chloroquine (CQ, 10mg/kg body weight) were injected through intra-peritoneal route in *C57BL6* mice 1h before total body γ-irradiation (TBI). Upon irradiation, various symptoms including a gradual loss in body weight, food and water intake and ruffled fur were observed and consequently, 50% of the mice died within 15 days of exposure (8 Gy IR dose is the LD_{50} dose in these mice) in radiation alone as compared to the unirradiated control mice (Figure 5.1). Treatment with Rap provided more than 50% protection in 8 Gy irradiated mice as compared to the radiation control. In contrast to this, CQ+IR treated mice indicated enhanced reduction in body weight, food and water intake and enhanced ageing with lesser survival as compared to IR alone (20 % less) (Figure 5.1A and B).

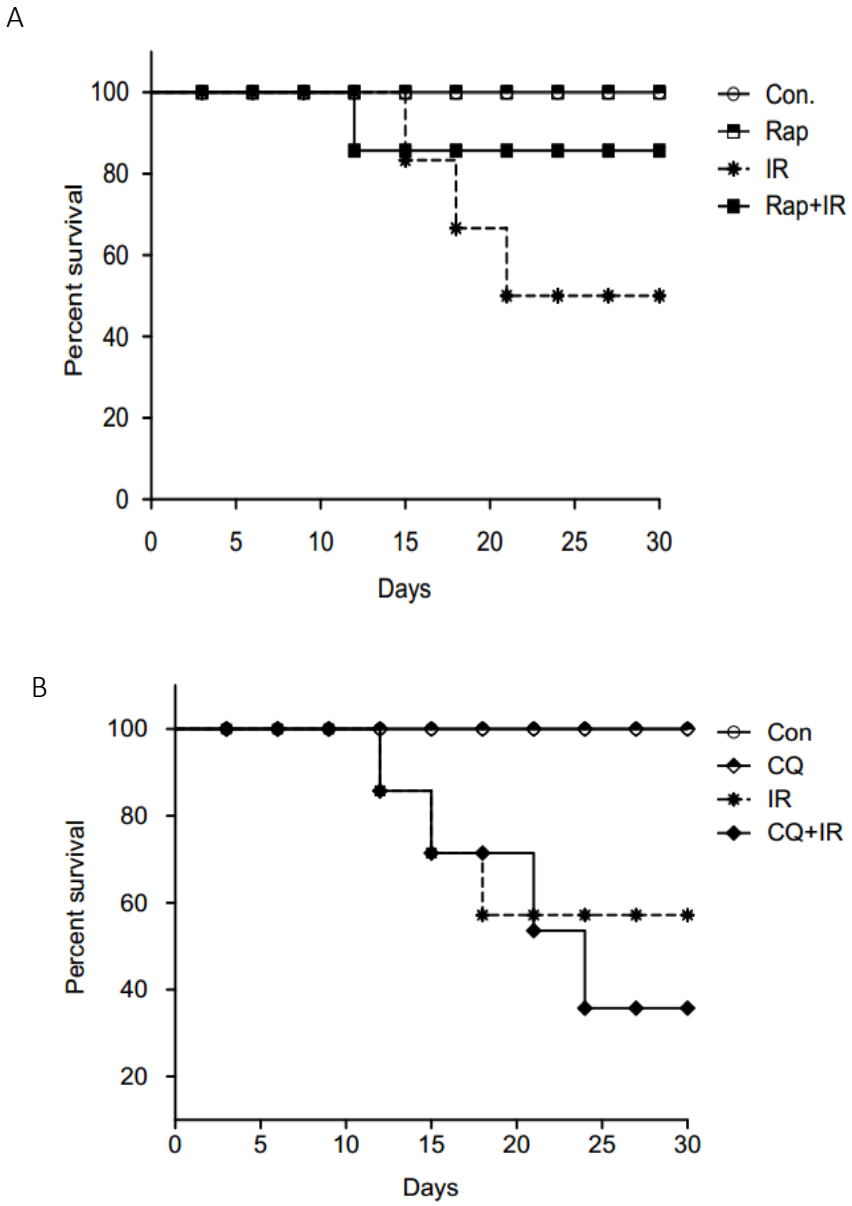


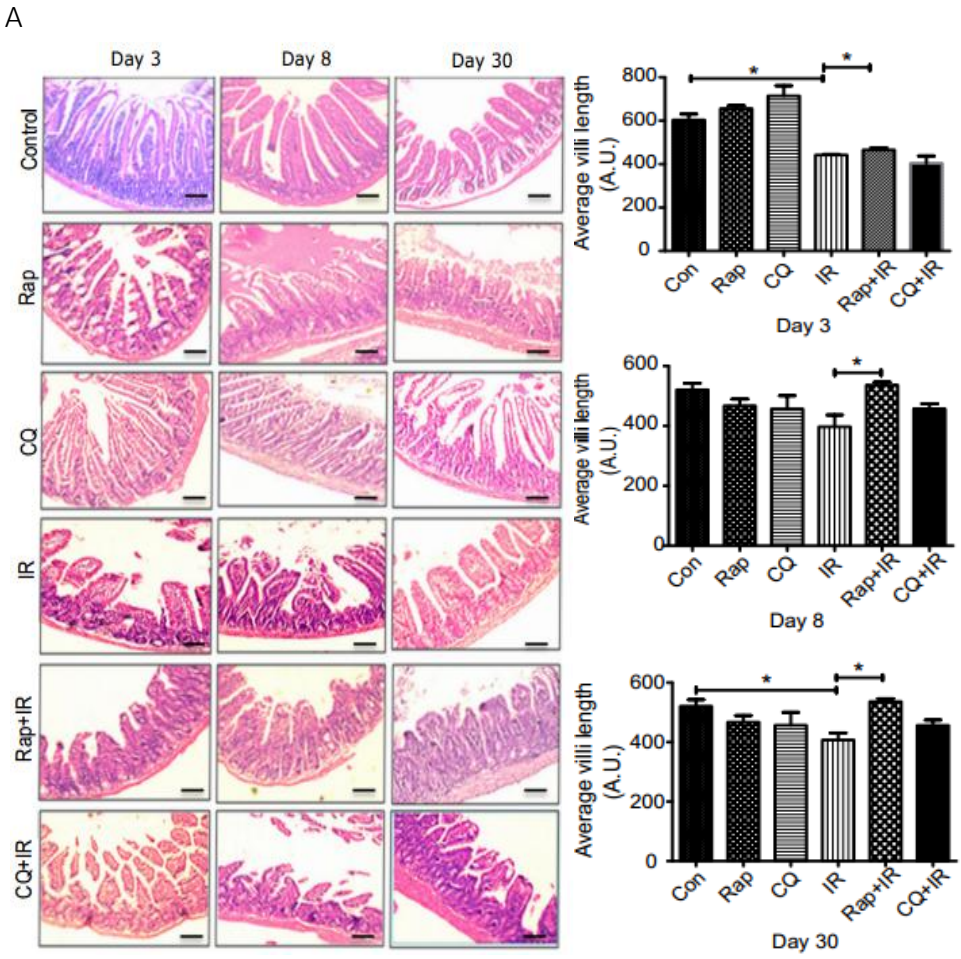
Figure 5.1: Radiation-induced autophagy is pro-survival under *in-vivo* conditions. **(A)** The effects of autophagy inducer Rapamycin (2mg/kg body weight) on mouse survival during the first 30 days after 8 Gy irradiation in mice. *C57BL6* mice were randomized into four groups: control, IR, Rap Rap+IR. Rapamycin was administered *via* IP route in a single dose 1h prior to irradiation. Mice were observed for their body weight, and lethality was scored daily for the first 30 days. Kaplan-Meier analysis was performed. Each treatment group contained at least ten animals. **(B)** The effect of autophagy inhibitor Chloroquine (10 mg/kg body weight) on animal survival was studied for the first 30 days after 8 Gy irradiation in mice. *C57BL6* mice were randomized into four groups: control, IR, CQ alone and CQ+IR. CQ was administered *via* intraperitoneal (IP) injection in a single dose, 1h prior to irradiation. Mice were observed for their body weight, and lethality was scored daily for the first 30 days. Kaplan-Meier analysis was performed for mice exposed to 8 Gy of total body irradiation.

5.3.2 Effect of autophagy modifiers in intestinal damage recovery of irradiated *C57BL6* mice

In order to explore radiation induced intestinal changes in drug treated mice, three mice per group were euthanized on day 3, 8 and 30 post irradiation, and H&E staining was performed. The villi from ileum and jejunum of healthy untreated controls were tall, cylindrical with adequate number of crypt cells having optimal length. In irradiated animals, there was reduction in the villi height, villi and crypt number, and cellularity (Figure 5.2A). At 72 h after irradiation, the crypts and villi number as well as villi height were significantly decreased. These were associated with other gross histological changes such as villi fusion and non-recoverable decreases in villi height (Figure 5.2A). In Rapamycin treated groups, the reduction in villi length and cellularity was not much affected, while severe decrease in villi height and cellularity was observed in CQ+IR group. The changes were further worse by Day 8, as indicated by more sterile crypts and further fall in villi and crypts cellularity. Contrary to this, better recovery in Rap+IR treated group was observed. Additionally, it was also observed that GI recovery in CQ+IR treatment was delayed till day 30 post-irradiation.

Ki67 is a widely used cell proliferation marker expressed in the nucleus of proliferating cells (Zhao WY., et al., 2014). In order to study intestinal crypt proliferation profile upon radiation exposure, Fluorescence based Immunohistochemistry (IHC-F) Ki67 staining of intestinal sections was performed on day 3, 8 and 30 post irradiation. IHC-F staining with Ki67-FITC antibody showed enhanced proliferation in rapamycin treated group while less proliferation was observed in autophagy inhibitor, chloroquine) treated groups (Figure 5.2B). Treatment with Rap 1 h before irradiation significantly countered radiation induced early histological changes (within 72 h), which further improved with

time. On the other hand, CQ treatment prior to irradiation decreased the number of Ki-67 positive cells as compared to the irradiated control. No alteration in the number of Ki-67 positive cells was observed in Rap/CQ alone when compared with the control group (data not shown). Radiation exposure induced hematopoietic injury causes rapid decline in hematopoietic progenitor cells, leading to pancytopenia (Singh V. K, et al., 2015a, Waselenko J. K., et al., 2004, Dainiak N, et al., 2002). Mice exposed to this level of total body irradiation displayed a rapid decline of all mature blood cell types, with a corresponding loss of hematopoietic progenitors in the bone marrow compartment. A better recovery in the bone marrow cells was observed in presence of Rap, suggesting that autophagy induction may aid in better hematopoietic recovery (Figure 5.1C).



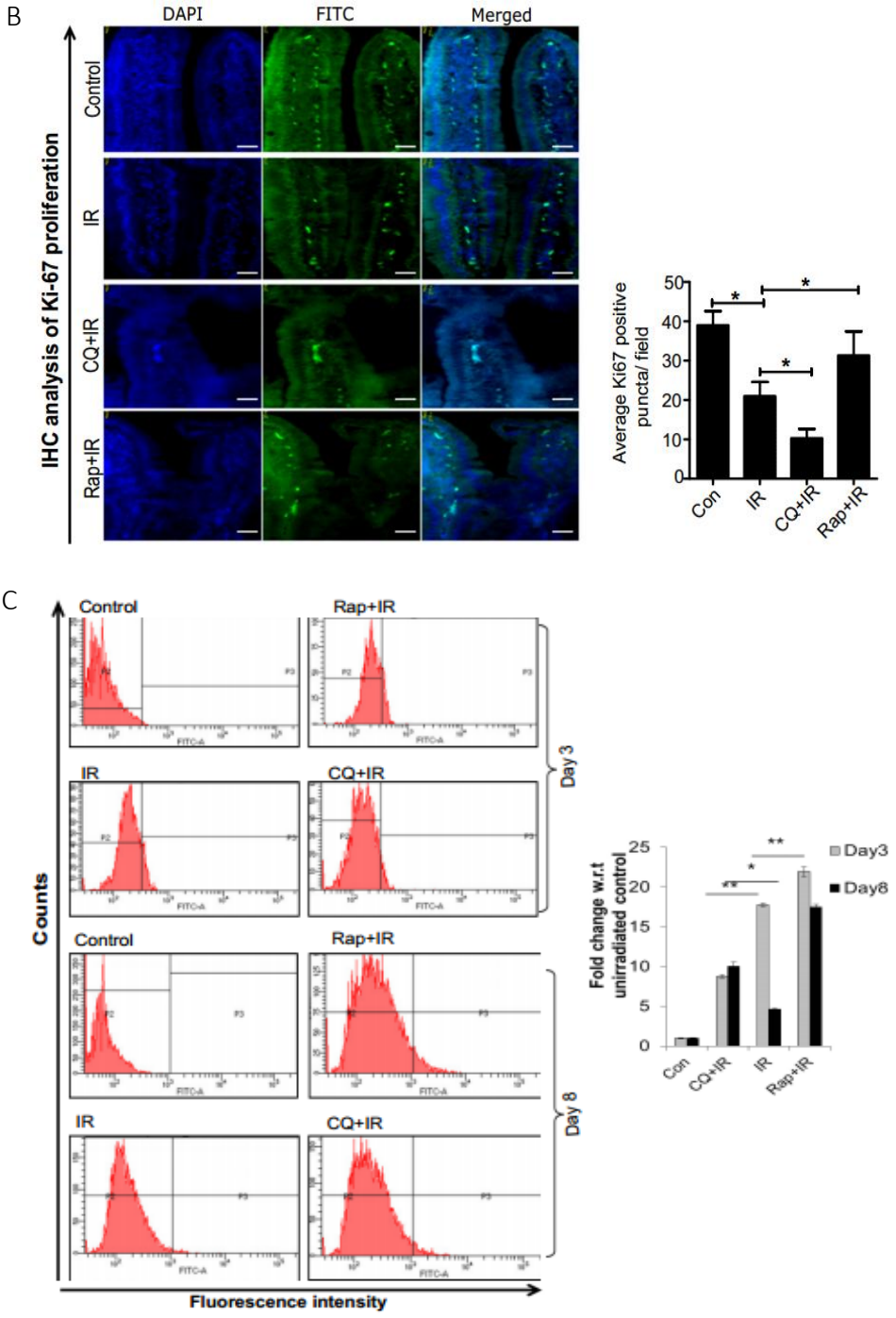


Figure 5.2: Effect of autophagy modifiers on irradiated mice intestinal damage recovery. **(A)** H&E-stained sections of *C57BL6* jejunum obtained at Day 3, Day 8 and Day 30, after exposure to whole body irradiation (8 Gy), after single dose administration of autophagy modulators (Rap or CQ) 1 h prior irradiation. Representative micrographs were taken at 10X magnification. **(B)** Fluorescence based Immunohistochemistry (IHC-F) analysis of Ki-67 positive cell proliferation in the presence of autophagy modifier drugs on day 8 post exposure. Representative micrographs were taken at 40X magnification. **(C).** Quantitative analysis of Ki-67 positive cells indication cell proliferation in the bone marrow in presence of autophagy modifier drugs, day 8 post radiation exposure.

5.3.3 Antioxidant status in murine GI tissue lysate

Highly reactive free radicals, ROS and RNS, generated after radiation exposure cause macromolecular damage in the GI cells, and are important contributing factors for acute GI injury (Kim YJ, et al., 2012). In order to understand the contribution of autophagy mediated reduction in radiation induced damage, the activity of antioxidant enzyme Superoxide dismutase (SOD), non-enzymatic GSH and lipid peroxidation was assessed. Radiation caused a notable decrease in GSH ($p < 0.05$) and SOD activities ($p < 0.05$), which Rap treatment was able to restore remarkably ($p < 0.01$) (for both GSH and SOD) in the GI of radiation exposed mice (Figures 3A and B). In contrast, CQ+IR treatment further reduced the levels of GSH and SOD activities as compared to the radiation control mice **(**Figures 5.3A and B). Ionizing radiation cause toxicity and multiple damages to vital biomolecules either directly through deposition of energy or indirectly *via* decomposition of water molecules present in human body which in-turn leads to the generation of ROS such as hydroxyl, hydrogen peroxide, superoxide radicals and RNS (Hall EJ, Giaccia AJ. eds. Radiobiology for the Radiologist, 6th ed. Philadelphia, PA, USA: Lippincott Williams & Wilkins, 2006). These ROS and RNS react with lipids and other molecules present in the cell and organelle membrane and initiate a chain reaction i.e. lipid peroxidation (LPO) by abstracting additional hydrogen atom. Thus formed LPO causes peroxidation of polyunsaturated fatty acid leading to production of Malone aldehyde (MDA) within the stresses cells (Shadyro OI., et al., 2002) Thus formed LPO adduct alters membrane integrity, permeability, fluidity and function of membrane bound enzymes (Sinha M, et al., 2012, Yong-Chul Kim, et al., 2014). Enhanced lipid peroxidation was observed in irradiated animal's GI as compared to unirradiated control group ($p<0.05$). Animals which were given combined

treatment of CQ+IR showed further enhanced levels of MDA formation ($p<0.05$). Significantly low levels of LPO adduct were formed in Rap+IR treatment as compared to the radiation control ($p<0.01$ for day 3 and 8) (Figure 5.3C).

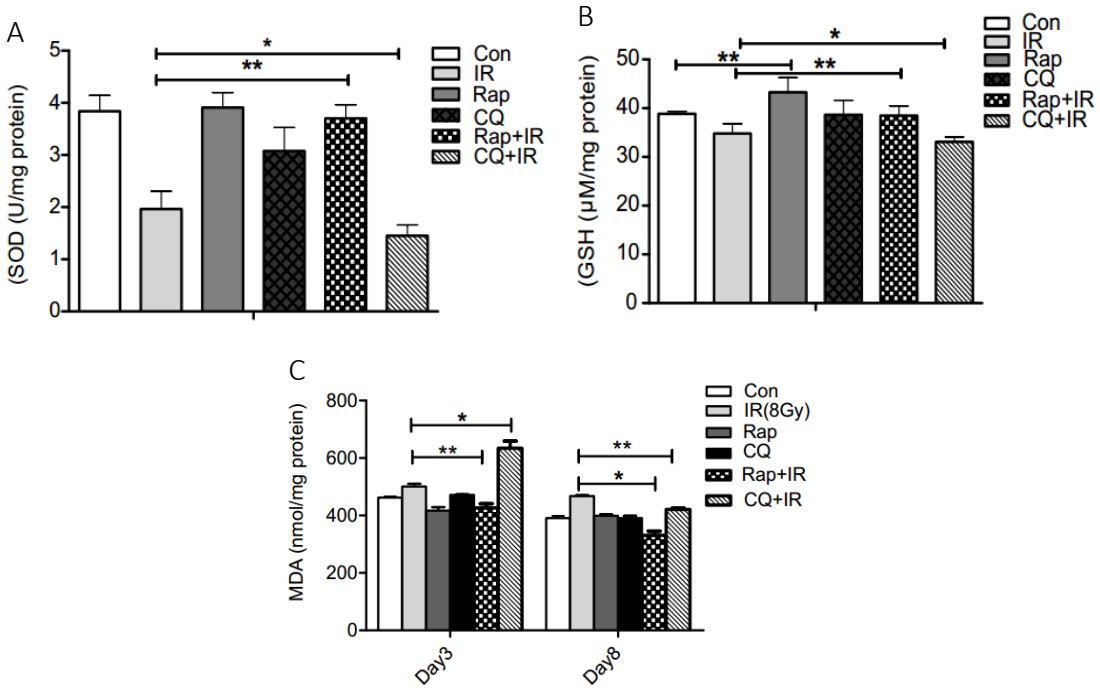


Figure 5.3: Antioxidant assays and lipid peroxidation in GI tissue lysates **(A)** ***SOD activity*** level in the GI tissues of mice at day 3 post t radiation exposure. **(B)** ***GSH*** level in the GI tissue of mice at day 3 post treatment. **(C)** ***Lipid peroxidation*** in the GI tissue of mice at day 3 post treatment**.** Induction of LPO in radiation exposed *C57BL6* mice in the presence of autophagy modulators on day 3 and day 8 post exposure. GI ileal- jejunal sections were harvested and used for LPO assay to determine MDA levels per mg protein.

5.3.4 Autophagic status in murine GI tissue

The radio protective effect of Rap treatment in mice intestine was evaluated at both day 3 and day 8 post radiation exposure. IHC with LC3-II antibody showed enhanced levels of lipidation of LC3, i.e. LC3-II formation in irradiated animals. Rap +IR treated mice showed relatively enhanced accumulation of LC3-II positive cells in mice GI tissue as compared to IR and CQ+IR. Drug alone groups didn't show comparative LC3-II lipidation (Figure 5.4A).

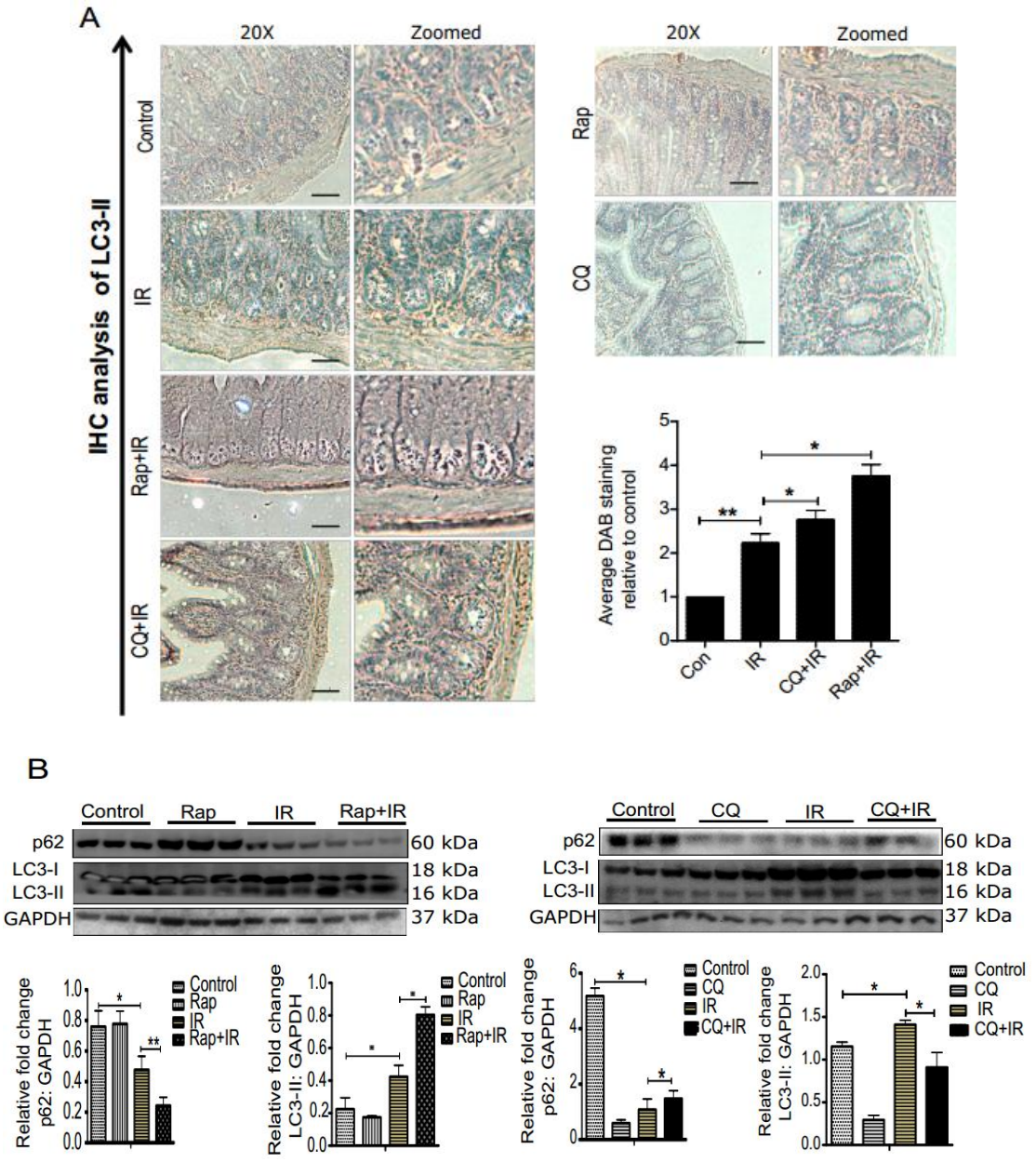


Figure 5.4: Autophagic status in mice GI tissue. **(A)** Autophagy status using IHC in mice jejunal tissue using LC3-II antibody. Effect of Rap and CQ treatments was evaluated on day 3 and day 8 post exposure. **(B)** Western blot analysis of LC3-II and p62 levels in mice jejunal tissue lysate. Effects of Rap and CQ treatments were evaluated on day3 post irradiation. The bands were quantitated by normalizing band intensities relative to GAPDH.

Further, immunoblot studies have clearly shown the effect of Rap and CQ pre-treatment on LC3-II lipidation and the levels of p62 expression in lethally irradiated mice jejunum. The levels of expression of LC3-II got significantly enhanced (> 2 fold; p <

0.05) at 72 h of 8 Gy exposure in IR treated group as compared to the unirradiated control. Further boost of LC3 lipidation and a decline in p62 expression were observed in Rap+IR treated groups as compared to IR alone (Figure 5.4B). In contrast, reduction in LC3-II and increase in p62 induction was observed in CQ+IR treated group as compared to IR (Figure 5.4B).

5.3.5 Programmed cell death studies in autophagy modifier treated murine GI tissue

Relationship between radiation exposure and apoptosis has been widely established. Apoptosis, being a programmed cell death phenomenon in which dead cells do not release toxic components to its surrounding micromilue (Galluzzi et al. 2015, Hacker 2013). The apoptotic process involved cleavage of cellular substrates via Caspase activation, DNA fragmentation, nuclear and cytoplasmic shrinkage and membrane blebbing etc (Boatright et al. 2003). Autophagy and apoptosis shows a complex connection based on the type and extent of stressor stimuli. From recent research it is has been observed that radiation exposure has a causal relationship with autophagy induction also (Chaurasia M, et al, 2019). Therefore it would be worth studying the link between these two processes (autophagy and apoptosis) in radiation exposed conditions. To evaluate the intestinal epithelial cell apoptosis, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay was performed (Figure 5.5A). TUNEL assay substrate i.e. terminal deoxynucleotidyl transferase (TdT)-mediated addition of labeled deoxyuridine triphosphate nucleotides (dUTPs) to the 3'-OH end of DNA strand breaks (generated as later stages of apoptosis) aids in measuring extent of apoptosis within those cells (Darzynkiewicz, et al., 2008). In our TUNEL studies,

Compared to the control, intestinal section from the irradiated mice showed a significant increase in the number of TUNEL positive apoptotic cells 3 day post-exposure as compared to the unirradiated control mice. In Rap pre-treated groups, the number of apoptotic cells were markedly decreased in comparison to the radiation treatment group while vice versa was observed in CQ+IR treated animals. No significant alteration in the number of TUNEL-positive cells was observed in Rap/CQ alone treated mice throughout the GI mucosa when compared with the control group. TUNEL-positive cells were observed throughout the GI mucosa of IR treated mice on day 3 post IR exposure. CQ+IR treatment significantly increased the number of TUNEL positive epithelial cells in small intestine of these mice (Figure 5.5A).

We examined the effect of Rap pre-treatment on cleaved PARP expression in lethally irradiated mice jejunum. Expression of cleaved PARP enhanced significantly at 72 h of IR exposure. The radio protective effect of Rap treatment in mice intestine was evaluated at both day 3 and day 8 post exposure. Interestingly, Rap administration resulted in the suppression of cleaved PARP expression. However, Rap alone did not induce any significant change in cleaved PARP at both the time intervals. On contrary to this, enhanced apoptosis was observed in CQ+IR treated mice on both the time points i.e. day 3 and 8 post exposure which was evident through the expression levels of cleaved PARP (Figure 5.5B). To summarize, Both TUNEL assay and Western blotting indicated reduced apoptosis in Rap+IR treated group while induction of apoptotic markers was observed in CQ+IR treated groups.

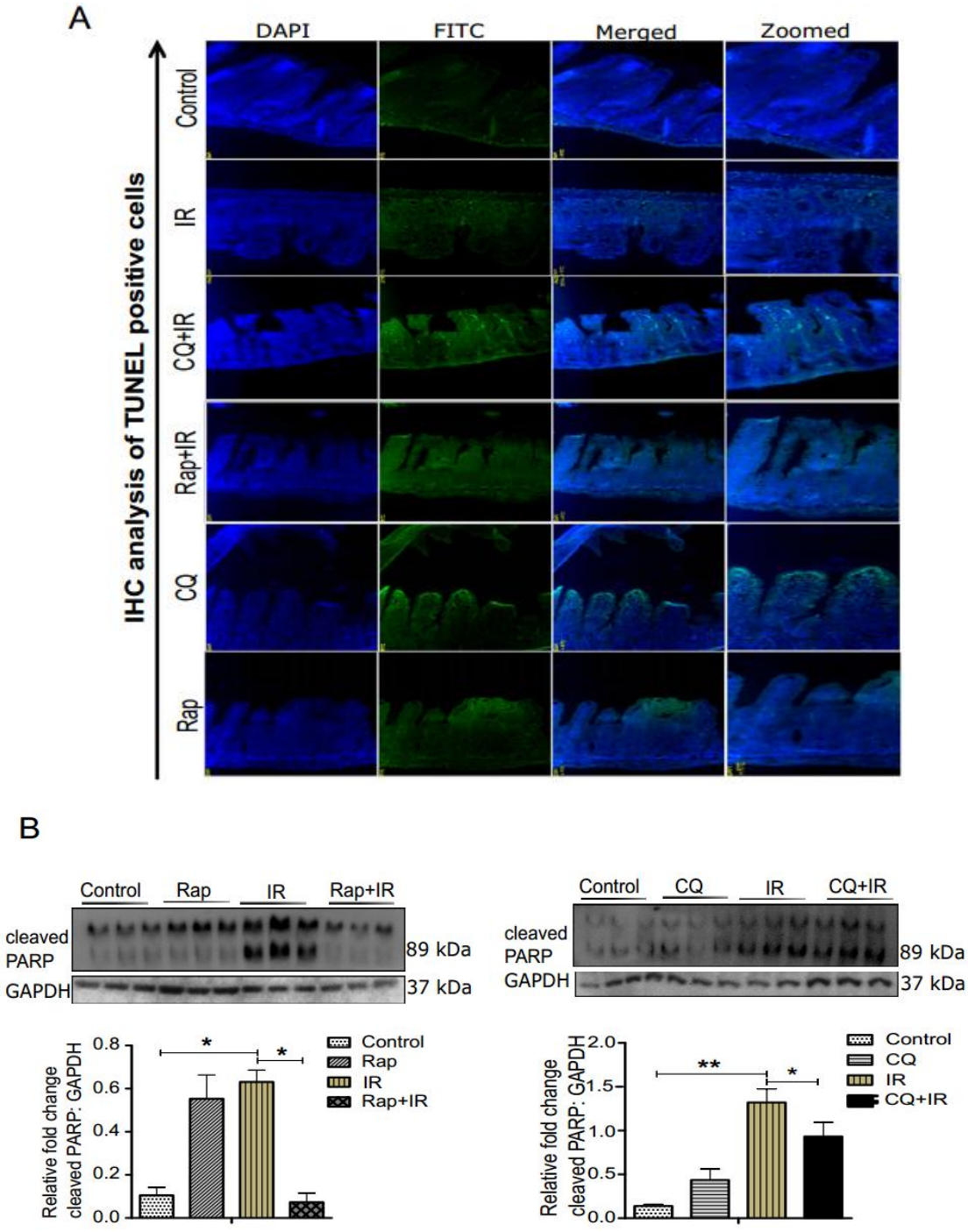


Figure 5.5: Programmed cell death studies in autophagy modifier treated murine GI tissue. **(A)** IHC analysis of TUNEL positive cells in GI tissue on day 3 post exposure. Stained sections were visualized under upright fluorescence microscope; images were captured at 40X microscopic magnification for quantification of TUNEL positive cells per crypt. Images were quantified using ImageJ software. **(B)** Western blot analysis of GI tissue lysate obtained at Day 3 and day 8 post-irradiation from Rap+IR and CQ+IR treated mice tissue. Blots were probed with intrinsic apoptosis marker, cleaved PARP; GAPDH was used as loading control.

5.4 Discussion

Growing incidence of possible exposure of humans to radiation has raised the need to develop countermeasures against radiation injuries. Radiation exposure causes toxicity and damages vital macromolecules such as nucleic acids, proteins, and lipids etc present in the cell (Fliedner TM, et al., 2007, Mac Naughton WK, et al., 2000). The resultant effects of radiation exposure are dose dependent. The haematopoietic syndrome can be subsequently taken care by administering growth factors, bone marrow transplantation, blood transfusion etc (López M., et al., 2011), but till now there is no approved medical countermeasure to recover from radiation induced GI damage. Only a handful of studies are available on medical countermeasures for IR induced GI damage recovery (Singh VK, et al., 2015a, Measey TJ, et al., 2018).

Autophagy being a context dependent phenomenon gets activated during stress conditions for cell survival or sensitization (Chen Y, et al., 2013, Chaurasia M. et al., 2016). It can have opposing effects in tumorigenesis i.e. both in tumour regression and promotion (White E, et al., 2009). Treatment strategies including autophagy induction has been a success in case of human gastric adenocarcinomas and hepatocellular carcinoma. MTOR expression has been shown to get increased, and tumour growth and angiogenesis is constricted in experimental models following treatment with rapamycin (Villanueva A, et al., 2008, Lang SA, et al., 2007). In contrast to its tumour-suppressing effects, autophagy may also help tumour cells survival under hypoxia and nutrient deprived conditions. Tumorigenic cells with defective apoptosis but functioning autophagy display a survival advantage under ischemic conditions compared with cells with blocked autophagy (Degenhardt K, et al., 2006). Due to the opposing effects of autophagy in tumorigenesis, targeting autophagic pathway in anticancer therapy may be

particularly difficult. For example, inhibition of autophagy increased sensitivity to radiation in radioresistant human cancer cell lines in one study (Apel A, et al., 2008) whereas another study showed that induction of autophagy radiosensitized prostate cancer cells (Cao C, et al., 2006). Treatment of lymphoma cells with the p53 stimulator tamoxifen led to tumour apoptosis and increased autophagy in surviving cells. Cotreatment with chloroquine enhanced p53-dependent apoptosis and tumour regression by blocking autophagy (Amaravadi RK, et al., 2007). Therefore, autophagy modulation as an adjuvant to standard chemotherapy may improve efficacy by shifting the balance from autophagy to apoptosis, but needs to be tumour and tumour-stage specific (Chen N, et al., 2010). Similar strategy can be employed for the survival of radiation exposed normal tissues by modulating autophagy levels.

Our thirty day survival study in mice has confirmed that Rap could help in better survival against 8 Gy dose of total body γ- radiation (TBI) as compared to IR alone (Chaurasia M, et al., 2019).

The manifestation of gastrointestinal syndrome, at doses above 6 Gy is the common cause of death of irradiated animals. Following exposure to ionizing radiation, the cells located at the base of the crypts undergo rapid apoptosis or undergo temporary or permanent senescence depending upon the absorbed radiation dose (Bala M, et al., 2015). This causes malabsorption, electrolyte imbalance, diarrhoea, inflammation, infections, weight loss, and ultimately results in mortality (C. S. Potten, et al., 1990). Our study showed that a single prophylactic dose of Rap (2mg /kg body weight) before sublethal irradiation (8 Gy) countered the radiation induced atrophy of mucosal layer, decrease in jejunum villi number and cellularity, crypts number etc and resulted in

reduction of apoptosis (Figure 5.2A, 5.2B, 5.5A and 5.5B) . We observed that radiation exposure (8 Gy) caused severe mucosal layer injury, mainly loss of viable crypt cells and disruption of villus integrity and functionality (Figure 5.2A). These pathologic developments were barred significantly by Rap treatment, demonstrating its effective protection against TBI inflicted GI injury.

These results demonstrate that the combination treatment with autophagy modulators can be used as an effective adjuvant strategy along with other antioxidants to augment the IR induced GI manifestations.

Chapter 6

Radiation Induced DNA Damage Response in Malignantly Transformed Tumorigenic Vs Non-Tumorigenic Intestinal Cells in the Presence of Autophagy Modifier Drugs

CHAPTER 6

RADIATION INDUCED DNA DAMAGE RESPONSE IN MALIGNANTLY TRANSFORMED TUMORIGENIC VS NON-TUMORIGENIC INTESTINAL CELLS IN THE PRESENCE OF AUTOPHAGY MODIFIER DRUGS

6.1 Introduction

Nucleus is a vital cell organelle, containing genetic information in the form of DNA. Exposure to ionizing radiation can lead to direct as well as indirect DNA damage depending on the dose of exposure. In the indirect mode of DNA damage, excessive ROS/RNS generated from ionizing radiation leads to genomic instability (Rodriguez-Rocha H et al., 2011). The cell follows various strategies to get rid of this DNA damage and genomic instability. One of these strategies includes a recently reported form of nuclear specific autophagy called nucleophagy wherein the elimination of damaged DNA occurs *via* autophagic vacuoles (Vessoni AT et al., 2013).

Fascinatingly, till now autophagy has been considered as stringently a cytosolic process, however, several autophagy-related proteins e.g. p62 undergo fast nuclear-cytosolic shuttling indicating the probable role of this clearance mechanism in the nuclear damage clearance (Filimonenko M, et al., 2010, Pankiv S, et al., 2010). Another protein, ALFY (autophagy-linked FYVE protein) has been shown to be involved in autophagy and localize predominantly in the nucleus (Simonsen A, et al., 2004, Clausen TH, et al., 2010, Isakson P, et al., 2013). However, following stress, ALFY is extruded from the nucleus to cytoplasm and interacts with p62 bodies. Micronuclei containing whole chromosomes or parts of the chromosomes are also suggested to be removed by

autophagy; thus facilitating the maintenance of genomic stability (Rello-Varona S, et al., 2012). Collectively, these circumstantial evidences suggests direct or indirect role of autophagy in the DDR and ROS/ RNS-mediated genotoxic stress. The precise mechanisms underlying DDR mediated autophagy are still not very clear. The nature and functional significance of this nuclear sequestration of autophagy-related proteins is not clear, although the nuclear-cytosolic shuttling of Beclin1 has been shown to be important for its autophagic and tumor suppressor functions (Liang XH, et al., 2001).

Autophagy appears to play a crucial role in regulating cellular fate following the induction of DNA damage (Rello-Varona S, et al., Liang XH, et al., 2001). For instance, in cells with DNA damage and defective apoptosis, autophagy facilitates cell death; thereby acting as a tumor suppressor (Maiuri MC, et al., 2007). Accumulating evidences suggest that radiation induced DNA damage induces autophagy. In response to DNA double strand breaks (generally considered lethal), two repair pathways are mainly activated. Homologous recombination (HR) and Non-homologous end joining (NHEJ) (Mao Z, et al., 2008, Brandsma I, et al., 2012). These two pathways differ in their template requirements (Figure 6.1). HR uses DNA template on the sister chromatid to repair the break, thus leading to the reconstitution of the original sequence and with more fidelity (Thompson LH et al., 2001). On contrary to this, NHEJ modifies the damaged DNA ends, and ligates them together with little or no homology with less fidelity, thus leading to deletions or insertions within the genome (Lieber MR., 2008). Major players of HR are MRE11, Rad 51, NBS1, BRACA1 and 2 (Bai Y. et al., 1996., Yang H. et al., 2005, San Filippo, J. et al. 2006). The NHEJ is initiated by Ku70 and Ku80, DNA-dependent protein kinase (DNA-PK), XRCC4 and DNA-Ligase IV (Anthony J. Davis et

al., 2013). Cells deficient in autophagy have been shown to accumulate higher levels of mutated DNA suggesting deficiency in the HR repair (Mathew R, et al., 2007).

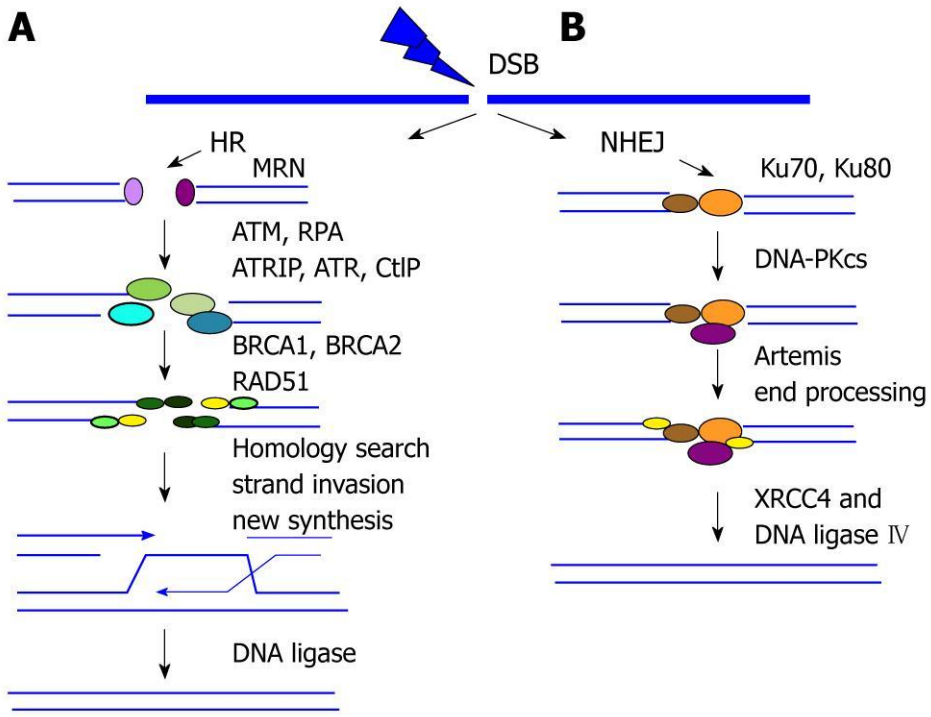


Figure 6.1 DNA double strand break repair pathways. (Guang Peng et al., 2011)

Besides aiding the metabolic precursors for the generation of ATP, as well as regulating the supply of dNTPs required for repair, autophagy has also been shown to influence the dynamics of DNA repair wherein it helps in recycling of key proteins involved in the processing of lesions (Dyavaiah M, et al., 2011. ATM can activate autophagy by both p53 dependent as well as independent mechanism. Cytosolic ATM can activate TSC2 tumor suppressor to inhibit mTORC1 through the LKB1/AMPK pathway and induce autophagy during ROS-mediated cellular damage (Pang XL, et al., 2013). Thus, these new findings integrate different stress response pathways taking place in different cellular compartments. Poly ADP-ribose polymerase 1 (PARP1) is another protein directly linking DDR and autophagy. PARP1 is hyperactivated upon radiation-induced

DNA damage that consumes NAD^+ resulting in ATP depletion (Schmukler E, et al., 2013).

Autophagy shows a pleomorphic role in the context of DNA damage response. Majority of the studies indicate that autophagy inhibition in cells treated with DNA damaging agents leads to enhanced cell death, supporting a pro-survival role for autophagy. This part of our study has been devised with the rationale to understand the differential response of tumorigenic colon carcinoma (HCT 116) and non-tumorigenic intestinal cell (INT 407) towards radiation exposure induced autophagy, and the association of this clearance mechanism with radiation induced DNA damage response.

6.2 Aim

In this chapter, we addressed the differential response of tumorigenic (colon carcinoma cells, HCT 116) and non-tumorigenic intestinal cell (INT 407) towards radiation exposure induced autophagy. The specific aims of the current chapter were:

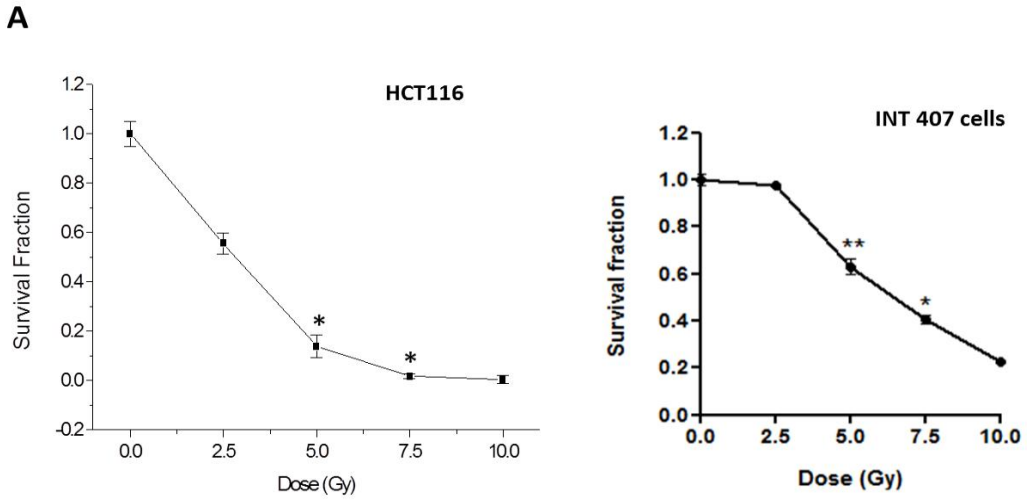
1. To study autophagy status of HCT 116 and INT 407 cells in radiation expossed condition.
2. To explore relationship between radiation induced DNA damage response and autophagy in these cells.

6.3 Results

6.3.1 Radiation induced autophagy status in malignantly transformed colon carcinoma vs non-tumorigenic cells

To investigate the status of radiation induced autophagy in malignantly transformed and untransformed cells; we first determined dose-dependent lethality of both the cells (HCT 116 and INT 407) exposed to various doses of IR (0 to 10 Gy). This was

achieved by analyzing their macrocolony formation ability following exposure to ionizing radiation. The LD_{50} was found to be approx. 5 Gy in both of these cell types (Figure 6.2A). Unless specified otherwise, all further investigations to understand the relationship between radiation-induced cell death and autophagy were carried at an absorbed radiation dose of 5 Gy. In order to explore the autophagy status in HCT 116 and INT 407 cells, we collected the cell lysate from both the cells exposed to varying doses of ionizing radiation and processed for Western blotting. We observed a dose dependent induction of LC3-II lipidation in both the cell types (Figure 6.2B). After looking at the dose dependent induction of autophagy, we decided to analyze if the extent of autophagy induction post radiation exposure is beneficial to these cell types. Further, dose dependent induction of autophagy using Western blotting suggested that the levels of autophagy starts building up soon after irradiation and the lipidation of LC3 occurred in dose dependent manner in both the cell lines (Figure 6.2B). In addition to Western blotting, we quantified LC3 punctas post radiation exposure in both the cell lines transiently transfected with a pEGFP-LC3 plasmid (addgene, plasmid no. 21073). After 16 h post-irradiation, we observed an increased EGFP-LC3 puncta formation as compared to non-irradiated control cells (Figure 6.2C).



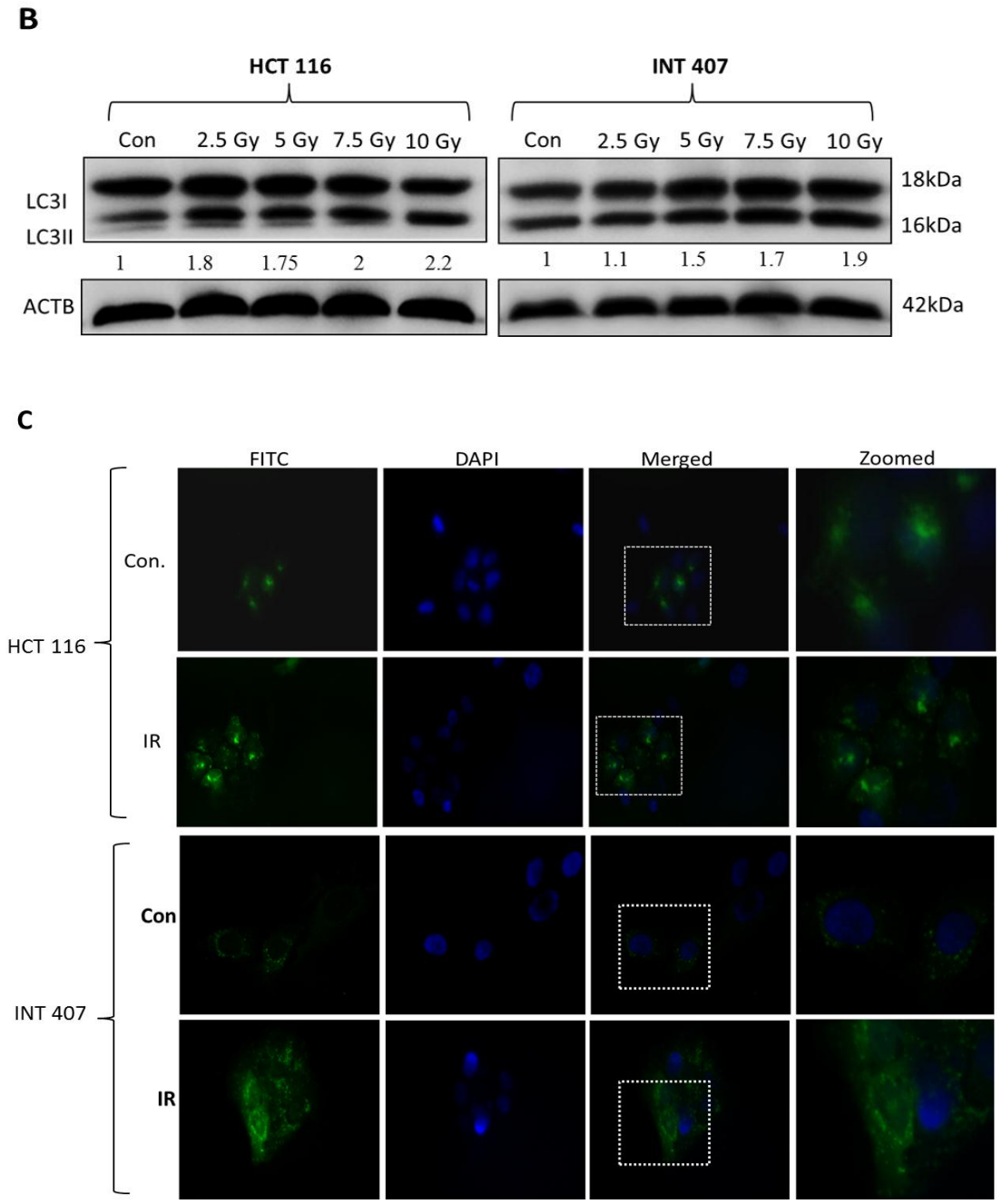
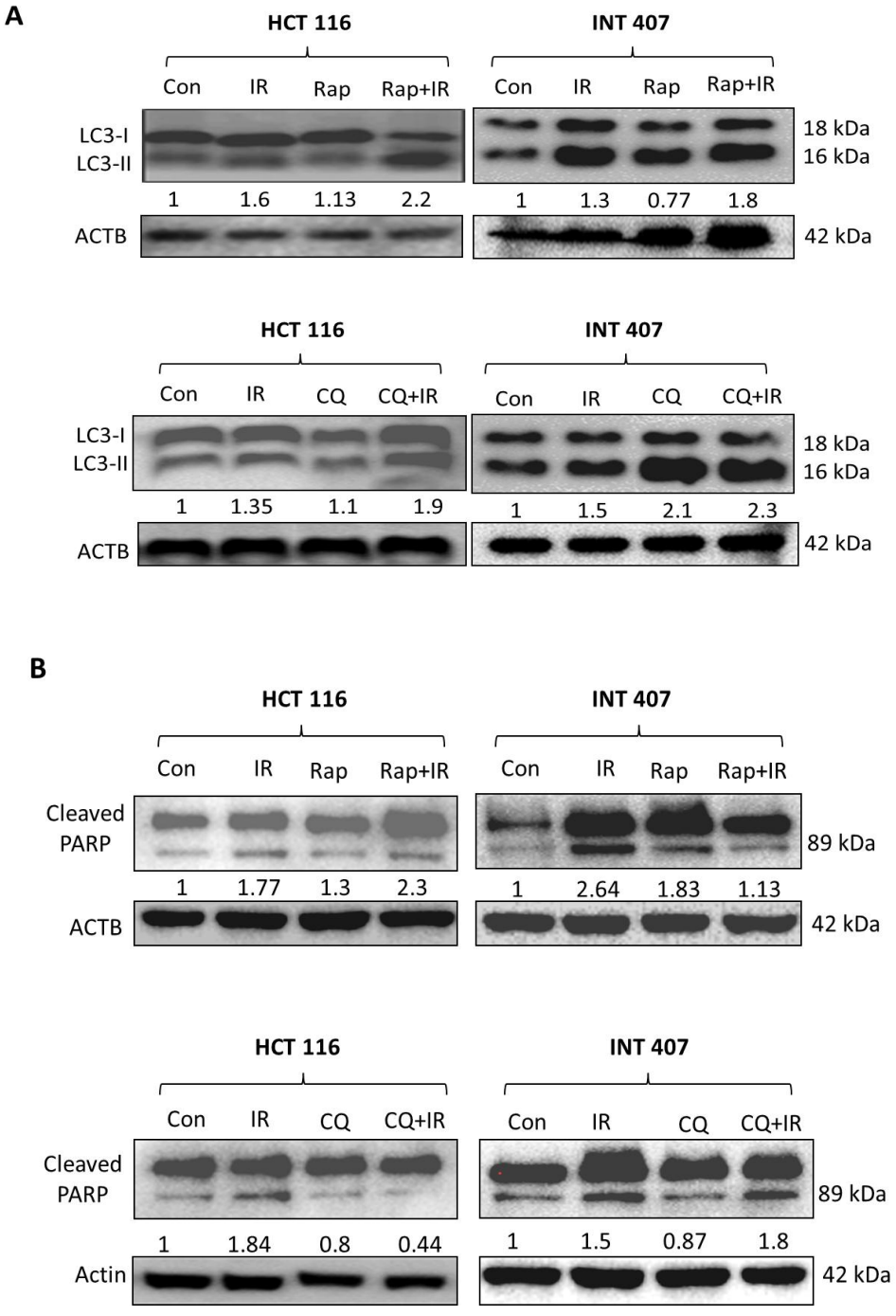


Figure 6.2: (A) Dose optimization in HCT 116 and INT 407 cells via macro colony formation assay), **(B)** Dose dependent induction of autophagy in HCT 116 and INT 407 cells, **(C)** fluorescence microscopy in presence of pEGFP-LC3 expressing construct in HCT 116 and INT 407 cells.

6.3.2 Radiation induced autophagy status in malignantly transformed and untransformed cells in the presence of autophagy modifier drugs

Both HCT 116 and INT 407 cells were pre-treated with rapamycin and chloroquine to increase or inhibit the levels of autophagy respectively (1 h prior exposure) at 50 nM and 5 μM concentrations respectively in radiation exposed conditions. Rap treatment enhanced the induction of autophagy in both the cells as compared to IR alone whereas CQ treatment (late autophagy inhibitor and affects clearance of autophagic vacuoles within cells) was showing efficient autophagy flux (accumulation of LC3-II) in these cells (Figure 6.3A). Thus, obtained results suggest similar levels of autophagy modulation in these cells. Next, we explored the fate of these different cell types post radiation exposure. We observed that autophagy inducer treatment induced significant enhancement in apoptosis in HCT 116 cells while autophagy inhibitor (CQ+IR) treatment made these cells more radio-resistant i.e. helping in their survival upon radiation exposure (Figure 6.3B). Thus, better sensitization is achieved in the presence of autophagy inducer drug. Hence, it is observed that better radio sensitization can be achieved in these cells via combined effect of both autophagy and apoptosis. On the other hand, in case of non tumorigenic INT 407 cells, autophagy inducer treatment caused reduction in induced apoptosis (PARP cleavage). However, enhanced apoptosis was observed in CQ+IR treatment as compared to IR alone (Figure 6.3B). To further investigate the ultimate fate of both the cell lines (HCT 116 and INT 407) in presence of autophagy modifiers, we performed macro colony formation assay and analyzed the clonogenic cell survival of both the cell lines. Rap significantly reduced the clonogenic potential of HCT 116 cells while enhanced colonies were obtained in INT 407 cells. On contrary to this, CQ treatment enhanced colony forming ability of HCT 116 while

reverse was observed for INT 407 cells (Figure 6.3C). Further we checked radiation induced ROS production in HCT 116 cells in presence of CQ (Figure 6.3D), enhanced ROS production in CQ+IR group as compared to IR alone.



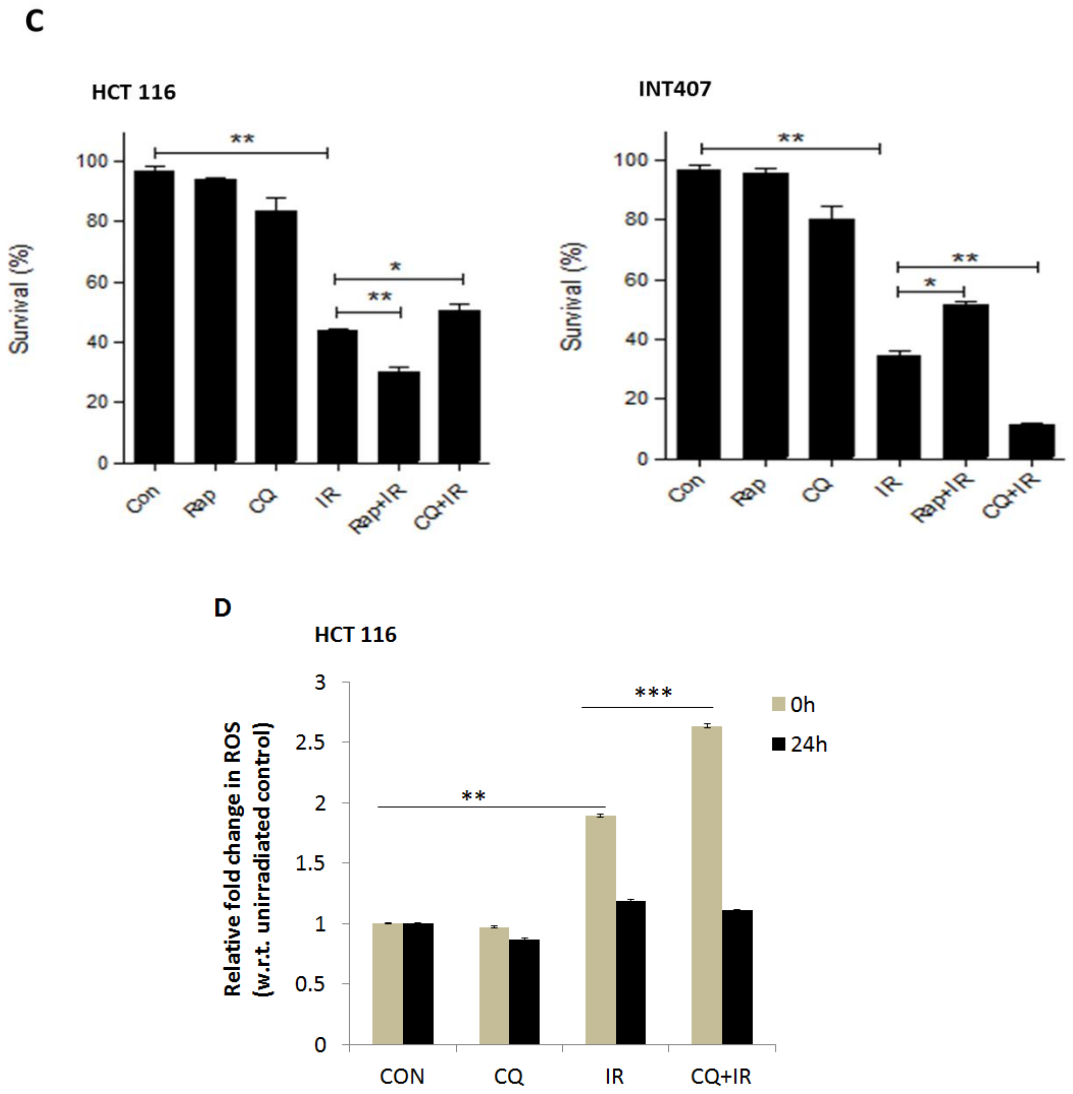


Figure 6.3: (A) Autophagy status of HCT 116 and INT 407 cells at 24 h post IR exposure in presence of autophagy modifier drugs, **(B)** Programmed cell death profile of HCT 116 and INT 407 cells at 24 h post exposure in presence of autophagy modifier drugs, **(C)** Clonogenic cell survival assay in radiation exposed HCT 116 and INT 407 cells, **(D)** Radiation induced oxidative stress in HCT 116 cells.

6.3.3 Radiation induced DNA damage response in malignantly transformed and untransformed cells in presence of autophagy modifier drugs

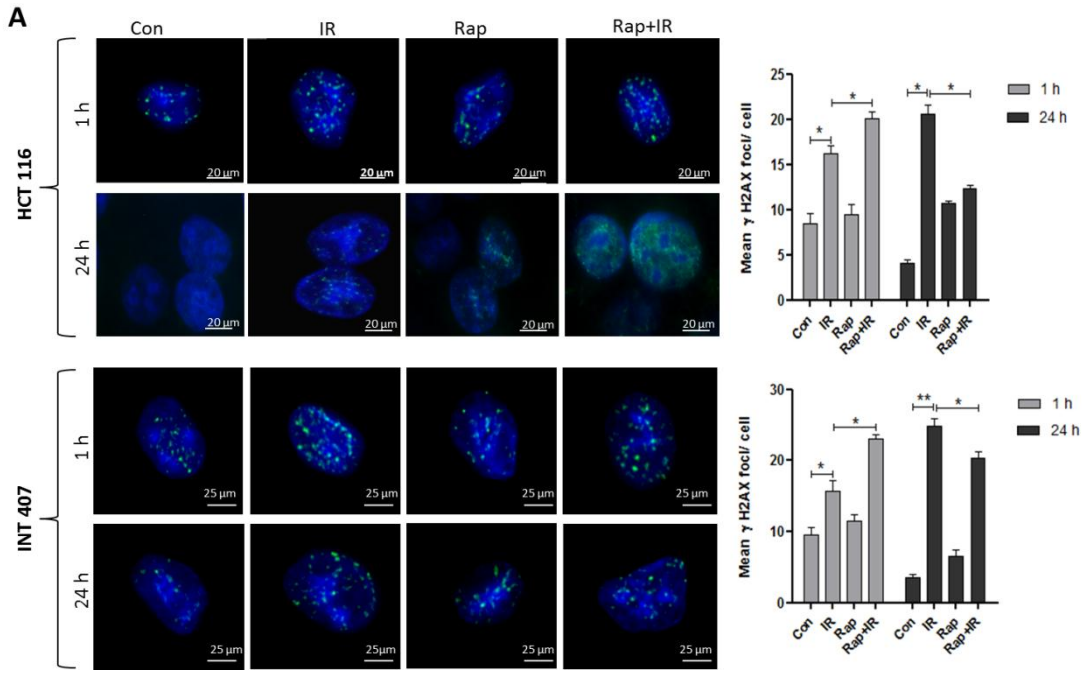
Numerous cytotoxic agents and ionizing radiation have been known to induce DNA double strand breaks (DSB) in a dose dependent manner (Kuo LJ et al., 2008). DSBs act as signal for phosphorylation of the histone H2A protein family variant i.e. H2AX.

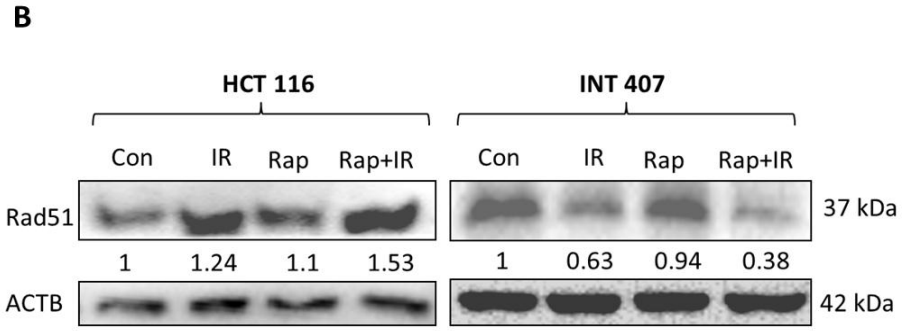
Phosphorylation of H2AX via cellular kinases; ataxia telangiectasia mutated (ATM) and ATM-Rad-related (ATR) at the Serine 139 moiety. This newly phosphorylated protein, gamma-H2AX, is the recruiting site for DNA repair proteins (Emmy P. Rogakou et al., 1998). Radiation induced DNA damage repair studies *via* gamma H2AX assay in HCT 116 cells shows enhanced damage in Rap+IR group as compared to radiation control at early time point (1 h post exposure); while at 24 h γH2AX foci formation assay suggests less number (almost two folds reduction in γH2AX foci) of foci in Rap+IR group as compared to IR alone. Similarly, INT 407 cells γH2AX data shows more number of foci at earlier time points which got reduced by 24 h post irradiation, indicating autophagy may be helping in DNA damage repair possibly through the removal of damaged cargos and thus resulting into better recovery in INT 407 cells too (Figure 6.4A). But in comparison to HCT 116 cells, INT 407 cells shows almost one folds less H2AX foci in Rap+IR group with respect to IR alone.

As ionizing radiation induces both DNA double strand and single strand breaks but DNA double strand breaks are more lethal and difficult to repair, specific DNA double strand break repair pathways gets activated post exposure to radiation. These are Homologous repair (HR) and Non homologous end joining (NHEJ) (Thompson LH et al., 2001). Literature, suggest that HR is the most promiscuous and less error prone as compared to NHEJ (Thompson LH et al., 2001, Lieber MR., 2008). From our Western blot studies, we checked the expression profile of Rad51 protein which is specifically involved in HR pathway. It was observed that HCT 116 cells have more expression of Rad51 in IR treated samples which was further enhanced in Rap+IR treated samples

(Figure 6.4B). In contrary to this, less Rad51 expression was observed in IR in INT 407 cells, which got further reduced after Rapamycin treatment. From our Western blot studies, it was indicative that radiation induced DNA damage repair *via* HR pathway is more prevalent in tumorigenic HCT 116 cells as compared to INT 407 cells. To summarize, we found that both the cells (non-tumorigenic/tumorigenic) were positive for autophagy induction following irradiation. Furthermore, the basal levels of autophagy helped in survival of both the cell types, enhancing radio-resistance in both the cell types. However, the expression of autophagy proteins was enhanced in carcinogenic cells. Moreover, autophagy inhibition enhances ROS production in HCT 116 cells, suggesting the role of autophagy in reducing ROS by removal of of damaged cargos i.e. damaged mitochondria, peroxisomes and misfolded/ damaged proteins which acts as sink for ROS generation within irradiated cells (Figure 6.3D). DNA damage studies (γH2AX and western blotting) indicated that the average damaged DNA repair frequency was higher in HCT 116 as compared to INT 407 cells in the presence of IR+Rap (autophagy inducer) treatment. In terms of DNA repair pathway activation, the expression of HR DNA repair protein (Rad 51)was enhanced in colon carcinoma cells as compared to normal counterparts indicating better and efficient DNA damage recovery with autophagy inducer drug Rapamycin (Figure 6.4B). Since involvement of HR mediators was not indicated by Western blotting in Rap+IR treated INT 407 cells still their Clonogenic cell survival potential was high in Rap combination treatment as compared to IR alone. We hypothesized that different kind of cell cycle perturbations were occurring in Rap+IR treatment as compared to IR alone in INT 407

cells which can provide us some substantial evidence about getting high survival in INT 407 cells. We observed a prominent G1/S arrest in case of INT 407 cells in Rap+IR treatment as compared to IR and unirradiated Control groups. Additionally, from our cell cycle studies also we got clear enhancement in Sub G1 population in IR alone as compared to Rap+IR combination (Figure 6.4C).





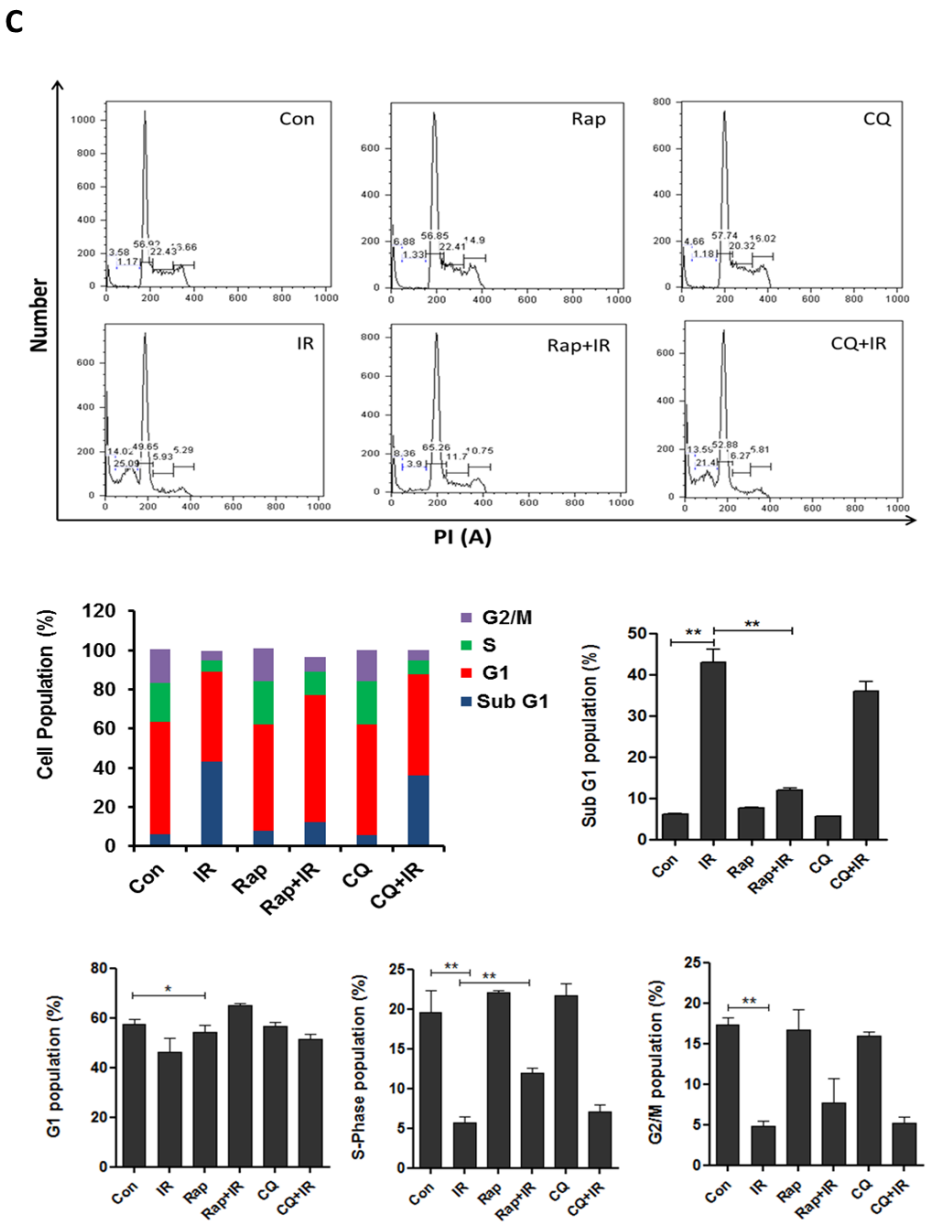


Figure 6.4: (A) DNA double strand break repair profile using γH2AX foci formation assay at early (1 h) and late (24 h) time points post irradiation. **(B)** Expression profile of Rad 51 protein in HCT 116 and INT 407 cells at 24 h post exposure. **(C)** Cell cycle assay in INT 407 cells at 24 h post irradiation.

6.4 Discussion

The above mentioned study was performed to understand context dependent role of autophagy in normal vs carcinogenic cells. After doing this empirical study we are now in a state to comment that the cells of different origin (based on their tumorigenic

potential) respond differently to the same radiation dose and autophagy modulator drugs. In carcinogenic cells, autophagy induction is helping in survival but in a temporal manner and there is an inverse relationship in autophagy induction, DDR and apoptosis. On the other side, in case of INT 407 cells, it seems that HR pathway of DDR is less active but still cell survival is high. This could possibly be due to NHEJ being predominantly active in these cells, thus providing survival. Further experimentation to understand the molecular mechanism and the status of NHEJ in these experiment setup will be interesting and will add on knowledge to the role of autophagy in DNA damage repair in tumorigenic vs non-tumorigenic cells.

Chapter 7

Elucidation of Role of Mitophagy in Radiation Exposed Conditions

CHAPTER 7

ELUCIDATION OF ROLE OF MITOPHAGY IN RADIATION EXPOSED CONDITIONS

7.1 Introduction

The role of autophagy is well established in the context of cancer. The specific removal of damaged mitochondria through the process of autophagy is known as mitochondrial autophagy or simply mitophagy. Mitochondria are considered to be the energy house of eukaryotic cells. To ensure functionality of these crucial organelles under a variety of stress conditions, cells have evolved a highly structured mechanism for recycling damaged mitochondria *via* Mitophagy (Valente EM, et al., 2004, Kitada T, et al., 1998). Mitophagy aids in selective degradation of damaged/dysfunctional and old mitochondria produced in response to certain deleterious stresses such as hypoxia and starvation, thereby helping in the maintenance of cellular stability (Valente EM, et al., 2004, Kitada T, et al., 1998). The damaged, dysfunctional mitochondria have been linked with numerous patho-physiological conditions and neurodegenerative diseases (Kitada T, et al., 1998, Aita VM, et al., 1999). Mitochondrial oxidative phosphorylation leads to the generation of toxic by-products involving ROS, particularly superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), which cause oxidative damage to mitochondrial lipids, DNA and proteins, making mitochondria further prone to the production of excessive ROS. The damaged mitochondria releases huge amount of calcium ions and cytochrome-c to the cytosol thus triggers apoptotic cascade within stressed cells (Saraste M., 1999, Wallace DC., 2005).

There are two major pathways that result into the induction of mitophagy. One of these pathways depends on the interaction between PINK1 and PARKIN, an E3-ubiquitin ligase with PINK1 over depolarized mitochondria (Wallace DC, et al., 2010). Under reduced mitochondrial potential, PINK1 accumulates over depolarized mitochondria where it interacts with PARKIN and causes its phosphorylation thereby inducing mitophagic response within stressed cells (Parsons MJ, et al., 2010). Other pathways of mitophagy induction are independent of PARKIN and are mainly mediated via ER-associated E3 ubiquitin ligase GP78 (Glycoprotein 78) and NIX/BNIP3L in a context-dependent manner (Chen Y, et al., 2013, Fu M, et al., 2013). A great knowledge about its role in removal of damaged/ superfluous mitochondria has come from Parkinson's disease patients. But now a days scientists have keenly started working on this pathway in context of other cellular insults like; ischemic reperfusion injury, diabetes mellitus and cancers etc. In context of radiation exposure, specific mitophagy induction has not yet been studied; additionally, main mitophagic pathways involved in radiation exposure elicited autophagy has not been known. We are yet to acquire details of mitophagy in context of radiation exposure and the final fate of cells inducing mitophagy following radiation exposure.

It is well-known that radiation exposure leads to massive mitochondrial biogenesis providing additional advantage for the cell survival (Chen Y, et al., 2013). Oxidative damage to mitochondria elicits mitochondria to undergo fission/fusion thus helping to ensure proper organization of the mitochondrial network during biogenesis (Hoppins S, et al., 2007, Chan DC. et al., 2006). A cell with damaged mitochondria undergoes mitochondrial fission leading to segregation of polarized and depolarized daughter mitochondria (Lionaki E, et al., 2015). The polarized daughter mitochondria may undergo fusion, thereby leading to mitochondrial biogenesis (Abrahams JP, et al., 1994, Boyer

PD. Et al., 1993). However, under conditions of extensive mitochondrial damage, the cell adapts mitophagy in order to exterminate the damaged and dysfunctional mitochondria (Lionaki E, et al., 2015). Most of the radiation exposure induced ROS/RNS is largely produced in the mitochondria (Chen Y, et al., 2013). Mitochondria are known to play an important role in radiation-induced cellular response, but the underlying mechanisms by which cytoplasmic stimuli modulate mitochondrial dynamics and functions are largely unknown. Numerous studies have pointed out the effect of radiation on mitochondrial dysfunction. Gamma-rays induce a p53-independent mitochondrial biogenesis in human colorectal carcinoma cells (Langer T, et al., 2001).

7.2 Aim

In this chapter, we addressed the induction of radiation induced mitophagy and its influence in context of colon carcinoma. The main aims of the current chapter were:

1. To study the radiation induced changes in mitochondrial membrane potential, mitochondrial mass and mitochondrial ROS production.
2. To study the kinetics of autophagy and mitophagy induction following radiation exposure in HCT 116 cells.
3. To understand the dependency of radiation induced mitophagy on Parkin.

7.3 Results

7.3.1 Radiation exposure severly affects mitochondrial physiology

To understand the changes in mitochondrial physiology in irradiated colon carcinoma (HCT 116) cells, we performed kinetics of diminution in mitochondrial potential from 30 min to 24 h post irradiation using Mitotracker red (stains depolarized mitochondria). Time dependent decline in MMP was observed from 30 min post irradiation till 24 h (Figure

7.1A). Depolarized mitochondria serve as sink for ROS generation, therefore kinetics of radiation-induced mitochondrial ROS was studied using flow cytometric analysis of Mitosox Red fluorescence. Kinetics of mitochondrial ROS generation was studied from 30 min and 24 h post IR exposure (Figure 7.1B). Additionally, we performed mitotracker green staining in HCT 116 cells at 24 h post irradiation. Increase in MFI, as compared to unirradiated control suggests increase in mitochondrial content at 24 h post exposure.

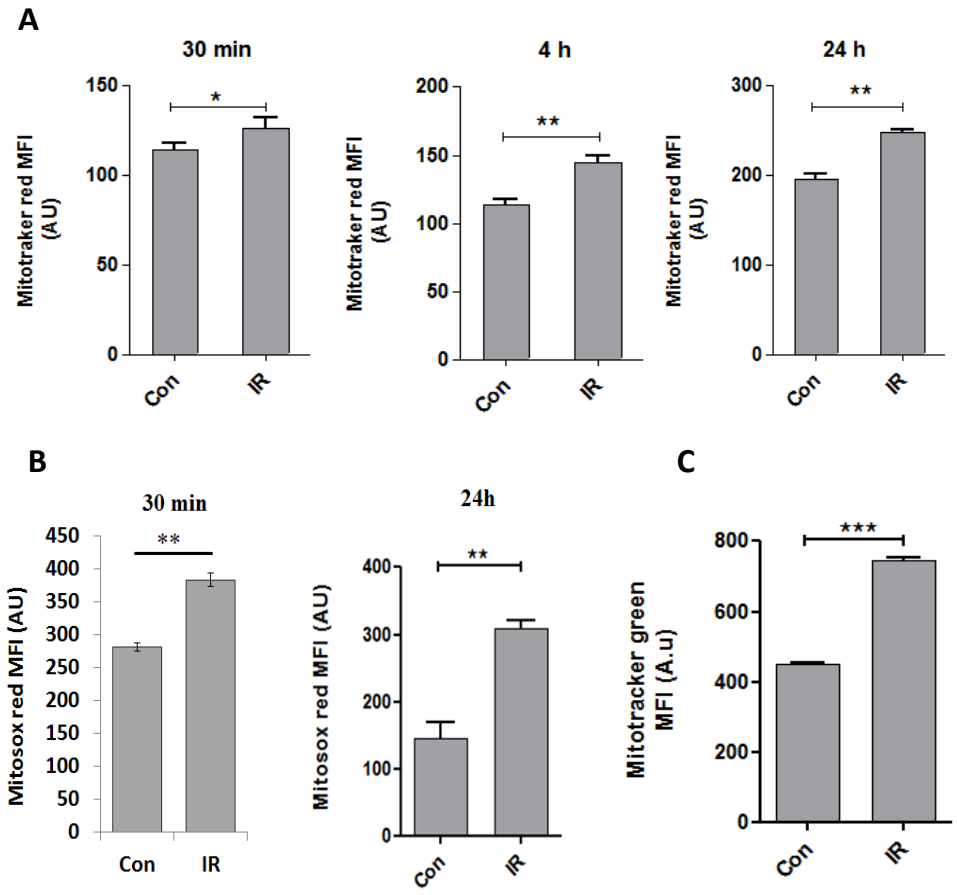


Figure 7.1: Radiation exposure affects mitochondrial physiology. **(A)** Mitotracker assay; HCT116 cells were irradiated with 5 Gy, cells were harvested at 30 min , 4 h and 24 h post irradiation to measure mitochondrial potential using mitotracker red. **(B)** Kinetics of radiation-induced mitochondrial ROS in stress in HCT 116 cells. The line graph shows relative fold change in fluorescence intensity for mitochondrial ROS production with respect to non-irradiated control cells after Mitosox Red staining (1µM) in response to radiation exposure (5 Gy). **P< 0.01, at 30 min and 24 h post-IR respectively. **(C)** Mitochondrial content analysis via Mitotracker green staining in HCT 116 cells at 24 h post radiation exposure, MFI normalized with respect to unirradiated control cells.

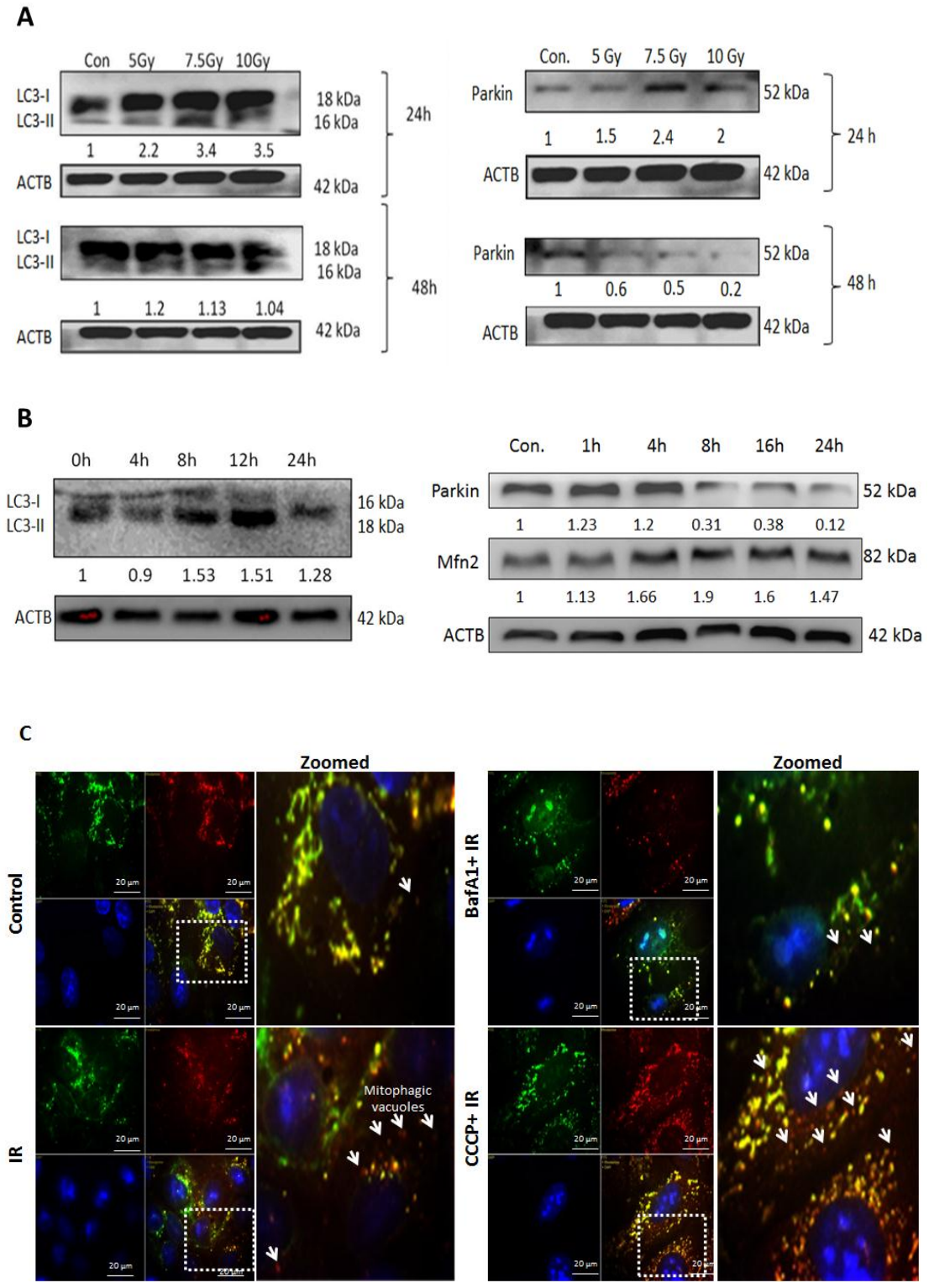
7.3.2 Radiation exposure induces autophagy as well as mitophagy in stressed cells

From our earlier studies based on *RAW 264.7* cells, it was confirmed that autophagy induction occurs in cells on exposure to radiation. We exposed HCT116 cells to various IR doses ranging from 0-10 Gy. We observed a dose dependent induction of both autophagy (indicated by the levels of LC3-II) and mitophagy (indicated by theexpression of Parkin) in these cells (Figure 7.2A). Since 5 Gy radiation dose is the LD_{50} for HCT 116 cells and this dose is suitable to induce enough levels of mitophagy in these cells, we selected 5 Gy IR dose for our further mitophagy related experiments. Both autophagy and mitophagy induction kinetics shows similar pattern of mitophagy induction as that of autophagy in these cells (Figure 7.2B left and right panel) with enhanced levels of both autophagy and mitophagy between 4-16 h post exposure.

In order to specifically study mitophagy induction following radiation exposure, we exposed U2OS cells tagged a mitochondrial matrix protein in fusion to GFP and cherry to radiation. These cells were a kind gift from Dr Anne Simonsen, University of Oslo, Norway. The protein, if processed through autophagy/mitophagy will give a red signal after fusion with lysosomes (due to low pH causing quenching of GFP). The mitochondria which are healthy and not being processed through mitophagy will be indicated by yellow fluorescence.

Our microscopy data indicated enormous mitophagy induction following radiation exposure. For autophagic flux, cells were treated with BafA1 (200 nM) 2 h before fixation. These BafA1 treated cells were used as negative control for mitophagy, while 1 µM CCCP (for 6 h) treated cells were used as positive control. Cells were fixed 24 h post-irradiation, and the images were captured using a Zeiss Axio fluorescence microscope. In presence of positive inducer of mitophagy i.e. CCCP, we found enhanced number of red flourescent

puncta as compared to yellow puncta in control group. This experiment confirmed the induction of mitophagy induction following radiation exposure (Figure 7.2C).



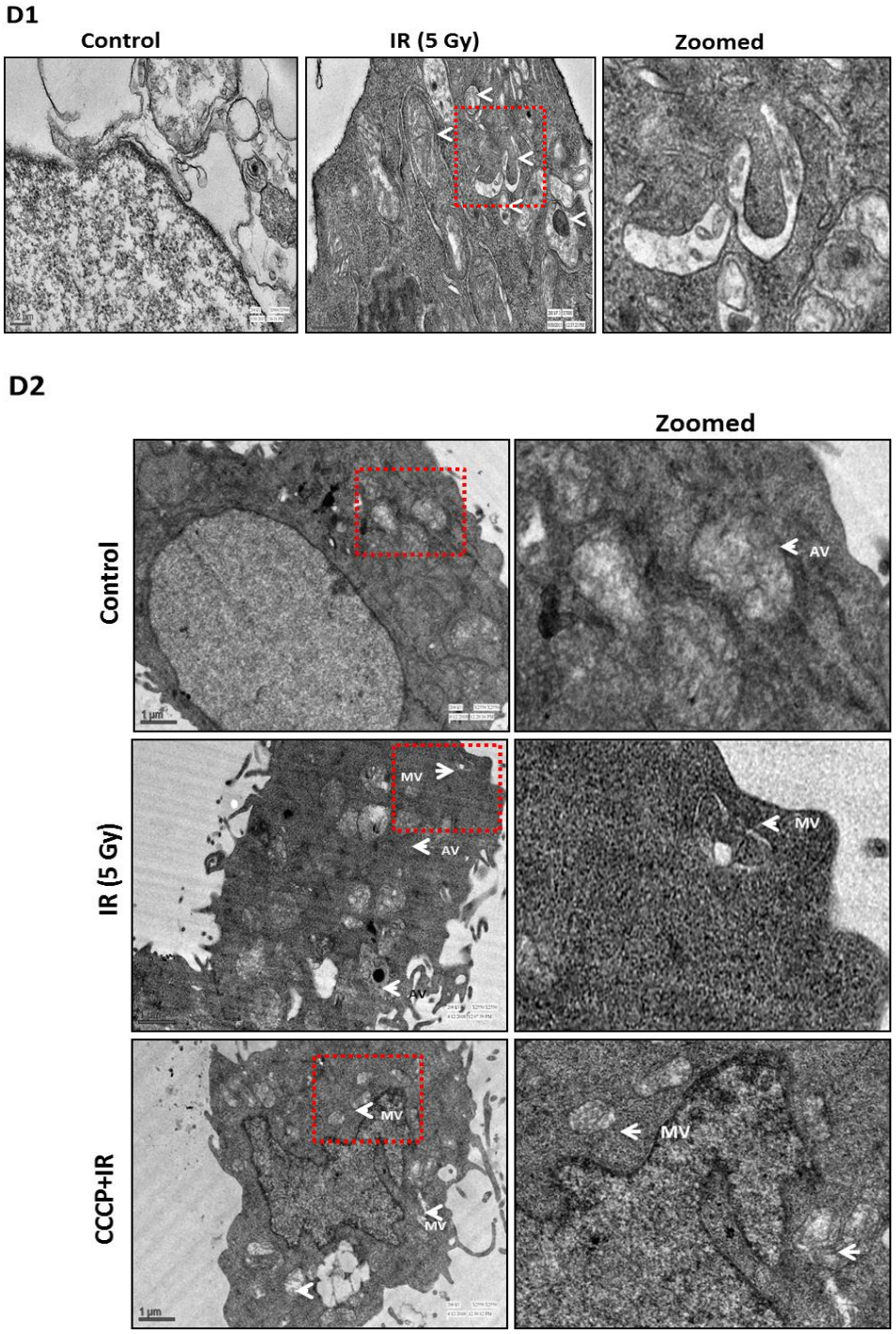


Figure 7.2: Radiation exposure induces autophagy and mitophagy in cells. **(A)** HCT 116 cells were exposed to different doses of IR ranging from 0 to 10 Gy, 24 h post-irradiation.Western blot analysis of LC3-II levels (relative to ACTB) and Parkin levels (relative to ACTB) in whole cell lysates. Data is representative of two independent experiments**. (B)** Western blot analysis of autophagy and mitophagy induction in radiation (5 Gy) exposed HCT 116 cells whole cell lysate. **(C)** Fluorescence microscopy studies in U2OS-doubletag-MLS doxycycline inducible system containing cells. The cells were irradiated with 4 Gy radiation dose. Blue color shows DAPI stained nuclei. **(D) D1, D2**: TEM images of mitophagic vacuoles in radiation exposed *RAW 264.7* and HCT 116 cells respectively.

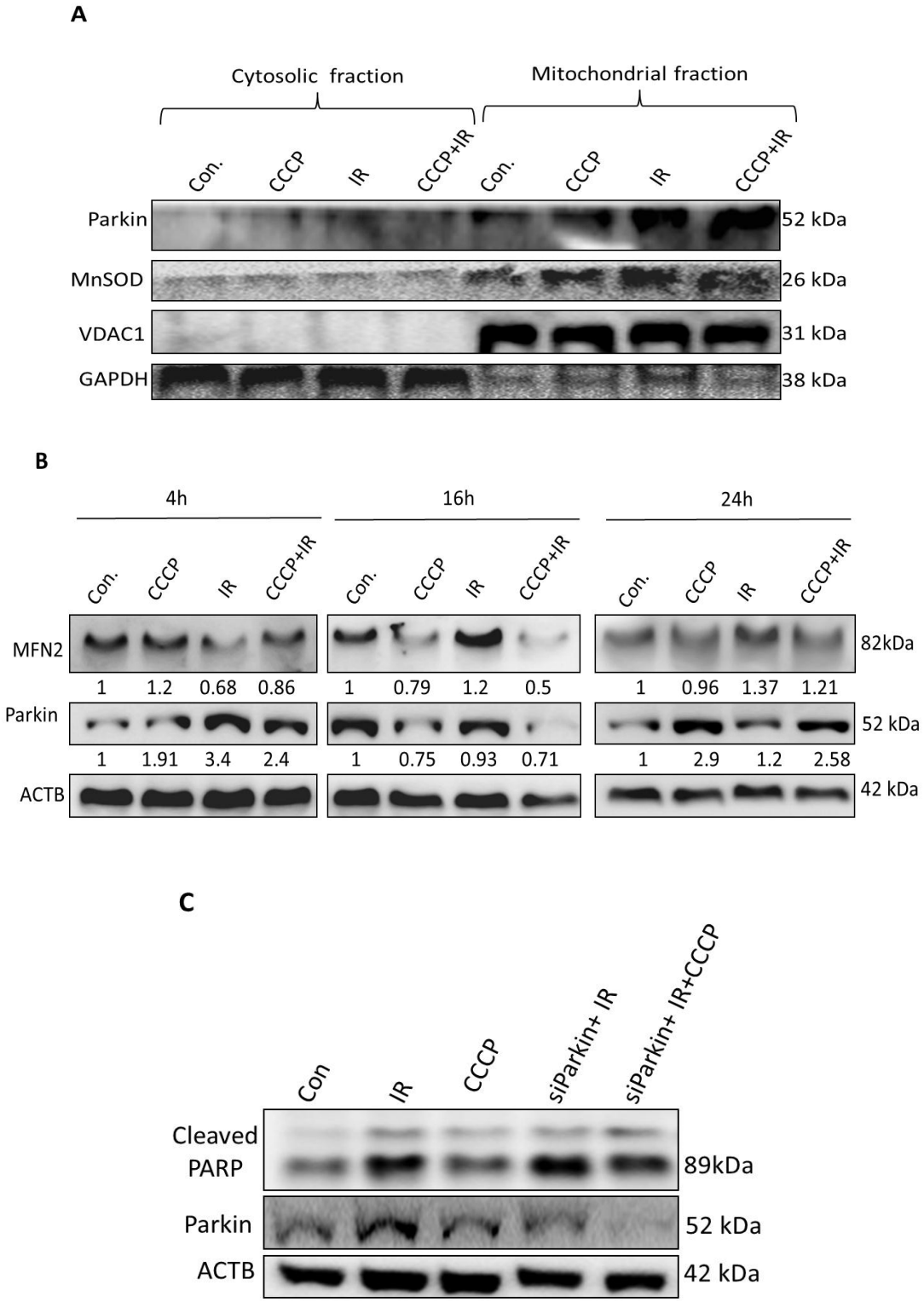
Mitophagy induction was further confirmed by using transmission electron microscopy (TEM) where, enhanced mitophagosomes/ mitophagic vacuoles were found in IR exposed cells as compared to unirradiated control. Results obtained from TEM also confirmed that mitophagy induction is not specifie to cell type, as similar data was obtained in *RAW 264.7* cells (which are of monocyte-mcrophage origin) as well as HCT 116 (GI epithelial) cells (Figure 7.2D).

7.3.3 Radiation induced mitophagy is Parkin dependent

Immunoblotting in mitochondrial and cytosolic fractions of IR esposed HCT 116 cells was performed to examine the localization of Parkin. We observed enhanced expression of Parkin protein specifically in mitochondrial fractions. This gave clear confirmation of its localization over damaged mitochiondria post irradiation (at 6 h) (Figure 7.3A). In addiation to mitochondral localization of Parkin, enhanced levels of mitochondrial SOD (MnSOD) in mitochondrial fractions of IR and CCCP+IR treatment show levels of induced antioxidant repair mechanism in the presence of IR and further enhancement in the same, in presence of CCCP+IR combination. These results suggest the recovery of antioxidant potential in the presence of mitophagy induction (Figure 7.3A).

Immunoblot studies at various time points 4, 16 and 24 h clearly shows enhanced level of mitophagy related protein Parkin while time points at which levels of Parkin were high i.e 4 h post IR levels of MFN2 (marker of mitochondrial fusion/ biogenesis) deacreased. From 16-24 h post IR, levels of Parkin started decreasing and MFN2 stars appearing indicating the fact that mitophagy and mitochindrial biogenesis show inverse relationship to a large extent (Figure 7.3B). In order to understand the fate of mitophagy inducing cells post-radiation exposure, we tested the levels of apoptotic marker PARP in them. From our si-RNA Parkin studies, we got clear indication that radiation induced and Parkin

dependent mitophagy is predominately prosurvival in nature (Figure 7.3C and D). The levels of cleaved PARP got increased in si-Parkin+IR treated groups as compared to radiation control.



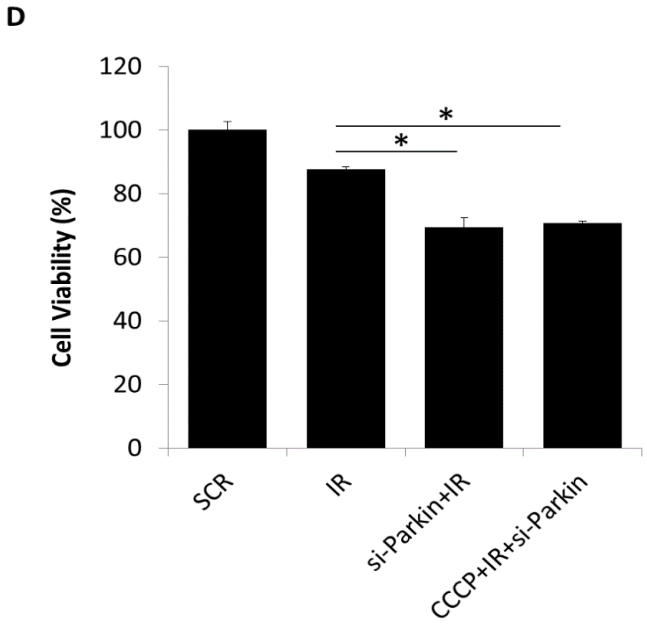


Figure 7.3: Radiation induced mitophagy is Parkin dependent. **(A)** Localization of Parkin post radiation exposure. The figure shows immunoblots of mitochondrial and cytosolic fractions from control, IR and CCCP alone and CCCP+IR samples harvested 14 h post-irradiation and probed with the indicated specific antibodies. Each blot is representative of two independent experiments. The graph shows quantitation of band intensities (relative to GAPDH for cytosolic fraction and relative to VDAC1 for mitochondrial fractions). **(B)** Immunoblot analysis of kinetics of mitophagy and mitochondrial biogenesis related protein Parkin and MFN1 respectively. The graph shows quantitation of band intensities (relative to ACTB). **(C)** Immunoblot analysis of cleaved PARP in presence of si-RNA-Parkin in HCT 116 cells. **(D)** SRB assay in presence of si-RNA Parkin in radiation exposed HCT 116 cells.

7.4 Discussion

Accumulating evidences suggest that dysfunctional mitochondrion has a pivotal role in modulating the metabolic reprogramming thus contributing to the process of tumorigenesis (Warburg O. 1956). Variations in the status of Warburg phenotype linked to the differences in mitochondrial status in cancer cells and/or tumor micro milieu (reverse Warburg phenotype) appear to be dependent on mitophagic potential of cells as well as the type and extent of stress (Pavlides S, et al., 2012). Poor prognosis and therapeutic resistance of highly glycolytic tumors suggest that mitophagy could be one of the contributing factors. Although the potential of targeting mitophagy as a

therapeutic strategy has so far remained elusive, emerging evidences suggest the potential of targeting this phenomenon for developing inhibitors of mitophagy as adjuvant in radio- and chemotherapy of tumors (Hughson LR, et al., 2012).

In our initial experiments based on fluorescence microscopy and FACS, we noticed that radiation exposure cause enormous changes in mitochondrial physiology i.e. decrease in mitochondrial membrane potential leading to generation of numerous ROS from mitochondria. All of these changes may finally induce oxidative stress within radiation exposed cells. Mitochondria with reduced potential serves as impending sites for mitochondrial division and mitophagy induction in stressed cells. From our western blotting and co-localization studies, we found a positive correlation between radiation exposure and mitophagy induction. We found that similar to autophagy, mitophagy induction is also dose dependent. From our preliminary si-RNA Parkin studies using SRB; Parkin dependent prosurvival role of radiation induced mitophagy was seen. We propose that mitophagy may initially get induced to aid in the removal of damaged mitochondrial from stressed cells and to lower down oxidative stress. However, the extent of mitochondrial damage and the release of cytochrome-c from these leaky mitochondria may be crucial factors in deciding the final decision of cell survival/ death.

Ionizing radiation apart from DNA damage may leads severe manifestations to other cellular contents mainly mitochondria, by causing its depolarization and thereby enhancing ROS levels within the stressed cells (Reisz JA, et al., 2014). Several studies have shown the patients with radio/ chemotherapy have more mitochondrial DNA mutations and deletions (Wardell TM, et al., 2003, Kam WW, et al., 2013). Change in mitochondrial physiology upon irradiation triggers mitophagy induction in tumor cells, thus induced

mitophagy helps in lowering oxidative stress by lowering down mitochondrial burden in these cells (Hu L, et al., 2016). In context of numerous tumorigenic conditions including breast, lung and erythroleukemias; Genetic inhibition of mitophagy aids in achieving better sensitization of these cells during anticancer treatments (Zheng R, et al., 2015, Wu HM, et al., 2016, Wang J, et al., 2016, Abdrakhmanov A, et al., 2019). Based on till now available knowledge and the data generated from our lab indicate that, unlike autophagy, mitophagy is a better cancer prevention/ sensitization strategy to target.

Chapter 8

Conclusion and Future Prospective

CHAPTER 8

CONCLUSION AND FUTURE PROSPECTIVE

This work started with background knowledge about ionizing radiation exposure, associated damage, linked possible fates and their potential manifestations using available drugs in context dependent manner. A major portion of scientific society works on development of better agents having anti-neoplastic and anti-cancer properties in combination with radiotherapy. These drugs involve DNA damaging agents, apoptosis inducers, agents helping in inducing cell senescence and autophagy inducers/ inhibitors (depending on cancer stage and specificity). In progression of cancer treatment modules, a large amount of normal tissue injury also occurs in vicinity of tumor burdened area. In contrast to this, if we talk about accidental/incidental exposure to normal healthy individual or tissue, we should have some radioprotective drugs for primary responders (army personnel's and trained doctors for radiation injury). Therefore today's world expect from scientific community, ability to handle both the situations i.e. what to do in order to achieve better radiotherapy of patients as well as radioprotection and mitigation of exposed individuals. In order to achieve better radioprotection, discovery of new drugs which aid in enhancing antioxidant defence system, narrow spectrum of reactivity i.e. specificity only towards cancer/ tumor cells and having differential effects towards cancerous vs non-cancerous cells is required. However, new drug discoveries and their clinical trial is a long path to achieve therefore, a new era of scientific community believes in repurposing of markedly available drugs for other treatment modalities and whether their repurposing can be done to achieve better drug development for other disorders/ diseases and situations.

Keeping these things in mind, this doctoral project was designed. In the initial part of our study, we explored the induction kinetics of autophagy following radiation exposure followed by ROS and UPR initiation kinetics in our model cell system i.e. macrophages. From available literature, we knew that radiation exposure can induce oxidative stress and ER stress but a precise relationship between the three pathways i.e. Autophagy induction, ROS generation and UPR was not known. We started with our *in-vitro* studies in murine macrophage cells and found a schematics relationship between the three processes. We found that radiation exposure induced primary ROS burst, which may cause severe macromolecular damage in exposed cells leading to ER stress response. Both ROS and UPR are capable of inducing autophagy upon radiation exposure (Malhotra JD, et al, 2007, Alexander A, et al., 2010, Ding W, et al., 2012, Chaurasia M, et al., 2019). In our study, we observed that primary signal was coming after ROS production, which was followed by UPR, which in-turn finally led to autophagy induction. Three major pathways have been reported after the induction of UPR; EIF2AK3/ PERK, ERN1/ IRE1 and ATF6. We found the involvement of EIF2AK3and ERN1 UPR pathways in autophagy induction post radiation exposure. Moreover, autophagy was found to play a crucial role in deciding the cell fate following radiation exposure. In murine macrophages, radiation induced autophagy was found to be pro-survival in both *in-vitro* and *ex-vivo* conditions which may be due to a better removal and recycling of damaged/ dysfunctional cellular cargos.

In the next part of our study, we further confirmed the pro-survival nature of autophagy induction in *in-vivo* conditions (murine *C57BL6* model). We observed that the radioprotection was enhanced in *C57BL6* mice when autophagy inducer drug, rapamycin was given prior to radiation treatment. Apart from autophagy inducers, we

have also used CQ, an inhibitor of autophagy, which is an FDA approved drug used primarily in the treatment of malaria. The key mode of action of this drug is that it inhibits fusion between autophagic vacuoles and lysosomes which are involved in clearance of damaged cargos; thus, the drug inhibits autophagy at later stages. In the presence of autophagy inhibitor CQ, stress burden was enhanced in radiation exposed mice, which finally affected the animal survival post radiation exposure. On the other hand, pre-treatment with rapamycin helped in eliciting autophagic response in combination treated mice as compared to radiation control. A better intestinal damage recovery, less apoptosis and survival enhancement was observed in the presence of rapamycin. Our data clearly indicates that pre-treatment of autophagy inducer aids in better intestinal recovery following radiation exposure in murine system and leads to overall survival advantage. In sum up, autophagy induction has a therapeutic potential as adjuvant therapy for both radioprotection as well as during radiotherapy, for the protection of normal cells in close vicinity of tumor microenvironment.

In the next section of our study, we explored a context and cell type dependent response of DNA damage repair in tumorigenic vs non-tumorigenic cells of intestinal origin. Autophagy induction kinetics was almost similar in both types of cells but the prime difference was in overall fate of irradiated cells in the context of cell type specificity. In terms of radiation induced DNA damage repair capability, tumorigenic (HCT 116) cell were more efficient as compared to the non–tumorigenic (INT 407) cells. Our preliminary data indicate that this may be due to the more efficient HR pathway in HCT 116 cells for DNA double strand break repair as compared to INT 407 cells.

In the last part of the study, we demonstrated that radiation exposure also induces mitophagy in a dose dependent manner. Majority of the mitophagy related studies have been performed in the context of neurodegenerative diseases (Kitada T, et al., 1998, Valente EM, et al., 2004, Lionaki E, et al., 2015). Late or deterministic effects of radiation exposure also involves, neurodegeneration, decreased cognitive ability and tumorigenesis. Radiation is known to affect CNS functions following exposure (Betlazar C, et al., 2016). Therefore, it was worthwhile to study relationship between radiation exposure and mitophagy induction. From our preliminary results, we found that radiation exposure causes enormous production of ROS. As damaged mitochondria act as primary sink for ROS production, induced ROS may act as a priming signal to initiate mitophagy response. This ROS induced mitophagy may be crucial for the removal of damaged mitochondrial burden from the cells in order to maintain cellular homeostasis. We observed a time dependent induction of mitophagy (similar to macroautophagy). Furthermore, the radiation induced mitophagy was found to be Parkin dependent and its inhibition restricts survival during radiation exposed conditions.

We speculate that the mitophagy induction following radiation exposure may contribute to the overall survival advantage received by autophagy during radiation-exposed conditions. However, further experiments are required to confirm our hypothesis. Overall, in this project, we have shown that both autophagy and mitophagy are induced following radiation exposure and both of these processes may contribute towards the survival of exposed cells. The mechanism involved has also been explored which may help in the identification of various novel targets for the diagnostic and therapeutic purpose in radiomodification, predominantly radiotherapy against cancer cells.

Future Prospective

The work conducted for this thesis make a remarkable contribution to the field of radio modification/ radiation exposure response. Before this work, only a handful of studies were published, that too in the context of cancer, majorly focussing on the role of autophagy in cancer radiotherapy scenarios.

Our work has contributed significantly in the field of radioprotection of normal cells after radiation exposure. Our data demonstrated how radiation induced autophagy has relationship with oxidative stress and we were able to find out the main ER stress pathway, which are contributing to its induction in radiation exposed conditions. Future studies should investigate how these UPR pathways induction help in the onset of autophagy (specifically a direct contact between ER stress pathways and autophagic machinery) under radiation exposure conditions. It will be interesting to explore the molecular mechanism responsible for the balance between necroptosis and autophagy under radiation stress. As we observed some indication of autophagy aiding in DNA damage repair, it will be interesting to explore how a cytosolic process aids in removal of nuclear damage. Specifically, the group plan to explore the relationship between DNA damage repair pathways *viz* NHEJ and HR and autophagic machinery. We will also explore if autophagy may assist in the removal of micronuclei formed after radiation exposure.

From, from our *in-vivo* mice autophagy study, we have identified the specific role of autophagy induction in GI damage recovery following radiation exposure. In future, the group will try to identify the radio-mitigative strategy using autophagy modifiers both under *in-vitro* and *in-vivo* conditions.

Our mitophagy studies in radiation exposed conditions has established that in addition to autophagy, specific activation of mitochondrial autophagy also occurs following radiation exposure, which may help in providing survival advantage to exposed cells by specifically removing ROS generating damaged mitochondria. Furthermore, to confirm our preliminary results that radiation induced mitophagy may be specifically Parkin dependent phenomena under radiation exposed conditions, we plan to perform experiments where Parkin may be silenced or CRISPR knocked out and then the effect on mitophagy under radiation exposed conditions will be studied. A major obstacle that needs to be overcome for future studies related to mitophagy is is the unavailability of a specific mitophagy inducer as well as inhibitor. The availability of these drugs will help to explore the specific role of mitophagy without disturbing/ altering other cellular signalling going on in the cells after radiation exposure. These studies will aid in understanding the in-depth mechanism of cancer and neurodegenerative diseases and may help in devising novel strategies under clinical scenarios. Additionally radiation exposure has been shown to lead to the formation of lot of protein aggregates in exposed organisms, specifically in brain, finally leading to neurodegeneration. Therefore, it will be very interesting to study the role of radiation induced mitophagy in brain tissues using specific mitophagy modifier drugs as well as peptides.

The importance of understanding all aspects of autophagy is highlighted by numerous diseases linked to autophagy dysfunction for which there are few currently available treatment options. The gradually accumulating knowledge about molecular details of autophagy/ mitophagy will hopefully lead to more targeted treatment option.

Bibliography

BIBLIOGRAPHY

Abdrakhmanov A, Kulikov AV, Luchkina EA, et al. Involvement of mitophagy in cisplatin-induced cell death regulation. Biol Chem 2019;400(2):161-170.

Abe T, Toyota M, Suzuki H, et al. Upregulation of BNIP3 by 5-aza-2'-deoxycytidine sensitizes pancreatic cancer cells to hypoxia-mediated cell death. J Gastroenterol 2005; 40(5): 504-10.

Abrahams JP, Leslie AG, Lutter R, et al. Structure at 2.8 A resolution of F1-ATPase from bovine heart mitochondria. Nature 1994;370(6491):621-8.

Aita VM, Liang XH, Murty V, et al. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 1999;59(1):59-65.

Alexander A, Cai SL, Kim J, et al. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc Natl Acad Sci U S A 2010;107(9):4153-8.

Alnasser HA, Guan Q, Zhang F, et al. Requirement of clusterin expression for prosurvival autophagy in hypoxic kidney tubular epithelial cells. Am J Physiol Renal Physiol. 2016 Jan 15;310(2):F160-73.

Amaravadi RK, Yu D, Lum JJ, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 2007;117(2):326-36.

Apel A, Herr I, Schwarz H, et al. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res 2008;68(5):1485-94.

Avivar-Valderas A, Bobrovnikova-Marjon E, Alan Diehl J, et al. Regulation of autophagy during ECM detachment is linked to a selective inhibition of mTORC1 by PERK. Oncogene. 2013 Oct 10;32(41):4932-40.

Bae H, Guan JL. Suppression of autophagy by FIP200 deletion impairs DNA damage repair and increases cell death upon treatments with anticancer agents. Mol Cancer Res 2011;9(9):1232-41.

Bartoletti-Stella A, Mariani E, Kurelac I, et al. Gamma rays induce a p53-independent mitochondrial biogenesis that is counter-regulated by HIF1alpha. Cell Death Dis 2013;4:e663.

Batista LF, Roos WP, Kaina B, et al. p53 mutant human glioma cells are sensitive to UV-C-induced apoptosis due to impaired cyclobutane pyrimidine dimer removal. Mol Cancer Res 2009;7(2):237-46.

Betlazar C, Middleton RJ, Banati RB, Liu GJ. The impact of high and low dose ionising radiation on the central nervous system. Redox Biol 2016;9:144-156.

Bhandary B, Marahatta A, Kim HR, et al. An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. Int J Mol Sci 2012;14(1):434-56.

Binks W. Protection against x rays and gamma rays in the industrial field. Br J Ind Med 1955;12(2):153-62.

Black HS. ROS: a step closer to elucidating their role in the etiology of light-induced skin disorders. J Invest Dermatol 2004;122(6):xiii-xiv

Black HS. ROS: a step closer to elucidating their role in the etiology of light-induced skin disorders. J Invest Dermatol. 2004 Jun;122(6):xiii-xiv.

Blanco R, Sole J, Montesinos J, Mesia C, et al., . Induction chemotherapy with cisplatin and gemcitabine followed by concurrent chemoradiation with twice-weekly gemcitabine in unresectable stage III non-small cell lung cancer: final results of a phase II study. Lung Cancer 2008;62(1):62-71.

Bommareddy A, Hahm ER, Xiao D, et al. Atg5 regulates phenethyl isothiocyanate-induced autophagic and apoptotic cell death in human prostate cancer cells. Cancer Res 2009;69(8):3704-12.

Bourgier C, Levy A, Vozenin MC, et al. Pharmacological strategies to spare normal tissues from radiation damage: useless or overlooked therapeutics? Cancer Metastasis Rev 2012;31(3-4):699-712.

Boyer PD. The binding change mechanism for ATP synthase--some probabilities and possibilities. Biochim Biophys Acta 1993;1140(3):215-50.

Brady NR, Hamacher-Brady A, Yuan H, et al. The autophagic response to nutrient deprivation in the hl-1 cardiac myocyte is modulated by Bcl-2 and sarco/endoplasmic reticulum calcium stores. Febs J 2007;274(12):3184-97.

Brandsma I, Gent DC. Pathway choice in DNA double strand break repair: observations of a balancing act. Genome Integr 2012;3(1):9.

Briganti S, Picardo M. Antioxidant activity, lipid peroxidation and skin diseases. What's new. J Eur Acad Dermatol Venereol 2003;17(6):663-9.

Briganti S, Picardo M. Antioxidant activity, lipid peroxidation and skin diseases. What's new. J Eur Acad Dermatol Venereol. 2003 Nov;17(6):663-9.

Brookes PS, Yoon Y, Robotham JL, et al. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol 2004;287(4):C817-33.

Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell 2008;134(3):451-60.

Buytaert E, Callewaert G, Hendrickx N, et al. Role of endoplasmic reticulum depletion and multidomain proapoptotic BAX and BAK proteins in shaping cell death after hypericin-mediated photodynamic therapy. Faseb J 2006;20(6):756-8.

Buytaert E, Callewaert G, Vandenheede JR, et al. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. Autophagy 2006;2(3):238-40.

Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochim Biophys Acta 2007; 1776(1):86-107.

Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochim Biophys Acta. 2007 Sep;1776(1):86-107.

Cao SS, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. Antioxid Redox Signal. 2014 Jul 20;21(3):396-413.

Cardenas C, Foskett JK. Mitochondrial Ca^{2+} signals in autophagy. Cell Calcium 2012;52(1):44-51.

Cardenas C, Miller RA, Smith I, et al. Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria. Cell 2010;142(2):270-83.

Casey TM, Eneman J, Crocker A, et al. Cancer associated fibroblasts stimulated by transforming growth factor beta1 (TGF-beta 1) increase invasion rate of tumor cells: a population study. Breast Cancer Res Treat 2008; 110(1): 39-49.

Castedo M, Perfettini JL, Roumier T, et al. Cell death by mitotic catastrophe: a molecular definition. Oncogene 2004;23(16):2825-37.

Castino R, Davies J, Beaucourt S, et al. Autophagy is a prosurvival mechanism in cells expressing an autosomal dominant familial neurohypophyseal diabetes insipidus mutant vasopressin transgene. FASEB J. 2005 Jun;19(8):1021-3.

Castro M, Grau L, Puerta P, et al. Multiplexed methylation profiles of tumor suppressor genes and clinical outcome in lung cancer. J Transl Med 2010; 8: 86.

Cesari R, Martin ES, Calin GA, et al. Parkin, a gene implicated in autosomal recessive juvenile parkinsonism, is a candidate tumor suppressor gene on chromosome 6q25-q27. Proc Natl Acad Sci U S A 2003; 100(10): 5956-61.

Chan DC. Mitochondria: dynamic organelles in disease, aging, and development. Cell 2006;125(7):1241-52.

Chatterjee A, Mambo E, Sidransky D. Mitochondrial DNA mutations in human cancer. Oncogene 2006; 25(34): 4663-74.

Chaurasia M, Bhatt AN, Das A, et al. Radiation-induced autophagy: mechanisms and consequences. Free Radic Res. 2016;50(3):273-90.

Chaurasia M, Gupta S, Das A, et al. Radiation induces EIF2AK3/PERK and ERN1/IRE1 mediated pro-survival autophagy. Autophagy 2019:1-16.

Chaurasia M, Misra S, Bhatt A N, et al. Metabolic imbalance associated mitophagy in tumor cells: genesis and implications. J Cancer Res Updates. 2015; 4, 95-107.

Chazotte B. Labeling mitochondria with MitoTracker dyes. Cold Spring Harb Protoc; 2011(8):990-2.

Chen N, Debnath J. Autophagy and tumorigenesis. FEBS Lett 2010;584:1427-35.

Chen X, Li M, Chen D, et al. Autophagy induced by calcium phosphate precipitates involves endoplasmic reticulum membranes in autophagosome biogenesis. PLoS One 2012;7(12):e52347.

Chen X, Qian Y, Wu S. The Warburg effect: evolving interpretations of an established concept. Free Radical Biology & Medicine 2015; 79: 253-63.

Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death Differ 2009;16(7):1040-52.

Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death Differ. 2009 Jul;16(7):1040-52.

Chen Y, Dorn GW 2nd. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science 2013;340(6131):471-5.

Chen Y, Li X, Guo L, et al. Combining radiation with autophagy inhibition enhances suppression of tumor growth and angiogenesis in esophageal cancer. Mol Med Rep. 2015 Aug;12(2):1645-52.

Cheong H, Yorimitsu T, Reggiori F, et al. Atg17 regulates the magnitude of the autophagic response. Mol Biol Cell 2005;16(7):3438-53.

Cho YS, Challa S, Moquin D, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 2009;137(6):1112-23.

Choudhary S. Deterministic and Stochastic Effects of Radiation. Cancer therapy and oncology international journal 2018.

Citrin D, Cotrim AP, Hyodo F, et al. Radioprotectors and mitigators of radiation-induced normal tissue injury. Oncologist 2010;15(4):360-71.

Claerhout S, Verschooten L, Van Kelst S, et al. Concomitant inhibition of AKT and autophagy is required for efficient cisplatin-induced apoptosis of metastatic skin carcinoma. Int J Cancer 2010;127(12):2790-803.

Clausen TH, Lamark T, Isakson P, et al. p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy. Autophagy 2010;6(3):330-44.

Codogno P, Meijer AJ. Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ 2005;12 Suppl 2:1509-18.

Coleman CN, Stone HB, Moulder JE, et al. Medicine. Modulation of radiation injury. Science 2004;304(5671):693-4.

Criollo A, Maiuri MC, Tasdemir E, et al. Regulation of autophagy by the inositol trisphosphate receptor. Cell Death Differ 2007;14(5):1029-39.

Dainiak N. Hematologic consequences of exposure to ionizing radiation. Exp Hematol 2002;30(6):513-28.

Dainiak N. Medical management of acute radiation syndrome and associated infections in a high-casualty incident. J Radiat Res 2018;59(suppl_2):ii54-ii64.

Dalby KN, Tekedereli I, Lopez-Berestein G, et al. Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. Autophagy. 2010 Apr;6(3):322-9.

Decuypere JP, Bultynck G, Parys JB. A dual role for Ca^{2+} in autophagy regulation. Cell Calcium 2011;50(3):242-50.

Degterev A, Hitomi J, Germscheid M, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 2008;4(5):313-21.

Degterev A, Huang Z, Boyce M, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 2005; 1(2):112-9.

Desmouliere A, Geinoz A, Gabbiani F, et al., Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 1993; 122(1): 103-11.

Ding W, Yang L, Zhang M, et al. Reactive oxygen species-mediated endoplasmic reticulum stress contributes to aldosterone-induced apoptosis in tubular epithelial cells. Biochem Biophys Res Commun 2012;418(3):451-6.

Ding W, Yang L, Zhang M, et al. Reactive oxygen species-mediated endoplasmic reticulum stress contributes to aldosterone-induced apoptosis in tubular epithelial cells. Biochem Biophys Res Commun. 2012 Feb 17;418(3):451-6.

Ding WX, Ni HM, Gao W, et al . Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival. J Biol Chem 2007;282(7):4702-10.

Dotiwala F, Eapen VV, Harrison JC, et al. DNA damage checkpoint triggers autophagy to regulate the initiation of anaphase. Proc Natl Acad Sci U S A 2013;110(1):E41-9.

Dwarakanath B, Jain V. Targeting glucose metabolism with 2-deoxy-D-glucose for improving cancer therapy. Future Oncol 2009;5(5):581-5.

Dwarakanath B, Singh D, Banerji AK, et al. Clinical studies for improving radiotherapy with 2-deoxy-D-glucose: present status and future prospects. J Cancer Res Ther 2009;5 Suppl 1:S21-6.

Dwarakanath BS. Cytotoxicity, radiosensitization, and chemosensitization of tumor cells by 2-deoxy-D-glucose in vitro. J Cancer Res Ther 2009;5 Suppl 1:S27-31.

Dwarkanath BS, Zolzer F, Chandana S, et al. Heterogeneity in 2-deoxy-D-glucose-induced modifications in energetics and radiation responses of human tumor cell lines. Int J Radiat Oncol Biol Phys 2001;50(4):1051-61.

Dyavaiah M, Rooney JP, Chittur SV, et al. Autophagy-dependent regulation of the DNA damage response protein ribonucleotide reductase 1. Mol Cancer Res 2011;9(4):462-75.

Eriksson D, Stigbrand T. Radiation-induced cell death mechanisms. Tumour Biol 2010;31(4):363-72.

Fajardo LG LF. et al., Principles & Practice of Radiation Oncology, 3rd ed. Philadelphia, New York : Lippincott-Raven, 1998;143-154.

Fan XY, Tian C, Wang H, et al. Activation of the AMPK-ULK1 pathway plays an important role in autophagy during prion infection. Sci Rep. 2015 Oct 1;5:14728. Yang ZJ, Chee CE, Huang S, et al. The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther. 2011 Sep;10(9):1533-41.

Farrukh MR, Nissar UA, Afnan Q, et al. Oxidative stress mediated Ca(2+) release manifests endoplasmic reticulum stress leading to unfolded protein response in UV-B irradiated human skin cells. J Dermatol Sci 2014;75(1):24-35.

Farrukh MR, Nissar UA, Afnan Q, et al. Oxidative stress mediated Ca(2+) release manifests endoplasmic reticulum stress leading to unfolded protein response in UV-B irradiated human skin cells. J Dermatol Sci. 2014 Jul;75(1):24-35.

Filimonenko M, Isakson P, Finley KD, et al. The selective macroautophagic degradation of aggregated proteins requires the PI3P-binding protein Alfy. Mol Cell 2010;38(2):265-79.

Fliedner TM, Graessle D, Meineke V, et al. Pathophysiological principles underlying the blood cell concentration responses used to assess the severity of effect after accidental whole-body radiation exposure: an essential basis for an evidence-based clinical triage. Exp Hematol 2007;35(4 Suppl 1):8-16.

Fu M, St-Pierre P, Shankar J, et al. Regulation of mitophagy by the Gp78 E3 ubiquitin ligase. Mol Biol Cell 2013;24(8):1153-62.

Fuchs Y, Steller H. Programmed cell death in animal development and disease. Cell 2011;147(4):742-58.

Fujiwara K, Iwado E, Mills GB, et al. Akt inhibitor shows anticancer and radiosensitizing effects in malignant glioma cells by inducing autophagy. Int J Oncol 2007;31(4):753-60.

Fujiwara M, Marusawa H, Wang HQ, et al. Parkin as a tumor suppressor gene for hepatocellular carcinoma. Oncogene 2008; 27(46): 6002-11.

Fulda S, Gorman AM, Hori O, et al. Cellular stress responses: cell survival and cell death. Int J Cell Biol;2010:214074.

Fulda S, Gorman AM, Hori O, et al. Cellular stress responses: cell survival and cell death. Int J Cell Biol. 2010;2010:214074.

Gamerdinger M, Hajieva P, Kaya AM, et al. Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. Embo J 2009;28(7):889-901.

Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, et al. HIF-dependent antitumorigenic effect of antioxidants in vivo. Cancer Cell 2007; 12(3): 230-8.

Gao W, Ding WX, Stolz DB, et al. Induction of macroautophagy by exogenously introduced calcium. Autophagy 2008;4(6):754-61.

Gao W, Shen Z, Shang L, et al. Upregulation of human autophagy-initiation kinase ULK1 by tumor suppressor p53 contributes to DNA-damage-induced cell death. Cell Death Differ 2011;18(10):1598-607.

Gargini R, Garcia-Escudero V, Izquierdo M. Therapy mediated by mitophagy abrogates tumor progression. Autophagy 2011; 7(5): 466-76.

Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria in cancer cells: what is so special about them? Trends Cell Biol 2008; 18(4): 165-73.

Golden EB, Pellicciotta I, Demaria S, et al. The convergence of radiation and immunogenic cell death signaling pathways. Front Oncol 2012;2:88.

Gorbunov NV, Kiang JG. Up-regulation of autophagy in small intestine Paneth cells in response to total-body gamma-irradiation. J Pathol 2009;219(2):242-52.

Gorbunov NV, Kiang JG. Up-regulation of autophagy in small intestine Paneth cells in response to total-body gamma-irradiation. J Pathol. 2009 Oct; 219(2):242-52.

Gordon PB, Holen I, Fosse M, et al. Dependence of hepatocytic autophagy on intracellularly sequestered calcium. J Biol Chem 1993;268(35):26107-12.

Gozuacik D, Bialik S, Raveh T, et al. DAP-kinase is a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death. Cell Death Differ 2008;15(12):1875-86.

Grammaticos P, Giannoula E, Fountos GP. Acute radiation syndrome and chronic radiation syndrome. Hell J Nucl Med 2013;16(1):56-9.

Griffiths EJ, Rutter GA. Mitochondrial calcium as a key regulator of mitochondrial ATP production in mammalian cells. Biochim Biophys Acta 2009; 1787(11): 1324-33.

Guido C, Whitaker-Menezes D, Lin Z, et al. Mitochondrial fission induces glycolytic reprogramming in cancer-associated myofibroblasts, driving stromal lactate production, and early tumor growth. Oncotarget 2010; 3(8): 798-810.

Guo JY, Chen HY, Mathew R, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev 2011; 25(5): 460-70.

Guo JY, Karsli-Uzunbas G, Mathew R, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. Genes Dev 2013; 27(13): 1447-61.

Gupta A, Roy S, Lazar AJ, et al. Autophagy inhibition and antimalarials promote cell death in gastrointestinal stromal tumor (GIST). Proc Natl Acad Sci U S A 2010;107(32):14333-8.

Gupta S, Mathur R, Dwarakanath BS. The glycolytic inhibitor 2-deoxy-D-glucose enhances the efficacy of etoposide in ehrlich ascites tumor-bearing mice. Cancer Biol Ther 2005;4(1):87-94.

Haglund K, Dikic I. Ubiquitylation and cell signaling. Embo J 2005;24(19):3353-9.

Hall EJ, Giaccia AJ. eds. Radiobiology for the Radiologist, 6th ed. Philadelphia, PA, USA: Lippincott Williams & Wilkins, 2006

Hall LL, Th'ng JP, Guo XW, et al. A brief staurosporine treatment of mitotic cells triggers premature exit from mitosis and polyploid cell formation. Cancer Res 1996;56(15):3551-9.

Hattori N, Mizuno Y. Pathogenetic mechanisms of parkin in Parkinson's disease. Lancet 2004;364(9435):722-4.

He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 2009;43:67-93.

Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67:425-79.

Hoppins S, Lackner L, Nunnari J. The machines that divide and fuse mitochondria. Annu Rev Biochem 2007;76:751-80.

Hosseinimehr SJ. Trends in the development of radioprotective agents. Drug Discov Today. 2007; 12(19-20):794-805.

Hoyer-Hansen M, Bastholm L, Szyniarowski P, et al. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol Cell 2007;25(2):193-205.

Hsieh CH, Pai PY, Hsueh HW, et al. Complete induction of autophagy is essential for cardioprotection in sepsis. Ann Surg. 2011 Jun;253(6):1190-200.

Hu JL, He GY, Lan XL, et al. Inhibition of ATG12-mediated autophagy by miR-214 enhances radiosensitivity in colorectal cancer. Oncogenesis. 2018 Feb 20;7(2):16.

Hu L, Wang H, Huang L, Zhao Y, et al. The Protective Roles of ROS-Mediated Mitophagy on (125)I Seeds Radiation Induced Cell Death in HCT116 Cells. Oxid Med Cell Longev 2016; 9460462.

Hughson LR, Poon VI, Spowart JE, et al. Implications of therapy-induced selective autophagy on tumor metabolism and survival. Int J Cell Biol 2012: 872091.

Huo Y, Fan L, Yin S, et al. Involvement of unfolded protein response, p53 and Akt in modulation of porcine reproductive and respiratory syndrome virus-mediated JNK activation. Virology. 2013 Sep;444(1-2):233-40.

Ikeda F, Dikic I. Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO Rep 2008;9(6):536-42.

Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003;115(5):577-90.

Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003 Nov 26;115(5):577-90.

Ionescu L, Cheung KH, Vais H, et al. Graded recruitment and inactivation of single InsP3 receptor Ca2+-release channels: implications for quantal [corrected] Ca2+release. J Physiol 2006;573(Pt 3):645-62.

Isakson P, Holland P, Simonsen A. The role of ALFY in selective autophagy. Cell Death Differ 2013;20(1):12-20.

Itakura E, Kishi C, Inoue K, et al. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell 2008;19(12):5360-72.

Jager S, Bucci C, Tanida I, et al. Role for Rab7 in maturation of late autophagic vacuoles. J Cell Sci 2004;117(Pt 20):4837-48.

Jain V: Modifications of radiation responses by 2-deoxy-Dglucose in normal and cancer cells. Ind J Nucl Med 1996; 11, 8-17

Johnke RM, Sattler JA, Allison RR. Radioprotective agents for radiation therapy: future trends. Future Oncol 2014;10(15):2345-57.

Kabeya Y, Kamada Y, Baba M, et al. Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. Mol Biol Cell 2005;16(5):2544-53.

Kam WW, Banati RB. Effects of ionizing radiation on mitochondria. Free Radic Biol Med 2013;65:607-619.

Khan MT, Joseph SK. Role of inositol trisphosphate receptors in autophagy in DT40 cells. J Biol Chem 2010;285(22):16912-20.

Kiang JG, Bradley R. Garrison, et al. Gorbunov. Radiation Combined Injury : DNA Damage, Apoptosis and Autophagy. Adaptive Med 2010; 2(1): 1-10.

Kiang JG, Garrison BR, and Gorbunov NV. Radiation Combined Injury: DNA Damage, Apoptosis and Autophagy. Adaptive Medicine. 2010.

Kiang JG, Smith JT, Agravante NG. Geldanamycin analog 17-DMAG inhibits iNOS and caspases in gamma-irradiated human T cells. Radiat Res 2009;172(3):321-30.

Kiang JG, Smith JT, Agravante NG. Geldanamycin analog 17-DMAG inhibits iNOS and caspases in gamma-irradiated human T cells. Radiat Res. 2009 Sep;172(3):321-30.

Kihara A, Noda T, Ishihara N, et al. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. J Cell Biol 2001;152(3):519-30.

Kim EJ, Lee YJ, Kang S, et al. Ionizing radiation activates PERK/eIF2alpha/ATF4 signaling via ER stress-independent pathway in human vascular endothelial cells. Int J Radiat Biol 2014;90(4):306-12.

Kim HJ, Soyombo AA, Tjon-Kon-Sang S, et al. The Ca(2+) channel TRPML3 regulates membrane trafficking and autophagy. Traffic 2009;10(8):1157-67.

Kim I, Lemasters JJ. Mitophagy selectively degrades individual damaged mitochondria after photoirradiation. Antioxid Redox Signal 2011;14(10):1919-28.

Kim JH, Kim HY, Lee YK, et al. Involvement of mitophagy in oncogenic K-Ras-induced transformation: overcoming a cellular energy deficit from glucose deficiency. Autophagy 2011; 7(10): 1187-98.

Kim KW, Moretti L, Mitchell LR, et al. Combined Bcl-2/mammalian target of rapamycin inhibition leads to enhanced radiosensitization via induction of apoptosis and autophagy in non-small cell lung tumor xenograft model. Clin Cancer Res 2009;15(19):6096-105.

Kim KW, Moretti L, Mitchell LR, et al. Endoplasmic reticulum stress mediates radiation-induced autophagy by perk-eIF2alpha in caspase-3/7-deficient cells. Oncogene 2010;29(22):3241-51.

Kim KW, Moretti L, Mitchell LR, et al. Endoplasmic reticulum stress mediates radiation-induced autophagy by perk-eIF2alpha in caspase-3/7-deficient cells. Oncogene. 2010 Jun 3;29(22):3241-51.

Kim YJ, Kim EH, Hahm KB. Oxidative stress in inflammation-based gastrointestinal tract diseases: challenges and opportunities. J Gastroenterol Hepatol 2012;27(6):1004-10.

Kirisako T, Ichimura Y, Okada H, et al. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol 2000;151(2):263-76.

Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392(6676):605-8.

Klionsky DJ, Abdalla FC, Abeliovich H, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2012 Apr;8(4):445-544.

Kojima Y, Acar A, Eaton EN, et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumorpromoting mammary stromal myofibroblasts. Proc Natl Acad Sci U S A 2010; 107(46): 20009-14.

Kolesnick R, Fuks Z. Radiation and ceramide-induced apoptosis. Oncogene. 2003; 22(37):5897-906.

Komatsu M, Waguri S, Ueno T, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol. 2005 May 9;169(3):425-34.

Komatsu M, Wang QJ, Holstein GR, et al. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. Proc Natl Acad Sci U S A. 2007 Sep 4;104(36):14489-94.

Kong EY, Cheng SH, Yu KN. Induction of autophagy and interleukin 6 secretion in bystander cells: metabolic cooperation for radiation-induced rescue effect? J Radiat Res. 2018 Mar 1;59(2):129-140.

Kongara S, Karantza V. The interplay between autophagy and ROS in tumorigenesis. Front Oncol 2012; 2: 171.

Koop EA, van Laar T, van Wichen DF, et al. Expression of BNIP3 in invasive breast cancer: correlations with the hypoxic response and clinicopathological features. BMC Cancer 2009; 9: 175.

Kouroku Y, Fujita E, Tanida I, et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ 2007;14(2):230-9.

Kouroku Y, Fujita E, Tanida I, et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ. 2007 Feb;14(2):230-9.

Krick R, Muhe Y, Prick T, et al. Piecemeal microautophagy of the nucleus: genetic and morphological traits. Autophagy 2009;5(2):270-2.

Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. Circ Res 2012; 111(9): 1208-21.

Kubota H. Quality control against misfolded proteins in the cytosol: a network for cell survival. J Biochem 2009;146(5):609-16.

Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. Nature. 2004 Dec 23;432(7020):1032-6.

Kumar S, Singh RK, Meena R. Emerging targets for radioprotection and radiosensitization in radiotherapy. Tumour Biol 2016;37(9):11589-11609.

Langer T, Kaser M, Klanner C, et al. AAA proteases of mitochondria: quality control of membrane proteins and regulatory functions during mitochondrial biogenesis. Biochem Soc Trans 2001;29(Pt 4):431-6.

Lee H, Noh JY, Oh Y, et al. IRE1 plays an essential role in ER stress-mediated aggregation of mutant huntingtin via the inhibition of autophagy flux. Hum Mol Genet. 2012 Jan 1;21(1):101-14.

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

Levine B. Cell biology: autophagy and cancer. Nature 2007;446(7137):745-7.

Levy JM, Thorburn A. Targeting autophagy during cancer therapy to improve clinical outcomes. Pharmacol Ther 2011;131(1):130-41.

Li J, Hou N, Faried A, et al. Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. Eur J Cancer 2010;46(10):1900-9.

Li J, Qin Z, Liang Z. The prosurvival role of autophagy in Resveratrol-induced cytotoxicity in human U251 glioma cells. BMC Cancer 2009;9:215.

Li J, Qin Z, Liang Z. The prosurvival role of autophagy in Resveratrol-induced cytotoxicity in human U251 glioma cells. BMC Cancer. 2009 Jun 30;9:215.

Li M, Jiang X, Liu D, et al. Autophagy protects LNCaP cells under androgen deprivation conditions. Autophagy 2008;4(1):54-60.

Li T, Su L, Zhong N, et al . Salinomycin induces cell death with autophagy through activation of endoplasmic reticulum stress in human cancer cells. Autophagy 2013;9(7):1057-68.

Li T, Su L, Zhong N, et al. Salinomycin induces cell death with autophagy through activation of endoplasmic reticulum stress in human cancer cells. Autophagy. 2013 Jul;9(7):1057-68.

Liang J, Shao SH, Xu ZX, et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol 2007;9(2):218-24.

Liang J, Shao SH, Xu ZX, et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol. 2007 Feb;9(2):218-24.

Liang XH, Yu J, Brown K, et al. Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. Cancer Res 2001;61(8):3443-9.

Lin JH, Walter P, Yen TS. Endoplasmic reticulum stress in disease pathogenesis. Annu Rev Pathol 2008;3:399-425.

Lin JH, Walter P, Yen TS. Endoplasmic reticulum stress in disease pathogenesis. Annu Rev Pathol. 2008;3:399-425.

Lionaki E, Markaki M, Palikaras K, et al. Mitochondria, autophagy and age-associated neurodegenerative diseases: New insights into a complex interplay. Biochim Biophys Acta 2015;1847(11):1412-23.

Liu EY, Xu N, O'Prey J, et al. Loss of autophagy causes a synthetic lethal deficiency in DNA repair. Proc Natl Acad Sci U S A 2015;112(3):773-8.

Lomax ME, Folkes LK, O'Neill P. Biological consequences of radiation-induced DNA damage: relevance to radiotherapy. Clin Oncol (R Coll Radiol) 2013;25:578-85.

Lomonaco SL, Finniss S, Xiang C, et al. The induction of autophagy by gamma-radiation contributes to the radioresistance of glioma stem cells. Int J Cancer 2009;125(3):717-22.

Lu H, Li G, Liu L, Feng L, et al. Regulation and function of mitophagy in development and cancer. Autophagy 2013; 9(11): 1720-36.

Ma Q, Cavallin LE, Yan B, et al. Antitumorigenesis of antioxidants in a transgenic Rac1 model of Kaposi's sarcoma. Proc Natl Acad Sci U S A 2009; 106(21): 8683-8.

Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 2004;4(12):966-77.

Macdonald JS, Smalley SR, Benedetti J, et al. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. N Engl J Med 2001;345(10):725-30.

Macia IGM, Lucas Calduch A, Lopez EC. Radiobiology of the acute radiation syndrome. Rep Pract Oncol Radiother 2011;16(4):123-30.

MacNaughton WK. Review article: new insights into the pathogenesis of radiation-induced intestinal dysfunction. Aliment Pharmacol Ther 2000;14(5):523-8.

Mahalingam D, Mita M, Sarantopoulos J, et al. Combined autophagy and HDAC inhibition: a phase I safety, tolerability, pharmacokinetic, and pharmacodynamic analysis of hydroxychloroquine in combination with the HDAC inhibitor vorinostat in patients with advanced solid tumors. Autophagy 2014;10(8): 1403-14.

Maiuri MC, Tasdemir E, Criollo A, et al. Control of autophagy by oncogenes and tumor suppressor genes. Cell Death Differ 2009;16(1):87-93.

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

Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 2007;9(12):2277-93.

Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal. 2007 Dec;9(12):2277-93.

Mammucari C, Milan G, Romanello V, et al. FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab 2007;6(6):458-71.

Mansilla S, Priebe W, Portugal J. Mitotic catastrophe results in cell death by caspase-dependent and caspase-independent mechanisms. Cell Cycle 2006;5(1):53-60.

Mao K, Wang K, Liu X, Klionsky DJ. The scaffold protein Atg11 recruits fission machinery to drive selective mitochondria degradation by autophagy. Dev Cell 2013;26(1):9-18.

Mao Z, Bozzella M, Seluanov A, et al. DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. Cell Cycle 2008;7(18):2902-6.

Marino G, Niso-Santano M, Baehrecke EH, et al. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol 2014;15(2):81-94.

Marino G, Niso-Santano M, Baehrecke EH, et al. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol. 2014 Feb;15(2):81-94.

Marquez RT, Xu L. Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. Am J Cancer Res. 2012;2(2):214-21.

Martinez-Outschoorn UE, Pavlides S, et al. Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: implications for breast cancer and DCIS therapy with autophagy inhibitors. Cell Cycle 2010; 9(12): 2423-33.

Mathew R, Kongara S, Beaudoin B, et al. Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev 2007;21(11):1367-81.

Matsuda N, Sato S, Shiba K, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol 2010;189(2):211-21.

McGee MD, Weber D, Day N, et al. Loss of intestinal nuclei and intestinal integrity in aging C. elegans. Aging Cell 2014;10(4):699-710.

McLelland GL, Soubannier V, Chen CX, et al., Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control. Embo J 2014; 33(4): 282-95.

Meistrich ML, Kangasniemi M. Hormone treatment after irradiation stimulates recovery of rat spermatogenesis from surviving spermatogonia. J Androl 1997;18(1):80-7.

Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene 2003;22(37):5734-54.

Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene. 2003 Sep 1;22(37):5734-54.

Mishra KN, Moftah BA, Alsbeih GA. Appraisal of mechanisms of radioprotection and therapeutic approaches of radiation countermeasures. Biomed Pharmacother 2018;106:610-617.

Mishra PJ, Mishra PJ, Humeniuk R, et al. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. Cancer Res 2008; 68(11): 4331-9.

Mizushima N, et al. Mol Biol Cell 2004;15:1101 Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell 2004;15(3):1101-11.

Mizushima N, Levine B, Cuervo AM, et al.Autophagy fights disease through cellular self-digestion", Nature 2008; 28; 451(7182): 1069–1075.

Mizushima N, Noda T, Yoshimori T, et al. A protein conjugation system essential for autophagy. Nature. 1998 Sep 24;395(6700):395-8.

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

Mizushima N. Autophagy: process and function. Genes Dev. 2007 Nov 15;21(22): 2861-73.

Moretti L, Cha YI, Niermann KJ, et al. Switch between apoptosis and autophagy: radiation-induced endoplasmic reticulum stress? Cell Cycle 2007;6(7):793-8.

Moretti L, Cha YI, Niermann KJ, et al. Switch between apoptosis and autophagy: radiation-induced endoplasmic reticulum stress? Cell Cycle. 2007 Apr 1;6(7):793-8.

Morselli E, Tasdemir E, Maiuri MC, et al. Mutant p53 protein localized in the cytoplasm inhibits autophagy. Cell Cycle 2008;7(19):3056-61.

Muller M. Cellular senescence: molecular mechanisms, in vivo significance, and redox considerations. Antioxid Redox Signal 2009;11(1):59-98.

Munoz-Gamez JA, Rodriguez-Vargas JM, Quiles-Perez R, et al. PARP-1 is involved in autophagy induced by DNA damage. Autophagy 2009;5(1):61-74.

Nadif Kasri N, Bultynck G, Sienaert I, et al. The role of calmodulin for inositol 1,4,5-trisphosphate receptor function. Biochim Biophys Acta 2002;1600(1-2):19-31.

Nair U, Klionsky DJ. Molecular mechanisms and regulation of specific and nonspecific autophagy pathways in yeast. J Biol Chem 2005;280(51):41785-8.

Nakai A, Yamaguchi O, Takeda T, et al. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med 2007;13(5):619-24.

Nakai A, Yamaguchi O, Takeda T, et al. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med. 2007 May;13(5):619-24.

Narendra D, Tanaka A, Suen DF, et al. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 2008;183(5):795-803.

Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 2008; 183(5): 795-803.

Narendra DP, Jin SM, Tanaka A, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 2010;8(1):e1000298.

Nehs MA, Lin CI, Kozono DE, et al. Necroptosis is a novel mechanism of radiation-induced cell death in anaplastic thyroid and adrenocortical cancers. Surgery 2011;150(6):1032-9.

Nishioka T, Shirato H, Yasuda M. X-Ray Irradiation Induces Mitophagy and Cancer Metabolic Reprogramming. Int J Oncol 2014; Vol 90, Issue 1, S778.

Noda T, Ohsumi Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem 1998;273(7):3963-6.

Ogata M, Hino S, Saito A, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol. 2006 Dec;26(24):9220-31.

Okami J, Simeone DM, Logsdon CD. Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer Res 2004; 64(15): 5338-46.

Olzmann JA, Li L, Chudaev MV, et al. Parkin-mediated K63-linked polyubiquitination targets misfolded DJ-1 to aggresomes via binding to HDAC6. J Cell Biol 2007;178(6):1025-38.

Otera H, Wang C, Cleland MM, et al. Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol 2010; 191(6): 1141-58.

Paglin S, Hollister T, Delohery T, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res. 2001 Jan 15;61(2):439-44.

Pang XL, He G, Liu YB, et al. Endoplasmic reticulum stress sensitizes human esophageal cancer cell to radiation. World J Gastroenterol 2013;19(11):1736-48.

Panganiban RA, Snow AL, Day RM. Mechanisms of radiation toxicity in transformed and non-transformed cells. Int J Mol Sci 2013;14(8):15931-58.

Pankiv S, Lamark T, Bruun JA, et al. Nucleocytoplasmic shuttling of p62/SQSTM1 and its role in recruitment of nuclear polyubiquitinated proteins to promyelocytic leukemia bodies. J Biol Chem 2010;285(8):5941-53.

Park C, Suh Y, Cuervo AM. Regulated degradation of Chk1 by chaperone-mediated autophagy in response to DNA damage. Nat Commun 2015;6:6823.

Park YE, Hayashi YK, Bonne G, et al. Autophagic degradation of nuclear components in mammalian cells. Autophagy 2009;5(6):795-804.

Parsons MJ, Green DR. Mitochondria in cell death. Essays Biochem 2010;47:99-114.

Pavlides S, Vera I, Gandara R, et al. Warburg meets autophagy: cancerassociated fibroblasts accelerate tumor growth and metastasis via oxidative stress, mitophagy, and aerobic glycolysis. Antioxid Redox Signal 2012; 16(11): 1264-84.

Pawlik TM, Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. Int J Radiat Oncol Biol Phys 2004;59(4):928-42.

Pearcey R, Brundage M, Drouin P, et al. Phase III trial comparing radical radiotherapy with and without cisplatin chemotherapy in patients with advanced squamous cell cancer of the cervix. J Clin Oncol 2002;20(4):966-72.

Pfisterer SG, Mauthe M, Codogno P, et al. Ca^{2+}/calmodulin-dependent kinase (CaMK) signaling via CaMKI and AMP-activated protein kinase contributes to the regulation of WIPI-1 at the onset of autophagy. Mol Pharmacol 2011;80(6):1066-75.

Picchio MC, Martin ES, Cesari R, et al. Alterations of the tumor suppressor gene Parkin in non-small cell lung cancer. Clin Cancer Res 2004; 10(8): 2720-4.

Pinheiro C, Longatto-Filho A, Scapulatempo C, et al. Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas. Virchows Arch 2008; 452(2): 139-46.

Polager S, Ofir M, Ginsberg D. E2F1 regulates autophagy and the transcription of autophagy genes. Oncogene 2008;27(35):4860-4.

Presley AD, Fuller KM, Arriaga EA. MitoTracker Green labeling of mitochondrial proteins and their subsequent analysis by capillary electrophoresis with laser-induced fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci 2003;793(1):141-50.

Qin L, Wang Z, Tao L, et al. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. Autophagy 2010;6(2):239-47.

Qin L, Wang Z, Tao L, et al. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. Autophagy. 2010 Feb;6(2):239-47.

Rahbar Saadat Y, Saeidi N, Zununi Vahed S, et al. An update to DNA ladder assay for apoptosis detection. Bioimpacts 2015;5(1):25-8.

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

Raviraj J, Bokkasam VK, Kumar VS, Reddy US, Suman V. Radiosensitizers, radioprotectors, and radiation mitigators. Indian J Dent Res 2014;25(1):83-90.

Reeves GI, Ainsworth EJ. Description of the chronic radiation syndrome in humans irradiated in the former Soviet Union. Radiat Res 1995;142(2):242-3.

Reggiori F, Komatsu M, Finley K, et al. Selective types of autophagy. Int J Cell Biol. 2012;2012:156272.

Reisz JA, Bansal N, Qian J, et al. Effects of ionizing radiation on biological molecules--mechanisms of damage and emerging methods of detection. Antioxid Redox Signal 2014;21(2):260-92.

Rello-Varona S, Lissa D, Shen S, et al. Autophagic removal of micronuclei. Cell Cycle 2012;11(1):170-6

Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol 2007;8(5):405-13.

Rimessi A, Bonora M, Marchi S, et al. Perturbed mitochondrial Ca2+ signals as causes or consequences of mitophagy induction. Autophagy 2013; 9(11): 1677-86.

Ristow M, Schmeisser K. Mitohormesis: Promoting Health and Lifespan by Increased Levels of Reactive Oxygen Species (ROS). Dose Response 2014; 12(2): 288-341.

Robert T, Vanoli F, Chiolo I, et al. HDACs link the DNA damage response, processing of double-strand breaks and autophagy. Nature 2011;471(7336):74-9.

Roberts DJ, Tan-Sah VP, Ding EY, et al. Hexokinase-II positively regulates glucose starvation-induced autophagy through TORC1 inhibition. Mol Cell 2014;53(4): 521-33.

Rodriguez-Rocha H, Garcia-Garcia A, Panayiotidis MI, et al. DNA damage and autophagy. Mutat Res 2011;711(1-2):158-66.

Rodriguez-Vargas JM, Ruiz-Magana MJ, Ruiz-Ruiz C, et al. ROS-induced DNA damage and PARP-1 are required for optimal induction of starvation-induced autophagy. Cell Res 2012;22(7):1181-98.

Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007 Jul;8(7):519-29.

Rong YP, Aromolaran AS, Bultynck G, et al. Targeting Bcl-2-IP3 receptor interaction to reverse Bcl-2's inhibition of apoptotic calcium signals. Mol Cell 2008;31(2):255-65.

Rosenfeldt MT, O'Prey J, Morton, et al. p53 status determines the role of autophagy in pancreatic tumour development. Nature 2013; 504(7479): 296-300.

Ryan KM. p53 and autophagy in cancer: guardian of the genome meets guardian of the proteome. Eur J Cancer 2011;47(1):44-50.

Saddoughi SA, Ogretmen B. Diverse functions of ceramide in cancer cell death and proliferation. Advances in cancer research 2013; 117: 37-58.

Saglar E, Unlu S, Babalioglu I, et al. Assessment of ER Stress and autophagy induced by ionizing radiation in both radiotherapy patients and ex vivo irradiated samples. J Biochem Mol Toxicol 2014;28(9):413-7.

Sailaja GS, Bhoopathi P, Gorantla B, et al. The secreted protein acidic and rich in cysteine (SPARC) induces endoplasmic reticulum stress leading to autophagy-mediated apoptosis in neuroblastoma. Int J Oncol. 2013 Jan;42(1):188-96.

Sakaki K, Wu J, Kaufman RJ. Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum. J Biol Chem 2008;283(22): 15370-80.

Salazar M, Carracedo A, Salanueva IJ, et al. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. J Clin Invest 2009;119(5):1359-72.

Schmukler E, Grinboim E, Schokoroy S, et al. Ras inhibition enhances autophagy, which partially protects cells from death. Oncotarget 2013;4(1):142-52.

Schweers RL, Zhang J, Randall MS, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc Natl Acad Sci USA 2007;104:19500–19505.

Scriven P, Brown NJ, Pockley AG, et al. The unfolded protein response and cancer: a brighter future unfolding? J Mol Med (Berl) 2007;85(4):331-41.

Scriven P, Brown NJ, Pockley AG, et al. The unfolded protein response and cancer: a brighter future unfolding? J Mol Med (Berl). 2007 Apr;85(4):331-41.

Selvakumaran M, Amaravadi RK, Vasilevskaya IA, et al. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. Clin Cancer Res 2013;19(11):2995-3007.

Shaid S, Brandts CH, Serve H, et al. Ubiquitination and selective autophagy. Cell Death Differ 2013;20(1):21-30.

Sharma K L, Tiwari M, Mishra K S. Mitochondrial Alteration: A Major Player in Carcinogenesis. Cell Biology 2015; 3(2-1): 8-16.

Shoji JY, Kikuma T, Arioka M, et al. Macroautophagy-mediated degradation of whole nuclei in the filamentous fungus Aspergillus oryzae. PLoS One 2010;5(12): e15650.

Simonsen A, Birkeland HC, Gillooly DJ, et al. Alfy, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes. J Cell Sci 2004;117(Pt 18):4239-51.

Singh VK, Newman VL, Berg AN, et al. Animal models for acute radiation syndrome drug discovery. Expert Opin Drug Discov 2015a;10(5):497-517.

Singh VK, Romaine PL, Seed TM. Medical Countermeasures for Radiation Exposure and Related Injuries: Characterization of Medicines, FDA-Approval Status and Inclusion into the Strategic National Stockpile. Health Phys 2015b;108(6):607-30.

Sinha K, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol 2013;87(7): 1157-80.

Sotgia F, Martinez-Outschoorn UE, Pavlides S, et al. Understanding the Warburg effect and the prognostic value of stromal caveolin-1 as a marker of a lethal tumor microenvironment. Breast Cancer Res 2011; 13(4): 213.

Sowter HM, Ferguson M, Pym C, et al. Expression of the cell death genes BNip3 and NIX in ductal carcinoma in situ of the breast; correlation of BNip3 levels with necrosis and grade. J Pathol 2003; 201(4): 573-80.

Spalding AC, Lawrence TS. New and emerging radiosensitizers and radioprotectors. Cancer Invest 2006;24(4):444-56.

Srivastava S. The Mitochondrial Basis of Aging and Age-Related Disorders. Genes (Basel). 2017 Dec; 8(12): 398.

Stansborough RL, Bateman EH, Al-Dasooqi N, et al. Fractionated abdominal irradiation induces intestinal microvascular changes in an in vivo model of radiotherapy-

induced gut toxicity. Support Care Cancer. 2017 Jun;25(6):1973-1983.

Stewart F.A, Akleyev A.V, M. Hauer-Jensen, et al., ICRP Statement on Tissue Reactions / Early and Late Effects of Radiation in Normal Tissues and Organs – Threshold Doses for Tissue Reactions in a Radiation Protection Context. ICRP Publication 118. Ann. ICRP 41(1/2). ICRP, 2012.

Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352(10):987-96.

Sui X, Chen R, Wang Z, et al. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 2013;4:e838.

Sui X, Kong N, Wang X, et al. JNK confers 5-fluorouracil resistance in p53-deficient and mutant p53-expressing colon cancer cells by inducing survival autophagy. Sci Rep 2014;4:4694.

Sun WT, Wang XC, Mak SK, et al. Activation of PERK branch of ER stress mediates homocysteine-induced BKCa channel dysfunction in coronary artery via FoxO3a-dependent regulation of atrogin-1. Oncotarget. 2017 Aug 1;8(31): 51462-51477.

Szabadkai G, Duchen MR. Mitochondria: the hub of cellular Ca^{2+} signaling. Physiology (Bethesda) 2008;23:84-94.

Tan EY, Campo L, Han C, et al. BNIP3 as a progression marker in primary human breast cancer; opposing functions in in situ versus invasive cancer. Clin Cancer Res 2007; 13(2 Pt 1): 467-74.

Tan JM, Wong ES, Kirkpatrick DS, et al.. Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. Hum Mol Genet 2008;17(3):431-9.

Tanaka Y, Guhde G, Suter A, et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 2000;406(6798):902-6.

Tasdemir E, Maiuri MC, Galluzzi L, et al. Regulation of autophagy by cytoplasmic p53. Nat Cell Biol 2008;10(6):676-87.

Taylor CW, Laude AJ. IP3 receptors and their regulation by calmodulin and cytosolic Ca2+. Cell Calcium 2002;32(5-6):321-34.

Tchkonia T, Zhu Y, van Deursen J, et al. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest 2013;123(3):966-72.

Thorburn J, Horita H, Redzic J, et al. Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. Cell Death Differ 2009;16(1):175-83.

Vakifahmetoglu H, Olsson M, Zhivotovsky B. Death through a tragedy: mitotic catastrophe. Cell Death Differ 2008;15(7):1153-62.

Valente EM, Abou-Sleiman PM, Caputo V, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 2004;304(5674):1158-60.

Verma A, Bhatt AN, Farooque A, et al. Calcium ionophore A23187 reveals calcium related cellular stress as “I-Bodies”: an old actor in a new role. Cell Calcium 2011;50(6):510-22.

Vessoni AT, Filippi-Chiela EC, Menck CF, et al. Autophagy and genomic integrity. Cell Death Differ 2013;20(11):1444-54.

Vicencio JM, Ortiz C, Criollo A, et al . The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin 1. Cell Death Differ 2009;16(7):1006-17.

Vingtdeux V, Giliberto L, Zhao H, et al. AMP-activated protein kinase signaling activation by resveratrol modulates amyloid-beta peptide metabolism. J Biol Chem 2010;285(12):9100-13.

Vogl DT, Stadtmauer EA, Tan KS, et al. Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. Autophagy 2014;10(8):1380-90.

Volkmann K, Lucas JL, Vuga D, et al. Potent and selective inhibitors of the inositol-

requiring enzyme 1 endoribonuclease. J Biol Chem. 2011 Apr 8;286(14):12743-55.

Waldman T, Lengauer C, Kinzler KW, et al. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. Nature 1996;381(6584):713-6.

Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 2005;39:359-407.

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

Wang J, Fang Y, Yan L, et al. Erythroleukemia cells acquire an alternative mitophagy capability. Sci Rep 2016;6:24641.

Wang K, Klionsky DJ. Mitochondria removal by autophagy. Autophagy. 2011 Mar;7(3):297-300.

Wang K, Liu R, Li J, et al. Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR- and hypoxia-induced factor 1alpha-mediated signaling. Autophagy 2011;7(9):966-78.

Wang SH, Shih YL, Ko WC, et al. Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway. Cell Mol Life Sci 2008;65(22):3640-52.

Warburg O. On the origin of cancer cells. Science 1956: 123(3191): 309-14.

Wardell TM, Ferguson E, Chinnery PF, et al. Changes in the human mitochondrial genome after treatment of malignant disease. Mutat Res 2003;525(1-2):19-27.

Waselenko JK, MacVittie TJ, Blakely WF, et al, Seed T and others. Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. Ann Intern Med 2004;140(12): 1037-51.

Waselenko JK, MacVittie TJ, Blakely WF, et al. Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. Ann Intern Med 2004;140(12):1037-51.

Whitaker-Menezes D, Martinez-Outschoorn UE, Lin Z, et al. Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts. Cell Cycle 2011; 10(11): 1772-83.

White E, Karp C, Strohecker AM, et al. Role of autophagy in suppression of inflammation and cancer. Curr Opin Cell Biol 2010;22(2):212-7.

Williams A, Sarkar S, Cuddon P, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol 2008;4(5):295-305.

Witkiewicz AK, Whitaker-Menezes D, Dasgupta A, et al. Using the "reverse Warburg effect" to identify high-risk breast cancer patients: stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers. Cell Cycle 2012; 11(6): 1108-17.

Wu HM, Shao LJ, Jiang ZF, et al. Gemcitabine-Induced Autophagy Protects Human Lung Cancer Cells from Apoptotic Death. Lung 2016;194(6):959-966.

Wu LJ, Randers-Pehrson G, Xu A, et al. Targeted cytoplasmic irradiation with alpha particles induces mutations in mammalian cells. Proc Natl Acad Sci U S A 1999;96(9):4959-64.

Wu Z, Chang PC, Yang JC, et al. Autophagy Blockade Sensitizes Prostate Cancer Cells towards Src Family Kinase Inhibitors. Genes Cancer 2010;1(1):40-9.

Xue X, Piao JH, Nakajima A, et al. Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha. J Biol Chem. 2005 Oct 7;280(40):33917-25.

Yamamori T, Yasui H, Yamazumi M, et al. Ionizing radiation induces mitochondrial reactive oxygen species production accompanied by upregulation of mitochondrial electron transport chain function and mitochondrial content under control of the cell cycle checkpoint. Free Radic Biol Med 2012;53(2):260-70.

Yang Z, Xu Y, Xu L, et al. Regulation of autophagy via PERK-eIF2alpha effectively relieve the radiation myelitis induced by iodine-125. PLoS One 2013;8(11):e76819.

Yang Z, Xu Y, Xu L, et al. Regulation of autophagy via PERK-eIF2alpha effectively relieve the radiation myelitis induced by iodine-125. PLoS One. 2013;8(11): e76819.

Yao KC, Komata T, Kondo Y, et al. Molecular response of human glioblastoma multiforme cells to ionizing radiation: cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. J Neurosurg 2003;98(2):378-84.

Yen YP, Tsai KS, Chen YW, et al. Arsenic induces apoptosis in myoblasts through a reactive oxygen species-induced endoplasmic reticulum stress and mitochondrial dysfunction pathway. Arch Toxicol. 2012 Jun;86(6):923-33.

Yi H, Liang B, Jia J, et al. Differential roles of miR-199a-5p in radiation-induced autophagy in breast cancer cells. FEBS Lett 2013;587(5):436-43.

Yorimitsu T, Nair U, Yang Z, et al. Endoplasmic reticulum stress triggers autophagy. J Biol Chem. 2006 Oct 6;281(40):30299-304.

Youle RJ, Narendra DP. Mechanisms of mitophagy. Nat Rev Mol Cell Biol 2011;12(1):9-14.

Yue Z, Jin S, Yang C, et al. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A 2003;100(25):15077-82.

Zalckvar E, Berissi H, Mizrachy L, et al. DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep 2009;10(3):285-92.

Zhang B, Davidson MM, Zhou H, et al. Cytoplasmic irradiation results in mitochondrial dysfunction and DRP1-dependent mitochondrial fission. Cancer Res 2013;73(22):6700-10.

Zhang C, Lin M, Wu R, et al. Parkin, a p53 target gene, mediates the role of p53 in glucose metabolism and the Warburg effect. Proc Natl Acad Sci U S A 2011; 108(39): 16259-64.

Zhang X, Li W, Wang C, et al. Inhibition of autophagy enhances apoptosis induced by proteasome inhibitor bortezomib in human glioblastoma U87 and U251 cells. Mol Cell Biochem 2014;385(1-2):265-75.

Zhang Y, Qi H, Taylor R, et al. The role of autophagy in mitochondria maintenance: characterization of mitochondrial functions in autophagy-deficient S. cerevisiae strains. Autophagy. 2007 Jul-Aug;3(4):337-46.

Zhao W, Diz DI, Robbins ME. Oxidative damage pathways in relation to normal tissue injury. Br J Radiol 2007;80 Spec No 1:S23-3.

Zheng R, Yao Q, Xie G, et al. TAT-ODD-p53 enhances the radiosensitivity of hypoxic breast cancer cells by inhibiting Parkin-mediated mitophagy. Oncotarget 2015;6(19):17417-29.

Zhou W, Yuan J. Necroptosis in health and diseases. Semin Cell Dev Biol 2014;35:14-23.

Zois CE, Koukourakis MI. Radiation-induced autophagy in normal and cancer cells: towards novel cytoprotection and radio-sensitization policies? Autophagy 2009;5(4):442-50.

Zong Y, Feng S, Cheng J, et al. Up-Regulated ATF4 Expression Increases Cell Sensitivity to Apoptosis in Response to Radiation. Cell Physiol Biochem. 2017;41(2):784-794.

Publications

AUTOPHAGY
https://doi.org/10.1080/15548627.2019.1582973

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Radiation induces EIF2AK3/PERK and ERN1/IRE1 mediated pro-survival autophagy

Madhuri Chaurasia [a,b], Swapnil Gupta [c], Asmita Das [b], B.S. Dwarakanath [d], Anne Simonsen [c], and Kulbhushan Sharma [a,c]

[a]Division of Metabolic Cell Signaling Research, Institute of Nuclear Medicine and Allied Sciences, Delhi, India; [b]Department of Biotechnology, Delhi Technological University, Delhi, India; [c]Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway; [d]Shanghai Proton and Heavy Ion Center, Shanghai, China

ABSTRACT

Cellular effects of ionizing radiation include oxidative damage to macromolecules, unfolded protein response (UPR) and metabolic imbalances. Oxidative stress and UPR have been shown to induce macroautophagy/autophagy in a context-dependent manner and are crucial factors in determining the fate of irradiated cells. However, an in-depth analysis of the relationship between radiation-induced damage and autophagy has not been explored. In the present study, we investigated the relationship between radiation-induced oxidative stress, UPR and autophagy in murine macrophage cells. A close association was observed between radiation-induced oxidative burst, UPR and induction of autophagy, with the possible involvement of EIF2AK3/PERK (eukaryotic translation initiation factor 2 alpha kinase 3) and ERN1/IRE1 (endoplasmic reticulum [ER] to nucleus signaling 1). Inhibitors of either UPR or autophagy reduced the cell survival indicating the importance of these processes after radiation exposure. Moreover, modulation of autophagy affected lethality in the whole body irradiated *C57BL/6* mouse. These findings indicate that radiation-induced autophagy is a pro-survival response initiated by oxidative stress and mediated by EIF2AK3 and ERN1.

Abbreviations: ACTB: actin, beta; ATF6: activating transcription factor 6; ATG: autophagy-related; BafA1: bafilomycin A_1; CQ: chloroquine; DBSA: 3,5-dibromosalicylaldehyde; EIF2AK3: eukaryotic translation initiation factor 2 alpha kinase 3; ERN1: endoplasmic reticulum (ER) to nucleus signaling 1; IR: ionizing radiation; MAP1LC3/LC3: microtubule-associated protein 1 light chain 3; 3-MA: 3-methyladenine; MTOR: mechanistic target of rapamycin kinase; NAC: N-acetyl-L-cysteine; PARP1: poly (ADP-ribose) polymerase family, member 1; 4-PBA: 4-phenylbutyrate; Rap: rapamycin; ROS: reactive oxygen species; UPR: unfolded protein response; XBP1: x-box binding protein 1

ARTICLE HISTORY
Received 11 March 2018
Revised 22 January 2019
Accepted 29 January 2019

KEYWORDS
Autophagy; EIF2AK3/PERK; ER stress; ERN1/IRE1; oxidative stress; radiation exposure

Introduction

Accumulating evidence suggests that acute exposure to ionising radiation, mainly low-linear energy transfer/LET causes macromolecular damage as well as reduced mitochondrial potential, leading to the generation of reactive oxygen species (ROS) and reactive nitrogen species. These events finally lead to activation of different signaling pathways including apoptosis, cell-growth and autophagic induction [1–4].

Autophagy is an evolutionarily conserved, lysosome-mediated degradation process. It helps in maintaining cellular homoeostasis upon various cellular traumas [5–10]. During macroautophagy (hereafter autophagy), a unique double-membrane autophagosome is formed, which engulfs cytoplasmic cargos and fuses with the lysosome to facilitate degradation of the sequestered cargo [11]. The core proteins involved in autophagosome formation are known as autophagy-related (ATG) proteins [12,13].

Radiation exposure causes macromolecular damage both by direct interaction and indirectly through the generation of reactive oxygen/nitrogen species [6]. Radiation-induced damage involves ROS generation leading to oxidative stress. In turn, oxidative stress may lead to various imbalances in the cell, including DNA damage, compromized mitochondrial functioning, protein misfolding, etc. In contrast to other stresses, autophagy induction following exposure of cells to radiation has received little attention [6–10]. Although, various studies have shown the induction of autophagy during radiation exposure, an in-depth analysis of the relationship has not been explored [14–19]. Recently, increasing doses of radiation have been shown to induce acidic vacuole formation, suggesting autophagy induction [4,6,20]. Autophagy affects the survival of various cancer types when exposed to radiation [17–19,21]. The endoplasmic reticulum (ER) is a crucial intracellular Ca^{2+} reservoir that serves as a platform for numerous cellular processes including translation, post-translational modification and proper folding. The ER is also the starting point for sorting and trafficking of proteins and lipids to various organelles and the cell surface. During ER stress, newly synthesized proteins are unable to fold properly, leading to a process collectively known as the unfolded protein response (UPR) [22]. During the UPR, protein synthesis shuts down until removal of all unfolded proteins from the cell system. It has been well established that stress-induced ROS formation causes indirect macromolecular damage (to DNA, proteins and lipids) [23,24]. It also elicits an activation signal to boost the cytosolic calcium load

CONTACT Kulbhushan Sharma kulbhushan@inmas.drdo.in; kulhushan.sharma@medisin.uio.no Division of Metabolic Cell Signaling Research, Institute of Nuclear Medicine and Allied Sciences (INMAS), Brig. S.K. Mazumdar Marg, Timarpur, Delhi, India
Supplemental data for this article can be accessed here.

released from ER [7]. ROS generation thus causes activation of ER stress leading to the induction of UPR [25–27]. Although studies have shown a correlation between radiation, UPR and autophagy, the mechanisms are not very clear [2,3,14,15,28]. Therefore, it is considered worthwhile to study the possible association between ROS, ER stress and autophagy following irradiation.

Because radiation-induced macromolecular damage is associated with ROS generation, we hypothesized that autophagy is induced to recycle damaged macromolecules (cargos) thereby protecting the cell against the radiation stress. Macrophages serve as an important line of defense under most of the stress conditions in our body. Therefore, in the present study, we have investigated the induction of autophagy following irradiation in murine macrophage cell line (*RAW 264.7*) as well as peritoneal macrophages *ex vivo*. Our results demonstrate a dose- and time-dependent induction of autophagy following radiation exposure, which was ROS-dependent and preceded by UPR, specifically through the activation of EIF2AK3/PERK (eukaryotic translation initiation factor 2 alpha kinase 3) and ERN1/IRE1 (endoplasmic reticulum [ER] to nucleus signaling 1) UPR pathways. Further, the induced autophagy facilitated the survival of irradiated cells by attenuating apoptotic cell death.

Results

Radiation induces autophagy in RAW 264.7 cells

Autophagy has been suggested to play a pro-survival role under various stress conditions [29–33]. To investigate the role of autophagy in radiation-induced cellular stress and cell death; we first determined dose-dependent lethality of *RAW 264.7* cells exposed to IR (0 to 10 Gy) by analyzing growth inhibition. The LD_{50} was found to be approximately 2.5 Gy in these cells (Figure 1(a)). Unless specified otherwise, all further investigations to understand the relationship between radiation-induced cell death and autophagy were carried at an absorbed radiation dose of 2.5 Gy, 12 or 24 h post-irradiation. A time-dependent growth inhibition (relative cell number at 24 h after irradiation) was accompanied by loss of cell as well as metabolic viability, and a significant loss of clonogenic survival at 2.5 Gy (Figures 1(b) and S1), clearly suggesting cell death. Next, we examined the induction of cell death by analyzing phosphatidyl externalization using multi-parametric flow cytometry with ANXA5/annexin V and propidium iodide (PI; apoptosis), as well as uptake of PI (necroptosis) (Figure 1(c), upper left and right panel, respectively) and found 30% to 40% increase in ANXA5- and PI-positive cells (apoptosis and necroptosis) at 12 and 24 h post-irradiation. Radiation-induced apoptosis was confirmed by the enhanced CASP3 (caspase 3) cleavage (Figure 1(c), lower panel).

To understand the role of autophagy under radiation stress, we examined its status and functional relevance in irradiated *RAW 264.7* cells by comparing the level of the autophagosomal membrane-bound form of MAP1LC3A/B (microtubule-associated protein 1 light chain 3 alpha/beta (referred to hereafter as LC3-II) relative to ACTB (actin, beta) [34]. A dose-dependent increase in autophagy levels was observed in irradiated cells, which correlated well with a significant decrease in the autophagy substrate SQSTM1/p62 (sequestosome 1) with increasing dose of radiation suggesting the induction of autophagy (Figure 1(d)). Further, the kinetics of autophagy induction was studied by harvesting irradiated *RAW 264.7* cells at different times post-exposure. The levels of LC3-II peaked at approximately 12 h post-irradiation and stabilized after that (Figure 1(e)). The autophagic flux was studied using the lysosomal proton-pump inhibitor bafilomycin A_1 (BafA1), which further confirmed radiation-induced autophagy in these cells (Figure 1(f)). Cells of human origin (U2OS, human osteosarcoma) also exhibited a similar response after radiation exposure (Figure S2). Furthermore, we quantified the number of LC3 puncta post-irradiation in *RAW 264.7* cells transiently transfected with a pEGFP-LC3 plasmid, 12 h post-irradiation, and observed a nearly 4–5 fold increase in EGFP-LC3 puncta formation as compared to non-irradiated control cells (Figure 1(g)). This observation was complemented by transmission electron microscopy quantifications, where significantly more autophagic vacuoles (autophagosomes) were observed 12 h post-irradiation (Figure 1(h)). Taken together, these results indicate the induction of autophagy as well as apoptosis in irradiated cells.

Radiation-induced autophagy is ROS dependent

Generation of reactive oxygen species (ROS) has been linked with the autophagy regulation [1,2,35], and we, therefore, investigated whether radiation-induced autophagy in *RAW 264.7* cells is ROS dependent. The kinetics of radiation-induced ROS was studied using flow cytometric analysis of DCFDA fluorescence and showed an initial burst immediately after irradiation (marked as 0 h) followed by a delayed ROS at approximately 12 h (Figure 2(a)). To scavenge radiation-induced ROS production, we determined the optimum dose of the antioxidant N-acetylcysteine (NAC) in *RAW 264.7* cells (Figure S3). ROS production was significantly abolished by incubation with 30 mM freshly prepared NAC (pH 7.4) for 1 h prior to irradiation (Figure 2(b)). NAC treatment also attenuated irradiation-induced autophagy, as suggested by a decrease in the levels of lipidated LC3, indicating oxidative stress to be upstream of autophagy induction (Figure 2(c)). Interestingly, we also observed a reduction in the levels of LC3-I along with a decline in LC3-II in NAC treated cells post exposure. The drug alone group have also shown some reduction in LC3 lipidation probably due to the effect of these drugs on the basal levels of autophagy (Figure S4, upper panel). Taken together, these results suggest that radiation-induced ROS is involved in the induction of autophagy in *RAW 264.7* cells.

Radiation-induced autophagy induction is mediated through ER stress (the UPR)

Since ER signaling is one of the major processes involved in regulation of autophagy, we hypothesized that ER stress might play an important role in radiation-induced autophagy [36–38]. We explored the induction profile of the classical UPR marker, HSPA5/GRP78 (heat shock protein 5), post-irradiation and observed a time-dependent induction (Figure 3(a)), which resembled the pattern of LC3 lipidation (Figure 1(e)) and oxidative burst (Figure 2(a)).

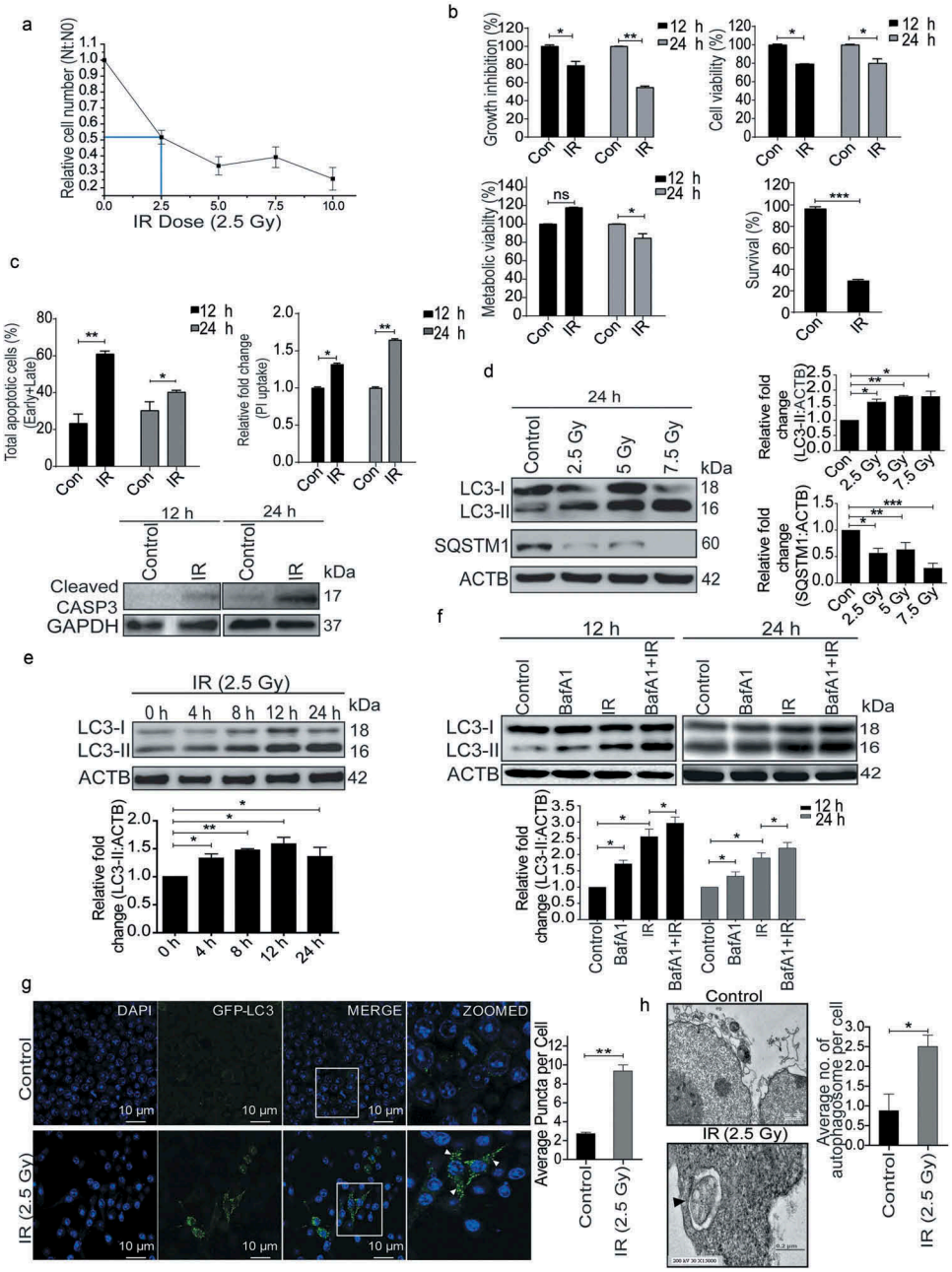


Figure 1. Radiation induces autophagy in *RAW 264.7* cells. (a) *RAW 264.7* cells were exposed to different doses of IR ranging from 0 to 10 Gy, 24 h post-irradiation cells were counted, and relative growth was calculated with respect to unirradiated control cells. The graph represents growth of irradiated cells relative to unirradiated control. LD_{50} of these cells was found to be 2.5 Gy. Data is representative of 3 independent experiments. (b) *RAW 264.7* cells were exposed to 2.5 Gy IR and processed for growth inhibition kinetics, cell viability and metabolic viability. Cells were counted for growth inhibition or processed by sulphorhodamine-B (SRB) or MTT for cell and metabolic viability respectively after 12 and 24 h. For the clonogenic assay, cells were exposed to 2.5 Gy radiation and were incubated at 37°C to form colonies. Upper left, and right panel represents growth inhibition and cell viability (by SRB assay) whereas lower left and right panel represent metabolic viability and clonogenicity. (c) Upper left panel: A bar graph showing results from ANXA5-PI assay in irradiated vs normal cells. Upper right panel: A bar graph showing mean fluorescence intensity of propidium iodide uptake in irradiated samples as compared to unirradiated control cells using flow cytometry at 12 and 24 h post-IR exposure. Lower Panel: Western blot analysis of cell lysate obtained at 12 and 24 h post-irradiation. Blots were probed with intrinsic apoptosis marker, cleaved CASP3, GAPDH was used as loading control. (d) Lysates from *RAW 264.7* cells exposed to increasing IR doses ranging from 0–7.5 Gy were resolved by SDS-PAGE and blotted onto PVDF membranes, followed by immunoblotting with LC3- and SQSTM1-specific antibodies. Each blot is representative of 3 independent experiments. The graphs show quantitation of band intensities (relative to ACTB) from 3 independent experiments. (e) The kinetics of autophagy induction in *RAW 264.7* cells was explored by Western blot analysis of LC3-II levels (relative to ACTB) in whole cell lysates. The graph shows quantification of band intensities (relative to ACTB) from 3 independent experiments. (f) In order to study autophagic flux, cells were irradiated, and BafA1 (100 nM) was added 2 h before harvesting. Samples were analyzed after 12 and 24 h. The graph shows quantification of band intensities (relative to ACTB) from 3 independent experiments. (g) EGFP-LC3 puncta were analyzed through confocal microscopy (63X magnification) in irradiated *RAW 264.7* cells. Briefly, after 24 h post-transfection with pEGFP-LC3, *RAW 264.7* cells were treated with 2.5 Gy ionizing radiation and images were captured 12 h post-irradiation. Puncta were counted using ImageJ from at least 3 fields per experiment. Arrows indicate puncta post-irradiation. Also, the difference in morphology of irradiated cells can be seen in the represented image. The graph on the right represents EGFP-LC3-positive puncta per cell after radiation exposure as compared to control (**P < 0.01, IR vs control). (h) Electron microscopy-based detection of autophagosome in *RAW 264.7* cells. Electron micrographs of control and radiation-exposed cells were taken at 12 h post-irradiation. Arrowhead in the representative micrograph shows the autophagosome. Autophagosomes were counted manually from at least 3 fields per experiment. The graph on the right represents an average number of autophagosomes/cell after radiation exposure as compared to control (*P < 0.05, IR vs control). Molecular mass is represented by kDa (kilodalton).

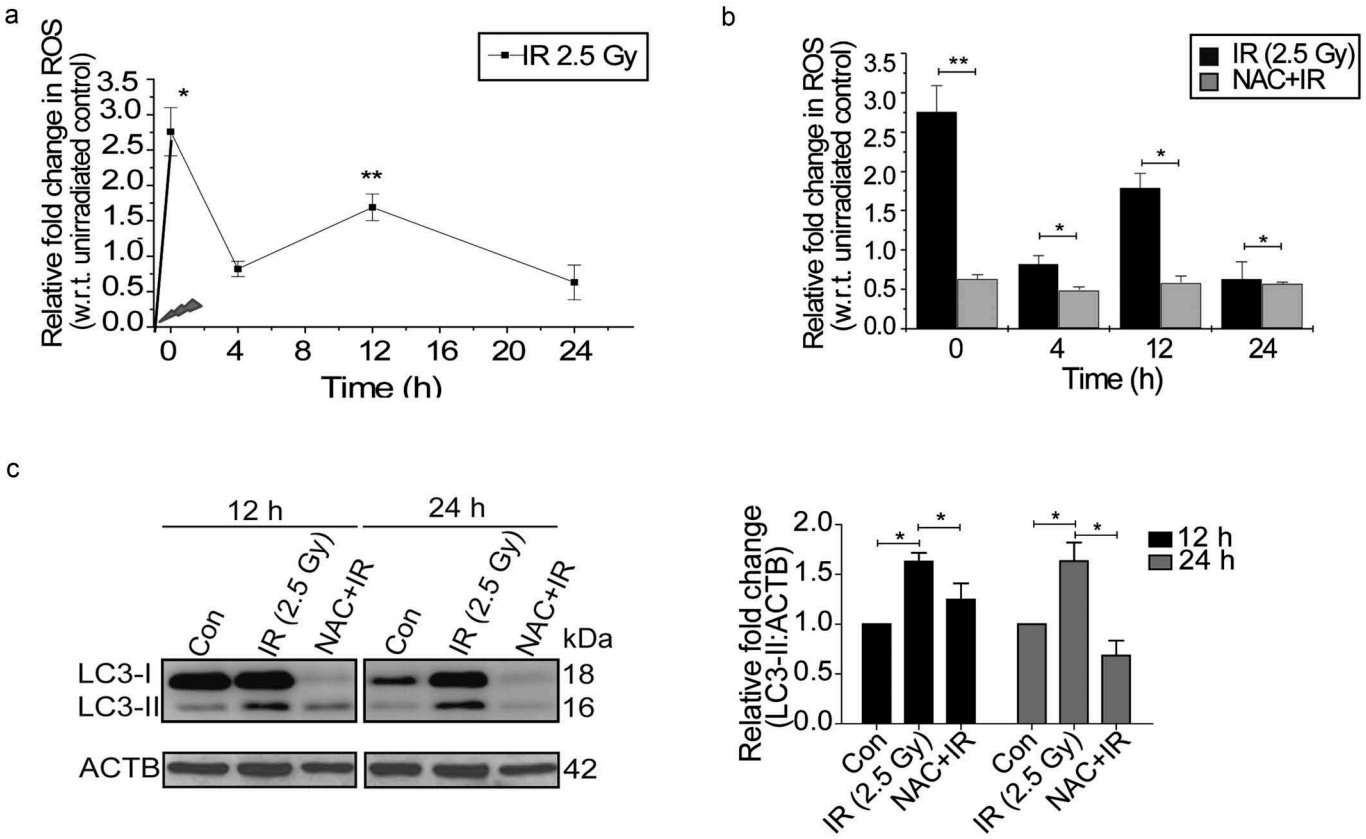


Figure 2. Radiation induces oxidative stress in *RAW 264.7* cells. (a) Kinetics of radiation-induced oxidative stress in *RAW 264.7* cells. The line graph shows relative fold change in fluorescence intensity for intracellular ROS production with respect to non-irradiated control cells after DCFDA staining (10 μM) in response to radiation exposure (2.5 Gy). *P < 0.05, at 0 h and **P < 0.01, at the 12 h post-IR respectively. (b) Effect of ROS scavenger NAC (freshly prepared, given 1 h prior irradiation) on the IR-induced oxidative burst. The values shown are relative to unirradiated control (considered as zero). The 0 h time point represents samples processed immediately after radiation. (c) Radiation-induced changes in autophagy levels are ROS-dependent. Immunoblots of total protein lysates from control, IR and NAC+IR samples harvested at 12 h or 24 h post-irradiation and probed with the indicated specific antibodies. For LC3 blotting, Sigma-Aldrich, L7543 was used. Each blot is representative of 3 independent experiments. The graph shows quantitation of band intensities (relative to ACTB) from 3 independent experiments.

Available evidence suggests a link between oxidative stress and ER stress [27,39–41]. To examine the relationship between radiation-induced ROS and UPR, we suppressed ROS with freshly prepared NAC and monitored the levels of HSPA5. NAC significantly reduced the levels of HSPA5 suggesting that radiation-induced UPR is ROS-dependent (Figure 3(b)).

In order to find out whether radiation-induced autophagy is UPR dependent, we suppressed UPR with its inhibitor 4-phenylbutyrate (4-PBA) and found a significant reduction in HSPA5 (Figure 3(c)). Reduction in the levels of radiation-induced LC3-II (more significant 12 h post-irradiation) in PBA-treated cells (Figure 3(d)), strongly suggests that the irradiation-induced autophagy is indeed UPR dependent and is linked to ROS. Decreased LC3 lipidation in cells treated with drug alone indicates the effect of these drugs on the basal levels of autophagy (Figure S4).

EIF2AK3 and ERN1 gets activated after radiation exposure and lead to autophagy induction

UPR is mediated by 3 major signaling pathways, namely ERN1, EIF2AK3 and ATF6 [22,25,27,35]. All these pathways have also been found to play a role in the induction of autophagy during diverse stress conditions [36,42]. However, specific UPR pathways activated in radiation-exposed condition are not well known. The UPR levels (HSPA5) started building post-radiation quickly, with p-EIF2AK3 being specifically activated very rapidly after irradiation (0 h) (Figure 4(a)). Furthermore, proteins like SQSTM1 and LC3 were also altered immediately after irradiation suggesting the possibility of p-EIF2AK3 mediated UPR linked to autophagy induction (Figure S5). As compared to early time points, the levels of both phosphorylated EIF2AK3 and ERN1 were elevated at 12 and 24 h post-IR exposure, while ATF6 remained unaltered, suggesting that EIF2AK3 and ERN1 are the major ER stress pathways involved in the activation of radiation-induced autophagy (Figure 4(b)).

To examine the role of the EIF2AK3 pathway in radiation-induced autophagy in *RAW 264.7* cells, we used GSK2606414, a specific pharmacological inhibitor of EIF2AK3 phosphorylation [43], and investigated the levels of autophagy. Reduction in the levels of p-EIF2AK3 in irradiated cells treated with GSK2606414 was accompanied by a significant decrease in the levels of LC3-II, indicating the involvement of EIF2AK3 signaling in radiation-induced autophagy (Figure 4(c)). To investigate the role of ERN1 in radiation-induced autophagy, we used 3,5-dibromosalicylaldehyde (an inhibitor of ERN1

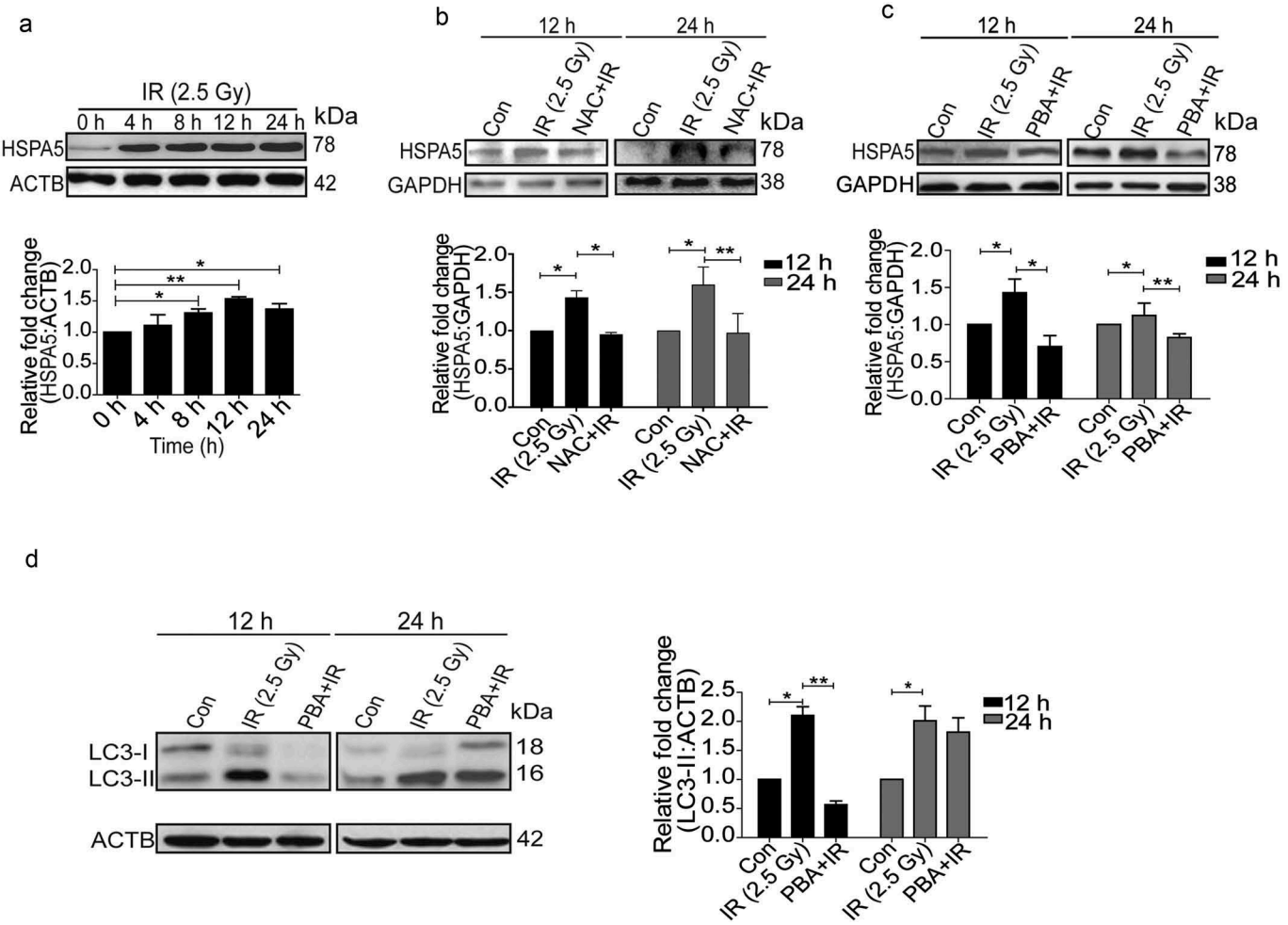


Figure 3. Radiation induces ER stress in *RAW 264.7* cells. (a) Kinetics of UPR induction after radiation exposure was studied through the expression profile of the UPR marker, HSPA5. The experiment was performed at least 3 independent times. The graph shows quantification of band intensities (relative to ACTB) from 3 independent experiments. (b) Immunoblots of total protein isolated from control, IR and NAC+IR samples at 12 or 24 h post-irradiation for analysing HSPA5 expression. NAC treatment was given 1 h prior to radiation followed by IR exposure. Blots were probed with the indicated specific antibodies. Each blot is representative of 3 independent experiments. The graph shows quantification of band intensities (relative to GAPDH) from 3 independent experiments. (c) Western blot analysis of UPR marker HSPA5 in cell lysate obtained from *RAW 264.7* cells irradiated in the presence of the UPR inhibitor 4-PBA (3.5 mM). The data shown are representative of at least 3 separate experiments. The graph shows quantification of band intensities (relative to GAPDH) from 3 independent experiments. (d) LC3 levels were analyzed in the samples treated with 4-PBA. The graph shows quantification of band intensities (relative to ACTB) from 3 independent experiments.

endoribonuclease activity) [44,45]. A significant decrease in spliced XBP1 protein coupled with a reduction in the levels of LC3-II in irradiated cells strongly suggested that ERN1 was also involved in radiation-induced autophagy (Figure 4(d)). Taken together, these observations advocate that the radiation-induced autophagy is mediated through ER stress and is specifically dependent upon EIF2AK3 and ERN1 pathways.

Radiation-induced UPR mediated autophagy is pro-survival and anti-apoptotic

After establishing the induction of autophagy post-irradiation, we investigated its role in determining cell fate. The cellular and metabolic viability of irradiated *RAW 264.7* cells were analyzed with the SRB and MTT assays, respectively, in the presence of autophagy inhibitors 3-MA (phosphatidylinositol 3-kinase [PtdIns3K] inhibitor, blocks the early steps of autophagy) and BafA1, late autophagy inhibitor (vacuolar-type H^+-ATPase inhibitor; blocks lysosomal degradation) [34]. Both, cell and metabolic viability of irradiated cells (examined *via* SRB and MTT assay respectively) were significantly reduced in the presence of these autophagy inhibitors (Figure 5(a,b)). As expected, both the inhibitors significantly reduced the clonogenic survival of irradiated cells (Figure 5(c)), whereas rapamycin (autophagy inducer) enhanced the clonogenic survival (Figure 5(d)). Cell death induced by some of the drugs used may be attributed to the blockage of the basal levels of autophagy.

Next, we sought to clarify if the pro-survival role of autophagy is a consequence of the inhibition of radiation-induced apoptosis and necroptosis. Increase in PI uptake (suggestive of necroptosis, Figure 5(e), left panel), as well as the levels of cleaved PARP1 (poly [ADP-ribose] polymerase family, member 1) and cleaved CASP3 (apoptosis; Figure 5(e), right panel), indicate that loss of clonogenic survival following irradiation is indeed linked to apoptosis and necroptosis. Importantly, suppression of autophagy using siRNA against *Atg7* and *Ulk1* (both

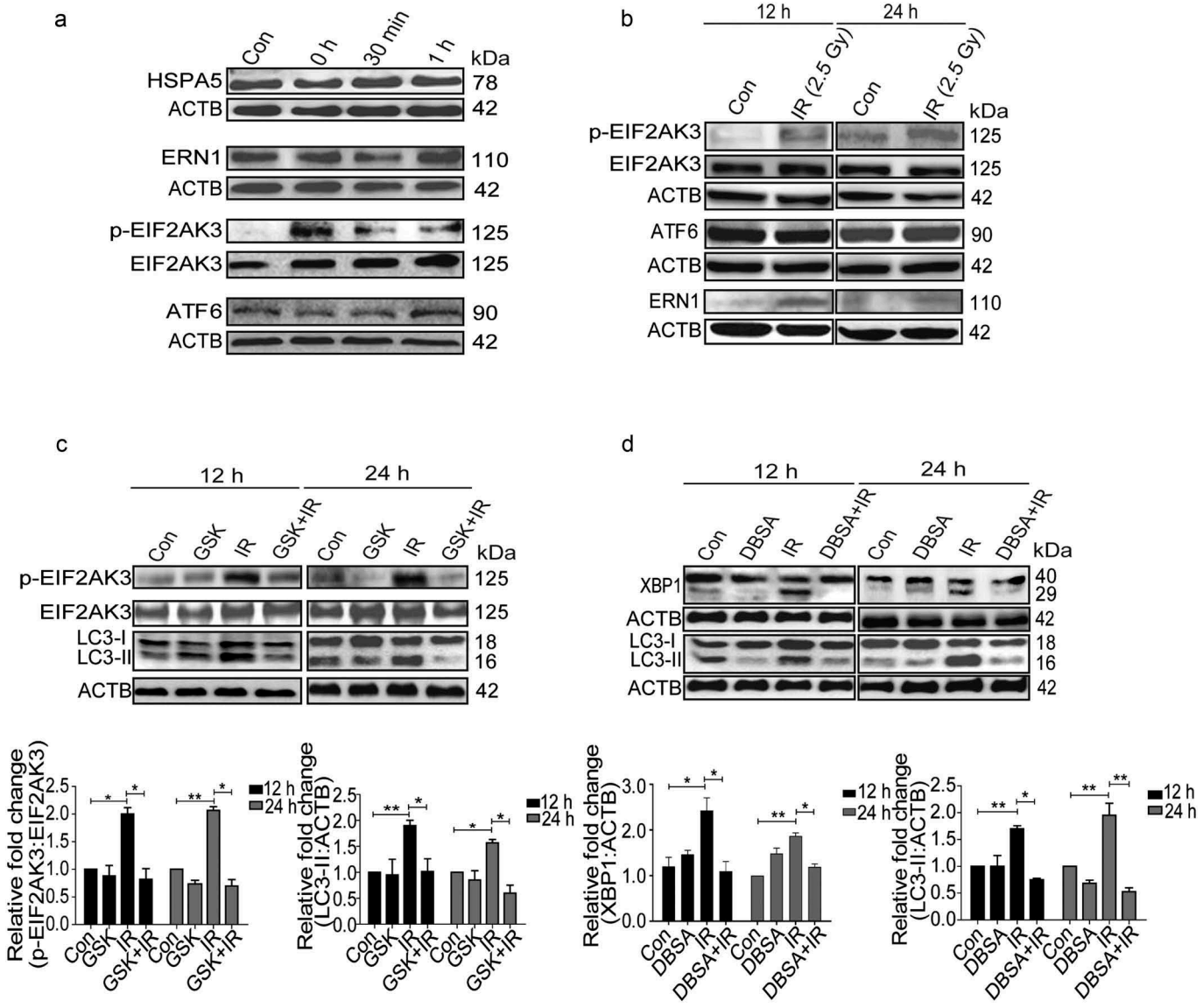


Figure 4. Radiation-induced activation profile of specific UPR pathways. (a) Immunoblot analysis of radiation-induced changes in specific UPR branch proteins at the early time point (0–1 h post-irradiation); blots were probed with HSPA5, ERN1, ATF6, p-EIF2AK3 and total EIF2AK3 specific antibodies. The time point of 0 h was taken immediately after radiation. (b) Western blot analysis of ERN1, ATF6 and p-EIF2AK3 levels at 12 and 24 h post-irradiation. (c) Immunoblot analysis of radiation-induced changes in EIF2AK3 phosphorylation and autophagy levels (LC3-II), in the presence of the EIF2AK3 inhibitor GSK2606414 at 12 and 24 h post-irradiation. The graph shows quantification of band intensities (relative to ACTB). (d) Immunoblot analysis of radiation-induced changes in the cleavage of XBP1 and autophagy levels (LC3-II), in the presence of the ERN1 inhibitor DBSA at 12 and 24 h post-irradiation. The graph shows quantification of band intensities (relative to ACTB) from 3 independent experiments.

important for autophagosome biogenesis [46–50]) (Figure 5(f)), compromized the cell viability (Figure 5(g)), showing that autophagy can rescue the cells from radiation-induced lethality. The effectiveness of siRNA was confirmed by LC3B lipidation analysis under both starvation and radiation exposure conditions (Figure 5(f)). Furthermore, the autophagic flux in the presence of *Atg7* siRNA was confirmed using BafA1 in irradiated cells. A significant increase in LC3-II was observed in the presence of BafA1 confirming that autophagic flux is induced during irradiation. This is not the case in cells depleted of ATG7, showing the autophagy-specific effect of irradiation (Figure 5(f), right panel). These observations lend further support to our proposition that radiation-induced autophagy is pro-survival and anti-apoptotic in nature.

Pro-survival nature of radiation-induced autophagy is ROS and UPR dependent

To further investigate whether the radiation-induced pro-survival role of autophagy is ROS-dependent and UPR mediated, irradiated *RAW 264.7* cells pre-treated with freshly prepared NAC or 4-PBA were analyzed for clonogenic survival. Both NAC and PBA significantly reduced the clonogenicity post-irradiation (Figure 6(a,b) respectively). Also, the levels of cleaved PARP1 were enhanced in the presence of these drugs after radiation (Figure 6(c,d)) indicating that blocking ROS or ER stress which reduces autophagy induction (Figures 2(c) and 3(d)), lead to enhanced apoptosis, which was supported by ANXA5-PI assay as well as the DNA ladder observed under these conditions (Figures 6(e)

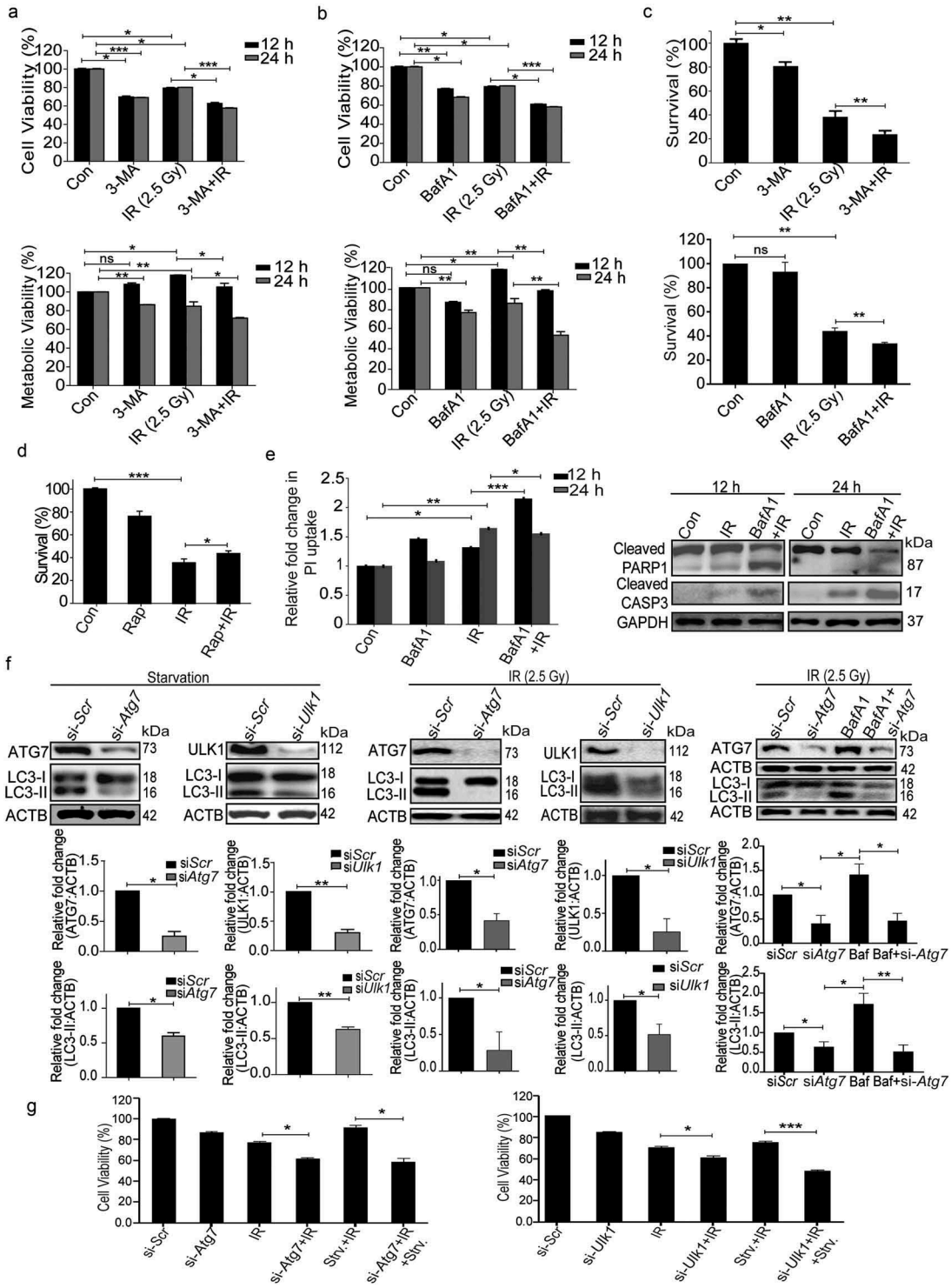


Figure 5. Radiation-induced autophagy is pro-survival and anti-apoptotic. (a) Cells were irradiated in the presence of the autophagy inhibitor, 3-MA (0.5 mM). SRB data (cell viability, shown in upper graph, *P < 0.05, ***P < 0.001, at 12 and 24 h post-irradiation respectively) and MTT data (metabolic viability, shown in lower graph, *P < 0.05, at 12 and 24 h post-irradiation respectively) at 12 and 24 h post-irradiation shows the reduced viability of *RAW 264.7* cells. (b) Cells were irradiated in the presence of the autophagy inhibitor, BafA1 (2.5 nM). Shown in the upper graph is cell viability (SRB) in the presence of BafA1 (*P < 0.05, ***P < 0.001, at 12 and 24 h post-irradiation respectively) whereas in the lower graph, metabolic viability in the presence of BafA1 is shown (**P < 0.01, at 12 and 24 h post-irradiation respectively). (c) The clonogenic assay was performed to study the cell survival in the presence of 3-MA and BafA1. The bar graph shows the survival fraction of cells irradiated in the presence of 3-MA (upper panel) or BafA1 (lower panel). (**P < 0.01, *P < 0.05, for 3-MA+IR vs IR and BafA1+ IR vs IR, respectively). (d) Cell survival studies in the presence of the autophagy inducer rapamycin. The clonogenic assay was performed in the presence of 25 nM rapamycin. The bar graph shows the survival percentage of cells treated with rapamycin in the absence or presence of radiation. (e) Left panel: A bar graph showing mean fluorescence intensity of propidium iodide uptake in irradiated samples in the presence of BafA1 as compared to unirradiated control cells using flow cytometry at 12 and 24 h post-IR exposure. ***P < 0.001, *P < 0.05, at 12 and 24 h post-irradiation respectively. Right panel: Western blot analysis of cell lysate obtained at 12 and 24 h post-irradiation from BafA1+ IR treated cells. Blots were probed with intrinsic apoptosis markers, cleaved PARP1 and cleaved CASP3; GAPDH was used as a loading control. (f) Effect of *Atg7* and *Ulk1* siRNA on the levels of autophagy. Cells were reverse transfected with *Atg7*- and *Ulk1*-specific siRNAs (50 nM) and incubated for 24 h. Next, cells were either starved for 3 h or exposed to radiation, harvested after 24 h and immunoblotted with specific antibodies against ATG7, ULK1 and LC3. The effect of si-*Atg7* on autophagic flux was further studied in the presence of BafA1. (g) Effect of genetic downregulation of autophagy on cell viability. Cells were reverse transfected with si-*Atg7* and *Ulk1* and incubated for 24 h. Next, cells were either starved for 3 h or kept in complete medium and exposed to 2.5 Gy radiation. After 24 h post-irradiation, SRB assay was performed to study cell viability. *P < 0.05, for IR vs si-*Atg7*, Strv.+IR vs si-*Atg7*+ IR+Strv., *P < 0.05 IR vs si-*Ulk1*+ IR and ***P < 0.001 Strv.+IR vs si-*Ulk1*+ IR+Strv. respectively. Strv., starvation.

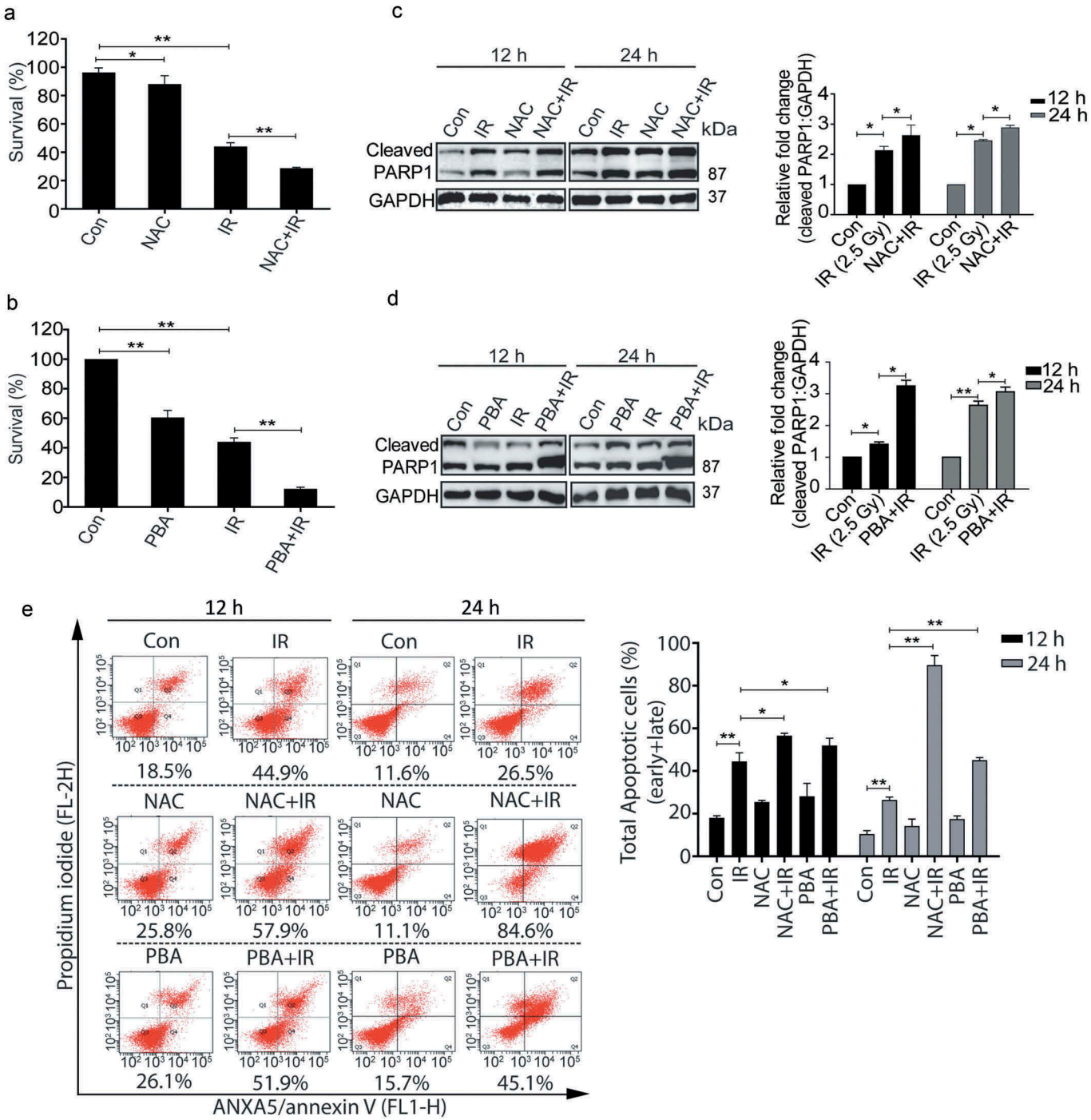


Figure 6. The pro-survival nature of radiation-induced autophagy is ROS and UPR dependent. (a) The clonogenic assay was performed after exposing cells to radiation in the presence of the ROS quencher NAC. The graph represents percent survival after irradiation compared to control. **P < 0.01, for IR and NAC-treated radiation control. (b) The clonogenic assay was performed after exposing cells to radiation in the presence of 4-PBA. The graph represents percent survival after irradiation compared to control. **P < 0.01, for IR and PBA-treated radiation control. (c) Western blot of cell lysates obtained 12 and 24 h post-irradiation from NAC alone, and NAC+IR treated cells was performed for the apoptotic marker, cleaved PARP1. Each blot is representative of 3 independent experiments. The graph shows quantitation of band intensities (relative to GAPDH) from 3 independent experiments. (d) Immunoblotting of PBA and PBA+IR treated samples at 12 and 24 h post-irradiation was performed to study apoptosis after ER stress inhibition. Each blot is representative of 3 independent experiments. The graph shows quantitation of band intensities (relative to GAPDH) from 3 independent experiments. (e) Apoptosis was analyzed using ANXA5/annexin V-PI staining followed by flow cytometry in *RAW 264.7* cells treated with ROS and UPR inhibitors, NAC and PBA respectively. Numbers under each cytogram represents total apoptosis (early+late). The same has been represented by bar graph in the right panel. The experiment was performed in triplicates, and the values are represented with SD.

and S6 respectively). The possibility of cell death induced due to the other off-target effects of these drugs can't be excluded. Taken together, these results further strengthen the notion that radiation-induced autophagy is ROS and UPR dependent. In addition, our data show that blocking either ROS or UPR may not be sufficient to reduce the radiation-induced cell death in *RAW* cells indicating autophagic induction as obligatory for cell survival in these conditions.

Autophagy activation provides a survival advantage to the irradiated animals

In order to investigate the *in-vivo* relevance of our *in-vitro* findings, we studied the effects of modulators of autophagy on the survival of whole body irradiated *C57BL/6* female mice at an absorbed dose of 8 Gy, which is the LD_{50} dose for the animals used. The MTOR inhibitor and autophagy inducer rapamycin (Rap) provided the survival advantage in radiation-exposed animals (Figure 7(a)), whereas the autophagy inhibitor chloroquine (CQ) reduced animal survival (Figure 7(b)). Changes in the body weight, as well as splenic weight, also complemented the observations on animal survival under these conditions (Table S1 and Figure S7). The induction of autophagy in these animals was confirmed at the cellular level by reduced expression of SQSTM1 in peritoneal macrophages of mice irradiated in the presence of rapamycin (Figures S7 and 7(c)). Further, we also noted reduced levels of apoptosis (cleaved CASP3) in rapamycin-treated animals (Figure 7(c), upper panel). In contrast, there was accumulation in the levels of SQSTM1 in animals irradiated in the presence of chloroquine as compared to those exposed to radiation alone. As expected, this was accompanied by an increase in the levels of cleaved CASP3 (Figure 7(c), lower panel). Taken together, these results indicate a pro-survival role of radiation-induced autophagy both *in vitro* and *in vivo*.

Discussion

In this study, we have shown that radiation induces ROS dependent autophagy in macrophages through UPR activation. Specific inhibition of EIF2AK3 and ERN1 pathways blocked autophagy, suggesting them as the key players for radiation-induced autophagy activation. Furthermore, the induction of autophagy resulted in a decline in cell death in both cellular as well as animal model system (summarised in Figure 8).

Ionizing radiation generates ROS and reactive nitrogen species (causing oxidative stress) causing macromolecular damage in the form of protein nitration, carbonylation and lipid peroxidation, besides many oxidative products of DNA [1–3,23,35,38]. Accumulation of these macromolecular lesions results in cell death, while proper recycling is essential for cell survival. It has been well established that radiation-induced ROS generation causes activation of unfolded protein response (UPR) and ER stress [5,7,24,25]. Autophagy is activated during oxidative stress as well as endoplasmic reticulum stress and may be both protective and detrimental following radiation exposure [5,14,15,21,28]. Cancer cells are known to activate pro-survival autophagy to develop resistance against chemo or radiotherapy [51]. In line with this, the radiation-induced autophagy in macrophages was found to be pro-survival in nature (MTT, SRB and clonogenicity data). Apoptosis is one of the major cell death pathways activated post-irradiation, initiated by the accumulation of various types of macromolecular as well as organelle damages caused mainly by oxidative stress [51,52]. The survival advantage provided by radiation-induced autophagy may stem from the efficient recycling of damaged mitochondria preventing CYCS (cytochrome c, somatic) release (mitophagy), or due to the degradation of pro-apoptotic protein complexes [53]. It will be interesting to understand the role of selective autophagy, e.g. mitophagy, lipophagy, ribophagy, aggrephagy etc. after radiation-induced stress conditions [54].

ROS and ER stress are associated events induced by many cytotoxic agents including ionizing radiation. During stress, unfolded proteins accumulate in the ER, leading to the activation of distinct ER stress sensors and elevation in the levels of HSPA5. Endoplasmic reticulum stress has been shown to mediate radiation-induced autophagy by EIF2AK3-EIF2S1/eIF2alpha (eukaryotic translation initiation factor 2, subunit 1 alpha) in CASP3- and CASP7-deficient MCF-7 breast cancer cells [15]. Notably, the time-dependent UPR induction (HSPA5 levels) correlated well with the secondary ROS as well as autophagy, which was attenuated by NAC, suggesting that ROS generation is an earlier event to ER stress and the radiation-induced UPR is ROS dependent. Reduction in the levels of LC3-II in the presence of 4-PBA (4-phenylbutyrate, a chemical chaperone and ER stress inhibitor) lent further support to the notion that radiation-induced autophagy is UPR dependent. Interestingly, NAC also reduced the levels of LC3-I, suggesting a possible effect of NAC on LC3 transcription and requires further investigations to understand its impact on the sustenance of autophagy. The activation of EIF2AK3 pathway is crucial for autophagic flux either through upregulation of ATG12 resulting in more LC3-II formation or through AMPK upregulation [42,55]. Results of the present study highlight the importance of EIF2AK3 and ERN1 pathways in the activation of radiation-induced autophagy. Our results are in line with the earlier studies suggesting the importance of EIF2AK3 and ERN1 during the induction of autophagy [14,15,21]. Interestingly, specific inhibition of ERN1 resulted in reduced lipidation of LC3, not only in the presence of radiation but also in control conditions indicating the importance of this pathway during basal autophagy. However, this is in contrast to the reports showing ERN1 signaling mediated impairment of autophagy flux in Huntington model [56]. The difference in the roles of the ERN1 pathway may be due to the difference in stress conditions. The early phosphorylation of EIF2AK3 (0 h sample, immediately after irradiation) and activation of both EIF2AK3, as well as ERN1 later (12 h and 24 h), are suggestive of a tight regulation of the activation of specific UPR signaling pathways in radiation-induced autophagy. The precise role of these pathways in radiation-induced autophagy needs to be explored further. The possibility of HSPA5 independent EIF2AK3 activation pathways responsible for its activation immediately after radiation may not be excluded.

Various signaling pathways are associated with autophagy induction including PtdIns3K-AKT1 and AMPK [12]. During starvation, PtdIns3K-AKT1 is unable to activate MTOR thus making ATG1 ready to initiate the autophagic process [5]. Similarly, cellular AMPK activation by a reduced ATP to AMP ratio inhibits MTOR activity and results in autophagy induction [57,58]. A fragile balance exists between autophagy and apoptosis. The anti-apoptotic protein BCL2 gets released from BECN1/Beclin 1 thus inhibiting apoptosis on autophagy induction [59]. Further, STK11/LKB1 (serine/threonine kinase 11)-AMPK may activate CDKN1B/p27Kip1 (cyclin-dependent kinase inhibitor 1B), a CDK inhibitor leading to

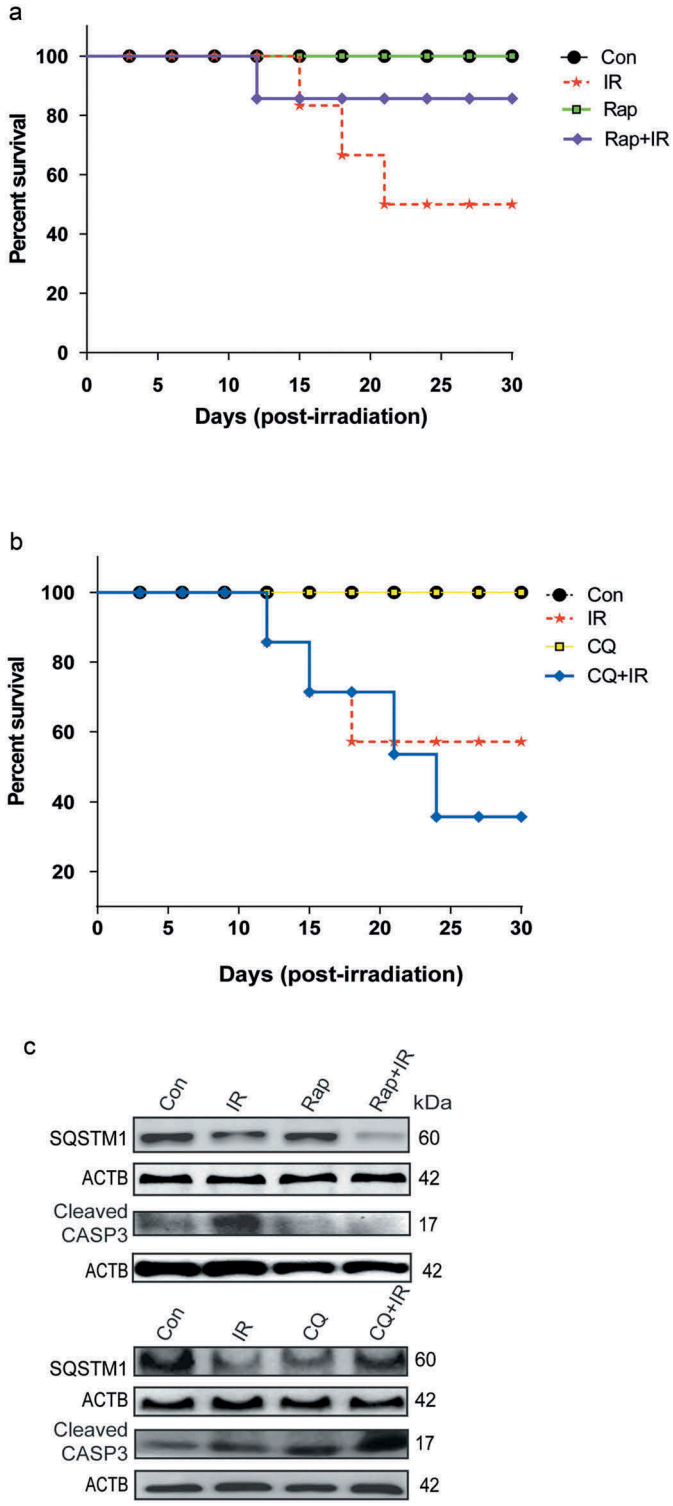


Figure 7. Radiation-induced autophagy is pro-survival under *in vivo* conditions. (a) The effects of autophagy inducer Rapamycin (2 mg/kg body weight) on survival during the first 30 d after 8 Gy irradiation in mice. *C57BL/6* mice were randomized into 4 groups: control, IR, Rap, Rap+IR. Rapamycin was administered *via* an intraperitoneal (i.p.) route in a single dose, 1 h prior to irradiation. Mice were observed for their body weight (Table S1), and lethality was scored daily for the first 30 d. Kaplan-Meier analysis was performed for mice receiving 8 Gy of total body irradiation. Each treatment group contained at least 6 animals. (b) The effect of the autophagy inhibitor chloroquine (10 mg/kg body weight) on animal survival was studied for the first 30 d after 8 Gy irradiation in mice. *C57BL/6* mice were randomized into 4 groups: control, IR, CQ alone and CQ+IR. CQ was administered *via* intraperitoneal (i.p.) injection in a single dose, 1 h prior to irradiation. Mice were observed for their body weight (Table S1), and lethality was scored daily for the first 30 d. Kaplan-Meier analysis was performed for mice receiving 8 Gy of total body irradiation. Each treatment group contained at least 6 animals. (c) Immunoblotting of isolated mice peritoneal cavity cells from rapamycin and chloroquine (CQ) treated mice was performed after day 8 of irradiation. Each mouse was given 4% thioglycolate treatment 72 h prior to peritoneal cavity cell isolation. Cells were lysed, and blots were probed with SQSTM1 (as a marker of autophagy) and cleaved CASP3 (as a marker of apoptosis). A total of 3 animals were sacrificed from each group for western blotting. Each blot is representative of 2 independent experiments.

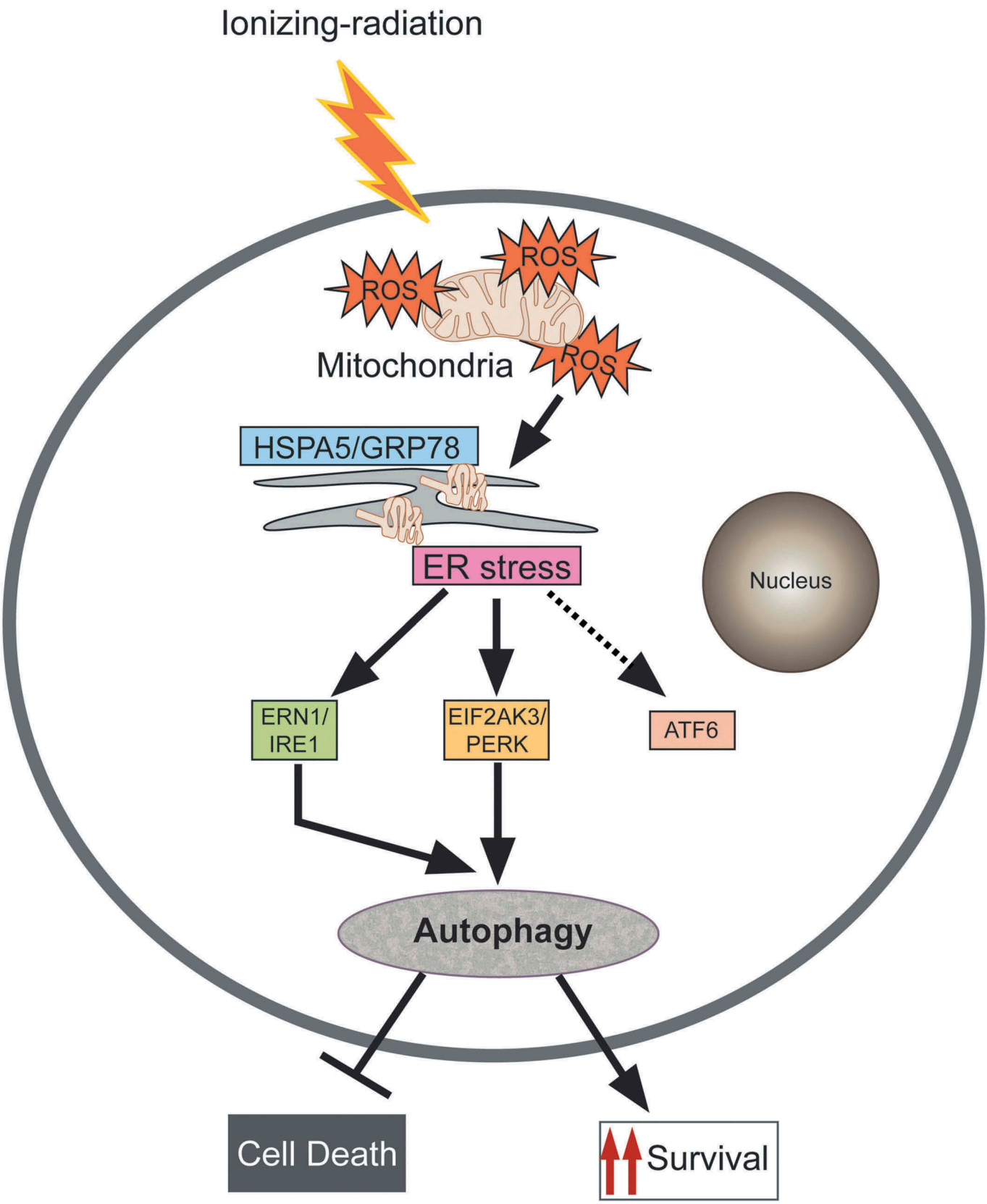


Figure 8. Proposed model for molecular signaling involved in radiation-induced autophagy. Radiation exposure results in the generation of numerous reactive oxygen species (ROS) mainly *via* mitochondrial potential disturbance. The formed ROS may cause damage to the macromolecules (primarily DNA, proteins and lipids) leading to protein misfolding and unfolding, resulting in ER stress. This stress is sensed through the UPR sensor HSPA5/GRP78 (which binds to the unfolded proteins) causing instigation of UPR through predominant activation of the EIF2AK3 and ERN1 branches of the UPR. The UPR results in the induction of autophagy in radiation-exposed conditions. This radiation-induced autophagy, which is dependent on ROS production and UPR for its induction, is a pro-survival stress response (which may be due to efficient recycling of damaged cellular cargos generated upon radiation exposure).

the cell cycle arrest, which prevents apoptosis and induces autophagy for cell survival [60]. Our study shows that autophagy counteracts necroptosis in radiation-exposed conditions. It will be interesting to explore the molecular mechanism responsible for the balance between necroptosis and autophagy under radiation stress.

Although autophagy and apoptosis have been recognized as important components of cellular responses to oxidative and other stress, the association between radiation-induced autophagy and animal survival has not been studied thoroughly [61]. Induction of autophagy (SQSTM1 clearance) with a concomitant decrease in apoptosis (reduced cleaved CASP3) in macrophages isolated from irradiated mice that were administered Rapamycin, lent support to the proposition that the induction of autophagy in critical cell components contributes to the survival of irradiated mice. It will be interesting to explore the effect of these autophagy modifiers in other cell types of the irradiated mice. Improved radioprotection after Rapamycin and enhanced radio-sensitization after chloroquine treatment indicate that autophagy is a potential target for the modification of systemic response to radiation that may be utilized for developing radiation countermeasure as well as improved tumor radiotherapy. In conclusion, our results suggest that radiation-induced autophagy is a pro-survival response initiated by oxidative stress and mediated by UPR, and emphasize that autophagy is a protection strategy deployed by the irradiated cells for survival.

Materials and methods

Cell lines and cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (American Type Culture Collection, TIB-71). The cells were maintained in high glucose DMEM medium (Sigma-Aldrich, D5648) supplemented with 10% (v:v) heat-inactivated fetal bovine serum (Gibco, 10270) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were treated with various chemicals including 3-methyladenine (Sigma-Aldrich, M9281), sulphorhodamine-B (Sigma-Aldrich, S1402), trichloroacetic acid (MP Biomedicals, 0215259290), DCFDA (Sigma-Aldrich, D6883), BafA1 (Sigma-Aldrich, B1793), 4-phenylbutyrate (Sigma-Aldrich, P21005), N-acetyl-L-cysteine (Sigma-Aldrich, A9165), GSK2606414 (Cayman chemicals, 17376) and 3,5-dibromosalicylaldehyde (Sigma-Aldrich, 122130), PBS (Himedia, TS1006). *Atg7*- and *Ulk1*-specific siRNA were purchased from Dharmacon (L-020112-00-0005). The GFP-LC3 plasmid was purchased from Addgene (21073; deposited by Tamotsu Yoshimori, Osaka University). The incubation time and concentration of agents used is stated in the figure legends.

Animals

C57BL/6 female mice (10–12 wk old) were injected with FDA approved autophagy modulators; chloroquine (a late autophagy inhibitor) (Sigma-Aldrich, C6628) or rapamycin (Sigma-Aldrich, PZ0020). Chloroquine was reconstituted in PBS and administered 10 mg/kg dose *via* an intraperitoneal (i.p.) route. Rapamycin was reconstituted in DMSO at 20 mg/ml and further diluted in PBS containing 5% DMSO to get the desired 2 mg/kg body weight dose, which was administered through an intraperitoneal (i.p.) route. The autophagy modifiers were administered 1 h prior to irradiation until otherwise mentioned. All proper controls were included in the study.

Macrophage isolation, culture and polarization

Peritoneal macrophages were attracted to the mouse peritoneal cavity by injecting 4% thioglycolate (chemoattractant; Sigma-Aldrich, B2551) solution into the cavity. After 72 h of thioglycolate stimulus, mice were euthanized, and peritoneal cavity macrophages were isolated by flushing the peritoneal cavity with PBS with the help of a 25 G needle. Peritoneal cavity cells were given one wash with PBS, and 0.2×10^6 cells per group were used to quantify the macrophage population in peritoneal fluid cells using an ADGRE1/F4/80-PE-Cy 5.5-conjugated antibody.

Western blot analysis

RAW 264.7 cells and mice peritoneal macrophages were washed with ice-cold PBS and lysed in RIPA buffer with protease and phosphatase inhibitor cocktail (Thermo Scientific/Pierce Protease Inhibitor Mini Tablets, 88665) on the ice. Cell lysates were centrifuged at 4°C (21952 g, 20 min), and the protein supernatant was transferred into new microcentrifuge tubes. The concentration of the protein samples was determined with BCA Protein Assay Kit (Thermo Scientific/Pierce, PI23227); 20 µg of the total protein was resolved using 10%, 12% or 15% SDS-PAGE, followed by protein transfer onto PVDF membranes. The membranes were blocked in PBS containing 0.1% Tween 20 (Himedia, MB067) with 3% bovine serum albumin (Sisco Research Laboratories, 85171) at room temperature for 1 h. The following primary antibodies were used; SQSTM1/p62 (Sigma-Aldrich, P0067), PARP1 (Thermo Fisher scientific/Pierce, MA5-15031), ATF6 (Santa Cruz Biotechnology, sc-22799), phospho-EIF2AK3/PERK (Santa Cruz Biotechnology, sc-32577), EIF2AK3/PERK (Cell Signaling Technology, 3192), IRE1/ERN1 (Santa Cruz Biotechnology, sc-20790), Cleaved CASP3 (Cell Signaling Technology, 9664), CASP3 (Cell Signaling Technology, 9662), ACTB (Santa Cruz Biotechnology, sc-47778), GRP78/HSPA5 (Santa Cruz Biotechnology, sc-1050), XBP1 (Santa Cruz Biotechnology, sc-7160), ATG7 (Cell Signaling Technology, 8558), ULK1 (Cell Signaling Technology, 4776) and GAPDH (Santa Cruz Biotechnology, sc-25778). For LC3 lipidation analysis, LC3A/B (Cell Signaling Technology, 4108) antibody was used in most of the experiments; otherwise Sigma-Aldrich, L7543 was used. Secondary antibodies were used from Santa Cruz Biotechnology. Detection of the blots was performed using ECL reagents (Amersham Pharmacia Biotechnology, RPN2232). Only the blots having band intensities within the linear range were included. The blots were quantified using ImageJ software.

Irradiation procedure

RAW 264.7 cells were irradiated with Tele-Cobalt Facility, Bhabhatron II (Panacea Medical Technologies, Bengaluru, Karnataka, India) at 2.5 Gy (a dose rate of 1.62 Gy/min) over an appropriate field size of 35 cm x 35 cm and at 80 SSD in the irradiation center. 3-MA (0.5 mM), BafA1 (2.5 nM), NAC (30 mM) or 4-PBA (3.5 mM) were added into culture medium 1 h before irradiation. As aqueous solutions of cysteine oxidize to cystine on contact with air at neutral or alkaline pH, NAC was prepared fresh for every experiment and pH was adjusted to 7.4. After irradiation, cells were incubated at atmospheric conditions of 5% CO_2 for the desired time points. For the 0 h time point, cells were processed immediately after radiation for the assay of interest.

For *in vivo* survival experiments, a group of at least 6 irradiated mice were followed up until 30 d in the presence of autophagy modulators. Mice in each group were exposed to whole-body radiation of 8 Gy from ^{60}Co γ-ray irradiator having a dose rate of 1.25 Gy/min. For Western blotting and other assays, 3 mice from each group were sacrificed on the third and eighth day after irradiation. All experiments were complied with the Institutional regulations on animal welfare protocols and were approved by the Institute's ethics committee of laboratory animals.

Transient transfection and microscopy

All DNA transfections were done using Lipofectamine 2000 (Invitrogen, 11668019), and cells were maintained in Opti-MEM medium (GIBCO, 31985062) devoid of serum and antibiotics. Four h post-transfection, the culture medium was replaced with fresh medium containing serum and antibiotics. Cells transfected with GFP-tagged proteins were observed at different time points, and photomicrographs were captured using a fluorescence microscope (Olympus, Center Valley, PA, USA) or confocal microscope (Zeiss LSM 710 ELYRA, Oberkochen, Germany). For siRNA transfection, cells were transfected with either *Atg7* or *Ulk1* siRNA (50 nM) using reverse transfection with RNAi max transfection reagent (Invitrogen, 13778075) in non-antibiotic 5% serum-supplemented opti-MEM media for 24 h. The next day, transfection media was replaced with high-glucose DMEM containing antibiotic and heat-inactivated serum and was processed for various assays including microscopy, western blotting or viability.

Transmission electron microscopy (TEM)

RAW 264.7 cells ($2x10^6$) were seeded in 90-mm dishes and allowed to attach overnight. Next day, cells were exposed to 2.5 Gy radiation dose. After 12 h, cells were washed twice with ice-cold PBS and fixed overnight in ice-cold Karnovsky's fixative (1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) at 4°C. Cells were then rinsed twice with ice-cold PBS, post-fixed in 1% osmium tetraoxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30–90%) and embedded in Epon (Sigma-Aldrich, 45345). Semi-thin sections (300 nm) were cut using a Reichart Ultracut (Leica Microsystems Inc., Chicago, IL, USA), stained with 0.5% toluidine blue, and examined under a light microscope. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and Reynold's lead citrate and examined using an FEI transmission electron microscope (Holland). The electron microscopy was performed at All India Institute of Medical Sciences, Delhi, India.

Analysis of ROS

DCFDA is a fluorogenic dye that measures ROS (hydroxyl, peroxyl) activity within the cells. After diffusion into the cells, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound. This compound is later oxidized by ROS into DCF, which is a highly fluorescent form.

The murine macrophage cells *RAW 264.7* were irradiated with a dose of 2.5 Gy at the indicated time point (0, 4, 12 and 24 h). Media was removed, and fresh media without serum was added. Next, 10 μM DCFDA (Sigma-Aldrich, D6883) was added to each well. The cells were incubated for 30 min at 37°C in the dark and processed using a BD FACS LSR-II flow cytometer. For 0 h reading, cells were pretreated with 10 μM DCFDA for 20 min and irradiated; immediately after irradiation, cells were washed, scraped in PBS and used for flow cytometry.

Cell viability assays

The effect of autophagy modulators (both chemical and genomic) on the metabolic viability of *RAW 264.7* cells was evaluated by the MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl-2H tetrazolium bromide] (Sigma-Aldrich, M2128) assay. Briefly, approx. 3×10^3 cells per well were seeded in 96-well microplates and were cultured for 12 h. The cells were then treated with the indicated concentrations of autophagy modulators for 12 and 24 h. Next, the medium in each well was replaced with 200 μl of fresh medium containing 0.5 mg/ml MTT. The cells were then incubated at 37°C for next 2 h, following which the medium was discarded, and 150 μl of DMSO was added to each well in order to dissolve formazan crystals. The optical density was read at 570 nm using an automated microplate reader (Bio-Tek, Winooski, USA). Experiments were carried out in triplicate, and the results are shown as mean ± SD of 3 independent experiments.

A similar protocol was followed for studying cell viability using sulphorhodamine-B (SRB) stain. After completion of indicated time points, cells were fixed in 10% (w:v) trichloroacetic acid for 45 min at 4°C followed by incubation with SRB for 30 min at 37°C. After completion of the desired incubation period, wells were washed to remove the excess stain by using 1% (v:v) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution, pH 10. The optical density was read at 510 nm using an automated microplate reader.

Growth inhibition kinetics

In order to study relative growth inhibition kinetics, 0.1×10^6 *RAW 264.7* cells were seeded in 35-mm dish in triplicates and allowed to grow at 37°C in CO_2 incubator. Next day, media

was changed, and cells were irradiated with the desired dose. Cells were processed for counting using hemocytometer. Relative cell number (N_t:N_0) was calculated with respect to the unirradiated control cells.

Colony-formation assay

Macro colony formation assay was performed to assess the effect of radiation-induced cell death in the presence of autophagy inhibitors. *RAW 264.7* cells were seeded in triplicates. Cells were treated with the drugs, 1 h prior to radiation. Media was replaced 24 h post-irradiation, and cells were incubated at 37°C to form colonies. After 10 d, colonies were washed with PBS, fixed in methanol and stained with 1% crystal violet for 10 min. Excess stain was removed with PBS. Stained colonies made up of more than 50 cells were scored and manually counted.

ANXA5/annexin V and PI staining

Apoptosis was studied using flow cytometry, 12 and 24 h post-irradiation using the ANXA5-PI staining assay kit according to the manufacturer's instructions (APOAF-Annexin V-FITC Apoptosis Detection Kit; Sigma-Aldrich, APOAF). Briefly, cells ($1x10^6$) were resuspended in 200 µl of binding buffer containing 5 µl ANXA5-FITC and 10 µl propidium iodide. After 15 min of incubation at room temperature in the dark, samples were acquired using a BD FACSCalibur flow cytometer. A minimum of 10,000 cells per sample were acquired and analyzed using BD FACS Diva software (Becton and Dickinson, San Jose, CA, USA). The percentage of ANXA5-positive and -negative cells were estimated by applying appropriate gates and using regional statistical analysis (Flow Jo software). Both early apoptotic (ANXA5-positive, PI-negative) and late apoptotic (ANXA5-positive, PI-positive) cells were considered positive.

Statistical analysis

All experiments were performed at least 3 times unless mentioned. All data are presented as mean ± S.D. of the average from triplicates unless mentioned. Statistical analysis was performed by Student's t-test (two-tailed) using the GraphPad Prism software for Windows (GraphPad Software, version 5.0, Inc., California Corporation). $P < 0.05$ was considered statistically significant.

Acknowledgments

The authors thank Dr. A. K. Singh, former Director, INMAS and the current Director, INMAS, Dr. Tarun Sekhri, for their constant encouragement. We also thank Dr.N. K. Chaudhary, Kailash Manda, Dr. Ravi Soni, Dr. Anant Narayan Bhatt and Mrs. Namita Kalra from INMAS for their technical support and valuable suggestions. We thank Carina Knudson for graphical illustrations, Mr. Anurag Singh and Dr. T.C. Das from AIIMS for electron microscopy and Prof. K. Natarajan and Ms. Aayushi Singh from ACBR, University of Delhi for Chemidoc facility and Flow cytometry sample acquisition, respectively.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Defence Research and Development Organisation (DRDO), Govt. of India [INM-311]. Ms Madhuri Chaurasia is a recipient of a fellowship from Indian Council of Medical Research (ICMR), Govt. of India.

ORCID

Madhuri Chaurasia http://orcid.org/0000-0001-8701-2255
Swapnil Gupta http://orcid.org/0000-0003-1902-7441
Asmita Das http://orcid.org/0000-0001-9846-1005
B.S. Dwarakanath http://orcid.org/0000-0001-6988-2601
Anne Simonsen http://orcid.org/0000-0003-4711-7057
Kulbhushan Sharma http://orcid.org/0000-0001-5226-4209

References

[1] Kiang JG, Garrison BR, Gorbunov NV. Radiation combined injury: DNA damage, apoptosis and autophagy. Adapt Med. 2010;2(1):1–10.
[2] Gorbunov NV, Kiang JG. Up-regulation of autophagy in small intestine Paneth cells in response to total-body gamma-irradiation. J Pathol. 2009 Oct;219(2):242–252. PubMed PMID: 19681094.
[3] Kiang JG, Smith JT, Agravante NG. Geldanamycin analog 17-DMAG inhibits iNOS and caspases in gamma-irradiated human T cells. Radiat Res. 2009 Sep;172(3):321–330. PubMed PMID: 19708781.
[4] Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene. 2003 Sep 1;22(37):5734–5754. PubMed PMID: 12947383.
[5] Chaurasia M, Bhatt AN, Das A, et al. Radiation-induced autophagy: mechanisms and consequences. Free Radic Res. 2016;50(3):273–290. PubMed PMID: 26764568.
[6] Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochim Biophys Acta. 2007 Sep;1776(1):86–107. PubMed PMID: 17693025.
[7] Farrukh MR, Nissar UA, Afnan Q, et al. Oxidative stress mediated Ca(2+) release manifests endoplasmic reticulum stress leading to unfolded protein response in UV-B irradiated human skin cells. J Dermatol Sci. 2014 Jul;75(1):24–35. PubMed PMID: 24794973.
[8] Nakai A, Yamaguchi O, Takeda T, et al. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med. 2007 May;13(5):619–624. PubMed PMID: 17450150.
[9] Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death Differ. 2009 Jul;16(7):1040–1052. PubMed PMID: 19407826.
[10] Fulda S, Gorman AM, Hori O, et al. Cellular stress responses: cell survival and cell death. Int J Cell Biol. 2010;2010:214074. PubMed PMID: 20182529; PubMed Central PMCID: PMCPMC2825543.
[11] Zhang Y, Qi H, Taylor R, et al. The role of autophagy in mitochondria maintenance: characterization of mitochondrial functions in autophagy-deficient S. cerevisiae strains. Autophagy. 2007 Jul-Aug;3(4):337–346. PubMed PMID: 17404498.
[12] Mizushima N. Autophagy: process and function. Genes Dev. 2007 Nov 15;21(22):2861–2873. PubMed PMID: 18006683.
[13] Marino G, Niso-Santano M, Baehrecke EH, et al. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol. 2014 Feb;15(2):81–94. PubMed PMID: 24401948; PubMed Central PMCID: PMCPMC3970201.

[14] Yang Z, Xu Y, Xu L, et al. Regulation of autophagy via PERK-eIF2alpha effectively relieve the radiation myelitis induced by iodine-125. PLoS One. 2013;8(11):e76819. PubMed PMID: 24223705; PubMed Central PMCID: PMCPMC3818370.
[15] Kim KW, Moretti L, Mitchell LR, et al. Endoplasmic reticulum stress mediates radiation-induced autophagy by perk-eIF2alpha in caspase-3/7-deficient cells. Oncogene. 2010 Jun 3;29 (22):3241–3251. PubMed PMID: 20348950; PubMed Central PMCID: PMCPMC2953962.
[16] Kong EY, Cheng SH, Yu KN. Induction of autophagy and interleukin 6 secretion in bystander cells: metabolic cooperation for radiation-induced rescue effect? J Radiat Res. 2018 Mar 1;59 (2):129–140. PubMed PMID: 29385614; PubMed Central PMCID: PMCPMC5951087.
[17] Wang F, Tang J, Li P, et al. Chloroquine enhances the radiosensitivity of bladder cancer cells by inhibiting autophagy and activating apoptosis. Cell Physiol Biochem. 2018;45(1):54–66. PubMed PMID: 29316551.
[18] Chen Y, Li X, Guo L, et al. Combining radiation with autophagy inhibition enhances suppression of tumor growth and angiogenesis in esophageal cancer. Mol Med Rep. 2015 Aug;12 (2):1645–1652. PubMed PMID: 25891159; PubMed Central PMCID: PMCPMC4464360.
[19] Hu JL, He GY, Lan XL, et al. Inhibition of ATG12-mediated autophagy by miR-214 enhances radiosensitivity in colorectal cancer. Oncogenesis. 2018 Feb 20;7(2):16. PubMed PMID: 29459645; PubMed Central PMCID: PMCPMC5833763.
[20] Paglin S, Hollister T, Delohery T, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res. 2001 Jan 15;61(2):439–444. PubMed PMID: 11212227.
[21] Sailaja GS, Bhoopathi P, Gorantla B, et al. The secreted protein acidic and rich in cysteine (SPARC) induces endoplasmic reticulum stress leading to autophagy-mediated apoptosis in neuroblastoma. Int J Oncol. 2013 Jan;42(1):188–196. PubMed PMID: 23123816; PubMed Central PMCID: PMCPMC3538858.
[22] Scriven P, Brown NJ, Pockley AG, et al. The unfolded protein response and cancer: a brighter future unfolding? J Mol Med (Berl). 2007 Apr;85(4):331–341. PubMed PMID: 17216204.
[23] Black HS. ROS: a step closer to elucidating their role in the etiology of light-induced skin disorders. J Invest Dermatol. 2004 Jun;122(6):xiii–xiv. PubMed PMID: 15175053.
[24] Briganti S, Picardo M. Antioxidant activity, lipid peroxidation and skin diseases. What's new. J Eur Acad Dermatol Venereol. 2003 Nov;17(6):663–669. PubMed PMID: 14761133.
[25] Ding W, Yang L, Zhang M, et al. Reactive oxygen species-mediated endoplasmic reticulum stress contributes to aldosterone-induced apoptosis in tubular epithelial cells. Biochem Biophys Res Commun. 2012 Feb 17;418(3):451–456. PubMed PMID: 22281495.
[26] Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007 Jul;8 (7):519–529. PubMed PMID: 17565364.
[27] Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal. 2007 Dec;9(12):2277–2293. PubMed PMID: 17979528.
[28] Moretti L, Cha YI, Niermann KJ, et al. Switch between apoptosis and autophagy: radiation-induced endoplasmic reticulum stress? Cell Cycle. 2007 Apr 1;6(7):793–798. PubMed PMID: 17377498.
[29] Castino R, Davies J, Beaucourt S, et al. Autophagy is a prosurvival mechanism in cells expressing an autosomal dominant familial neurohypophyseal diabetes insipidus mutant vasopressin transgene. FASEB J. 2005 Jun;19(8):1021–1023. PubMed PMID: 15781609.
[30] Alnasser HA, Guan Q, Zhang F, et al. Requirement of clusterin expression for prosurvival autophagy in hypoxic kidney tubular epithelial cells. Am J Physiol Renal Physiol. 2016 Jan 15;310(2): F160–73. PubMed PMID: 26561650.
[31] Hsieh CH, Pai PY, Hsueh HW, et al. Complete induction of autophagy is essential for cardioprotection in sepsis. Ann Surg. 2011 Jun;253(6):1190–1200. PubMed PMID: 21412148.
[32] Li J, Qin Z, Liang Z. The prosurvival role of autophagy in Resveratrol-induced cytotoxicity in human U251 glioma cells. BMC Cancer. 2009 Jun 30;9:215. PubMed PMID: 19566920; PubMed Central PMCID: PMCPMC2717978.
[33] Dalby KN, Tekedereli I, Lopez-Berestein G, et al. Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. Autophagy. 2010 Apr;6(3):322–329. PubMed PMID: 20224296; PubMed Central PMCID: PMCPMC2914492.
[34] Klionsky DJ, Abdalla FC, Abeliovich H, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2012 Apr;8(4):445–544. PubMed PMID: 22966490; PubMed Central PMCID: PMCPMC3404883.
[35] Lin JH, Walter P, Yen TS. Endoplasmic reticulum stress in disease pathogenesis. Annu Rev Pathol. 2008;3:399–425. PubMed PMID: 18039139; PubMed Central PMCID: PMCPMC3653419.
[36] Ogata M, Hino S, Saito A, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol. 2006 Dec;26(24):9220–9231. PubMed PMID: 17030611; PubMed Central PMCID: PMCPMC1698520.
[37] Yorimitsu T, Nair U, Yang Z, et al. Endoplasmic reticulum stress triggers autophagy. J Biol Chem. 2006 Oct 6;281(40):30299–30304. PubMed PMID: 16901900; PubMed Central PMCID: PMCPMC1828866.
[38] Li T, Su L, Zhong N, et al. Salinomycin induces cell death with autophagy through activation of endoplasmic reticulum stress in human cancer cells. Autophagy. 2013 Jul;9(7):1057–1068. PubMed PMID: 23670030; PubMed Central PMCID: PMCPMC3722315.
[39] Cao SS, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. Antioxid Redox Signal. 2014 Jul 20;21(3):396–413. PubMed PMID: 24702237; PubMed Central PMCID: PMCPMC4076992.
[40] Xue X, Piao JH, Nakajima A, et al. Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha. J Biol Chem. 2005 Oct 7;280(40):33917–33925. PubMed PMID: 16107336.
[41] Yen YP, Tsai KS, Chen YW, et al. Arsenic induces apoptosis in myoblasts through a reactive oxygen species-induced endoplasmic reticulum stress and mitochondrial dysfunction pathway. Arch Toxicol. 2012 Jun;86(6):923–933. PubMed PMID: 22622864.
[42] Kouroku Y, Fujita E, Tanida I, et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ. 2007 Feb;14(2):230–239. PubMed PMID: 16794605.
[43] Sun WT, Wang XC, Mak SK, et al. Activation of PERK branch of ER stress mediates homocysteine-induced BKCa channel dysfunction in coronary artery via FoxO3a-dependent regulation of atrogin-1. Oncotarget. 2017 Aug 1;8(31):51462–51477. PubMed PMID: 28881660; PubMed Central PMCID: PMCPMC5584261.
[44] Huo Y, Fan L, Yin S, et al. Involvement of unfolded protein response, p53 and Akt in modulation of porcine reproductive and respiratory syndrome virus-mediated JNK activation. Virology. 2013 Sep;444(1–2):233–240. PubMed PMID: 23850458.
[45] Volkmann K, Lucas JL, Vuga D, et al. Potent and selective inhibitors of the inositol-requiring enzyme 1 endoribonuclease. J Biol Chem. 2011 Apr 8;286(14):12743–12755. PubMed PMID: 21303903; PubMed Central PMCID: PMCPMC3069474.
[46] Komatsu M, Waguri S, Ueno T, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol. 2005 May 9;169(3):425–434. PubMed PMID: 15866887; PubMed Central PMCID: PMCPMC2171928.

[47] Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. Nature. 2004 Dec 23;432(7020):1032–1036. PubMed PMID: 15525940.

[48] Mizushima N, Noda T, Yoshimori T, et al. A protein conjugation system essential for autophagy. Nature. 1998 Sep 24;395 (6700):395–398. PubMed PMID: 9759731.

[49] Komatsu M, Wang QJ, Holstein GR, et al. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. Proc Natl Acad Sci U S A. 2007 Sep 4;104(36):14489–14494. PubMed PMID: 17726112; PubMed Central PMCID: PMCPMC1964831.

[50] Fan XY, Tian C, Wang H, et al. Activation of the AMPK-ULK1 pathway plays an important role in autophagy during prion infection. Sci Rep. 2015 Oct 1;5:14728. PubMed PMID: 26423766; PubMed Central PMCID: PMCPMC4589734.

[51] Yang ZJ, Chee CE, Huang S, et al. The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther. 2011 Sep;10 (9):1533–1541. PubMed PMID: 21878654; PubMed Central PMCID: PMCPMC3170456.

[52] Zong Y, Feng S, Cheng J, et al. Up-regulated ATF4 expression increases cell sensitivity to apoptosis in response to radiation. Cell Physiol Biochem. 2017;41(2):784–794. PubMed PMID: 28214891.

[53] Wang K, Klionsky DJ. Mitochondria removal by autophagy. Autophagy. 2011 Mar;7(3):297–300. PubMed PMID: 21252623; PubMed Central PMCID: PMCPMC3359476.

[54] Reggiori F, Komatsu M, Finley K, et al. Selective types of autophagy. Int J Cell Biol. 2012;2012:156272. PubMed PMID: 22956958; PubMed Central PMCID: PMCPMC3432363.

[55] Avivar-Valderas A, Bobrovnikova-Marjon E, Alan Diehl J, et al. Regulation of autophagy during ECM detachment is linked to a selective inhibition of mTORC1 by PERK. Oncogene. 2013 Oct 10;32(41):4932–4940. PubMed PMID: 23160380; PubMed Central PMCID: PMCPMC3600386.

[56] Lee H, Noh JY, Oh Y, et al. IRE1 plays an essential role in ER stress-mediated aggregation of mutant huntingtin via the inhibition of autophagy flux. Hum Mol Genet. 2012 Jan 1;21 (1):101–114. PubMed PMID: 21954231.

[57] Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003 Nov 26;115 (5):577–590. PubMed PMID: 14651849.

[58] Qin L, Wang Z, Tao L, et al. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. Autophagy. 2010 Feb;6(2):239–247. PubMed PMID: 20104019.

[59] Marquez RT, Xu L. Bcl-2:beclin1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch. Am J Cancer Res. 2012;2(2):214–221. PubMed PMID: 22485198; PubMed Central PMCID: PMCPMC3304572.

[60] Liang J, Shao SH, Xu ZX, et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol. 2007 Feb;9(2):218–224. PubMed PMID: 17237771.

[61] Stansborough RL, Bateman EH, Al-Dasooqi N, et al. Fractionated abdominal irradiation induces intestinal microvascular changes in an in vivo model of radiotherapy-induced gut toxicity. Support Care Cancer. 2017 Jun;25(6):1973–1983. PubMed PMID: 28175996.

FREE RADICAL RESEARCH, 2016
http://dx.doi.org/10.3109/10715762.2015.1129534

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

Radiation-induced autophagy: mechanisms and consequences

Madhuri Chaurasia[a,b], Anant Narayan Bhatt[a], Asmita Das[b], Bilikere S. Dwarakanath[a,c]* and Kulbhushan Sharma[a]*

[a]Division of Metabolic Cell Signaling Research, Institute of Nuclear Medicine and Allied Sciences, Delhi, India; [b]Department of Biotechnology, Delhi Technological University, Delhi, India; [c]Central Research Facility, Sri Ramachandra University, Porur, Tamil Nadu, Chennai, India

ABSTRACT

Autophagy is an evolutionary conserved, indispensable, lysosome-mediated degradation process, which helps in maintaining homeostasis during various cellular traumas. During stress, a context-dependent role of autophagy has been observed which drives the cell towards survival or death depending upon the type, time, and extent of the damage. The process of autophagy is stimulated during various cellular insults, e.g. oxidative stress, endoplasmic reticulum stress, imbalances in calcium homeostasis, and altered mitochondrial potential. Ionizing radiation causes ROS-dependent as well as ROS-independent damage in cells that involve macromolecular (mainly DNA) damage, as well as ER stress induction, both capable of inducing autophagy. This review summarizes the current understanding on the roles of oxidative stress, ER stress, DNA damage, altered mitochondrial potential, and calcium imbalance in radiation-induced autophagy as well as the merits and limitations of targeting autophagy as an approach for radioprotection and radiosensitization.

ARTICLE HISTORY
Received 2 July 2015
Revised 9 November 2015
Accepted 4 December 2015
Published online 14 January 2016

KEYWORDS
Calcium; DNA damage response; ER stress; mitophagy; oxidative stress

Introduction

Living beings are exposed to both environmental and man-made radiations (in the form of therapeutic modalities). In order to balance the detrimental effects induced on bio-molecules by high energy exposure, cells have evolved various defensive mechanisms and autophagy appears to be one of them. Autophagy is a process in which cell starts recycling its damaged constituents (including organelles and proteins) by delivering them to the lysosomes. A basal level of autophagy is maintained in a healthy cell to sustain cellular homeostasis, which gets modulated under stress conditions (like starvation, hypoxia, etc.). The process has been shown to promote cells towards survival but excessive autophagy may also lead to autophagic cell death. Various steps involved in autophagy include sequestration, transport of cargo to lysosomes, degradation, and utilization of the degraded products [1,2]. In autophagy, a unique double-membrane organelle, autophagosome is formed, which engulfs the cellular cargos (either damaged or destined to recycle). In addition to recycling of cargos, autophagy also plays other different roles including organelle and protein quality control [2]. As autophagy is involved in cell growth, survival, development, and death; its levels must be regulated properly. Ionizing radiation causes macromolecular (DNA, protein, and lipid) damage and imbalances in metabolism eliciting several intracellular responses that collectively determine the fate of the irradiated cell. This review focuses on the current understanding of mechanisms underlying radiation-induced autophagy and its association with macromolecular damage, oxidative stress, and ER stress.

Induction of autophagy

There are mainly three types of autophagy, namely macroautophagy, microautophagy, and chaperone-mediated autophagy. Among all these forms, macroautophagy is the most extensively studied [3]. In addition, specific terms have been given for the forms of autophagy found to be involved in the selective

CONTACT Kulbhushan Sharma kulsinmas@gmail.com Division of Metabolic Cell Signaling Research, Institute of Nuclear Medicine and Allied Sciences, Brig. S.K. Mazumdar Marg, Delhi 110054, India; Bilikere S. Dwarakanath dwarakanathdrbs@gmail.com Division of Metabolic Cell Signaling Research, Institute of Nuclear Medicine and Allied Sciences, Brig. S.K. Mazumdar Marg, Delhi 110054, India
*These authors contributed equally to this work.

removal of peroxisomes (peroxophagy; occurs when cells adapt to glucose metabolism), mitochondria (mitophagy), and other organelles [4,5]. Autophagy is a well-conserved process observed in various organisms including yeast as well as mammals. The proteins involved in autophagy are known as autophagy-related proteins (Atg). These include a series of proteins from Atg1 to Atg32. Under experimental conditions, autophagy initiation is marked by taking into account few parameters which include (i) LC3-II (Atg 8) to LC3-I ratio, (ii) increased levels of Atg5-Atg12 complex, (iii) increased levels of Beclin1, and (iv) decreased levels of p62 [6,7].

Various signaling pathways are associated with autophagy induction which include PI3K-Akt pathway and TORC1 and two pathways (target of rapamycin complexes 1 and 2) [6]. The TORC1 is rapamycin sensitive and gets inhibited in its presence, leading to the stimulation of autophagy. Under normal conditions, TORC1 remains active and keep a check on autophagy induction [8]. Autophagy is suppressed by the activation of TOR through PI3K Akt pathway. Activated TOR phosphorylates and thus inhibits Atg1 which ultimately results in the downregulation of autophagy [9,10]. During starvation on the contrary, Atg1 is dephosphorylated to take part in autophagosome formation. After activation, the binding affinity of Atg1 (ULK1) to Atg13 and Atg17 gets enhanced by several folds, leading to the stimulation of Atg1–Atg13–Atg17 scaffold, which further helps in the recruitment of numerous Atg proteins thus initiating the autophagosome formation [8,10–12]. AMPK (5′-AMP-activated protein kinase) has also been known to play a role in autophagy induction. During metabolic stress, reduced cellular ATP concentration is detected by AMPK. In mammals, cellular AMPK is activated by a reduced ATP to AMP ratio through the upstream molecules. Activated AMPK causes phosphorylation and activation of the Tuberous Sclerosis Complex1/2 (TSC1/2), which inhibits mTOR activity [13,14]. Further, LKB1–AMPK may also phosphorylate and activate p27 kip1, a cdk inhibitor leading to the cell cycle arrest, which prevents apoptosis and induces autophagy for cell survival [15].

One of the key components required for autophagy induction is a specific complex called class III phosphatidylinositol 3-kinase (PtdIns3K) complex. This complex composed of PtdIns3K, Vps34 (vacuolar protein sorting 34), Vps15 in myristoylated form (p150), Atg14 (Barkor), and Atg6/Vps30 (Beclin 1 in mammals) is required for the assembly and initiation of phagophore membrane [10,16,17]. PtdIns3K complex along with Atg proteins further recruits two ubiquitin-like conjugation complexes, Atg12–Atg5–Atg16 and Atg8-PE. Initially Atg12 is activated by Atg7, which is then transferred to Atg10 and finally covalently attaches with Atg5 protein. The Atg12–Atg5 further interacts with Atg16 to form Atg12–Atg5–Atg16 complex which finally gets attached to the phagophore [10]. Ultimately Atg8 in non-lipidated form (LC3-I) attaches to Atg12–Atg5–Atg16 complex and gets converted into its lipidated form (LC3-II), leading to the elongation of autophagosome. After complete autophagosome formation, its outer membrane fuses with the lysosome to form autophagolysosome, where lysosomal hydrolases degrade destined cargos. Earlier studies have revealed that part of the ATG8 gets degraded during the autophagic process which is responsible for higher Atg8 level in autophagosomes as compared with autolysosomes (highly acidic) [3,18]. Relatively weaker fluorescence signal of GFP-tagged LC3 in autophagolysosomes in comparison with the autophagosomes lends further support to the Atg8 degradation [3]. In mammalian cells, the autophagosome and lysosome fusion requires a lysosomal membrane protein namely LAMP-2 and a small GTPase Rab7 [19,20]. Processes involved in the induction of autophagy following exposure of cells to stress have been briefly summarized in Figure 1.

Deregulated autophagy has been associated with various human pathophysiological conditions such as cancer, myopathies, neurodegeneration, heart, liver, and gastrointestinal disorders [21–23]. In most of these pathogenic conditions, autophagy has been shown to play a provocative role as indicated by the presence of mutated autophagy-related genes like *Beclin1*, *PARKIN*, and *PINK1* in various cancers and neurodegenerative disorders [21,22]. Literature suggests a close association between cancer development and autophagy. Malignant cells with enhanced autophagy appear to be highly resistant to a variety of stress and chemotherapy in comparison with their normal counterparts. Thus, a combination of chemotherapy with inhibitors of autophagy has been suggested to be a better strategy in these cases [24,25]. In contrast, elevated levels of autophagy have also been shown to promote cell death through apoptosis [7,26]. Thus, in these cases, autophagy acts as a barrier in cancer induction as well as progression.

Autophagy versus proteasomal machinery

Autophagy and ubiquitin proteasomal system (UPS) are the two major machineries involved in the removal of misfolded or unfolded proteins and their aggregates. Due to its additional involvement in the recycling of damaged organelles, autophagic machinery is considered relatively more advanced in cargo recycling as compared with proteasomal machinery. Ubiquitination is the common step between proteasomal and autophagic degradation pathway. During this step, the

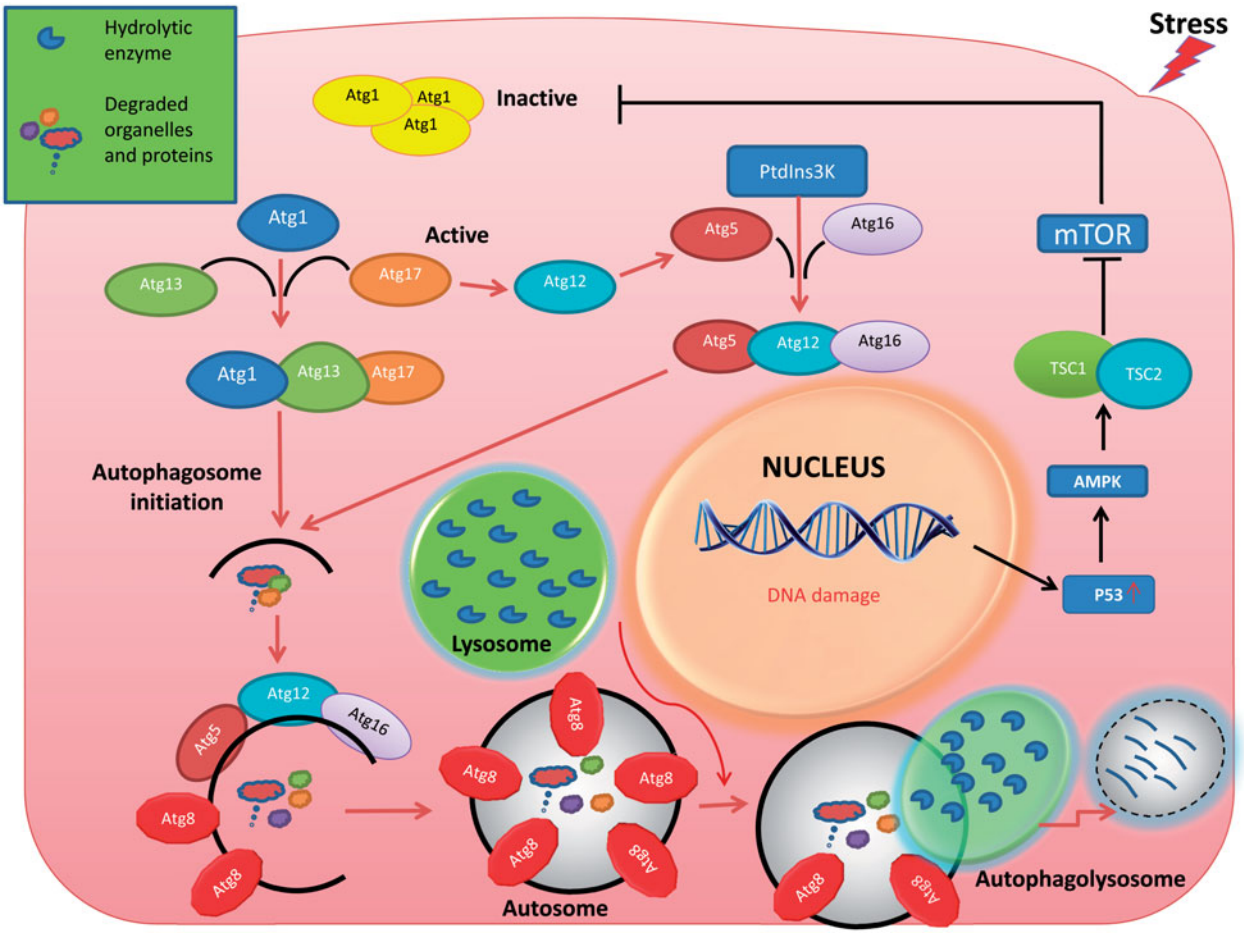


Figure 1. Schematic mechanism of autophagy induction. Autophagy is induced by a variety of stress stimuli (like for example the DNA damage in this representation) and involves a sequence of events comprising sequestration and degradation of damaged cytosolic cargos. The autophagic signaling starts with the inhibition of Akt/mTOR pathway, leading to the activation of Atg1, which in turn makes a complex with Atg13 and Atg17. In parallel, other autophagy-related molecules form another complex via PI3k class3 complex and are recruited over a double-membrane structure to form autophagosome, which ultimately fuses with lysosome and leads to the degradation of damaged cargos.

ubiquitin moiety gets covalently attached with the protein to be recycled. The enzymatic cascade involved in ubiquitination is termed E1 (activation), E2 (conjugation), and E3 (ligation) [27]. Based on the conjugation of ubiquitin moiety, ubiquitination can be mono, bi, and polyubiquitination. There are at least seven lysine residues on which ubiquitination can take place; these include K6, K11, K27, K29, K33, K48, and K63 [28].

The decision for the mode of degradation of a misfolded or damaged protein depends on several factors. One of these factors is the position of lysine residue to be ubiquitinated. For instance, K48 ubiquitin chains are considered as the classical signal to target proteins for proteasomal degradation [27]. On one hand, non-classical linkage type such as K63 ubiquitination signals for autophagic pathway [29,30]. During autophagy, p62 and PARKIN are the two important E3 ubiquitin ligases which help in the removal of aggregated proteins. Of these ubiquitin ligases, p62 has been found to be involved in K63-linked ubiquitination followed by the removal of cargos *via* both macro as well as specific autophagy. On the other hand, PARKIN can form K48-linked ubiquitin chains and cause removal of damaged protein via proteasomal machinery [31]. Conversely, when PARKIN forms K63-linked polyubiquitin chains on misfolded protein, it leads to the recruitment of ubiquitinated protein into aggresome finally helping in the removal of proteins via autophagic machinery [32,33]. Similarly, if the protein refolding is not successful, E3 ubiquitin ligase co-chaperone carboxyl terminus of heat-shock cognate70 (HSC70)-interacting protein (CHIP) may cause protein ubiquitination thereby selecting unfolded proteins for degradation preferentially through the proteasomal system. However, when chaperone-mediated refolding and proteasomal system is overloaded, protein aggregation ensues; thus formed

protein aggregates under UPS burdened condition are destined to autophagic machinery for removal [34,35]. The possibility of coexistence of UPS and autophagy cannot be ruled out.

The autophagic targeting of protein aggregates is determined by the ''LC3 Interacting Region'' (LIR) motif of p62 and NBR1. Co-chaperones such as BCL-2-associated athanogene 1 (Bag1) and Bag3 also play crucial regulatory role in determining protein degradation pathway. Bag1 helps in the removal of ubiquitinated proteins via UPS, whereas Bag3 helps in the autophagic removal of degraded proteins [36]. In young cells, Bag1 co-chaperone expression is relatively higher as compared with Bag3, whereas in aged cells, protein aggregation gets enhanced leading to enhanced Bag3 expression. In fact, Bag1/Bag3 ratio plays key role in determining the predominant pathway for the removal of misfolded protein [35,36].

The decision for removal of the complete organelles (mitochondria, peroxisomes, etc.) via proteasomal or autophagic machinery is also signaled through ubiquitinated proteins present over these organelles. For instance, degradation of damaged mitochondria can take place either through removal of misfolded mitochondrial proteins or via complete and specific removal of mitochondria (mitophagy). Interestingly, in both conditions, misfolded proteins serve as the main initiating signals.

Radiation-induced autophagy

There are a variety of radiations to which the mankind is exposed. The man made radiation includes clinical exposures as well as radiation incidents and accidents. Radiation exposure results in the damage of exposed organs and cells, leading to both acute radiation syndrome and delayed effects. After exposure, three different types of acute radiation syndromes may arise in a dose-dependent manner namely hematopoietic (HI), gastrointestinal (GI), and central nervous system (CNS) syndrome, besides the cutaneous syndrome (skin damage) independent of these three syndromes. Hematopoietic, gastrointestinal, skin, and vascular endothelium are among the most radio-sensitive organs [37–39]. Doses in the range of 1–7 Gy results in hematopoietic syndrome in humans, which is associated with overall decline in blood cells, increased susceptibility of radiation exposed persons to several infections and haemorrhage. GI syndrome occurs after a whole body exposure of more than 8 Gy [40,41].

Cellular effects caused by IR exposure include death, mutation, and transformation that arise from oxidative damage to macromolecules (DNA, protein, and lipids), alterations in cell and nuclear membrane permeability, chromosome aberrations, and metabolic imbalances. At the systemic level, decrease in lymphocytes, macrophages, neutrophils, stem cells, and disturbance in tissue integrity takes place finally leading to multiple organ failure, resulting in mortality, and morbidity depending on the level of exposure. A number of intracellular events are initiated/activated including generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), activation of p53/Bax pathway, increase in DNA double-strand breaks (DSB), single-strand breaks (SSB), and activation of different signaling pathways involved in apoptosis, growth, and autophagic induction [42–45]. Among the key molecules activated during radiation exposure, inducible nitric oxide synthase gene (iNOS) and nitric oxide (NO) have been shown to be involved in radiation induced apoptosis and autophagy [43,44]. As iNOS gene promotor region contains motifs of many transcription factors such as nuclear factor κB (NF-κB) and kruppel like factor 6 (KLF6), it results in increased NO production that causes caspase-mediated apoptosis and protein nitration-mediated autophagic induction [43].

Radiation-induced oxidative stress can cause compromised mitochondrial functioning, protein misfolding and ER stress, besides DNA damage. Most of these factors have been shown to induce autophagy [46–50]. However, detailed mechanisms underlying the induction of autophagy after radiation exposure have not been completely elucidated. Formation of acidic vacuoles has been found with increasing doses of radiation indicating an increased autophagic activity within these cells [51]. Autophagy regulation during various stress conditions, e.g., hypoxia, nutrient starvation, or ionizing radiation has also been linked to various micro-RNAs. More recent studies suggest the role of miR-199a-5p in autophagic regulation following irradiation [52]. Interestingly, autophagy has been reported to control miRNA biogenesis and activity, suggesting a feedback loop between miRNAs and autophagy [53]. Over expression of this miRNA has been shown to suppress radiation-induced autophagy in MCF7 breast cancer cell line [54,55].

Despite concerted efforts over the last few decades, the exact role of autophagy in cellular radiation response has remained controversial. Two schools of thought exist: one suggests that it is a cell survival phenomenon while the other nurtures the notion that autophagy is a type II-programmed cell death helping in the removal of affected cells. Current understanding suggests that the type, extent, and time of stress are important determinants of the fate of a cell following autophagy induction [56–60].

Mitochondrial association of autophagy

Mitochondrion is the energy currency for a cell and is indispensable for critical metabolic functions. The damaged, dysfunctional mitochondria have been linked with a series of patho-physiological conditions and neurodegenerative diseases [21–23]. In addition to canonical autophagy, other similar processes which are involved in the removal of specific damaged organelles do exist. Mitophagy (specific removal of mitochondria) is one of them [61]. Mitophagy is an important process involved in the development of reticulocytes to mature erythrocytes [62]. Mitochondrial oxidative phosphorylation leads to the generation of toxic by-products involving ROS particularly superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) which cause oxidative damage to mitochondrial lipids, DNA, and proteins, making mitochondria further prone to the production of excessive ROS. The damaged mitochondria, in turn, release huge amount of calcium ions (Ca^{2+}) and cytochrome-c to the cytosol and thereby trigger apoptosis [63,64].

Although the consequences of mitophagy and detailed pathways have been poorly understood, accumulating evidences reveal that there are three major pathways by which mitochondrial quality control can be regulated. The first two are mitochondrial proteolytic systems. In the first one, AAA (ATP associated with diverse cellular activities) protease complexes present in the inner mitochondrial membrane degrade misfolded membrane proteins; while in the other pathway, vesicular transport of degraded mitochondrial protein for removal to lysosomes takes place [65]. The third pathway known as mitophagy involves sequestration of damaged mitochondrion within a double-membrane vesicle, the autophagosome, followed by fusion with a lysosome [61,65].

There are two major pathways that results in the induction of mitophagy. One of these depends on the interaction between PTEN-induced putative kinase 1 (PINK1), a mitochondria specific kinase, and PARKIN, an E3-ubiquitin ligase (Figure 2) [65]. Under normal conditions, PINK1 binds with the mitochondrial outer membrane and gets translocated to inner mitochondrial membrane where PARL (presenselin associated, rhomboid-like) protease causes its proteolytic degradation [67]. Under reduced mitochondrial potential, PINK1 accumulates over mitochondria where it interacts with PARKIN and causes its phosphorylation [68]. Activated

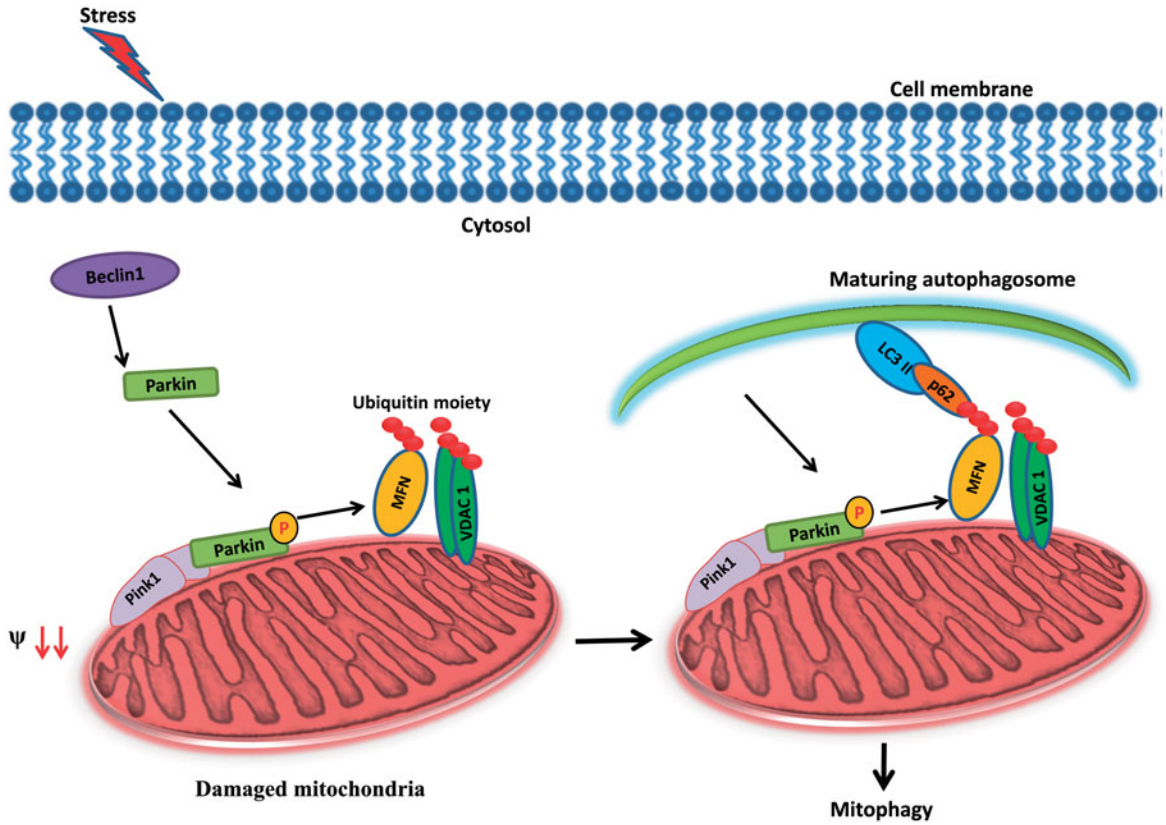


Figure 2. Mechanism of mitophagy induction. Radiation-induced decrease in the mitochondrial membrane potential causes induction of mitophagic cascade by the accumulation of PINK1. PINK1 helps in translocation of PARKIN to the damaged mitochondrial surface, where activated PARKIN adds ubiquitin moiety to mitochondrial fusion proteins MFN. This prevents the mitochondrial fusion and initiates the mitophagic process via engulfment of damaged mitochondria by the formation of autophagosomal membrane around it.

PARKIN causes ubiquitination of mitochondrial proteins. These ubiquitinated mitochondrial surface proteins act as a landing platform for p62/SQSTM1 which finally forms a functional link between ubiquitinated proteins, including MFN1/2 (Mitofusin1/2) and LC3, leading to the initiation of autophagosome with the help of Atg32. Additionally, the outer mitochondrial membrane voltage-dependent anion channel (VDAC) acts as a signal for the removal of damaged mitochondria [69]. Other pathways of mitophagy induction are independent of PINK1-PARKIN and are mainly mediated via ER-associated E3 ubiquitin ligase GP78 (glycoprotein 78) and NIX/BNIP3L in a context-dependent manner [61,66,70].

It is well established that radiation exposure leads to extensive mitochondrial biogenesis providing additional advantage for the cell survival [71,72]. However, under conditions of extensive mitochondrial damage, the cell adapts mitophagy in order to exterminate the damaged and dysfunctional mitochondria. In this way, mitophagy results in cell survival after radiation injury. As discussed above, ionizing radiation (IR) can generate excessive ROS/RNS leading to DNA damage and genomic instability [73]. Most of these IR-induced ROS/RNS is largely produced in the mitochondria [72,73]. Mitochondria are known to play an important role in radiation-induced cellular response, but the underlying mechanisms by which cytoplasmic stimuli modulate mitochondrial dynamics and functions are largely unknown. Numerous studies have pointed out the effect of radiation on mitochondrial dysfunction. Targeted cytoplasmic irradiation has been shown to cause mitochondrial fragmentation and a reduction in cytochrome-c oxidase followed by succinate dehydrogenase activity and a diminished respiratory chain function [74]. Gamma-rays also induce a p53-independent mitochondrial biogenesis in human colorectal carcinoma cells [71]. This radiation induced mitochondrial dysfunction and biogenesis has been shown to be associated with mitophagy induction [75]. Photo-irradiation of individual mitochondria from primary hepatocytes causes altered mitochondrial potential, inner membrane permeablization, excessive ROS generation, and mitophagy induction in a dose-dependent manner and phosphatidylinositol 3-kinase-independent manner [75].

Mitophagy shows a strong correlation with metabolic reprogramming in irradiated cancer cells [76]. A number of glycolytic regulatory genes along with mitophagy-specific markers are up-regulated in irradiated cells thus providing them a survival advantage [76]. Also, a reverse-Warburgian phenomenon has been recently proposed for the stromal cells present in the micro milieu of cancer cells that fulfill the energy requirement of cancer cells through metabolic modification and enhanced mitophagy [61]. While the role of mitophagy in neurodegenerative disorders, cardiac dysfunction, and lung fibrosis has been extensively studied, its role in radiation response has received much less attention. It would be interesting to investigate the activation of mitophagy following irradiation and examine its link with the ER stress, DNA damage response, and Ca^{2+} signaling, particularly in tumors.

ER stress and radiation-induced autophagy

The endoplasmic reticulum is a crucial intracellular Ca^{2+} reservoir which provides executive machinery for numerous cellular processes including translation, post-translational modification, and proper folding. ER is also involved in the initiation of several pathways of the vesicular movement of membrane and proteins to various organelles as well as the cell surface. It has been well established that ROS generated following stress conditions including radiation exposure causes indirect macromolecular damage to DNA, proteins, lipids, etc. [77,78]. In response to excessive unfolding of proteins (due to damage caused by radiation-induced ROS), a process collectively known as unfolded protein response (UPR) gets induced in ER [79]. Additionally, it also elicits an activation signal to boost the cytosolic calcium load released from ER (a store house of Ca^{2+}) [47]. ROS generation thus causes activation of ER stress which is mediated by UPR response [80,81]. UPR in-turn has been shown to have a strong correlation with autophagy [25]. These couplings indicate a possible association between ROS, ER stress, $[Ca^{2+}]i$, and autophagy.

Accumulating evidences suggest a strong association between ER stress and autophagy in various organisms including yeast and mammals [25]. In healthy conditions, Grp78 (the main marker of UPR activation) remains bound with IRE1, PERK (eukaryotic translation initiation factor 2-alpha kinase 3), and ATF6 (activating transcription factor 6) present over the endoplasmic reticulum membrane. In contrast, affinity of Grp78 for unfolded proteins increases several folds during UPR. It dissociates from its ER-sensing transducers and binds to unfolded proteins in the ER lumen leading to the activation of all three distinct ER stress sensors. PERK and IRE1 get activated by phosphorylation whereas ATF6 gets activated by its fragmentation and translocation from ER to golgi and finally to the nucleus. These three sensors initiate transcription of different target genes (Figure 3). One of the target genes is c-Jun N-terminal kinase (JNK) which is essential for lipid conjugation of LC3 and may act as a connecting link between ER stress and autophagy induction [82]. All these events prime the activation of ER stress. The specific ER stress markers like

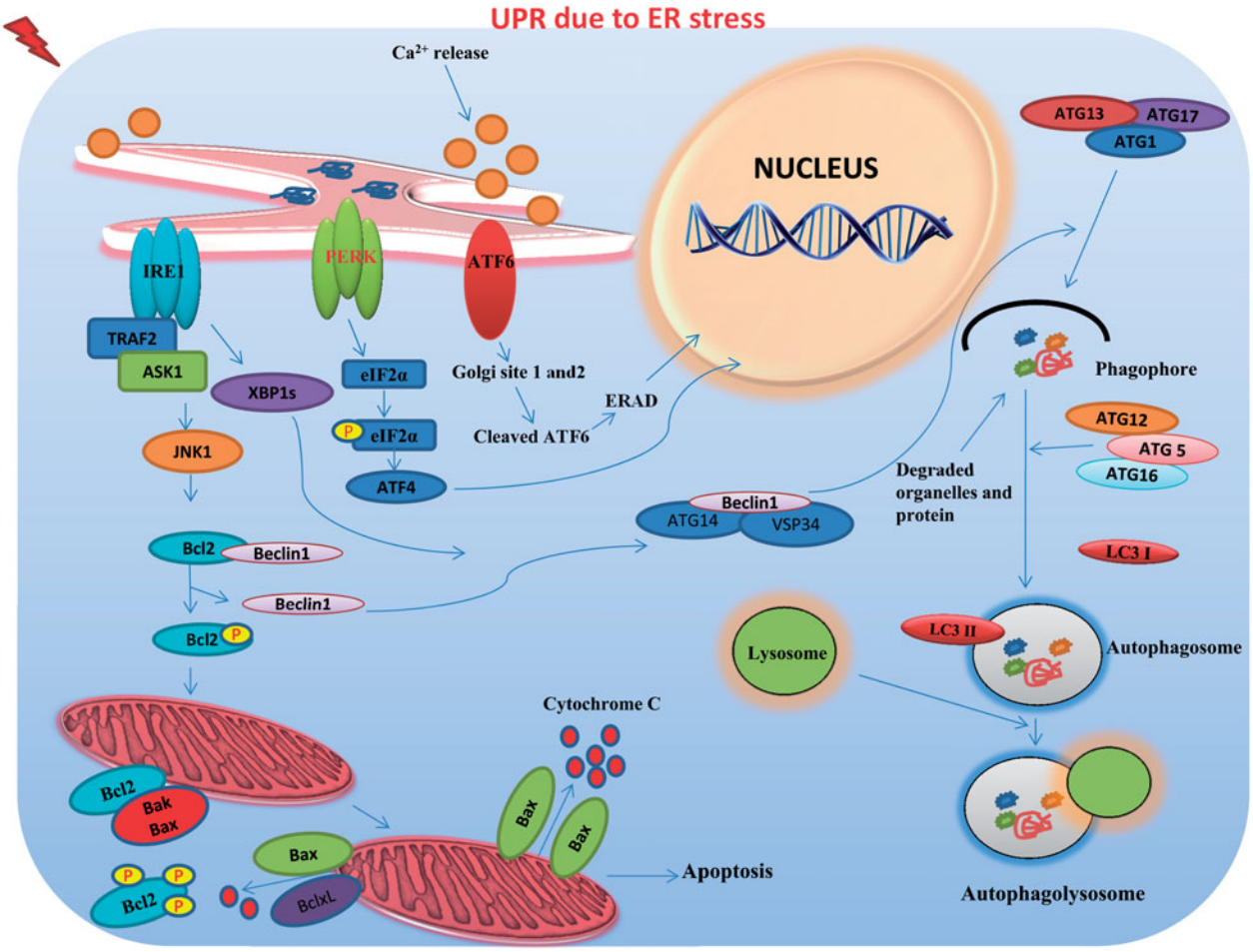


Figure 3. Schematic diagram showing signaling activated during UPR to promote ER stress, autophagy, and apoptosis induction in a stressed cell. During ER stress, GRP78 causes release of ER stress associated sensors mainly IRE1α, PERK, and ATF6 to get oligomerize over ER membrane. IRE1α causes cleavage of XBP1 to a spliced XBP1 which upon translation, upregulates other unfolded protein response associated genes. It also causes activation of JNK1, which further causes phosphorylation of Bcl2, thus leading to breakdown of Bcl2-Beclin1 complex. Beclin1 in-turn starts an anti-apoptotic cascade which induces autophagy through the formation of Vps34 complexes. On the other hand, Bcl2 goes and binds over stressed mitochondria to prevent binding of Bax. Also, ER stress sensor PERK causes phosphorylation of eIF2α, leading to global shutdown of protein synthesis except production of autophagy-related proteins thereby inducing the autophagic machinery within the stressed cells.

ERM1, XBP1, eIF2α, ATF4, DDIT3, or Chop II have been found to be up-regulated during UPR. Studies also suggest that in response to ER stress, the phosphorylation of eIF2α (eukaryotic initiation factor 2α) by PERK (an eIF2α kinase) causes global shutdown of protein synthesis except Atg4 which is required for mediating LC3I to LC3II conversion thus ultimately leading to autophagy induction [79,83–85].

ER stress has emerged as a novel traumatic condition to the cells which is involved in induction of autophagy by negatively regulating the levels of AKT/TSC/mTOR pathway [24]. Numerous ER stress inducers like tunicamycin (inhibitor of N-linked gycosylation), DTT (causes intervention in disulphide bond formation), MG132 (intrusion in proteasome function), cisplatin, thapsigargin (inhibitor of the sarcoplasmic calcium ATPase, namely SERCA2), etc. are proposed to induce autophagy. ER stress-induced autophagy has been predominantly shown to have a prosurvival role; but in parallel, there are studies suggesting that excessive ER stress may cause autophagic cell death followed by apoptosis [11,24,25]. Although it is known that ER stress is one of the autophagy-inducing pathways but the exact mechanism is still under elucidation. Moreover, only few reports exist on radiation exposure-induced autophagy through ER stress.

Exposure of cells to ionizing radiation causes oxidative stress which in turn may initiate unfolded protein response. Available evidences suggest an association between radiation exposure and ER stress, which finally results in the commencement of efficient autophagic machinery in the exposed cells [86,87]. Although the

main signaling pathway of ER stress getting activated following irradiation is still a debatable one; some recent evidences suggest that PERK-eIF2α and/or IRE1α may serve as the main executing pathways of ER stress in irradiated scenarios [87,88]. Furthermore, recent studies have also indicated the importance of further downstream molecules (i.e. eIF2α/ATF4) of PERK-mediated UPR pathway in irradiated endothelial cells (HUVEC and HCAEC). Significant alterations have not been observed in IRE1 and ATF6 branches in these cells [89]. Unpublished data from our lab also suggest predominant activation of PERK and IRE1 pathway in radiation-exposed conditions. Treatment of spinal metastasis with Iodine-125 has been shown to activate ER stress through the activation of PERK-eIF2α which finally causes induction of autophagy [87]. In line with this, IR-induced ER stress has also shown autophagy induction in a dose-dependent manner in the blood samples of human cancer patients [90].

The autophagic process induced in response to ER stress is found to be involved in providing survival advantage to the cells. However, if the exposure burden is too large to handle, the same PERK-eIF2α pathway can activate several cell death pathways like apoptosis and necrosis for the removal of damaged cells [87]. Further studies are required to understand the relationship between radiation-induced ER stress and autophagy.

Role of calcium signaling in radiation-induced autophagy

Intracellular calcium is distributed between several sub-domains like ER lumen and mitochondria. During stress conditions, the sub-cellular distribution of unbound $[Ca^{2+}]i$ gets altered and it gets released into the cytoplasm, promoting either cell proliferation or cell death [91,92]. Altered $[Ca^{2+}]i$ is indeed shown to regulate autophagy, mainly macroautophagy [93,94]. At a first glance, evidences in the literature seem mystifying and suggest that elevations of $[Ca^{2+}]i$ can both activate and inhibit autophagy [93,95]. However, emerging evidences and in-depth analysis suggest that the distribution of $[Ca^{2+}]i$ in different sub-domains and extent of Ca^{2+} release from the ER lumen leads to the activation of different signaling pathways causing either activation or inhibition of autophagy.

Evidences showing increased $[Ca^{2+}]i$ as an activator of autophagy have mainly used stress-inducing agents like anti-cancer drugs, radiation, photodynamic therapy (PDT), Ca^{2+} ionophore and SERCA inhibitor, thapsigargin, etc., which mobilize Ca^{2+} from one sub-domain to the other and also lead to elevated $[Ca^{2+}]i$ [96–98]. However, Ca^{2+} chelators viz. BAPTA-AM inhibits the induction of autophagy, confirming the involvement of cytosolic Ca^{2+} [70,98–100]. Autophagy induced by starvation and inhibition of mTOR using rapamycin is also reversed by BAPTA-AM, suggesting the indirect role of Ca^{2+} signaling in starvation-induced autophagy [100]. Autophagy induced by extracellular calcium is countered by extracellular and intracellular buffering, suggesting that ER is probably the main, but not the only source of free Ca^{2+} during Ca^{2+} -induced autophagy [101].

It is well established that stress induced elevated cytoplasmic Ca^{2+} influx, originating either from ER or extracellular environment is first buffered by mitochondria [102]. However, sustained elevation exceeding the buffering capacity of the mitochondria leads to accumulation in the cytoplasm, suppressing the mTOR activity in a CaMKK- and AMPK-dependent manner [92,93,98,102–104]. The Ca^{2+} overloaded mitochondria also become non-functional, which increase AMP/ATP ratio and activation of AMPK signaling [93]. Moreover, the elevated $[Ca^{2+}]i$ also activates calmodulin-dependent DAPK which phosphorylates Beclin1, thereby promoting its dissociation from Bcl-2 leading to the induction of autophagy [105]. Besides activation of these signaling cascades, the excess Ca^{2+} overloading into mitochondria also irreversibly damages them by precipitating all the inorganic phosphates (Pi) in to $Ca_3(PO_4)_2$ (calcium phosphate, insoluble) [92,103]. These damaged mitochondria get cleared from cells by mitophagy or macroautopahgy [76,103]. TRPML3, a Ca^{2+}-permeable channel, recruited to autophagosomes, has been shown to be important in autophagy [106]. Overexpression of TRPML3 positively correlates with induction of autophagy [93]. Further the role of Ca^{2+} signaling is not only limited to the induction of autophagy, but may be important for its progression as well.

Low levels of Ca^{2+} oscillations for shorter period of time have been suggested to inhibit autophagy that appears to act through the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R, three isoforms), a ubiquitously expressed intracellular Ca^{2+} release channel, located mainly in the ER [93,107–111]. IP3R forms channel in the mitochondria with the help of Beclin 1 and Bcl-2 thus, decreasing the level of free Beclin 1 to induce autophagy [111]. Therefore, the absence of IP3Rs would lead to the dissociation of Beclin 1 from Bcl-2, followed by autophagy stimulation. This hypothesis has indeed been verified with the IP3Rs triple knock out (TKO) chicken DT40 B lymphocytes, which exhibit enhanced autophagy levels [112,113]. Interestingly, the expression of other ER Ca^{2+} channels like the ryanodine receptor (RyR) does not restore the elevated autophagy levels [113], confirming the involvement of IP3Rs. Release of low levels of Ca^{2+} mainly from ER is directly buffered by mitochondria

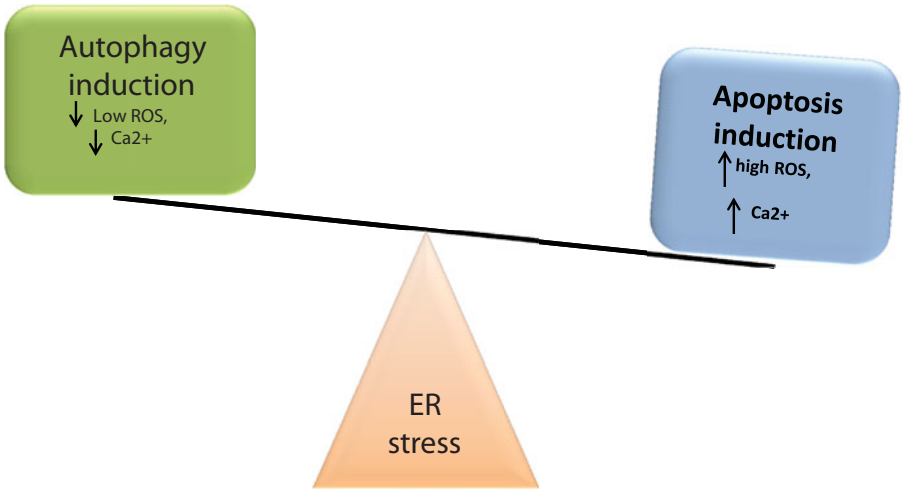


Figure 4. Triangular association among ER stress, ROS generation, and cytosolic calcium, and their role in the induction of autophagy/apoptotic cascade in a cell.

through IP3Rs channel. Low levels of Ca^{2+} accumulation in mitochondria induces majority of TCA cycle enzymes leading to enhanced production of ATP [92]. This reduces the ratio of AMP/ATP, which inhibits AMPK and, therefore, autophagy in cells [113]. There are also evidences to suggest that activation of calpain by intracellular Ca^{2+} can lead to increased IP3 production through cAMP and activate IP3R-mediated Ca^{2+} release, thereby inhibiting autophagy [114]. The anti-apoptotic Bcl-2 protein appears to be a critical regulator of Ca^{2+}-induced autophagy at the ER level as its over-expression in an ER-targeted manner is most effective in reducing Ca^{2+}-induced autophagy [98,100]. Bcl-2 inhibits autophagy by facilitating the buffering of Ca^{2+} by mitochondria through IP3Rs-Bcl-2-Beclin-1 channel, besides sequestering Beclin-1 and thus Ca^{2+} seems to regulate autophagy in both positive and negative manner depending on the degree of disturbance in Ca^{2+} homeostasis and cellular status (normal cells and stressed cells) [98,115].

Radiation-induced oxidative stress leads to ER stress thereby causing enhancement in $[Ca^{2+}]i$ apart from extracellular Ca^{2+} intake [116,117]. Keeping both radiation-induced calcium imbalance and Ca^{2+} imbalance-induced autophagy in consideration, it seems that calcium-induced autophagy must also be elicited during radiation exposed conditions and may be linked with ROS and ER stress, where increased cytosolic Ca^{2+} may cause suppression of mTOR activity in a CaMKK- and AMPK-dependent manner [93,98,104]. Moreover, the elevated $[Ca^{2+}]i$ may cause activation of calmodulin-dependent DAPK which by phosphorylation of Beclin1 helps in induction of autophagy. However, studies confirming triangular relationship between radiation exposure, autophagy and calcium imbalance are need of the hour. The possible effect of this triangular relationship on cellular fate has been depicted in Figure 4.

Radiation-induced DNA damage response and autophagy

Nucleus is an important part of a cell which contains genetic information in the form of DNA and, therefore, autophagic degradation of the entire nucleus appears to be intriguing. A novel form of nuclear-specific autophagy called nucleophagy has been recently reported wherein the elimination of damaged DNA occurs via autophagic vacuoles [118]. However, in certain multinucleated fungi and nematodes like filamentous fungi *Aspergillus oryzae* and *Caernohabiditis elegans*, nuclear DNA degradation by a highly selective form of autophagy called piecemeal microautophagy (PMN) has been reported under exceptional circumstances [119,120]. Specific removal of damaged nuclear DNA has also been reported in certain mammalian cells [121–123].

Interestingly, while autophagy is a strictly cytoplasmic process, several autophagy-related proteins, e.g., p62 are enriched in the nucleus or undergo fast nuclear-cytosolic shuttling [124,125]. Another protein, ALFY (autophagy-linked FYVE protein), has been shown to be involved in autophagy and localize predominantly in the nucleus [124,126–128]. However, following stress, ALFY is extruded from the nucleus to cytoplasm and interacts with p62 bodies in a similar manner certain nuclear proteins exit out of the nucleus for their removal via autophagic machinery. Recent reports suggest that compromised autophagy leads to delayed degradation

of damaged nuclear components (DNA, RNA, and nucleoproteins) [122]. In line with this, mutated lamins have been shown to induce deformations in the nuclear envelope that induces nucleophagy [123]. Micronuclei containing whole chromosomes or parts of the chromosomes are also suggested to be removed by autophagy; thus facilitating the maintenance of genomic stability [122]. The nature and functional significance of this nuclear sequestration of autophagy-related proteins is not clear, although the nuclear-cytosolic shuttling of Beclin1 has been shown to be important for its autophagic and tumor suppressor functions [129].

Autophagy appears to play a crucial role in regulating cellular fate following the induction of DNA damage [118,130]. For instance, in cells with DNA damage and defective in apoptosis, autophagy facilitates cell death; thereby acting as a tumor suppressor [26]. In line with this, the suppression of ULK1-interacting protein FIP200 has been reported to impair DDR, thus triggering cell death upon ionizing radiation-induced oxidative stress [131]. Collectively, these circumstantial evidences suggest direct or indirect role of autophagy in the DDR and ROS/RNS-mediated genotoxic stress. However, precise mechanisms underlying DDR-mediated autophagy are still not very clear.

Autophagy also takes care of the micronuclei as shown by a recent study where co-localization of micronuclei, autophagic vacuole with p62, and Υ-H2AX foci (a DNA damage marker) has been reported [122]. Non-autophagic micronuclei were p62-negative suggesting that the presence of DNA damage directly or indirectly signals for autophagic engulfment. Accumulating evidences suggest that radiation-induced DNA damage induces autophagy. In response to DNA double-strand breaks (generally considered lethal), two repair pathways are mainly activated. Homologous recombination (HR) which depends on sequence homology and restricted to the S and G2 phases of the cell cycle is associated with high fidelity while non-homologous end joining (NHEJ), independent of the sister chromatid is relatively error prone [132,133]. Cells deficient in autophagy have been shown to accumulate higher levels of mutated DNA suggesting deficiency in the HR repair [134,135]. The role of chaperone-mediated autophagy has been recently implicated in maintaining the genome stability. Chaperone-mediated autophagy plays an essential role in the degradation of Chk1 following exposure to DNA damaging agents (etoposide and gamma radiation). Furthermore, inactivation of chaperone-mediated autophagy under these conditions results in the accumulation of DNA damage [136].

Inhibition of NHEJ in these irradiated cells (thus lacking HR machinery and thus completely disabled in terms of DNA repair) results in enhanced apoptosis [135]. Autophagy has also been shown to influence the dynamics of DNA repair wherein it helps in recycling of key proteins involved in the processing of lesions; besides aiding the metabolic precursors for the generation of ATP as well as regulating the supply of dNTPs required for repair [137,138]. Studies carried out in yeast have shown that activation of autophagy following the induction of DSBs results in anaphase arrest, which persists till autophagy is blocked or vacuolar proteolysis is inhibited suggesting that DDR-induced autophagic process may also contribute to cytotoxicity [130,139].

Two essential proteins, p53 and ATM, serve as connecting links between radiation-induced DDR and autophagy. Following DNA damage, p53 provokes autophagy by transcriptionally inducing several genes including damage-regulated autophagy modulator (DRAM), ULK1/2, sestrin1/2, and bnip3 [140]. These genes can directly regulate autophagy, e.g., the lysosomal proteins DRAM1 and ULK1/2 interact with Atg13 and FIP200 to induce autophagy [141]. Similarly, Sestrin1 and 2 activate AMPK and the TSC1/2 complex, leading to the inactivation of mTORC1 and thus autophagy induction (Figure 5) [142]. ATM can activate autophagy by both p53 dependent and independent mechanisms. Cytosolic ATM can activate TSC2 tumor suppressor to inhibit mTORC1 through the LKB1/AMPK pathway and induce autophagy during ROS-mediated cellular damage [143]. On the other hand, nuclear ATM can initiate autophagy via AMPK by direct activation of LKB1 (the AMP kinase) [143]. Thus, these new findings integrate different stress response pathways taking place in different cellular compartments. From this perspective, ATM would be required for both initiation (nucleus) and mediation (cytosol) of DDR. Interestingly, induction of autophagy during starvation requires destruction of cytosolic p53 revealing a multifaceted role of p53 in autophagy regulation [144,145]. PolyADP-ribose polymerase 1 (PARP1) is another protein directly linking DDR and autophagy [146,147]. PARP1 is hyperactivated upon radiation-induced DNA damage that consumes NAD^+ resulting in ATP depletion. Such energetic imbalance can activate autophagy via AMPK pathway [146,147].

Autophagy shows a pleomorphic role in the context of DNA damage response. Majority of the studies indicate that autophagy inhibition in cells treated with DNA-damaging agents leads to enhanced cell death, supporting a prosurvival role for autophagy. In this scenario, transcription factors such as p53, p73, and E2F1, which not only promote DNA repair, cell cycle arrest, or apoptosis in response to different degrees of DNA damage but also control autophagy would have pivotal roles [148]. On the other hand, autophagy has

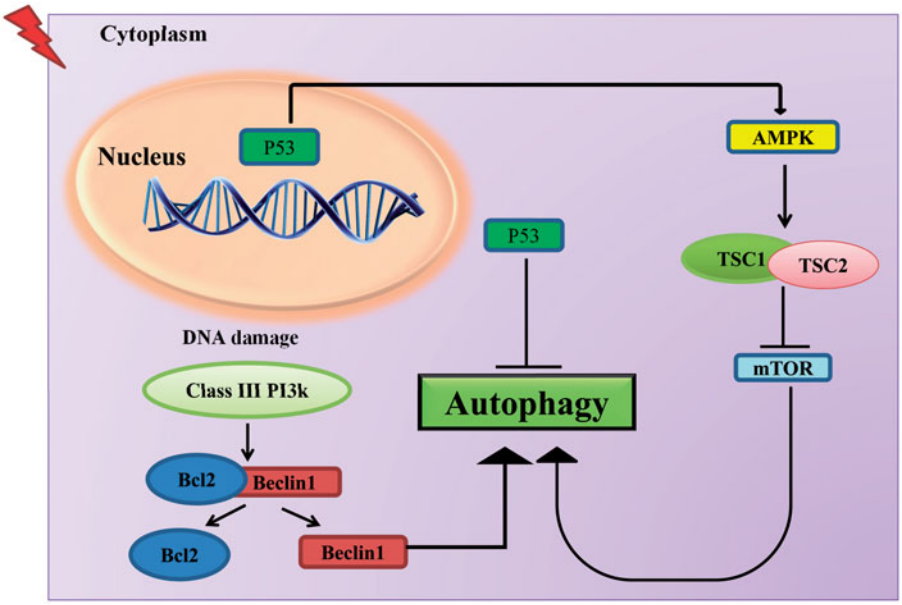


Figure 5. Schematic model illustrating the possible signaling pathways induced following irradiation and their involvement in the regulation of radiation-induced autophagy in cells.

also been shown to promote degradation of acetylated Sae2 in valproic acid-treated yeast cells in an intricate manner, wherein autophagy activation could contribute to perseverance of DNA damage and further enhancement of apoptotic signaling in mammalian cells by controlling turnover of certain DNA repair-related enzymes [137]. Taken together, these studies indicate that repair of radiation-induced DNA damage may be linked with autophagy, which may either enable the cell to overcome the radiation stress or may activate cell death in a context dependent manner.

Targeting autophagy for altering radiosensitivity

Most of the studies linking radiation with autophagy have been performed on cancer patients undergoing radiotherapy. Elevated levels of autophagy have been found to be associated with chemo as well as radio-resistance of various cancerous types [71,72,77,78,83–85]. Clinical trials combining chemotherapeutic agents with autophagy inhibitors such as chloroquine (CQ), hydroxychloroquine (HCQ), and 3-methyladenine (3-MA) provide survival benefits and increased life span in patients with breast cancer, myeloma, prostate cancer, and several other advanced tumors (Table 1) [149,150–161]. Similar finding are also reported with radiotherapy (Table 2) [56,161–164]. Reduced expression of Beclin1 protein has been associated with decreased cell survival in radio-resistant cancer cell lines exposed to low-dose irradiation during radiotherapy [165]. These studies support the role of autophagy in cell survival under radiation stress. In addition of its role in carcinogenesis, autophagy has also been reported to play a role in angiogenesis. Ionizing radiation induce contrasting effects on vascularisation by enhancing autophagic levels in cells, which in turn enhances production of pro-angiogenic factors, e.g., VEGF finally leading to enhanced radioresistance [164,166–168].

A dose-dependent correlation has been observed between radiation-induced autophagy and cell-cycle arrest [56]. A lower dose of ionizing radiation mainly induces G2/M arrest. Also, co-treatment of cells with ER stress and autophagy activators along with radiation further enhances the G2/M block extent [56,168]. This associated cell-cycle arrest plays a key role in overall radiation resistance in various cancer conditions.

In contrast to the role of autophagy in radio-resistance, there are evidences suggesting that autophagy can also promote cell death [169,170]. Various tumor suppressors have been shown to induce high levels of autophagy [73]. For instance, loss of Beclin1 gene function has been associated with various solid tumors including breast, ovarian, and prostate tumors [171–173]. Similarly, combined treatment of Akt inhibitors along with radiation has been shown to induce autophagy in numerous carcinoma conditions, thus enhancing radiosensitization of the cancer cells [169]. Molecular mechanisms through which autophagy helps tumor suppression are poorly understood. The best-determined mechanism is autophagy's ability to degrade damaged and mutated components of a cell

Table 1. Pre-clinical studies using modifiers of autophagy for enhancing the efficacy of anti-cancer therapeutics.

Cancer type	Modifiers of autophagy	Primary therapeutic	Ref.
Colorectal cancer	3-MA	5-FU	[150]
	CQ, siRNA against Beclin1	Bevacizumab, Oxaliplatin	[151]
	HCQ	HDAC inhibition	[152]
	HCQ	Temozolomide	[153]
Gastric cancer	CQ, siRNA against ATG5, Beclin1	Quercetin	[154]
Gastrointestinal stromal tumors (GISTs)	CQ, Quinacrine, siRNA against ATG7, ATG12	Imatinib	[155]
Multiple myeloma (MM)	HCQ	Bortezomib	[157]
Lymphoma	CQ, siRNA against ATG5	Tamoxifen	[170]
Prostate cancer	3-MA, CQ, siRNA against ATG7	Saracatinib	[157]
	3-MA, siRNA against ATG5	Phenethyl isothiocyanate	[158]
Skin cancer	3-MA, siRNA against ATG5	Cisplatin	[159]
Glioma	3-MA, HCQ, E64 + Pepstatin A	Cannabinoid	[160]

Table 2. Modifiers of autophagy as potential radiosensitizers in various cancers.

Cancer type	Modifier of autophagy	Type of ionizing radiation	Ref.
Breast cancer	siRNA against Beclin 1, ATG3, ATG4b, ATG5, ATG12	γ-Radiation	[161]
Glioma	3-MA, BFA, siRNA Beclin 1, ATG5	γ-Radiation	[163]
Colorectal cancer	Tunicamycin, siRNA against Beclin 1	γ-Radiation	[57]
	siRNA against Beclin 1, ATG5, ATG7, UVRAG	γ-Radiation	[162]
Lung cancer	ABT-737/rapamycin	X-Rays	[164]

which may otherwise gain oncogenic properties [174]. Metabolic inhibitors like 2-deoxy-D-glucose (2-DG) have been shown to induce autophagy under conditions of starvation like in hypoxic tumor cells [175]. Whether variations in the extent of autophagy induction is partly responsible for the heterogeneity in the response of tumor cells *in vitro* and *in vivo* to 2-DG alone and in combination with ionizing radiation or anticancer drugs needs to be investigated, so as to individualize the therapy using 2-DG as adjuvant [176–179].

Summary

Emerging knowledge suggests autophagy as one of the important recycling mechanisms for cell survival during a variety of cellular trauma including radiation exposure. The process of autophagy is an important determinant of the fate of irradiated cell which in-turn gets regulated through various pathways including altered mitochondrial membrane potential, elevated Ca^{2+} levels, DNA damage, and ER stress. Due to its pro-survival nature predominantly, autophagy is generally considered as a protection strategy deployed by the irradiated cells. Under certain conditions, autophagy appears to act as a double-edged sword as a successful execution promotes survival, while abortive autophagy promotes death by inducing apoptosis. Thus, the ultimate fate of irradiated cells appears to be dependent on a fine balance between autophagy and apoptosis. The relationship between the two appears to be determined by various factors including the nature and extent of stress, cell type, cellular micro milieu, and the post irradiation time (exposure to stress). The process of autophagy seems to have pleomorphic nature acting similar to p53, which is also involved in both cell survival (by promoting cell-cycle arrest and DNA repair following DNA damage) and cell death (by inducing apoptosis when the extent of damage is too high). Since autophagy appears to be an important determinant of the fate of an irradiated cell, modifiers of autophagy can be considered as potential radio-modifiers for use as countermeasure agents as well as adjuvant to radiotherapy of cancer.

Acknowledgements

The authors thank Dr. A. K. Singh, Director INMAS, for his constant encouragement.

Declaration of interest

The authors report that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper. Work in author's laboratories is supported by grants from DRDO, Govt. of India (INM-311). Ms Madhuri Chaurasia is a recipient of fellowship from ICMR, Govt. of India.

References

[1] Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008;132:27–42.
[2] Levine B. Cell biology: autophagy and cancer. Nature 2007;446:745–747.
[3] Mizushima N, MizushimaN, YamamotoA, MatsuiM, YoshimoriT, OhsumiY, et al. *In vivo* analysis of autophagy

in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell 2004;15:1101–1111.
[4] Nair U, Klionsky DJ. Molecular mechanisms and regulation of specific and nonspecific autophagy pathways in yeast. J Biol Chem 2005;280:41785–41788.
[5] Mao K, Wang K, Liu X, Klionsky DJ. The scaffold protein Atg11 recruits fission machinery to drive selective mitochondria degradation by autophagy. Dev Cell 2013;26:9–18.
[6] Mizushima N. Autophagy: process and function. Genes Dev 2007;21:2861–2873.
[7] Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol 2014;15:81–94.
[8] Noda T, Ohsumi Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem 1998;273:3963–3966.
[9] Cheong H, Yorimitsu T, Reggiori F, Legakis JE, Wang CW, Klionsky DJ. Atg17 regulates the magnitude of the autophagic response. Mol Biol Cell 2005;16:3438–3453.
[10] He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 2009;43:67–93.
[11] Gozuacik D, Bialik S, Raveh T, Mitou G, Shohat G, Sabanay H, et al. DAP-kinase is a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death. Cell Death Differ 2008;15:1875–1886.
[12] Kabeya Y, Kamada Y, Baba M, Takikawa H, Sasaki M, Ohsumi Y. Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. Mol Biol Cell 2005;16:2544–2553.
[13] Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003;115:577–590.
[14] Qin L, Wang Z, Tao L, Wang Y. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. Autophagy 2010;6:239–247.
[15] Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol 2007;9:218–224.
[16] Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell 2008;19:5360–5372.
[17] Kihara A, Noda T, Ishihara N, Ohsumi Y. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. J Cell Biol 2001;152:519–530.
[18] Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, et al. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol 2000;151:263–276.
[19] Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen EL. Role for Rab7 in maturation of late autophagic vacuoles. J Cell Sci 2004;117:4837–4848.
[20] Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 2000;406:902–906.
[21] Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 2004;304:1158–1160.
[22] Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, et al. Mutations in the Parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392:605–608.
[23] Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, et al. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 1999;59:59–65.
[24] Ding WX, Ni HM, Gao W, Hou YF, Melan MA, Chen X, et al. Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival. J Biol Chem 2007;282:4702–4710.
[25] Li T, Su L, Zhong N, Hao X, Zhong D, Singhal S, Liu X. Salinomycin induces cell death with autophagy through activation of endoplasmic reticulum stress in human cancer cells. Autophagy 2013;9:1057–1068.
[26] Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 2007;8:741–752.
[27] Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67:425–479.
[28] Ikeda F, Dikic I. Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO Rep 2008;9:536–542.
[29] Haglund K, Dikic I. Ubiquitylation and cell signaling. EMBO J 2005;24:3353–3359.
[30] Tan JM, Wong ES, Kirkpatrick DS, Pletnikova O, Ko HS, Tay SP, et al. Lysine63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. Hum Mol Genet 2008;17:431–439.
[31] Hattori N, Mizuno Y. Pathogenetic mechanisms of parkin in Parkinson's disease. Lancet 2004;364:722–724.
[32] Olzmann JA, Li L, Chudaev MV, Chen J, Perez FA, Palmiter RD, Chin LS. Parkin-mediated K63-linked polyubiquitination targets misfolded DJ-1 to aggresomes via binding to HDAC6. J Cell Biol 2007;178:1025–1038.
[33] Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 2008;183:795–803.
[34] Kubota H. Quality control against misfolded proteins in the cytosol: a network for cell survival. J Biochem 2009;146:609–616.
[35] Shaid S, Brandts CH, Serve H, Dikic I. Ubiquitination and selective autophagy. Cell Death Differ 2013;20:21–30.
[36] Gamerdinger M, Hajieva P, Kaya AM, Wolfrum U, Hartl FU, Behl C. Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. EMBO J 2009;28:889–901.
[37] Fliedner TM, Graessle D, Meineke V, Dorr H. Pathophysiological principles underlying the blood cell concentration responses used to assess the severity of effect after accidental whole-body radiation exposure: an essential basis for an evidence-based clinical triage. Exp Hematol 2007;35:8–16.
[38] Mac Naughton WK. Review article: new insights into the pathogenesis of radiation-induced intestinal dysfunction. Aliment Pharmacol Ther 2000;14:523–528.

[39] Meistrich ML, Kangasniemi M. Hormone treatment after irradiation stimulates recovery of rat spermatogenesis from surviving spermatogonia. J Androl 1997;18:80–87.
[40] Coleman CN, Stone HB, Moulder JE, Pellmar TC. Medicine. Modulation of radiation injury. Science 2004;304:693–694.
[41] Waselenko JK, Mac Vittie TJ, Blakely WF, Pesik N, Wiley AL, Dickerson WE, et al. Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. Ann Intern Med 2004;140:1037–1051.
[42] Kiang JG, Garrison BR, Gorbunov NV. Radiation combined injury: DNA damage, apoptosis and autophagy. Adaptive Med 2010;2:1–10.
[43] Gorbunov NV, Kiang JG. Up-regulation of autophagy in small intestine Paneth cells in response to total-body gamma-irradiation. J Pathol 2009;219:242–252.
[44] Kiang JG, Smith JT, Agravante NG. Geldanamycin analog 17-DMAG inhibits iNOS and caspases in gamma-irradiated human T cells. Radiat Res 2009;172:321–330.
[45] Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene 2003;22:5734–5754.
[46] Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochim Biophys Acta 2007;1776:86–107.
[47] Farrukh MR, Nissar UA, Afnan Q, Rafiq RA, Sharma L, Amin S, et al. Oxidative stress mediated Ca(2+) release manifests endoplasmic reticulum stress leading to unfolded protein response in UV-B irradiated human skin cells. J Dermatol Sci 2014;75:24–35.
[48] Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, et al. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med 2007;13:619–624.
[49] Chen Y, Azad MB, Gibson SB. Superoxide is the major reactive oxygen species regulating autophagy. Cell Death Differ 2009;16:1040–1052.
[50] Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: cell survival and cell death. Int J Cell Biol 2010;2010:214074.
[51] Zois CE, Koukourakis MI. Radiation-induced autophagy in normal and cancer cells: towards novel cytoprotection and radio-sensitization policies? Autophagy 2009;5:442–450.
[52] Yi H, Liang B, Jia J, Liang N, Xu H, Ju G, et al. Differential roles of miR-199a-5p in radiation-induced autophagy in breast cancer cells. FEBS Lett 2013;587:436–443.
[53] Codogno P, Meijer AJ. Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ 2005;12:1509–1518.
[54] Pawlik TM, Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. Int J Radiat Oncol Biol Phys 2004;59:928–942.
[55] Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM. Molecular response of human glioblastoma multiforme cells to ionizing radiation: cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. J Neurosurg 2003;98:378–384.
[56] Pang XL, He G, Liu YB, Wang Y, Zhang B. Endoplasmic reticulum stress sensitizes human esophageal cancer cell to radiation. World J Gastroenterol 2013;19:1736–1748.
[57] Schmukler E, Grinboim E, Schokoroy S, Amir A, Wolfson E, Kloog Y, Pinkas-Kramarski R. Ras inhibition enhances autophagy, which partially protects cells from death. Oncotarget 2013;4:142–152.
[58] Zhang X, Li W, Wang C, Leng X, Lian S, Feng J, et al. Inhibition of autophagy enhances apoptosis induced by proteasome inhibitor bortezomib in human glioblastoma U87 and U251 cells. Mol Cell Biochem 2014;385:265–275.
[59] Li J, Qin Z, Liang Z. The prosurvival role of autophagy in Resveratrol-induced cytotoxicity in human U251 glioma cells. BMC Cancer 2009;9:215.
[60] Sui X, Kong N, Wang X, Fang Y, Hu X, Xu Y, et al. JNK confers 5-fluorouracil resistance in p53-deficient and mutant p53-expressing colon cancer cells by inducing survival autophagy. Sci Rep 2014;4:4694.
[61] Chaurasia M, Misra S, Bhatt AN, Das A, Dwarakanath BS, Sharma K. Metabolic imbalance associated mitophagy in tumor cells: genesis and implications. J Cancer Res Updates 2015;4:95–107.
[62] Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc Natl Acad Sci USA 2007;104:19500–19505.
[63] Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 2005;39:359–407.
[64] Parsons MJ, Green DR. Mitochondria in cell death. Essays Biochem 2010;47:99–114.
[65] Langer T, Kaser M, Klanner C, Leonhard K. AAA proteases of mitochondria: quality control of membrane proteins and regulatory functions during mitochondrial biogenesis. Biochem Soc Trans 2001;29:431–436.
[66] Chen Y, Dorn GW II. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science 2013;340:471–475.
[67] Youle RJ, Narendra DP. Mechanisms of mitophagy. Nat Rev Mol Cell Biol 2011;12:9–14.
[68] Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, et al. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol 2010;189:211–221.
[69] Wang SH, Shih YL, Ko WC, Wei YH, Shih CM. Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway. Cell Mol Life Sci 2008;65:3640–3652.
[70] Fu M, St-Pierre P, Shankar J, Wang PT, Joshi B, Nabi IR. Regulation of mitophagy by the Gp78 E3 ubiquitin ligase. Mol Biol Cell 2013;24:1153–1162.
[71] Bartoletti-Stella A, Mariani E, Kurelac I, Maresca A, Caratozzolo MF, Iommarini L, et al. Gamma rays induce a p53-independent mitochondrial biogenesis that is counter-regulated by HIF1alpha. Cell Death Dis 2013;4:e663.
[72] Yamamori T, Yasui H, Yamazumi M, Wada Y, Nakamura Y, Nakamura H, Inanami O. Ionizing radiation induces mitochondrial reactive oxygen species production accompanied by upregulation of mitochondrial electron transport chain function and mitochondrial content under control of the cell cycle checkpoint. Free Radic Biol Med 2012;53:260–270.
[73] Wu LJ, Randers-Pehrson G, Xu A, Waldren CA, Geard CR, Yu Z, Hei TK. Targeted cytoplasmic irradiation with alpha

particles induces mutations in mammalian cells. Proc Natl Acad Sci USA 1999;96:4959–4964.
[74] Zhang B, Davidson MM, Zhou H, Wang C, Walker WF, Hei TK. Cytoplasmic irradiation results in mitochondrial dysfunction and DRP1-dependent mitochondrial fission. Cancer Res 2013;73:6700–6710.
[75] Kim I, Lemasters JJ. Mitophagy selectively degrades individual damaged mitochondria after photoirradiation. Antioxid Redox Signal 2011;14:1919–1928.
[76] Nishioka T, Shirato H, Yasuda M. X-ray irradiation induces mitophagy and cancer metabolic reprogramming. Int J Oncol 2014;90:S778
[77] Black HS. ROS: a step closer to elucidating their role in the etiology of light-induced skin disorders. J Invest Dermatol 2004;122:xiii–xxiv.
[78] Briganti S, Picardo M. Antioxidant activity, lipid peroxidation and skin diseases. What's new. J Eur Acad Dermatol Venereol 2003;17:663–669.
[79] Scriven P, Brown NJ, Pockley AG, Wyld L. The unfolded protein response and cancer: a brighter future unfolding? J Mol Med (Berl) 2007;85:331–341.
[80] Ding W, Yang L, Zhang M, Gu Y. Reactive oxygen species-mediated endoplasmic reticulum stress contributes to aldosterone-induced apoptosis in tubular epithelial cells. Biochem Biophys Res Commun 2012;418:451–456.
[81] Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 2007;9:2277–2293.
[82] Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol 2006;26:9220–9231.
[83] Lin JH, Walter P, Yen TS. Endoplasmic reticulum stress in disease pathogenesis. Annu Rev Pathol 2008;3:399–425.
[84] Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 2004;4:966–977.
[85] Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ 2007;14:230–239.
[86] Buytaert E, Callewaert G, Vandenheede JR, Agostinis P. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. Autophagy 2006;2:238–240.
[87] Yang Z, Xu Y, Xu L, Maccauro G, Rossi B, Chen Y, et al. Regulation of autophagy via PERK-eIF2alpha effectively relieve the radiation myelitis induced by iodine-125. PLoS One 2013;8:e76819.
[88] Kim KW, Moretti L, Mitchell LR, Jung DK, Lu B. Endoplasmic reticulum stress mediates radiation-induced autophagy by perk-eIF2alpha in caspase-3/7-deficient cells. Oncogene 2010;29:3241–3251.
[89] Kim EJ, Lee YJ, Kang S, Lim YB. Ionizing radiation activates PERK/eIF2alpha/ATF4 signaling via ER stress-independent pathway in human vascular endothelial cells. Int J Radiat Biol 2014;90:306–312.
[90] Saglar E, Unlu S, Babalioglu I, Gokce SC, Mergen H. Assessment of ER stress and autophagy induced by ionizing radiation in both radiotherapy patients and ex vivo irradiated samples. J Biochem Mol Toxicol 2014;28:413–417.
[91] Chen X, Li M, Chen D, Gao W, Guan JL, Komatsu M, Yin XM. Autophagy induced by calcium phosphate precipitates involves endoplasmic reticulum membranes in autophagosome biogenesis. PLoS One 2012;7:e52347.
[92] Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol 2004;287:C817–C833.
[93] Decuypere JP, Bultynck G, Parys JB. A dual role for Ca(2+) in autophagy regulation. Cell Calcium 2011;50:242–250.
[94] Cardenas C, Foskett JK. Mitochondrial Ca(2+) signals in autophagy. Cell Calcium 2012;52:44–51.
[95] Gordon PB, Holen I, Fosse M, Rotnes JS, Seglen PO. Dependence of hepatocytic autophagy on intracellularly sequestered calcium. J Biol Chem 1993;268:26107–26112.
[96] Pfisterer SG, Mauthe M, Codogno P, Proikas-Cezanne T. Ca^{2+}/calmodulin-dependent kinase (CaMK) signaling via CaMKI and AMP-activated protein kinase contributes to the regulation of WIPI-1 at the onset of autophagy. Mol Pharmacol 2011;80:1066–1075.
[97] Buytaert E, Callewaert G, Hendrickx N, Scorrano L, Hartmann D, Missiaen L, et al. Role of endoplasmic reticulum depletion and multidomain proapoptotic BAX and BAK proteins in shaping cell death after hypericin-mediated photodynamic therapy. FASEB J 2006;20:756–758.
[98] Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, et al. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol Cell 2007;25:193–205.
[99] Sakaki K, Wu J, Kaufman RJ. Protein kinase Ctheta is required for autophagy in response to stress in the endoplasmic reticulum. J Biol Chem 2008;283:15370–15380.
[100] Brady NR, Hamacher-Brady A, Yuan H, Gottlieb RA. The autophagic response to nutrient deprivation in the hl-1 cardiac myocyte is modulated by Bcl-2 and sarco/endoplasmic reticulum calcium stores. FEBS J 2007;274:3184–3197.
[101] Gao W, Ding WX, Stolz DB, Yin XM. Induction of macroautophagy by exogenously introduced calcium. Autophagy 2008;4:754–761.
[102] Szabadkai G, Duchen MR. Mitochondria: the hub of cellular Ca^{2+} signaling. Physiology (Bethesda) 2008;23:84–94.
[103] Verma A, Bhatt AN, Farooque A, Khanna S, Singh S, Dwarakanath BS. Calcium ionophore A23187 reveals calcium related cellular stress as ''actor in a new role'': an old actor in a new role. Cell Calcium 2011;50:510–522.
[104] Vingtdeux V, Giliberto L, Zhao H, Chandakkar P, Wu Q, Simon JE, et al. AMP-activated protein kinase signaling activation by resveratrol modulates amyloid-beta peptide metabolism. J Biol Chem 2010;285:9100–9113.
[105] Zalckvar E, Berissi H, Mizrachy L, Idelchuk Y, Koren I, Eisenstein M, et al. DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep 2009;10:285–292.

[106] Kim HJ, Soyombo AA, Tjon-Kon-Sang S, So I, Muallem S. The Ca(2+) channel TRPML3 regulates membrane trafficking and autophagy. Traffic 2009;10:1157–1167.
[107] Ionescu L, Cheung KH, Vais H, Mak DO, White C, Foskett JK. Graded recruitment and inactivation of single InsP3 receptor Ca2+-release channels: implications for quantal [corrected] Ca2 + release. J Physiol 2006;573:645–662.
[108] Taylor CW, Laude AJ. IP3 receptors and their regulation by calmodulin and cytosolic Ca2+. Cell Calcium 2002;32:321–334.
[109] NadifKasri N, Bultynck G, Sienaert I, Callewaert G, Erneux C, Missiaen L, et al. The role of calmodulin for inositol 1,4,5-trisphosphate receptor function. Biochim Biophys Acta 2002;1600:19–31.
[110] Vicencio JM, Ortiz C, Criollo A, Jones AW, Kepp O, Galluzzi L, et al. The inositol 1,4,5-trisphosphate receptor regulates autophagy through its interaction with Beclin1. Cell Death Differ 2009;16:1006–1017.
[111] Criollo A, Maiuri MC, Tasdemir E, Vitale I, Fiebig AA, Andrews D, et al. Regulation of autophagy by the inositol trisphosphate receptor. Cell Death Differ 2007;14:1029–1039.
[112] Khan MT, Joseph SK. Role of inositol trisphosphate receptors in autophagy in DT40 cells. J Biol Chem 2010;285:16912–16920.
[113] Cardenas C, Miller RA, Smith I, Bui T, Molgo J, Muller M, et al. Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2 + transfer to mitochondria. Cell 2010;142:270–283.
[114] Williams A, Sarkar S, Cuddon P, Ttofi EK, Saiki S, Siddiqi FH, et al. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat Chem Biol 2008;4:295–305.
[115] Rong YP, Aromolaran AS, Bultynck G, Zhong F, Li X, Mc Coll K, et al. Targeting Bcl-2-IP3 receptor interaction to reverse Bcl-2's inhibition of apoptotic calcium signals. Mol Cell 2008;31:255–265.
[116] Bhandary B, Marahatta A, Kim HR, Chae HJ. An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. Int J Mol Sci 2012;14:434–456.
[117] Moretti L, Cha YI, Niermann KJ, Lu B. Switch between apoptosis and autophagy: radiation-induced endoplasmic reticulum stress? Cell Cycle 2007;6:793–798.
[118] Vessoni AT, Filippi-Chiela EC, Menck CF, Lenz G. Autophagy and genomic integrity. Cell Death Differ 2013;20:1444–1454.
[119] Shoji JY, Kikuma T, Arioka M, Kitamoto K. Macroautophagy-mediated degradation of whole nuclei in the filamentous fungus Aspergillus oryzae. PLoS One 2010;5:e15650.
[120] Mc Gee MD, Weber D, Day N, Vitelli C, Crippen D, Herndon LA, et al. Loss of intestinal nuclei and intestinal integrity in aging C. elegans. Aging Cell 2014;10:699–710.
[121] Krick R, Muhe Y, Prick T, Bredschneider M, Bremer S, Wenzel D, et al. Piecemeal microautophagy of the nucleus: genetic and morphological traits. Autophagy 2009;5:270–272.
[122] Rello-Varona S, Lissa D, Shen S, Niso-Santano M, Senovilla L, Marino G, et al. Autophagic removal of micronuclei. Cell Cycle 2012;11:170–176.
[123] Park YE, Hayashi YK, Bonne G, Arimura T, Noguchi S, Nonaka I, Nishino I. Autophagic degradation of nuclear components in mammalian cells. Autophagy 2009;5:795–804.
[124] Filimonenko M, Isakson P, Finley KD, Anderson M, Jeong H, Melia TJ, et al. The selective macroautophagic degradation of aggregated proteins requires the PI3P-binding protein Alfy. Mol Cell 2010;38:265–279.
[125] Pankiv S, Lamark T, Bruun JA, Overvatn A, Bjorkoy G, Johansen T. Nucleocytoplasmic shuttling of p62/SQSTM1 and its role in recruitment of nuclear polyubiquitinated proteins to promyelocytic leukemia bodies. J Biol Chem 2010;285:5941–5953.
[126] Simonsen A, Birkeland HC, Gillooly DJ, Mizushima N, Kuma A, Yoshimori T, et al. Alfy, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes. J Cell Sci 2004;117:4239–4251.
[127] Clausen TH, Lamark T, Isakson P, Finley K, Larsen KB, Brech A, et al. p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy. Autophagy 2010;6:330–344.
[128] Isakson P, Holland P, Simonsen A. The role of ALFY in selective autophagy. Cell Death Differ 2013;20:12–20.
[129] Liang XH, Yu J, Brown K, Levine B. Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. Cancer Res 2001;61:3443–3449.
[130] Rodriguez-Rocha H, Garcia-Garcia A, Panayiotidis MI, Franco R. DNA damage and autophagy. Mutat Res 2011;711:158–166.
[131] Bae H, Guan JL. Suppression of autophagy by FIP200 deletion impairs DNA damage repair and increases cell death upon treatments with anticancer agents. Mol Cancer Res 2011;9:1232–1241.
[132] Mao Z, Bozzella M, Seluanov A, Gorbunova V. DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. Cell Cycle 2008;7:2902–2906.
[133] Brandsma I, Gent DC. Pathway choice in DNA double strand break repair: observations of a balancing act. Genome Integr 2012;3:9.
[134] Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, et al. Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev 2007;21:1367–1381.
[135] Liu EY, Xu N, O'Prey J, Lao LY, Joshi S, Long JS, et al. Loss of autophagy causes a synthetic lethal deficiency in DNA repair. Proc Natl Acad Sci USA 2015;112:773–778.
[136] Park C, Suh Y, Cuervo AM. Regulated degradation of Chk1 by chaperone-mediated autophagy in response to DNA damage. Nat Commun 2015;6:6823.
[137] Robert T, Vanoli F, Chiolo I, Shubassi G, Bernstein KA, Rothstein R, et al. HDACs link the DNA damage response, processing of double-strand breaks and autophagy. Nature 2011;471:74–79.
[138] Dyavaiah M, Rooney JP, Chittur SV, Lin Q, Begley TJ. Autophagy-dependent regulation of the DNA damage response protein ribonucleotide reductase 1. Mol Cancer Res 2011;9:462–475.
[139] Dotiwala F, Eapen VV, Harrison JC, Arbel-Eden A, Ranade V, Yoshida S, Haber JE. DNA damage checkpoint triggers autophagy to regulate the initiation of anaphase. Proc Natl Acad Sci USA 2013;110:E41–E49.

[140] Ryan KM. p53 and autophagy in cancer: guardian of the genome meets guardian of the proteome. Eur J Cancer 2011;47:44–50.
[141] Gao W, Shen Z, Shang L, Wang X. Upregulation of human autophagy-initiation kinase ULK1 by tumor suppressor p53 contributes to DNA-damage-induced cell death. Cell Death Differ 2011;18:1598–1607.
[142] Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell 2008;134:451–460.
[143] Alexander A, Cai SL, Kim J, Nanez A, Sahin M, Mac Lean KH, et al. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc Natl Acad Sci USA 2010;107:4153–4158.
[144] Morselli E, Tasdemir E, Maiuri MC, Galluzzi L, Kepp O, Criollo A, et al. Mutant p53 protein localized in the cytoplasm inhibits autophagy. Cell Cycle 2008;7:3056–3061.
[145] 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;10:676–687.
[146] Munoz-Gamez JA, Rodriguez-Vargas JM, Quiles-Perez R, Aguilar-Quesada R, Martin-Oliva D, de Murcia G, et al. PARP-1 is involved in autophagy induced by DNA damage. Autophagy 2009;5:61–74.
[147] Rodriguez-Vargas JM, Ruiz-Magana MJ, Ruiz-Ruiz C, Majuelos-Melguizo J, Peralta-Leal A, Rodriguez MI, et al. ROS-induced DNA damage and PARP-1 are required for optimal induction of starvation-induced autophagy. Cell Res 2012;22:1181–1198.
[148] Batista LF, Roos WP, Kaina B, Menck CF. p53 mutant human glioma cells are sensitive to UV-C-induced apoptosis due to impaired cyclobutane pyrimidine dimer removal. Mol Cancer Res 2009;7:237–246.
[149] Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, et al. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 2013;4:e838.
[150] 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;46:1900–1909.
[151] Selvakumaran M, Amaravadi RK, Vasilevskaya IA, O'Dwyer PJ. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. Clin Cancer Res 2013;19:2995–3007.
[152] Mahalingam D, Mita M, Sarantopoulos J, Wood L, Amaravadi RK, Davis LE, et al. Combined autophagy and HDAC inhibition: a phase I safety, tolerability, pharmacokinetic, and pharmacodynamic analysis of hydroxychloroquine in combination with the HDAC inhibitor vorinostat in patients with advanced solid tumors. Autophagy 2014;10:1403–1414.
[153] 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;10:1369–1379.
[154] Wang K, Liu R, Li J, Mao J, Lei Y, Wu J, et al. Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR-andhypoxia-induced factor 1alpha-mediated signaling. Autophagy 2011;7:966–978.
[155] Gupta A, Roy S, Lazar AJ, Wang WL, Mc Auliffe JC, Reynoso D, et al. Autophagy inhibition and antimalarials promote cell death in gastro intestinal stromal tumor (GIST). Proc Natl Acad Sci USA 2010;107:14333–14338.
[156] Vogl DT, Stadtmauer EA, Tan KS, Heitjan DF, Davis LE, Pontiggia L, et al. Combined autophagy and proteasome inhibition: a phase1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. Autophagy 2014;10:1380–1390.
[157] Wu Z, Chang PC, Yang JC, Chu CY, Wang LY, Chen NT, et al. Autophagy blockade sensitizes prostate cancer cells towards Src family kinase inhibitors. Genes Cancer 2010;1:40–49.
[158] Bommareddy A, Hahm ER, Xiao D, Powolny AA, Fisher AL, Jiang Y, Singh SV. Atg5 regulates phenethyl isothiocyanate-induced autophagic and apoptotic cell death in human prostate cancer cells. Cancer Res 2009;69:3704–3712.
[159] Claerhout S, Verschooten L, Van Kelst S, De Vos R, Proby C, Agostinis P, Garmyn M. Concomitant inhibition of AKT and autophagy is required for efficient cisplatin-induced apoptosis of metastatic skin carcinoma. Int J Cancer 2010;127:2790–2803.
[160] Salazar M, Carracedo A, Salanueva IJ, Hernandez-Tiedra S, Lorente M, Egia A, et al. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. J Clin Invest 2009;119:1359–1372.
[161] Levy JM, Thorburn A. Targeting autophagy during cancer therapy to improve clinical outcomes. Pharmacol Ther 2011;131:130–141.
[162] Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res 2008;68:1485–1494.
[163] Lomonaco SL, Finniss S, Xiang C, Decarvalho A, Umansky F, Kalkanis SN, et al. The induction of autophagy by gamma-radiation contributes to the radioresistance of glioma stem cells. Int J Cancer 2009;125:717–722.
[164] Kim KW, Moretti L, Mitchell LR, Jung DK, Lu B. Combined Bcl-2/mammalian target of rapamycin inhibition leads to enhanced radiosensitization via induction of apoptosis and autophagy in non-small cell lung tumor xenograft model. Clin Cancer Res 2009;15:6096–6105.
[165] Fujiwara K, Iwado E, Mills GB, Sawaya R, Kondo S, Kondo Y. Akt inhibitor shows anticancer and radiosensitizing effects in malignant glioma cells by inducing autophagy. Int J Oncol 2007;31:753–760.
[166] Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, et al. FoxO3 controls autophagy in skeletal muscle *in vivo*. Cell Metab 2007;6:458–471.
[167] Polager S, Ofir M, Ginsberg D. E2F1 regulates autophagy and the transcription of autophagy genes. Oncogene 2008;27:4860–4864.
[168] 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. Proc Natl Acad Sci USA 2003;100:15077–15082.
[169] Li M, Jiang X, Liu D, Na Y, Gao GF, Xi Z. Autophagy protects LNCaP cells under androgen deprivation conditions. Autophagy 2008;4:54–60.

[170] Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 2007;117:326–336.
[171] Thorburn J, Horita H, Redzic J, Hansen K, Frankel AE, Thorburn A. Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. Cell Death Differ 2009;16:175–183.
[172] Maiuri MC, Tasdemir E, Criollo A, Morselli E, Vicencio JM, Carnuccio R, Kroemer G. Control of autophagy by oncogenes and tumor suppressor genes. Cell Death Differ 2009;16:87–93.
[173] White E, Karp C, Strohecker AM, Guo Y, Mathew R. Role of autophagy in suppression of inflammation and cancer. Curr Opin Cell Biol 2010;22:212–217.
[174] Roberts DJ, Tan-Sah VP, Ding EY, Smith JM, Miyamoto S. Hexokinase-II positively regulates glucose starvation-induced autophagy through TORC1 inhibition. Mol Cell 2014;53:521–533.
[175] Dwarakanath B, Jain V. Targeting glucose metabolism with 2-deoxy-d-glucose for improving cancer therapy. Future Oncol 2009;5:581–585.
[176] Dwarakanath BS, Singh D, Banerji AK, Sarin R, Venkataramana NK, Jalali R, et al. Clinical studies for improving radiotherapy with 2-deoxy-D-glucose: present status and future prospects. J Cancer Res Ther 2009;5:S21–S26.
[177] Dwarkanath BS, Zolzer F, Chandana S, Bauch T, Adhikari JS, Muller WU, et al. Heterogeneity in 2-deoxy-d-glucose-induced modifications in energetics and radiation responses of human tumor cell lines. Int J Radiat Oncol Biol Phys 2001;50:1051–1061.
[178] Dwarakanath BS. Cytotoxicity, radiosensitization, and chemosensitization of tumor cells by 2-deoxy-d-glucose *in vitro*. J Cancer Res Ther 2009;5:S27–S31.
[179] Gupta S, Mathur R, Dwarakanath BS. The glycolytic inhibitor 2-deoxy-d-glucose enhances the efficacy of etoposide in ehrlich ascites tumor-bearing mice. Cancer Biol Ther 2005;4:87–94.

Metabolic Imbalance Associated Mitophagy in Tumor Cells: Genesis and Implications

Madhuri Chaurasia[1,2,#], Shashank Misra[1,#], Anant N. Bhatt[1], Asmita Das[2], Bilikere Dwarakanath[1,*] and Kulbhushan Sharma[1,*]

[1]*Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi 110054, India*

[2]*Department of Biotechnology, Delhi Technological University, Delhi 110042, India*

Abstract: Emerging knowledge supports the notion that metabolic reprogramming facilitates the progression of many cancers and in some it could be initiated by mutations in genes related to mitochondrial function. While dysfunctional mitochondria plays a pivotal role in driving metabolic reprogramming, mitophagy that recycles damaged mitochondria by selective and organized degradation appears to be vital for sustaining carcinogenesis. Although the potential of targeting mitophagy as a therapeutic strategy has still remained elusive, poor prognosis and therapeutic resistance of highly glycolytic tumors suggest that inhibitors of mitophagy could be potential adjuvant in radio- and chemotherapy of tumors. We briefly review the current status of knowledge on the interrelationship between mitophagy and metabolic reprogramming during carcinogenesis and examine mitophagy as a potential target for developing anticancer therapeutics and adjuvant.

Keywords: Warburg, PARKIN, Oxidative stress, Metabolic Reprogramming, Calcium.

Mitochondria are considered to be the energy house of eukaryotic cells. To ensure functionality of this crucial requirement under a variety of stress conditions, cells have evolved a highly structured mechanism for recycling damaged mitochondria known as Mitophagy [1, 2]. Mitophagy aids in selective degradation of damaged/dysfunctional and old mitochondria produced in response to certain deleterious stresses such as hypoxia and starvation, thereby helping in the maintenance of cellular homeostasis [1, 2]. Accumulating evidences suggest that dysfunctional mitochondrion has a pivotal role in modulating the metabolic reprogramming thus contributing to the process of tumorigenesis [3]. Variations in the status of Warburg phenotype linked to the differences in mitochondrial status in cancer cells and/or tumor micro milieu (reverse Warburg phenotype) appear to be dependent on mitophagic potential of cells as well as the type and extent of stress [4]. Poor prognosis and therapeutic resistance of highly glycolytic tumors suggest that mitophagy could be one of the contributing factors. Although the potential of targeting mitophagy as a therapeutic strategy has so far remained elusive, emerging evidences suggest the potential of targeting this phenomenon for developing inhibitors of mitophagy as adjuvant in radio- and chemotherapy of tumors [5]. This review will discuss the relationship between metabolic disturbance leading to calcium imbalance and mitophagy in both malignant as well as untransformed cells and critically examine the direct and collateral evidences for developing inhibitors of mitophagy as adjuvant in cancer therapy.

METABOLIC REPROGRAMMING AND TUMORIGENESIS

Metabolic reprogramming or altered bioenergetics has emerged as an important hallmark of cancer. The source of cancer initiation and maintenance which was earlier only restricted to genetic mutations is now gradually being attributed to metabolic reprogramming also. Glucose and ATP have been identified as key players in altered bioenergetics. Metabolic alternations in cancer cells were recognized as early as 1920, when Otto Warburg gave his hypothesis of "Warburg effect" stating that ''*Cancer, above all other diseases, has countless secondary causes".*

Warburg postulated that unlike normal cells, cancer cells produce lactate from glycolysis even in the presence of abundant oxygen. He termed it as aerobic glycolysis. Warburg attributed this phenomenon to dysfunctional mitochondria that impairs oxidative phosphorylation [3, 6]. High glycolytic rate might also result from a decreased mitochondrial mass in tumor cells [7]. The constant glycolysis in these cells is maintained by up regulation of glucose transporters (Glut1-4) that help in glucose uptake [8-11].

In addition to providing ATP, increased glucose uptake also provides cancer cells with building blocks of the cell i.e. macromolecules like nucleotides, amino

*Address correspondence to these authors at the Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi 110054, India; Tel: +91-11-23918838; Fax: +91-11-23919509;
E-mails: dwarakanathdrbs@gmail.com; kulsinmas@gmail.com

#These authors contributed equally.

ISSN: 1929-2260 / E-ISSN: 1929-2279/15

acids and lipids by diverting glucose to Pentose Phosphate Pathway [12-14]. The generation of biomass maintains rapid proliferation and provides a balance between the anabolic and catabolic activities of cells. Warburg effect also maintains the damaged reactive oxygen species (ROS) levels by generation of adequate NADPH *via* phosphoenol pyruvate pathway and through PKM2 isoform of the pyruvate kinase (PK), which catalyzes the conversion of phosphoenol pyruvate (PEP) to pyruvate as the last step of glycolysis. The PKM2 isoform also helps in maintaining rapid proliferation by up regulation of glucose transport and enhanced synthesis of early glycolytic intermediate in order to achieve metabolic balance among ATP production, biomass synthesis, as well as the control of oxidative stress due to ROS generation [15, 16].

Although Warburg's interpretation of the association of aerobic glycolysis with dysfunctional mitochondria in tumor cells has been challenged in recent times, subsequent studies revealed that tumor mitochondria do respire and produce ATP [17]. The Warburg effect specifically assigned to cancer cells has also been observed in rapidly proliferating normal cells such as stimulated lymphocytes and mitotic and proliferating fibroblasts [18-22]. Thus, it appears that phenomenon of aerobic glycolysis is a metabolically conserved process adapted to sustain growth of highly proliferating cells in order to fulfill their energy and metabolic demands. Impairment in growth of breast cancer even at high levels of glycolysis and promotion of tumorigenesis with enhanced basal autophagy leading to the maintenance of mitochondrial function in cells with activated Ras during periods of nutrient limitation suggest that aerobic glycolysis is not applicable to all cancer cells [23, 24].

MITOCHONDRIAL ALTERATIONS AND TUMORI-GENESIS

Mitochondrial dysfunction has been implicated in the pathogenesis of the various disorders including Parkinson's disease, diabetes mellitus and cancer [25-30]. Polymorphism in mitochondrial DNA enhancing the susceptibility to breast and prostate cancer, and recent identification of fumarate and succinate dehydro-genases as tumor suppressor genes have highlighted the relationship between mitochondria and tumorigenesis [31-34]. Besides genetic changes, enhanced ROS production leading to oxidative stress, suppression of mitochondrial outer membrane potential (MOMP) (that elicits apoptosis) and enhanced glycolysis in cancer cells also indicate involvement of mitochondria in tumorigenesis even at the functional level [35-37].

Several evidences support the mitochondrial association with tumorigenesis at the genetic level. Polymorphism in mitochondrial DNA promotes tumorigenesis *via* two ways i.e. by impeding steady-state oxidative phosphorylation (OXPHOS) and by facilitating cancer cell adaptation to changing bioenergetics environments [38]. Further, mutations in the genes encoding proteins such as succinate and fumarate inhibits α-ketoglutarate-dependent prolyl hydroxylases (PHDs), thus stabilizing hypoxia-inducible factor 1α (HIF1α) [39]. The stabilized HIF1α is then translocated to the nucleus causing a shift in energy metabolism from oxidative to glycolysis [40, 41]. Fumarate inhibition also potentiates tumorigenesis by succinylation of cysteine residues in Kelch-like ECH-associated protein 1 (KEAP-1), which activates nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway thereby up-regulating the level of stress response genes [42, 43].

Generation of ROS leading to the altered cellular redox state by both functional and dysfunctional mitochondria is also known to promote tumorigenesis. Increased ROS disrupts mitochondrial signaling by oxidizing thiol groups in cysteine residues of caspases as well as cysteine residues of phosphotases like PTEN tumor suppressor, the CDC25B oncogene, and MAPK phosphatases [44-46]. Increased ROS also stabilizes HIF1α which in-turn impairs respiration (TCA cycle) leading to the reduction in ROS levels and thus protecting tumor cells from apoptosis [47-50]. Promotion of tumorigenesis by ROS is also evident by the degradation of the KEAP-1 that activates NRF2 signaling [51]. NRF2 pathway endorses metabolic programming towards anabolic pathways that sustains tumor growth along with maintaining damaged ROS levels that further potentiates tumor cell proliferation [52]. MOMP suppression has also been shown to promote tumor growth by inhibiting apoptosis of tumor cells [36]. Thus, accumulating evidences indicate a strong relationship between mitochondria and tumorigenesis both at the genetic as well as functional levels.

MITOCHONDRIAL ALTERATIONS INDUCED META-BOLIC REPROGRAMMING

The mechanistic link between mitochondria and aerobic glycolysis is provided by Hexokinase II (HK-II) that gets up-regulated in cancer cells and translocates

to the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane. This interaction is facilitated by the phosphorylation of HK-II by protein kinase B (Akt) [53]. The mitochondrial HK-II being in close proximity to ATP source facilitates rapid phosphorylation of glucose leading to higher glycolysis and PPP. Another interconnecting link between Warburg effect and mitochondria is the pyruvate kinase M2 (PKM2), which maintains tumor growth by up-regulating HIF1α and ROS levels as well as providing biomass to the cancer cells [54]. However, this interaction is context and tumor-type dependent as under moderate hypoxia conditions, PKM2 is inhibited through its oxidation, leading to the promotion of PPP pathway and enhanced generation of cellular NADPH which prevents oxidative stress generation. On the other hand, during severe hypoxia, the dependency of cancer cells on PKM2 increases owing to the limited oxidative phosphorylation [54].

Tumor cells are heterogeneous in terms of metabolism and morphology. Metabolic heterogeneity includes variations in the levels of oxidative phosphorylation and Warburg effect due to fluctuations in the oxygen and nutrient supply. Besides the tumor cells, heterogeneity has also been shown in the stromal cells present in the tumor micro milieu consisting cells of hematopoietic (T cells, B cells, NK cells, macrophages and MDSC) and mesenchymal origin (fibroblasts, myofibroblasts, mesenchymal stem cells (MSCs), adipocytes and endothelial cells) [55]. However, the role of these cells in metabolic reprogramming of tumor cells has remained elusive. Recently, a new concept of “Reverse Warburg effect” or “Battery-operated tumor growth” (hereafter called as non-Warburgian phenotyope) has been proposed where the stromal cells appear to influence the metabolic reprogramming of tumor cells through a host-parasite relationship, with stromal cells acting as host and cancer cells as parasites [4]. The stromal cells surrounding tumor cells have also been shown to display efficient mechanism for recycling dysfunctional mitochondria acting as a nutrient supplier [4]. However, the implications of efficient recycling of mitochondria in the tumor cells and micro milieu on the resistance against chemo- and radiotherapies have not been well understood.

ROLE OF CALCIUM IN FUNCTIONAL ALTERATION OF MITOCHONDRIA

Hypoxia and/or altered metabolism are the major sources of oxidative stress in cancer cells. This persistent oxidative stress leads to the chain reaction of cellular lipid oxidation. Oxidized lipid metabolites (by-products) either alter the membrane fluidity or get released inside the cytoplasm and the respective organelles. The oxidized lipids alter the permeability of membrane to calcium or directly act as calcium ionophores leading to increased cytosolic calcium [56, 57]. Mitochondria buffer this overloaded cytosolic calcium by acting as a sink thus preventing cell death. However, calcium accumulation in mitochondria leads to hormesis effect, called mitohormesis [58, 59]. At low concentration, calcium enhances the oxidative phosphorylation capacity by activating many mitochondrial dehydrogenases leading to aggressive metabolic phenotype [58]. Majority of the cancer cells show mitochondrial accumulation in the close proximity of ER, creating the microdomain of high calcium for mitochondrial calcium uniporter leading to regulated increase in mitochondrial calcium, thus assisting in the development of the aggressive metabolic phenotype for enhanced growth and survival [60]. On the other hand, calcium overload in the mitochondria leads to mitochondrial damage [56].

Since the accumulation of damaged mitochondria is detrimental to cells and one of the major causes of cancers, mitochondrial quality control is essential for maintaining the cellular integrity and function [61]. Therefore, damaged and functionally compromised mitochondria undergo the process of degradation and regeneration of newer mitochondria called mitophagy and mitochondrial biogenesis respectively. As oxidative stress and calcium induced mitochondrial damage is continuous process in cancer cells, damaged mitochondria can be observed in them at any given time in the form of mitochondria derived vesicles (MDVs) and “I-Bodies” [56, 61, 62]. Taken together, all these events appear to be inter-dependent and work in a cyclic manner in the cancer cell. Oxidative stress leads to disturbance in cellular calcium homeostasis causing mitochondrial damage and altered metabolism, further resulting in to enhanced ROS production in cancer cells (Figure **1**).

MITOPHAGY: RECYCLING OF THE DAMAGED MITOCHONDRIA

Mitochondrial dysfunction has been shown in numerous patho-physiologcal conditions including cancer, metabolic disorders, neurodegeneration, diabetes and aging [1, 2, 63]. Being a critical component, various mitochondria quality control mechanisms exist in cells that include mitochondrial

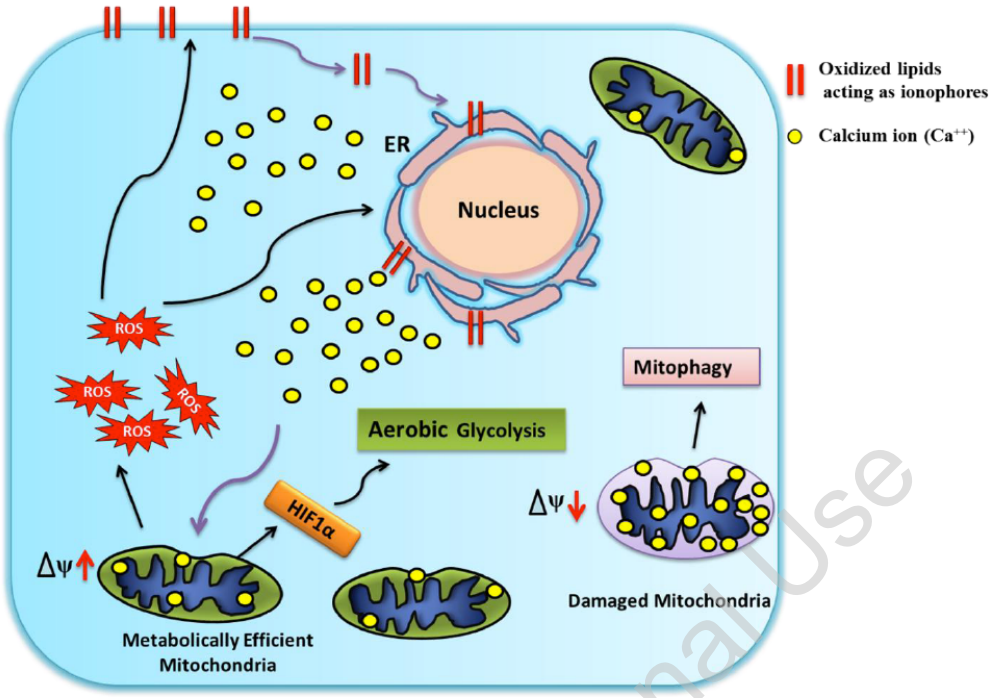


Figure 1: Schematic diagram showing oxidative stress induced alterations in calcium homeostasis during metabolic reprogramming of cancer cells. ROS induced lipid oxidation makes membrane permeable to calcium, which is buffered by mitochondria making it metabolically efficient. This leads to further ROS production and stabilization of HIF1α converting the cancer cell in to Warburg phenotype. Mitochondria overloaded with calcium develop irreversible damage and are cleared from cells through mitophagy.

fusion, fission, biogenesis and mitophagy (mitochondrial autophagy). Mitophagy is the primary mechanism responsible for the recycling of damaged and dysfunctional mitochondria with the help of autophagosome, which further fuses with lysosomes to form autophagolysosomes [64, 65]. Mitophagy and mitochondrial fusion are antagonistic, and decide the fate of dysfunctional mitochondria [66]. Mitochondrial fission takes place predominantly in the depolarized mitochondria lacking fusion protein optic atrophy 1 (Opa-1), whereas mitochondrial fusion takes place in polarized mitochondria *via* depletion of mitochondrial fission protein dynamin-related protein 1 (Drp1) with the help of protein kinase A (PKA) [67-72]. As mitochondria cannot be recycled in its original form due to its large size, mitochondrial fission is considered as the prerequisite for initiating mitophagy.

During mitophagy, numerous key adaptor molecules at the outer membrane of damaged and dysfunctional mitochondria, facilitates interaction with LC3 (autophagy related protein which helps in autophagosome elongation) present at the growing autophagosome membrane [64, 73]. The main adaptors involved in mitophagic induction include E3 ubiqutin ligase PARKIN, NIX, BNIP3, FUNDC1 and Mul1 [64, 73, 74]. Oxidative phosphorylation in damaged mitochondria leads to the generation of toxic by-products involving reactive oxygen species (ROS), which causes oxidative damage to mitochondrial lipids, DNA and proteins leading to further ROS production. The damaged mitochondria in turn, release huge amount of Ca^{2+} ions and cytochrome-c to the cytosol thereby triggering apoptosis [75-77]. Although specific mechanisms involved in mitophagic induction are not completely understood, two molecular pathways have been implicated. The first pathway depends upon PINK1 (PTEN induced putative kinase 1) and PARKIN interaction where PINK1 is a mitochondria specific kinase and PARKIN is an E3- ubiquitin ligase [78]. The second is mainly mediated *via* different molecules such as ER associated E3 ubiquitin ligase GP78 (glycoprotein 78) and NIX/BNIP3L in a context dependent manner [78, 79].

Accumulating evidences suggest the involvement of nearly 32 autophagy related (ATG) proteins in mitophagic progression. A new autophagy related protein i.e. ATG33, which is specifically involved in mitophagy has been recently identified as a mitochondrial outer membrane protein [80, 81]. The core mitophagic machinery is activated by the recruitment of ATG32-ATG11-ATG8 (LC3 in mammals), where ATG11 acts as an adaptor between

ATG8 and ATG32 and helps in the recruitment of autophagic machinery over mitochondria. Under normal conditions, PINK1 (63 kDa) gets translocated from outer mitochondrial membrane to the inner mitochondrial membrane where PARL (presenselin associated, rhomboid-like) protease causes its proteolytic cleavage into a short PINK1 isoform of approximately 52 kDa ($PINK1_{52}$) [82, 83]. However, under conditions of reduced mitochondrial potential (like oxidative stress), PINK1 accumulates in the outer mitochondrial membrane, where it interacts with PARKIN and causes its phosphorylation [84]. Activated PARKIN causes ubiquitination of various mitochondrial proteins, which act as a landing platform for p62/SQSTM1 forming a functional link between ubiquitinated proteins, including MFN1/2 (Mitofusin1/2) and LC3, leading to the initiation of autophagosome with the help of Atg32 (Figure **2**).

MITOPHAGY AND CANCER

During trauma, mitophagy supports tumor cell survival by providing substrates for mitochondrial metabolism [24, 85]. Aggressive tumor cells appear to harbour robust mitochondria, although due to severe mutations in tumor suppressor as well as TCA cycle genes, they rely more on aerobic glycolysis to meet their energy demands. Such mitochondria show 'Warburg phenomenon'. In addition to the mutations in metabolic regulatory genes, several mitophagy related genes have also been found to be mutated in many types of cancers during initial stages of tumor development [86, 87]. This results in the induction of defective mitophagy in these cells, leading to a higher accumulation of dysfunctional mitochondria ultimately leading to enhanced ROS generation and tumor induction [85]. Such mitochondria are more robust, having high antioxidant defence mechanism and can survive in highly hypoxic environment. We recently showed that Ca^{2+} rich structures formed by high intracellular Ca^{2+} induced dysfunction and aggregation of mitochondria in response to stress can be revealed by high density packing of the fluorescent calcium ionophore A23187 called "I-Bodies" [56]. Presence of endogenous "I-Bodies" in cancer cells indeed supports the association of mitochondrial dysfunction and carcinogenesis [56]. "I-Bodies" are suggested to provide a snapshot view of the ongoing mitophagy or genetic defect in the clearance of dysfunctional mitochondria in cancer cells similar to Parkinson's disease [56, 88]. Increase in radiation and anticancer drug (etoposide) induced "I-Bodies" validate the hypothesis that "I-Bodies" are mitophagic vacuoles encircling damaged mitochondria.

Cellular compositions of tumors are highly heterogeneous, with clonal variations of tumor cells

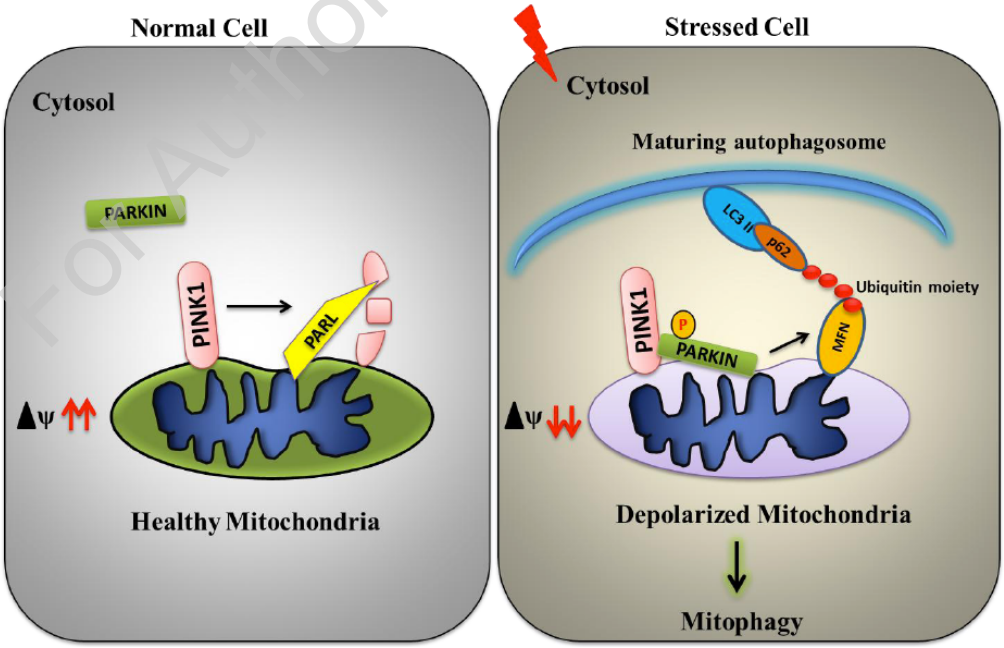


Figure 2: Mechanism of mitophagy induction. In normal cells, onset of mitophagy is abrogated by the proteolytic cleavage of PINK1 by PARL. On exposure to stress, same cell undergoes mitophagy in a sequential manner. Reduction in the mitochondrial membrane potential leads to the induction of mitophagic calcium *via* accumulation of PINK1, facilitating translocation and activation of PARKIN that adds ubiquitin moiety to mitochondrial fusion proteins Mfn, thereby inhibiting mitochondrial fusion. PINK1-PARKIN interaction initiates the mitophagic process *via* engulfment of damaged mitochondria.

and other tumor-associated cell types including fibroblasts, endothelial and immune cells. These cells constitute the tumor stroma and have also been shown to display efficient dysfunctional mitochondria recycling which acts as nutrient supplier thus fertilizing the tumor niche and thereby helping in tumor progression and resistance [1, 2, 63]. However, the effects of the efficient recycling of mitochondria (mitophagy) in tumor cells/micro milieu on their resistance against chemo- and radiotherapies have not been clearly understood.

The mitophagy related protein PARKIN has been identified as a p53 target gene and has been reported to prevent the Warburg effect by encouraging oxidative metabolism [87]. PARKIN has also been found to be deleted in numerous cancer conditions namely ovarian, lung, and breast cancer [89, 90]. Further, mice with severe PARKIN mutations have been found to be more vulnerable to spontaneous liver tumors [86, 87]. Mutations in other mitophagy related adaptor proteins like BNIP3 and NIX enhances tumor invasiveness and malignancies in lung, colorectal, hematologic, liver, and pancreatic cancers [91-97]. Thus, these studies suggest an inverse relationship between initiation, progression and resistance to the therapies vis-a-vis the mitophagy potential of tumors. In contrast, mitophagy has also been shown to be a tumor-promoting process which is supported by its ability to maintain a healthy mitochondrial pool required to fulfil the enhanced energy need of tumor cells [24, 98].

TUMOR ASSOCIATED MITOPHAGY AND AEROBIC GLYCOLYSIS

Although not well established, circumstantial evidences indicate a direct relationship between tumorigenesis and mitophagy [99, 100]. Similar to autophagy, mitophagy is also involved in maintaining functional (and thus energy generating) mitochondrion pool as well as nutrients for better cancer cell survival. A direct relationship between mitophagy and glycolysis is still lacking. Available evidences suggest that as functional mitochondria are a prerequisite for energy generation through glycolysis in a tumor cell (Warburg effect), mitophagy must add on to the survival and progression of tumorigenesis even during therapeutic stress [101, 102]. For instance, Ras oncogene positive tumors have been shown to activate mitophagy which is associated with enhanced glycolysis [103].

The impact of alterations in metabolic reprogramming and mitophagy in the tumor micro milieu has been recently explored. Many tumor cells appear to maintain their mitochondrial function of enhanced glycolysis *via* a complex mechanism wherein tumor cells indirectly derive energy from the neighbouring cells in the tumor microenvironment; the tumor stromal cells which exhibit a higher glycolytic phenotype i.e. Warburg Effect [4]. As a messenger, tumor cells generate enormous amounts of reactive oxygen species (ROS), which gets released into the tumor micro milieu. Tumor stromal cells gets influenced by this huge ROS supply, thus initiating the onset of stromal oxidative stress, autophagy and mitophagy due to the activation of key transcription factors, namely HIF1α (aerobic glycolysis) and NFkB (inflammation) [104-111]. Two types of mitochondria may exist in these stromal cells; those which are less robust and signal mitophagy initiation on sensing the ROS released into the micro environment followed by their altered membrane potential (Non-Warburgian), and those which are more robust and start L-lactate production after sensing oxidative stress (Warburgian). Mitophagic degradation of non-Warburgian mitochondria provides recycled products as well as raw materials for the Warburgian mitochondria to facilitate aerobic glycolysis and enhanced tumor stromal lactate production. This lactate produced by Warburgian mitochondria is released into the tumor microenvironment with the help of mono-carboxylate transporter 4 (MCT4) and MCT1 [112, 113]. In response to the nutrient (in form of lactate) released into the micro milieu, cancer cells exhibit 'reverse Warburg phenomena' where L-lactate functions as an onco-metabolite, stimulating mitochondrial biogenesis, glutaminolysis and OXPHOS in them, thereby directly providing energy for their growth and mitochondrial biogenesis [114]. In contrast, stromal cells have also been associated with tumor regression and tumor cell killing. Stromal cells of hematopoietic origin such as T cells, dendritic cells and NK cells have been found to suppress tumor progression and therefore projected as targets for developing anti-tumor therapeutics [115-118].

Cancer associated fibroblasts have also been shown to over express mitochondrial fission factor (MFF) which is considered as the prerequisite for mitophagy [119, 120]. The MFF over-expressing fibroblasts undergo oxidative stress with augmented ROS production and NF-kB activation, thus driving the onset of mitophagy and ultimately, glycolytic metabolism [120]. Similarly, MFF has been shown to promote a glycolytic phenotype *in vivo*, under conditions of hypoxia, where cancer associated

fibroblasts (MFF fibroblasts) become more glycolytic and display an efflux of high-energy mitochondrial fuels into the extracellular microenvironment which help drive mitochondrial biogenesis in cancer cells.

Mitophagy and glycolysis show strong interrelationship in stromal cells as well as cancer cells thereby promoting tumor cell survival even under adverse conditions of therapy [121]. Therefore, mitophagy appears to be a key quality control deciding the response of cancer cells to therapy and may thus be a potential target for adjuvant therapy.

THERAPEUTIC IMPLICATIONS OF TARGETING MITOPHAGY

Application of mitophagy inhibitors as primary or adjuvant tumor therapy has not yet been translated to the clinics. However, emerging knowledge suggests a potential for developing therapeutic strategies targeting mitophagy [122]. Inhibitors of glycolysis like 2-deoxy glucose (2-DG) and 3-bromopyruvate have been shown to selectively induce tumor cell death as well as enhance death induced by anticancer therapies like ionizing radiation and chemotherapeutic drugs [123-125]. However, a great deal of heterogeneity has been observed in both these effects among well-established tumor cell lines *in vitro*, animal tumors *in vivo* and clinical response [123, 126, 127]. This heterogeneity may be partly attributed to the presence of both Warburgian as well as non-Warburgian mitochondria in resistant tumors [4]. Mitophagy as well as enhanced glycolysis in these non-Warburgian mitochondria assists in providing nutrients to the Warburgian phenotype, thereby augmenting the tumor resistance. Thus, inhibition of mitophagy in combination with metabolic modifiers (like 2-deoxy-glucose, metformin etc.) can be a potential approach for improving the efficacy of radio- and chemotherapies (Figure **3**).

To what an extent variations in the treatment induced mitophagy (or autophagy) contributes to the heterogeneous responses observed in pre-clinical and clinical studies needs further investigations using genetically modified cell systems. Combinations of antioxidants like N- acetyl cystein and quercetin which can inhibit mitophagy as well as lactate production leading to the accumulation of more dysfunctional mitochondria ultimately driving the cell towards apoptosis could also be a potential strategy that requires systematic investigations [128, 129]. Furthermore, inhibitors of mitochondrial fission that inhibit mitophagy in stromal as well as tumor cells could also be potential adjuvants.

Mitophagy exhibits a double faceted role in tumorigenesis i.e. either survival-supporting or death-promoting [121, 128, 129]. Therefore, inducing prolonged or robust mitophagy using mitophagy modifiers along with the conventional anti-cancer therapies could also be explored as an anti-cancer strategy. Prolonged mitophagy in tumor cells would exhaust the metabolites required for sustaining the tumor growth ultimately leading to cell death. Induction of robust mitophagy using linamarase/linamarin/ glucose oxidase (lis/lin/GO) system leading to the loss of mitochondrial membrane potential and irreversible cell death of tumor cells has been reported recently. Similarly, induction of mitophagy by ceramide; and enhanced cell death of nasopharyngeal carcinoma (CNE2) during low-intensity ultrasound therapy in the presence of curcumin on induction of mitophagy further substantiate the potential of targeting robust or treatment induced prolonged mitophagy [130-132]. Various anticancer agents like ionophores and drugs which alter mitochondrial permeability transition pores (mPTPs) such as paclitaxel and doxorubicin that induce apoptosis, have been shown to enhance mitophagy and autophagy [133-135]. These observations suggest induction of mitophagy as an attractive anticancer approach. Further, administration of glycolytic inhibitors in combination with mitophagy inducing chemotherapies have been proposed to significantly enhance tumor cell death as a result of increased dependency of tumor cell on glycolysis following excessive mitophagy [5]. This also explains the enhanced efficacies of mPTP opening drugs when administered with glycolytic inhibitors like lonidamine (a hexokinase inhibitor) [136, 137]. Even though induction of prolonged or robust mitophagy appears reasonable, care must be taken as robust induction would depend upon the type and degree of stress. Moreover, the specificity of these approaches towards tumor cells needs to be investigated further.

The lack of specific biomarkers and understanding of the mitophagy associated tumor cell death is another hurdle that needs to be considered in order to make this strategy feasible in the clinics. Association of Glut-4 and over-expression of MCT as well as deletion in Caveolin-1 have been shown in resistant and aggressive tumors [138-140]. Since these are associated with reverse Warburgian phenotype as well as enhanced mitophagy, they may serve as markers for identifying tumors where mitophagy inhibitors could be useful in combination with other therapeutic agents. Since host factors also contribute to the responses of

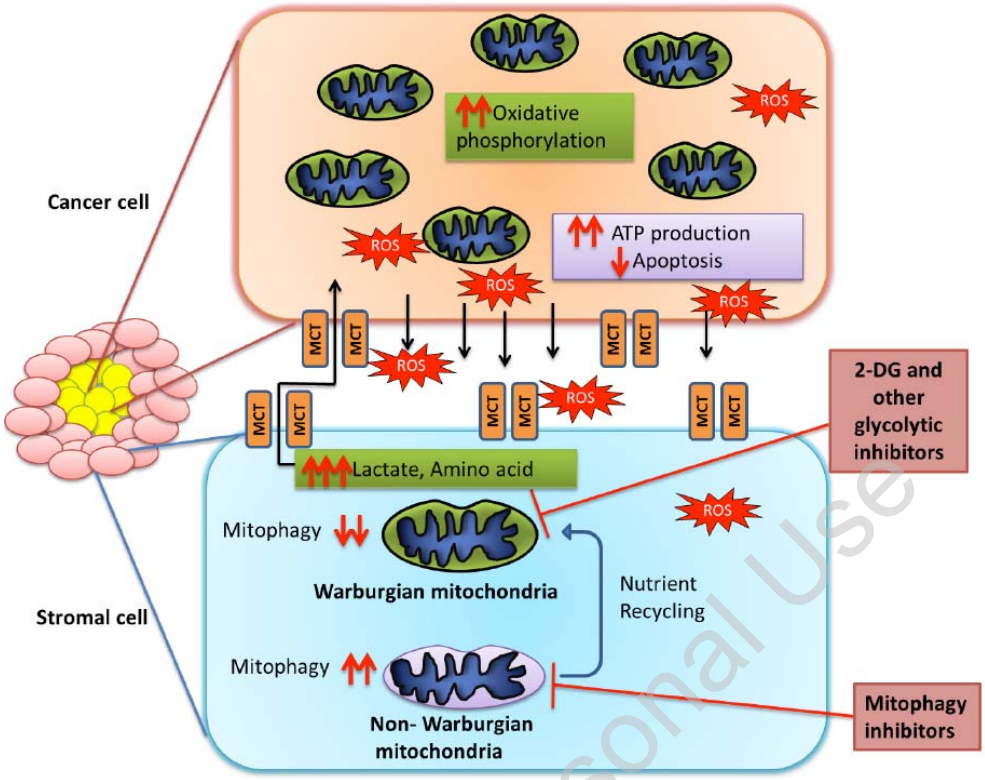


Figure 3: Host parasite relationship between stromal and cancer cells and the influence of metabolic reprogramming in tumorigenesis. The stromal cells (host) that display both Warburg & non-Warburgian mitochondria provide nutrition and biomass to the cancer cells (parasite) for their growth. Potential targets for developing therapeutics/adjuvant (with currently known inhibitors) are also shown.

tumors under *in vivo* conditions, identification (establishment) of appropriate surrogate markers will be helpful in individualizing therapies targeting mitophagy/autophagy for improving therapeutic gain.

ACKNOWLEDGEMENT

We thank Dr. RP Tripathi, Director INMAS for his constant encouragement. Work in author's laboratories is supported by grants from DRDO, Govt. of India (INM-311). Mr Shashank Misra and Ms Madhuri Chaurasia are recipients of fellowship from DST and ICMR, Govt. of India respectively.

REFERENCES

[1] Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, *et al*. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 2004; 304(5674): 1158-60. http://dx.doi.org/10.1126/science.1096284

[2] Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, *et al*. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392(6676): 605-8. http://dx.doi.org/10.1038/33416

[3] Warburg O. On the origin of cancer cells. Science 1956: 123(3191): 309-14. http://dx.doi.org/10.1126/science.123.3191.309

[4] Pavlides S, Vera I, Gandara R, Sneddon S, Pestell RG, Mercier I, *et al*. Warburg meets autophagy: cancer-associated fibroblasts accelerate tumor growth and metastasis *via* oxidative stress, mitophagy, and aerobic glycolysis. Antioxid Redox Signal 2012; 16(11): 1264-84. http://dx.doi.org/10.1089/ars.2011.4243

[5] Hughson LR, Poon VI, Spowart JE, Lum JJ. Implications of therapy-induced selective autophagy on tumor metabolism and survival. Int J Cell Biol 2012: 872091. http://dx.doi.org/10.1155/2012/872091

[6] Warburg O: The metabolism of tumors. Constable and Co., London 1930.

[7] Gogvadze V, Zhivotovsky B, Orrenius S. The Warburg effect and mitochondrial stability in cancer cells. Molecular Aspects of Medicine 2010; 31(1): 60-74. http://dx.doi.org/10.1016/j.mam.2009.12.004

[8] Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. Current Opinion in Genetics & Development 2008; 18(1): 5461. http://dx.doi.org/10.1016/j.gde.2008.02.003

[9] DeBerardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene 2010; 29(3): 313-24. http://dx.doi.org/10.1038/onc.2009.358

[10] Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. Cell 2008; 134(5): 703-7. http://dx.doi.org/10.1016/j.cell.2008.08.021

[11] Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. Genes Dev 2009; 23(5): 537-48. http://dx.doi.org/10.1101/gad.1756509

[12] Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic

requirements of cell proliferation. Science 2009; 324(5930): 1029-33.
http://dx.doi.org/10.1126/science.1160809

[13] Tong X, Zhao F, Thompson CB. The molecular determinants of de novo nucleotide biosynthesis in cancer cells. Current Opinion in Genetics & Development 2009; 19(1): 32-7.
http://dx.doi.org/10.1016/j.gde.2009.01.002

[14] Locasale JW, Grassian AR, Melman T, Lyssiotis CA, Mattaini KR, Bass AJ, *et al*. Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. Nature Genetics 2011; 43(9): 869-74.
http://dx.doi.org/10.1038/ng.890

[15] Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang JK, Shen M, *et al*. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. Science 2011; 334(6060): 1278-83.
http://dx.doi.org/10.1126/science.1211485

[16] Hamanaka RB, Chandel NS. Cell biology. Warburg effect and redox balance. Science 2011; 334(6060): 1219-20.
http://dx.doi.org/10.1126/science.1215637

[17] Weinhouse S. The Warburg hypothesis fifty years later. Z Krebsforsch Klin Onkol Cancer Res Clin Oncol 1976; 87(2): 115-26.
http://dx.doi.org/10.1007/BF00284370

[18] Darzynkiewicz Z, Staiano-Coico L, Melamed MR. Increased mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. Proc Natl Acad Sci U S A 1981; 78(4): 2383-7.
http://dx.doi.org/10.1073/pnas.78.4.2383

[19] Hedeskov CJ. Early effects of phytohaemagglutinin on glucose metabolism of normal human lymphocytes. The Biochemical Journal 1968; 110(2): 373-80.

[20] Wang T, Marquardt C, Foker J. Aerobic glycolysis during lymphocyte proliferation. Nature 1976; 261(5562): 702-5.
http://dx.doi.org/10.1038/261702a0

[21] Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annual Review of Cell and Developmental Biology 2011; 27: 441-64.
http://dx.doi.org/10.1146/annurev-cellbio-092910-154237

[22] Munyon WH, Merchant DJ. The relation between glucose utilization, lactic acid production and utilization and the growth cycle of L strain fibroblasts. Experimental Cell Research 1959; 17(3): 490-8.
http://dx.doi.org/10.1016/0014-4827(59)90069-2

[23] Fogal V, Richardson AD, Karmali PP, Scheffler IE, Smith JW, Ruoslahti E. Mitochondrial p32 protein is a critical regulator of tumor metabolism *via* maintenance of oxidative phosphorylation. Molecular and Cellular Biology 2010; 30(6): 1303-18.
http://dx.doi.org/10.1128/MCB.01101-09

[24] Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, *et al*. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev 2011; 25(5): 460-70.
http://dx.doi.org/10.1101/gad.2016311

[25] Exner N, Lutz AK, Haass C, Winklhofer KF. Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. EMBO J 2012; 31(14): 3038-62.
http://dx.doi.org/10.1038/emboj.2012.170

[26] Winklhofer KF, Haass C. Mitochondrial dysfunction in Parkinson's disease. Biochim Biophys Acta 2010; 1802(1): 29-44.
http://dx.doi.org/10.1016/j.bbadis.2009.08.013

[27] Schapira AH. Mitochondrial disease. Lancet 2006; 368(9529): 70-82.
http://dx.doi.org/10.1016/S0140-6736(06)68970-8

[28] Pieczenik SR, Neustadt J. Mitochondrial dysfunction and molecular pathways of disease. Experimental and Molecular Pathology 2007; 83(1): 84-92.
http://dx.doi.org/10.1016/j.yexmp.2006.09.008

[29] Boland ML, Chourasia AH, Macleod KF. Mitochondrial dysfunction in cancer. Front Oncol 2013; 3: 292.
http://dx.doi.org/10.3389/fonc.2013.00292

[30] King A, Selak MA, Gottlieb E. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. Oncogene 2006; 25(34): 4675-82.
http://dx.doi.org/10.1038/sj.onc.1209594

[31] Canter JA, Kallianpur AR, Parl FF, Millikan RC. Mitochondrial DNA G10398A polymorphism and invasive breast cancer in African-American women. Cancer Res 2005; 65(17): 8028-33.

[32] Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun CQ, Hall J, *et al*. mtDNA mutations increase tumorigenicity in prostate cancer. Proc Natl Acad Sci U S A 2005; 102(3): 719-24.
http://dx.doi.org/10.1073/pnas.0408894102

[33] Tomlinson IP, Alam NA, Rowan AJ, Barclay E, Jaeger EE, Kelsell D, *et al*. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nature Genetics 2002; 30(4): 406-10.
http://dx.doi.org/10.1038/ng849

[34] Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, *et al*. Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. Science 2000; 287(5454): 848-51.
http://dx.doi.org/10.1126/science.287.5454.848

[35] Sabharwal SS, Schumacker PT. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? Nature Reviews Cancer 2014; 14(11): 709-21.
http://dx.doi.org/10.1038/nrc3803

[36] Kroemer G. Mitochondria in cancer. Oncogene 2006; 25(34): 4630-2.
http://dx.doi.org/10.1038/sj.onc.1209589

[37] Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. Nature Reviews Cancer 2011; 11(5): 325-37.
http://dx.doi.org/10.1038/nrc3038

[38] Wallace DC. Mitochondria and cancer. Nature Reviews Cancer 2012; 12(10): 685-98.
http://dx.doi.org/10.1038/nrc3365

[39] Kurelac I, Romeo G, Gasparre G. Mitochondrial metabolism and cancer. Mitochondrion 2011; 11(4): 635-7.
http://dx.doi.org/10.1016/j.mito.2011.03.012

[40] Bardella C, Pollard PJ, Tomlinson I. SDH mutations in cancer. Biochim Biophys Acta 2011; 1807(11): 1432-43.
http://dx.doi.org/10.1016/j.bbabio.2011.07.003

[41] Wallace DC, Fan W. Energetics, epigenetics, mitochondrial genetics. Mitochondrion 2010; 10(1): 12-31.
http://dx.doi.org/10.1016/j.mito.2009.09.006

[42] Adam J, Hatipoglu E, O'Flaherty L, Ternette N, Sahgal N, Lockstone H, *et al*. Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. Cancer Cell 2011; 20(4): 524-37.
http://dx.doi.org/10.1016/j.ccr.2011.09.006

[43] Ooi A, Wong JC, Petillo D, Roossien D, Perrier-Trudova V, Whitten D, *et al*. An antioxidant response phenotype shared between hereditary and sporadic type 2 papillary renal cell carcinoma. Cancer Cell 2011; 20(4): 511-23.
http://dx.doi.org/10.1016/j.ccr.2011.08.024

[44] Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG. Reversible inactivation of the tumor suppressor PTEN by H2O2. J Biol Chem 2002; 277(23): 20336-42.
http://dx.doi.org/10.1074/jbc.M111899200

[45] Buhrman G, Parker B, Sohn J, Rudolph J, Mattos C. Structural mechanism of oxidative regulation of the phosphatase Cdc25B *via* an intramolecular disulfide bond. Biochemistry 2005; 44(14): 5307-16.
http://dx.doi.org/10.1021/bi047449f

[46] Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 2005; 120(5): 649-61. http://dx.doi.org/10.1016/j.cell.2004.12.041

[47] Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, *et al*. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem 2000; 275(33): 25130-8. http://dx.doi.org/10.1074/jbc.M001914200

[48] Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metabolism 2006; 3(3): 177-85. http://dx.doi.org/10.1016/j.cmet.2006.02.002

[49] Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metabolism 2006; 3(3): 187-97. http://dx.doi.org/10.1016/j.cmet.2006.01.012

[50] Choksi S, Lin Y, Pobezinskaya Y, Chen L, Park C, Morgan M, *et al*. A HIF-1 target, ATIA, protects cells from apoptosis by modulating the mitochondrial thioredoxin, TRX2. Molecular Cell 2011; 42(5): 597-609. http://dx.doi.org/10.1016/j.molcel.2011.03.030

[51] Hayes JD, McMahon M. NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. Trends in Biochemical Sciences 2009; 34(4): 176-88. http://dx.doi.org/10.1016/j.tibs.2008.12.008

[52] Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, *et al*. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. Cancer Cell 2012; 22(1): 66-79. http://dx.doi.org/10.1016/j.ccr.2012.05.016

[53] Roberts DJ, Tan-Sah VP, Smith JM, Miyamoto S. Akt phosphorylates HK-II at Thr-473 and increases mitochondrial HK-II association to protect cardiomyocytes. J Biol Chem 2013; 288(33): 23798-806. http://dx.doi.org/10.1074/jbc.M113.482026

[54] Zhang Y, Yang JM. Altered energy metabolism in cancer: a unique opportunity for therapeutic intervention. Cancer Biology & Therapy 2013; 14(2): 81-9. http://dx.doi.org/10.4161/cbt.22958

[55] Chen X, Qian Y, Wu S. The Warburg effect: evolving interpretations of an established concept. Free Radical Biology & Medicine 2015; 79: 253-63. http://dx.doi.org/10.1016/j.freeradbiomed.2014.08.027

[56] Verma A, Bhatt AN, Farooque A, Khanna S, Singh S, Dwarakanath BS. Calcium ionophore A23187 reveals calcium related cellular stress as "I-Bodies": an old actor in a new role. Cell Calcium 2011; 50(6): 510-22. http://dx.doi.org/10.1016/j.ceca.2011.08.007

[57] Serhan C, Anderson P, Goodman E, Dunham P, Weissmann G. Phosphatidate and oxidized fatty acids are calcium ionophores. Studies employing arsenazo III in liposomes. J Biol Chem 1981; 256(6): 2736-41.

[58] Griffiths EJ, Rutter GA. Mitochondrial calcium as a key regulator of mitochondrial ATP production in mammalian cells. Biochim Biophys Acta 2009; 1787(11): 1324-33. http://dx.doi.org/10.1016/j.bbabio.2009.01.019

[59] Ristow M, Schmeisser K. Mitohormesis: Promoting Health and Lifespan by Increased Levels of Reactive Oxygen Species (ROS). Dose Response 2014; 12(2): 288-341. http://dx.doi.org/10.2203/dose-response.13-035.Ristow

[60] Rimessi A, Bonora M, Marchi S, Patergnani S, Marobbio CM, Lasorsa FM, *et al*. Perturbed mitochondrial Ca2+ signals as causes or consequences of mitophagy induction. Autophagy 2013; 9(11): 1677-86. http://dx.doi.org/10.4161/auto.24795

[61] Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria in cancer cells: what is so special about them? Trends Cell Biol 2008; 18(4): 165-73. http://dx.doi.org/10.1016/j.tcb.2008.01.006

[62] McLelland GL, Soubannier V, Chen CX, McBride HM, Fon EA. Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control. Embo J 2014; 33(4): 282-95. http://dx.doi.org/10.1002/embj.201385902

[63] Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, *et al*. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 1999; 59(1): 59-65. http://dx.doi.org/10.1006/geno.1999.5851

[64] Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys 2007; 462(2): 245-53. http://dx.doi.org/10.1016/j.abb.2007.03.034

[65] Youle RJ, Narendra DP. Mechanisms of mitophagy. Nat Rev Mol Cell Biol 2011; 12(1): 9-14. http://dx.doi.org/10.1038/nrm3028

[66] Twig G, Hyde B, Shirihai OS. Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochim Biophys Acta 2008; 1777(9): 1092-7. http://dx.doi.org/10.1016/j.bbabio.2008.05.001

[67] Ehses S, Raschke I, Mancuso G, Bernacchia A, Geimer S, Tondera D, *et al*. Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. J Cell Biol 2009; 187(7): 1023-36. http://dx.doi.org/10.1083/jcb.200906084

[68] Head B, Griparic L, Amiri M, Gandre-Babbe S, van der Bliek AM. Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells. J Cell Biol 2009; 187(7): 959-66. http://dx.doi.org/10.1083/jcb.200906083

[69] Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, *et al*. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. Embo J 2008; 27(2): 433-46. http://dx.doi.org/10.1038/sj.emboj.7601963

[70] Graef M, Nunnari J. Mitochondria regulate autophagy by conserved signalling pathways. Embo J 2011; 30(11): 2101-14. http://dx.doi.org/10.1038/emboj.2011.104

[71] Gomes LC, Di Benedetto G, Scorrano L. During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. Nat Cell Biol 2011; 13(5): 589-98. http://dx.doi.org/10.1038/ncb2220

[72] Rambold AS, Kostelecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. Proc Natl Acad Sci U S A 2011; 108(25): 10190-5. http://dx.doi.org/10.1073/pnas.1107402108

[73] Liu L, Feng D, Chen G, Chen M, Zheng Q, Song P, *et al*. Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. Nat Cell Biol 2012; 14(2): 177-85. http://dx.doi.org/10.1038/ncb2422

[74] Zhang J, Ney PA. Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. Cell Death Differ 2009; 16(7): 939-46. http://dx.doi.org/10.1038/cdd.2009.16

[75] Saraste M. Oxidative phosphorylation at the fin de siecle. Science 1999; 283(5407): 1488-93. http://dx.doi.org/10.1126/science.283.5407.1488

[76] Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 2005; 39: 359-407. http://dx.doi.org/10.1146/annurev.genet.39.110304.095751

[77] Parsons MJ, Green DR. Mitochondria in cell death. Essays Biochem.2010; 47: 99-114. http://dx.doi.org/10.1042/bse0470099

[78] Chen Y, Dorn GW, 2nd. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science 2013; 340(6131): 471-5. http://dx.doi.org/10.1126/science.1231031

[79] Fu M, St-Pierre P, Shankar J, Wang PT, Joshi B, Nabi IR. Regulation of mitophagy by the Gp78 E3 ubiquitin ligase. Mol Biol Cell 2013; 24(8): 1153-62. http://dx.doi.org/10.1091/mbc.E12-08-0607

[80] Kanki T, Wang K, Baba M, Bartholomew CR, Lynch-Day MA, Du Z, *et al*. A genomic screen for yeast mutants defective in selective mitochondria autophagy. Mol Biol Cell 2009; 20(22): 4730-8. http://dx.doi.org/10.1091/mbc.E09-03-0225

[81] Welter E, Montino M, Reinhold R, Schlotterhose P, Krick R, Dudek J, *et al*. Uth1 is a mitochondrial inner membrane protein dispensable for post-log-phase and rapamycin-induced mitophagy. Febs J 2013; 280(20): 4970-82. http://dx.doi.org/10.1111/febs.12468

[82] Deas E, Plun-Favreau H, Gandhi S, Desmond H, Kjaer S, Loh SH, *et al*. PINK1 cleavage at position A103 by the mitochondrial protease PARL. Hum Mol Genet 2011; 20(5): 867-79. http://dx.doi.org/10.1093/hmg/ddq526

[83] Ivatt RM, Whitworth AJ. The many faces of mitophagy. EMBO Rep 2014; 15(1): 5-6. http://dx.doi.org/10.1002/embr.201338224

[84] Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, *et al*. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J Cell Biol 2010; 189(2): 211-21. http://dx.doi.org/10.1083/jcb.200910140

[85] Kongara S, Karantza V. The interplay between autophagy and ROS in tumorigenesis. Front Oncol 2012; 2: 171. http://dx.doi.org/10.3389/fonc.2012.00171

[86] Fujiwara M, Marusawa H, Wang HQ, Iwai A, Ikeuchi K, Imai Y, *et al*. Parkin as a tumor suppressor gene for hepatocellular carcinoma. Oncogene 2008; 27(46): 6002-11. http://dx.doi.org/10.1038/onc.2008.199

[87] Zhang C, Lin M, Wu R, Wang X, Yang B, Levine AJ, *et al*. Parkin, a p53 target gene, mediates the role of p53 in glucose metabolism and the Warburg effect. Proc Natl Acad Sci U S A 2011; 108(39): 16259-64. http://dx.doi.org/10.1073/pnas.1113884108

[88] Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, *et al*. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol 2010; 12(2): 119-31. http://dx.doi.org/10.1038/ncb2012

[89] Picchio MC, Martin ES, Cesari R, Calin GA, Yendamuri S, Kuroki T, *et al*. Alterations of the tumor suppressor gene Parkin in non-small cell lung cancer. Clin Cancer Res 2004; 10(8): 2720-4. http://dx.doi.org/10.1158/1078-0432.CCR-03-0086

[90] Cesari R, Martin ES, Calin GA, Pentimalli F, Bichi R, McAdams H, *et al*. Parkin, a gene implicated in autosomal recessive juvenile parkinsonism, is a candidate tumor suppressor gene on chromosome 6q25-q27. Proc Natl Acad Sci U S A 2003; 100(10): 5956-61. http://dx.doi.org/10.1073/pnas.0931262100

[91] Koop EA, van Laar T, van Wichen DF, de Weger RA, Wall E, van Diest PJ. Expression of BNIP3 in invasive breast cancer: correlations with the hypoxic response and clinicopathological features. BMC Cancer 2009; 9: 175. http://dx.doi.org/10.1186/1471-2407-9-175

[92] Okami J, Simeone DM, Logsdon CD. Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer Res 2004; 64(15): 5338-46. http://dx.doi.org/10.1158/0008-5472.CAN-04-0089

[93] Sowter HM, Ferguson M, Pym C, Watson P, Fox SB, Han C, *et al*. Expression of the cell death genes BNip3 and NIX in ductal carcinoma in situ of the breast; correlation of BNip3 levels with necrosis and grade. J Pathol 2003; 201(4): 573-80. http://dx.doi.org/10.1002/path.1486

[94] Tan EY, Campo L, Han C, Turley H, Pezzella F, Gatter KC, *et al*. BNIP3 as a progression marker in primary human breast cancer; opposing functions in in situ versus invasive cancer. Clin Cancer Res 2007; 13(2 Pt 1): 467-74. http://dx.doi.org/10.1158/1078-0432.CCR-06-1466

[95] Abe T, Toyota M, Suzuki H, Murai M, Akino K, Ueno M, *et al*. Upregulation of BNIP3 by 5-aza-2'-deoxycytidine sensitizes pancreatic cancer cells to hypoxia-mediated cell death. J Gastroenterol 2005; 40(5): 504-10. http://dx.doi.org/10.1007/s00535-005-1576-1

[96] Castro M, Grau L, Puerta P, Gimenez L, Venditti J, Quadrelli S, *et al*. Multiplexed methylation profiles of tumor suppressor genes and clinical outcome in lung cancer. J Transl Med 2010; 8: 86. http://dx.doi.org/10.1186/1479-5876-8-86

[97] Murai M, Toyota M, Suzuki H, Satoh A, Sasaki Y, Akino K, *et al*. Aberrant methylation and silencing of the BNIP3 gene in colorectal and gastric cancer. Clin Cancer Res 2005; 11(3): 1021-7.

[98] Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM, *et al*. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. Genes Dev 2013; 27(13): 1447-61. http://dx.doi.org/10.1101/gad.219642.113

[99] Fujiwara M, Marusawa H, Wang HQ, Iwai A, Ikeuchi K, Imai Y, *et al*. Parkin as a tumor suppressor gene for hepatocellular carcinoma. Oncogene 2008; 27(46): 6002-11. http://dx.doi.org/10.1038/onc.2008.199

[100] Lu H, Li G, Liu L, Feng L, Wang X, Jin H. Regulation and function of mitophagy in development and cancer. Autophagy 2013; 9(11): 1720-36. http://dx.doi.org/10.4161/auto.26550

[101] Chatterjee A, Mambo E, Sidransky D. Mitochondrial DNA mutations in human cancer. Oncogene 2006; 25(34): 4663-74. http://dx.doi.org/10.1038/sj.onc.1209604

[102] Rosenfeldt MT, O'Prey J, Morton JP, Nixon C, MacKay G, Mrowinska A, *et al*. p53 status determines the role of autophagy in pancreatic tumour development. Nature 2013; 504(7479): 296-300. http://dx.doi.org/10.1038/nature12865

[103] Kim JH, Kim HY, Lee YK, Yoon YS, Xu WG, Yoon JK, *et al*. Involvement of mitophagy in oncogenic K-Ras-induced transformation: overcoming a cellular energy deficit from glucose deficiency. Autophagy 2011; 7(10): 1187-98. http://dx.doi.org/10.4161/auto.7.10.16643

[104] Casey TM, Eneman J, Crocker A, White J, Tessitore J, Stanley M, *et al*. Cancer associated fibroblasts stimulated by transforming growth factor beta1 (TGF-beta 1) increase invasion rate of tumor cells: a population study. Breast Cancer Res Treat 2008; 110(1): 39-49. http://dx.doi.org/10.1007/s10549-007-9684-7

[105] Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 1993; 122(1): 103-11. http://dx.doi.org/10.1083/jcb.122.1.103

[106] Direkze NC, Hodivala-Dilke K, Jeffery R, Hunt T, Poulsom R, Oukrif D, *et al*. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. Cancer Res 2004; 64(23): 8492-5.
http://dx.doi.org/10.1158/0008-5472.CAN-04-1708

[107] Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, *et al*. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proc Natl Acad Sci U S A 2010; 107(46): 20009-14.
http://dx.doi.org/10.1073/pnas.1013805107

[108] Martinez-Outschoorn UE, Pavlides S, Whitaker-Menezes D, Daumer KM, Milliman JN, Chiavarina B, *et al*. Tumor cells induce the cancer associated fibroblast phenotype *via* caveolin-1 degradation: implications for breast cancer and DCIS therapy with autophagy inhibitors. Cell Cycle 2010; 9(12): 2423-33.
http://dx.doi.org/10.4161/cc.9.12.12048

[109] Mishra PJ, Mishra PJ, Humeniuk R, Medina DJ, Alexe G, Mesirov JP, *et al*. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. Cancer Res 2008; 68(11): 4331-9.
http://dx.doi.org/10.1158/0008-5472.CAN-08-0943

[110] Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, *et al*. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005; 121(3): 335-48.
http://dx.doi.org/10.1016/j.cell.2005.02.034

[111] Waghray M, Cui Z, Horowitz JC, Subramanian IM, Martinez FJ, Toews GB, *et al*. Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts. Faseb J 2005; 19(7): 854-6.
http://dx.doi.org/10.1096/fj.04-2882fje

[112] Whitaker-Menezes D, Martinez-Outschoorn UE, Lin Z, Ertel A, Flomenberg N, Witkiewicz AK, *et al*. Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts. Cell Cycle 2011; 10(11): 1772-83.
http://dx.doi.org/10.4161/cc.10.11.15659

[113] Pinheiro C, Longatto-Filho A, Scapulatempo C, Ferreira L, Martins S, Pellerin L, *et al*. Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas. Virchows Arch 2008; 452(2): 139-46.
http://dx.doi.org/10.1007/s00428-007-0558-5

[114] Sharma K L, Tiwari M, Mishra K S. Mitochondrial Alteration: A Major Player in Carcinogenesis. Cell Biology 2015; 3(2-1): 8-16.

[115] Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, *et al*. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011; 29(15): 1949-55.
http://dx.doi.org/10.1200/JCO.2010.30.5037

[116] Lou Y, Liu C, Kim GJ, Liu YJ, Hwu P, Wang G. Plasmacytoid dendritic cells synergize with myeloid dendritic cells in the induction of antigen-specific antitumor immune responses. J Immunol 2007; 178(3): 1534-41.
http://dx.doi.org/10.4049/jimmunol.178.3.1534

[117] Kundu N, Ma X, Holt D, Goloubeva O, Ostrand-Rosenberg S, Fulton AM. Antagonism of the prostaglandin E receptor EP4 inhibits metastasis and enhances NK function. Breast Cancer Res Treat 2009; 117(2): 235-42.
http://dx.doi.org/10.1007/s10549-008-0180-5

[118] Moretta L, Pietra G, Montaldo E, Vacca P, Pende D, Falco M, *et al*. Human NK cells: from surface receptors to the therapy of leukemias and solid tumors. Front Immunol 2014; 5: 87.
http://dx.doi.org/10.3389/fimmu.2014.00087

[119] Otera H, Wang C, Cleland MM, Setoguchi K, Yokota S, Youle RJ, *et al*. Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol 2010; 191(6): 1141-58.
http://dx.doi.org/10.1083/jcb.201007152

[120] Guido C, Whitaker-Menezes D, Lin Z, Pestell RG, Howell A, Zimmers TA, *et al*. Mitochondrial fission induces glycolytic reprogramming in cancer-associated myofibroblasts, driving stromal lactate production, and early tumor growth. Oncotarget 2010; 3(8): 798-810.

[121] Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. Circ Res 2012; 111(9): 1208-21.
http://dx.doi.org/10.1161/CIRCRESAHA.112.265819

[122] Chourasia AH, Boland ML, Macleod KF. Mitophagy and cancer. Cancer Metab 2015; 3: 4.
http://dx.doi.org/10.1186/s40170-015-0130-8

[123] Dwaraknath BS. Cytotoxicity, radiosensitization and chemosensitization of tumor cells by 2-deoxy-D-glucose *in vitro*. J. Cancer Res Ther.2009; 5: S27-S31.
http://dx.doi.org/10.4103/0973-1482.55137

[124] Dwarakanath BS and Jain V. Targeting glucose metabolism with 2-deoxy-D-glucose for improving cancer therapy (Invited Editorial). Future Oncol 2009; 5**:** 581-585.
http://dx.doi.org/10.2217/fon.09.44

[125] Jain V: Modifications of radiation responses by 2-deoxy-D-glucose in normal and cancer cells. Ind J Nucl Med 1996; 11, 8-17

[126] Dwarakanath BS, Zolzer F, Chandna S, Bauch T, Adhikari JS, Muller WU, Streffer C and Jain V: Heterogeneity in 2-deoxy-D-glucose induced modifications in energetic and radiation responses of human tumor cell lines. Int. J. Radiation Oncology Biology Phys 2001; 51, 1151-1161.
http://dx.doi.org/10.1016/S0360-3016(01)01534-6

[127] Mohanti BK, Rath GK, Anantha N *et al.*: Improving cancer radiotherapy with 2-deoxy-D-glucose: phase I/II clinical trials on human cerebral gliomas. Int. J. Radiat. Oncol. Biol. Phys 1996; 35: 103-11.
http://dx.doi.org/10.1016/S0360-3016(96)85017-6

[128] Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, *et al*. HIF-dependent antitumorigenic effect of antioxidants *in vivo*. Cancer Cell 2007; 12(3): 230-8.
http://dx.doi.org/10.1016/j.ccr.2007.08.004

[129] Ma Q, Cavallin LE, Yan B, Zhu S, Duran EM, Wang H, *et al*. Antitumorigenesis of antioxidants in a transgenic Rac1 model of Kaposi's sarcoma. Proc Natl Acad Sci U S A 2009; 106(21): 8683-8.
http://dx.doi.org/10.1073/pnas.0812688106

[130] Gargini R, Garcia-Escudero V, Izquierdo M. Therapy mediated by mitophagy abrogates tumor progression. Autophagy 2011; 7(5): 466-76.
http://dx.doi.org/10.4161/auto.7.5.14731

[131] Saddoughi SA, Ogretmen B. Diverse functions of ceramide in cancer cell death and proliferation. Advances in cancer research 2013; 117: 37-58.
http://dx.doi.org/10.1016/B978-0-12-394274-6.00002-9

[132] Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 2008; 183(5): 795-803.
http://dx.doi.org/10.1083/jcb.200809125

[133] Ligeret H, Barthelemy S, Bouchard Doulakas G, Carrupt PA, Tillement JP, Labidalle S, *et al*. Fluoride curcumin derivatives: new mitochondrial uncoupling agents. FEBS Lett 2004; 569(1-3): 37-42.
http://dx.doi.org/10.1016/j.febslet.2004.05.032

[134] Galluzzi L, Larochette N, Zamzami N, Kroemer G. Mitochondria as therapeutic targets for cancer chemotherapy. Oncogene 2006; 25(34): 4812-30.
http://dx.doi.org/10.1038/sj.onc.1209598

[135] Milane L, Duan Z, Amiji M. Therapeutic efficacy and safety of paclitaxel/lonidamine loaded EGFR-targeted nanoparticles

for the treatment of multi-drug resistant cancer. PLoS One 2011; 6(9): e24075.
http://dx.doi.org/10.1371/journal.pone.0024075

[136] Floridi A, Bruno T, Miccadei S, Fanciulli M, Federico A, Paggi MG. Enhancement of doxorubicin content by the antitumor drug lonidamine in resistant Ehrlich ascites tumor cells through modulation of energy metabolism. Biochem Pharmacol 1998; 56(7): 841-9.
http://dx.doi.org/10.1016/S0006-2952(98)00054-9

[137] Wang X, Leung AW, Luo J, Xu C. TEM observation of ultrasound-induced mitophagy in nasopharyngeal carcinoma cells in the presence of curcumin. Experimental and therapeutic medicine 2012; 3(1): 146-8.

[138] Pavlides S, Tsirigos A, Vera I, Flomenberg N, Frank PG, Casimiro MC, *et al*. Loss of stromal caveolin-1 leads to oxidative stress, mimics hypoxia and drives inflammation in the tumor microenvironment, conferring the "reverse Warburg effect": a transcriptional informatics analysis with validation. Cell Cycle 2010; 9(11): 2201-19.
http://dx.doi.org/10.4161/cc.9.11.11848

[139] Sotgia F, Martinez-Outschoorn UE, Pavlides S, Howell A, Pestell RG, Lisanti MP. Understanding the Warburg effect and the prognostic value of stromal caveolin-1 as a marker of a lethal tumor microenvironment. Breast Cancer Res 2011; 13(4): 213.
http://dx.doi.org/10.1186/bcr2892

[140] Witkiewicz AK, Whitaker-Menezes D, Dasgupta A, Philp NJ, Lin Z, Gandara R, *et al*. Using the "reverse Warburg effect" to identify high-risk breast cancer patients: stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers. Cell Cycle 2012; 11(6): 1108-17.
http://dx.doi.org/10.4161/cc.11.6.19530

Received on 20-04-2015 Accepted on 05-04-2015 Published on 12-05-2015

DOI: http://dx.doi.org/10.6000/1929-2279.2015.04.02.8