Characterisation of the role of autophagy in DNA damage repair

By Emma Yu Liu

Beatson Institute

Cancer Research UK

Institute of Cancer Sciences

Garscube Estate, Switchback Road

Glasgow G61 1BD UK

Abstract:

Autophagy is an evolutionarily conserved process that is important for the maintenance of cellular homeostasis and also genomic integrity. Autophagy is a self-digestive process that takes place in the cytoplasm, however recent studies by Eileen White and colleagues demonstrated that defective autophagy leads to accumulation of DNA damage in vitro and in vivo [1] [2]. This discovery leads to a question: whether there is increased DNA damage incidences or defective DNA damage repair in autophagy deficient cells. Autophagy and DNA damage are two important areas for cancer research. The aim of this project is to provide a better understanding of the role of autophagy plays in DNA damage and DNA damage response.

The activation of Chk1 facilitates its degradation. The results presented in this project illustrate that autophagy deficient cells exhibit elevated proteasomal activities and autophagy inhibition leads to activation of Chk1. These combined factors contribute to increased degradation of Chk1 in autophagy deficient cells. This was manifested first as decreased phospho-Chk1 in response to DNA damage, later on when the loss of autophagy effect is more pronounced; decrease in total Chk1 protein level was observed. Chk1 is a crucial DNA damage response mediator that plays roles in cell cycle checkpoints and DNA damage repair. Cells without autophagy appear to have intact cell cycle checkpoints in response to starvation or DNA damaging agents; however they show deficiency in homologous recombination (HR) repair pathways. Autophagy deficient cells display increased spontaneous cell death and formation of micronuclei. Defective HR pathways in autophagy deficient cells lead to hyper-dependency on non-homologous end-joining (NHEJ) process. Since HR and NHEJ are the two main ways of repairing double strand breaks (DSB), it is not surprising that inhibition of NHEJ following DSB inducing agents in autophagy deficient cells results in persistence of damage lesions and increased cell death.

This project demonstrated that loss or inhibition of autophagy leads to defective DNA damage response pathways. We established that Chk1 is de-regulated in autophagy deficient cells and this has differential downstream effects on DNA damage response.

These findings potentially provide a novel synthetic lethal strategy to selectively kill autophagy-deficient cells, which are implicated in a number of diseases including certain cancers.

Table of contents

List of figures

List of tables

Acknowledgement

My heartfelt appreciation goes to my supervisor Prof. Kevin Ryan whose enormous support and insightful comments were invaluable during the course of my study.

I feel very privileged to have the opportunity to work in a wonderful lab where I have received everyday assistance and useful discussions from everyone. I'm grateful that Jim O'Prey, Jaclyn Long and Sanket Joshi who helped me throughout my study and proofread the manuscript.

I want to thank Prof. David Gillespie and Naihan Xu for many useful discussions throughout the project.

I would also like to express my gratitude to my family and my friends for their moral support and warm encouragements. I'll miss my commute buddy @Ericuk and lunch buddy @zjuliaw and thank you for your company and friendship. I would like to express appreciation to my wonderful partner Marcus, and my parents who have always been supportive and patient with me, backing me for all the decisions I have made. I simply can not believe how lucky I am having you all as part of my Phd.

I would like to acknowledge Dr Steven Tait and and Dr Paolo Salomoni for their valuable advice and constructive comments to the manuscript.

Finally, I would like to acknowledge The Beatson Institute and Cancer Research UK for the funding that made it possible to complete this study.

Author’s declaration

Unless otherwise stated, I declare that all the work presented in this thesis is my own.

Abbreviations

3-MA – 3‑Methyladenine

AMPK – AMP-Activated Protein Kinase

AMBRA - autophagy/beclin-1 regulator 1

Atg– autophagy related gene

AT - ataxia-telangiectasia

ATM - ataxia-telangiectasia-mutated

ATP - Adenosine triphosphate

ATR - ataxia and rad3 related

ATRIP - ATR-Interacting Protein

BARKOR (Beclin 1-associated autophagy-related key regulator)

Bcl-2 – B Cell Lymphoma 2

BH3 - Bcl-2 homology 3

BER - base excision repair

BNIP3 - Bcl-2/adenovirus E1B 19-kDa interacting protein 3

BrdU - 5-bromo-2′deoxyuridine

CDK - Cyclin depedant kinase

Chk1 and Chk2 – Check Point 1 and 2

CQ – Chloroquine

DDR - DNA damage response

DMSO – dimethylsulphoxide

DRAM - damage-regulated autophagy modulator

DSB – Double strand breaks

ECM – Extracellular Matrix

EDU - 5-ethynyl-2´-deoxyuridine

EGF - Epidermal growth factor

ER - Endoplasmic Reticulum

FAK200 - Focal adhesion kinase family-interacting protein

FCS - fetal calf serum

FIP200 - 200 kDa FAK-family interacting protein

GFP -Green Fluorescence Protein

HBS - HEPES buffered solution

HCQ – hydroxylchloroquine

HCl – hydrochloric acid

HR – Homologous recombination

IR - ionising irradiation

Keap1 - kelch-like ECH-associated protein 1

LAMP2 - lysosome-associated membrane protein

LB - L-Broth

Mcl1 - myeloid leukemia cell differentiation protein

MEF – Mouse embryonic fibroblast

MHC II - (major histocompatibility complex class II)

MMR - DNA mismatched repair

MRN - MRE11, Rad50 and NBS1

MS - multiple sclerosis

mTOR – Mammalian Target of Rapamycin

mTORC1- mTOR complex 1

NER - nucleotide excision repair

NHEJ – Non-homologous end joining

NRF2 - nuclear factor-erythroid 2-related factor-2

PARP - poly ADP ribose polymerase

PBS - Phosphate Buffered Saline

PDGF - platelet-derived growth factor

PE – phosphatidylethanolamine

PE buffer – PBS-EDTA buffer

PFA - paraformaldehyde

pH3 – phospho – Histone H3

PI (chemical) – Propidium Iodide

PI (biomolecule) - phosphoinositide

PI3K - phosphoinositide-3-kinase

PI3P - phosphatidylinositol 3-phosphate

PINK1 - PTEN-induced putative kinase protein 1

PIP3 - phosphatidylinositol-3,4,5-trisphosphate

RIPA - radio immunoprecipitation assay buffer

ROS – Reactive oxygen species

RPA – Replication protein A

qRT-PCR - Quantitative Real Time Polymerase chain reaction

RTK – Receptor Tyrosine Kinase

SCID – severe combined immunodeficiency

TAE - Tris-acetate-EDTA

TE – Tris-EDTA

TBS - Tris-Buffered Saline

TRAIL - tumor necrosis factor-related apoptosis-inducing ligand

TSC2 –tuberous sclerosis 2

mTOR kinase - TOR, mammalian target of rapamycin

ULK - Unc-51-like kinases

UV - ultraviolet

UVRAG - UV-irradiation-resistance-associated gene

VEGF - Vascular endothelial growth factor

VPS15 - vacuolar protein sorting 15

VPS34- vacuolar protein sorting 34

XP - xeroderma pigmentosum

Chapter 1. Introduction

1.1 Autophagy – Definition and Classification.

Autophagy is a fundamental catabolic process that is well conserved from yeast to higher eukaryotic cells. It is a self-digestive mechanism where metabolites are recycled in the cells. During the process, cellular proteins, organelles (e.g. mitochondria and endoplasmic reticulum (ER) membranes) and cytoplasm are sequestered and degraded in the lysosomes by hydrolases.

Autophagy is constitutively active at basal levels in the cells; it is thought to degrade long-lived proteins and organelles in bulk as a mean of recycling cellular building blocks and maintaining cellular homeostasis and integrity. Autophagic activities are up-regulated when the cells undergo metabolic stress such as starvation and hypoxia [3]. In this case it plays the role of an adaptive survival mechanism; autophagy acts as a 'cannibalistic' process that fuels cell bioenergetics through self-digestion and recycling of cellular contents. Because of its self-eating nature, autophagy mediated survival is temporary and provides an opportunity for the cells to deal with the stressful environment. Prolonged activation of autophagy due to continuous metabolic stress can contribute towards cell death [4]. It is thought that autophagy is an accelerator rather than effector in programmed cell death under normal physiological conditions. Dying cells very often display accumulation of autophagosomes and autophagy is considered to be an important mediator of the clearance of cell corpses [5]. In cancer cells, there is evidence that autophagy can act as a cell death mechanism, alternative to apoptosis [6]. It is well acknowledged that defective apoptosis is one of the major transformations during tumourigensis [7], therefore autophagic cell death can be potentially utilised to eliminate tumour cells. In fact, Autophagy is required for a number of cancer therapeutic drugs to achieve sufficient killing [8] [9].

The relationships between apoptosis and autophagy is complex and the two processes frequently overlap each other. ATG1 overexpression in *Drosophila* leads to cell death which display apoptotic characteristics [10]. Autophagy has been found to be a apoptosis-independent cell death mechanism in *Dictyostelium*, which does not have genes that encode proteins in the apoptotic pathways [11].

Three main types of autophagy have been described; they are macroautophagy, microautophagy, and chaperone-mediated autophagy. The three processes share common molecular machinery to a certain degree, the protein/organelle cargoes are degraded in the lysosomes for all three types. The three processes are well coordinated and complement each other's functions to maintain cellular homeostasis [12] [13]. Macroautophagy (hereafter autophagy) is the most characterized process which degrades long-lived proteins and removes damaged organelles. The formation of autophagosomes that contain bulk proteins and organelles distinguishes this type of autophagy from other autophagic processes. Microautophagy is a process where cytoplasm is directly engulfed by the lysosome through invagination [14]. Chaperone-mediated autophagy is able to degrade cytosolic proteins selectively. Specific substrate proteins containing the amino acid motif KFERQ are recognised by the hsc70 chaperone in the cytoplasm [15]. Hsc70 and its substrate protein bind to lysosome-associated membrane protein type 2A (LAMP-2A) which is localized on the lysosomal membrane. The substrate protein is unfolded by hsc70 and translocated into lysosomes where it is degraded [16]. Selective autophagy is a process where specific organelles, pathogens and proteins are post tranlationsl modified and recognised by adaptor proteins such as p62 and NBR1, which bind to autophagosome proteins including LC3. The cargo is tethered to the site of autophagosome and engulfed into the vesicle [17] .

1.2 The process of autophagy

Autophagy is a highly dynamic process where a series of membrane-trafficking events take place. As illustrated in Figure 1.1, the autophagy process consists of

four major steps, namely initiation, elongation, maturation and fusion. mTOR (mammalian target of Rapamycin) is an inhibitory regulator of autophagy in response to a number of stimuli, phosphorylating and inhibiting the ULK complex. The ULK complex activates Beclin 1, which is the key component of a complex which generates double membranes to form phagophores. Phagophores grow to enclose cellular contents in the cytoplasm such as proteins and organelles and fuse at both ends forming vesicular autophagsosomes. Autophagy is completed when autophagosomes fuse with lysosomes forming autolysosomes where the contents are degraded.

Autophagy is a housekeeping process that takes place in nearly all cell types, and is up-regulated by a range of stimuli, such as starvation, hypoxia and DNA damage. These signalling pathways are described as follows.

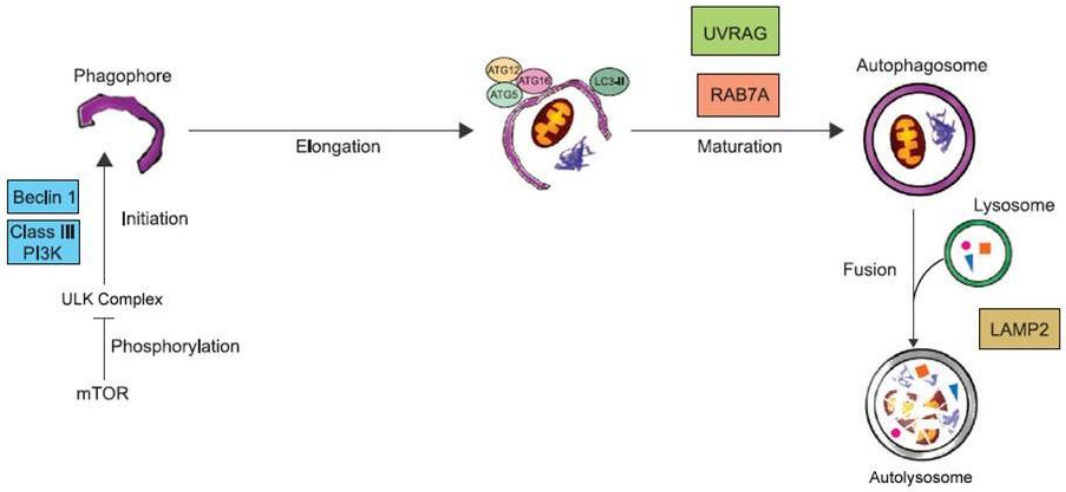


Figure 1.1 Autophagy is a multi-step process.

(Figure adapted from Liu and Ryan, 2012.) Double membraned phagophores elongate to enclose cytoplasm and organelles into vesicles called autophagosomes, which are eventually fused with lysosomes where the contents are degraded. mTOR kinase is a sensor initiating autophagy in response to growth factors and nutrients. Double membraned structures called phagophores elongate to enclose cellular contents and fuse to form autophagosomes. Autophagosomes are trafficked to fuse with lysosomes which contain acidic hydrolases that degrade the contents of autophagosomes.

1.2.1 Autophagy levels are modulated in response to a variety of stimuli in mammalian cells.

mTOR kinase [18] is a key inhibitory regulator of autophagy in response to a spectrum of signals including growth factors (e.g. insulin-like factors, PDGF (platelet-derived growth factor), VEGF (vascular endothelial growth factor) and EGF (epidermal growth factor)), nutrients (e.g. amino acids and glucose), energy (ATP) and oxygen as demonstrated in figure 1.2. When there are sufficient nutrients in the cells, autophagy is kept at low levels. mTOR constitutively inhibits the initiation of autophagy through the phosphorylation of the Atg13-ULK-FIP200 complex. Autophagy can be regulated in both mTOR dependent or independent manners, depending on the stimuli, as illustrated in Figure 1.2. Besides autophagy, mTOR is also a master regulator of protein synthesis, cell cycle control and cell proliferation, in accordance with nutrient availability [19]. mTOR kinase exits in two structurally and functionally distinct complexes mTORC1 and mTORC2. mTOR signalling pathways are frequently hyper-activated in human cancers and deregulated in metabolic diseases [20].

In recent years, it was discovered that nutrient-availability signals are relayed to mTORC1 by different pathways. AMPK mediates mTORC activities in response to glucose availability [21] and Class III PI3K regulates mTORC according to the availability of amino acids and growth factors [22].

Growth factors are molecules secreted by cells, which stimulate the proliferation and/or differentiation of the secreting cells by autocrine signalling, or stimulate neighbouring cells by paracrine interactions. Growth factors bind to receptor tyrosine kinases (RTKs) on the cell surface and in turn PI(3)K is recruited to the cell membrane and activated generating PIP3 (phosphatidylinositol-3,4,5-trisphosphate). This leads to a signalling cascade at the membrane. Akt, Rheb and mTORC1 complex are in turn activated [23].

The concentrations of amino acids are the dominating signals for the activation of the multi-protein complex mTORC1 [24]. During the final steps of autophagy, autophagosomes containing proteins and organelles fuse with lysosomes where the macromolecules are broken down to small basic parts. Proteins are digested into amino acids by the acidic hydrolases. Recent studies have demonstrated that mTORC1 can localise to the outer surfaces of lysosomes and sense amino acids within the vesicles [25]. mTOR lysosomal activation pathways are conserved from yeast to mammalian cells [26]. The pathway is regulated by Rag GTPases, GATOR1 and GATOR2 complexes [27] [28]. Since mTOR is a key inhibitory regulator of autophagy, the cells may utilise this mechanism to lower autophagic activity in response to lysosomal amino acids.

Lysosomes have been thought to be a cellular factory of degradation and recycling; in recent years, works from Sabatini and Ballabio's labs have demonstrated that lysosomal surfaces are also a signalling hub regulating mTOR and autophagy activities [27] [29]. In addition, the positioning of lysosomes is regulated by nutrient availability and this also plays a role in mTORC1 signalling and autophagy activation [30]. mTOR is the best characterised nutrient sensing autophagy regulator; other mTOR independent amino acid signalling pathways that negatively regulate autophagy have also been reported [31] [32]. The molecular details of these mTOR-independent pathways remain to be explored.

Glucose is the primary energy source for mammalian cells [33] and autophagy activities are modulated in accordance with glucose availability. AMPK is activated when the cells are under conditions of glucose starvation. AMPK activates autophagy through two mechanisms - it activates the ULK1 complex through phosphorylation and it inactivates mTORC1 by phosphorylating Raptor and TSC2 (tuberous sclerosis 2) [34]. The activation of the ULK1 complex marks the initiation of autophagy (Chapter 1.2.2), which promotes cell survival in the presence of energy stress.

Besides nutrient availability as outlined above, autophagy can also be up-regulated by genotoxic stress such as DNA damage and reactive oxygen species (ROS) [35]. The p53 tumour suppressor plays important roles in cell cycle regulation, DNA damage responses and programmed cell death. It is reported that p53 is lost in over 50% of cancers [36]. Nuclear p53 and cytosolic p53 have been found to play opposing roles in autophagy regulation. In the nucleus, p53 plays the role of a transcription factor [37], and in the presence of genotoxic stress, p53 can activate a number of autophagy genes including AMPK, ULK1 and ULK 2 [38] [39] [40]. Nuclear p53 also activates damage-regulated autophagy modulator 1 (DRAM1) [41], which consists of multiple splice variants that regulate autophagy [42]. The exact mechanisms through which DRAM1 modulates autophagy remain to be elucidated. In contrast, cytoplasmic p53 mainly inhibits autophagy [37]. Moreover, p73 is a tumour suppressor transcription factor that belongs to the p53 protein family. p73 can also induce autophagy, in a DRAM-independent way [43].

Other autophagy-modulating molecules include Bcl-2 [44] and BNIP3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3) [45]. These are also frequently involved in cancer. Bcl-2 protein is an important regulator of programmed cell death, inhibiting autophagy indirectly through its interaction with Beclin-1, a protein essential for autophagy initiation, through BH3 (Bcl-2 homology 3) domains. Bcl-2, as an anti-apoptotic protein, is considered to modulate autophagy levels to prevent over-eating of the cells and cell death [44]. Autophagy is also activated in response to hypoxia. BNIP3 and BNIP3L are two essential elements for hypoxia-induced autophagy; they contain BH3 domains that disrupt the interactions between Beclin-1 and Bcl-2 [46]. Hypoxia induced autophagy is thought to be a pro-survival mechanism utilized by cancer cells.

To summarise as shown in Fig. 1.2, autophagy is constitutively kept at low levels when there are sufficient nutrient signals and can be activated by energy stress or genotoxic stress.

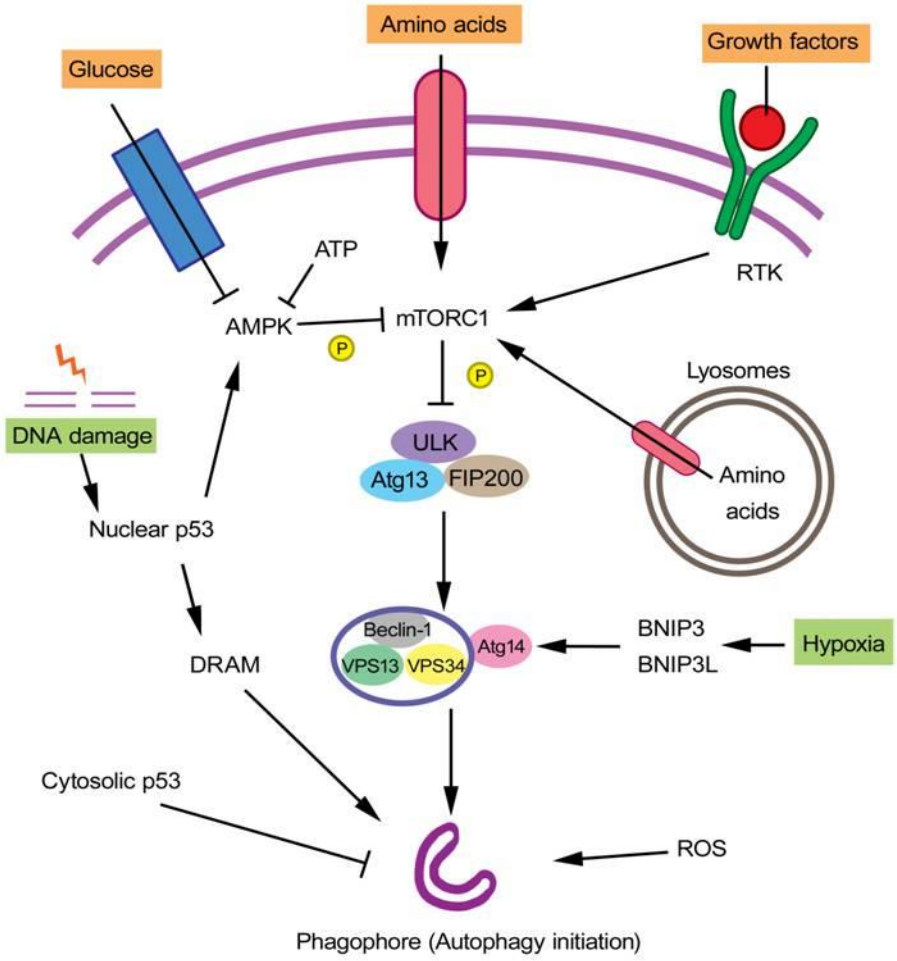


Figure 1.2 Autophagy levels are modulated in response to a variety of stimuli in mammalian cells.

Autophagy is inhibited in the presence of sufficient glucose, amino acids and growth factors and the nutrient-sensing kinase mTOR is a master inhibitor of autophagy. Autophagy is up-regulated by stress signals such as hypoxia, ROS and DNA damage.

A number of small molecules have been found to activate autophagy; some of them have shown potential to be used to treat protein aggregate diseases (as reviewed in [47] and [48]). Rapamycin is an mTOR inhibitor and has been shown to reduce protein aggregates through up-regulation of autophagy [49]. Trehalose, an mTOR-independent autophagy activator, is a chemical chaperone and how it activates autophagy is not fully understood. Resveratrol induces autophagy through deacetylase sirtuin 1. Other molecules such as Ca2+ channel inhibitors, calpain inhibitors and dopamine antagonists have also been shown to inhibit autophagy in an mTOR-independent way [48]. Autophagy is an important mechanism that mediates the clearance of protein aggregates. These molecules have shown protective effects against protein neurodegenerative diseases such as Huntington's [47].

1.2.2 Initiation of autophagy

To date more than 30 genes in yeast [50] have been identified to be directly involved in the execution of autophagy, they are known as the Atg gene family. Most Atg proteins are well conserved from yeast to mammalian cells and it is likely that there are more autophagy related genes expressed in higher eukaryotic cells [51]. ULK1 and ULK2 (Unc-51 like kinase) are the mammalian homologues of yeast Atg1. They are found in a stable complex with Atg13 and FIP200 (focal adhesion kinase family-interacting protein). As demonstrated in Fig. 1.2, mTORC1 is a molecular switch for autophagy in response to glucose, amino acids, growth factors and DNA damage. mTORC1 phosphorylates all members of the Atg13-ULK-FIP200 complex during nutrient replete conditions and this leads to the inhibition in the kinase activities of the complex. The initiation of autophagy is constitutively kept at low levels. The activity of the Atg13-ULK-FIP200 complex is controlled by phosphorylation at different sites. During starvation, mTORC1 kinase activity is suppressed in response to the lack of growth factor signalling, ULK1/2 complex is activated through auto-phosphorylation and it in turn phosphorylates

Atg13 and FIP200 [52]. Activated Atg13-ULK-FIP200 complex drives the induction of autophagy.

During the initiation of autophagy, double membranes are isolated to form a unique structure termed a phagophore. Phagophores elongate to sequester cellular contents such as proteins and organelles. There has been much debate on where the membrane forming phagophore / autophagosome originates. Based on studies of specific markers on autophagosome membranes, it was speculated that it came from plasma membrane and/or mitochondrial membrane and/or ER [53] [54]. There were no direct experiments demonstrating the origin of autophagosomes, until recently, Hamasaki and colleagues have shown compelling direct evidence that autophagosomes form at the contact sites between mitochondria and ER [55].

There are a number of genes critical for phagophore formation (Fig. 1.1). Beclin 1 protein is the mammalian ortholog of yeast Atg6; it provides structural scaffolding for autophagosome biogenesis. Beclin 1 was first discovered as an interacting partner of anti-apoptotic protein Bcl-2 [56]. Bcl-2 sequesters Beclin 1 through interaction with the Beclin 1 BH3 domain. This interaction can be disrupted either by competitive replacement by other proteins containing BH3 domains or by phosphorylation of Bcl-2 [57]. During starvation, Beclin 1 is released from the Bcl-2 complex. Free Beclin 1 then forms a core complex with Vps34 and Vps15, two proteins involved in vacuolar sorting pathways. Vps34 is a class III phosphoinositide-3-kinase (PI3K) [58] and Vps15 is a non-catalytic regulatory unit in the complex.

In the nutrient replete state, AMBRA (autophagy/beclin-1 regulator 1) protein binds to the PI3K core complex through interaction with Beclin 1, and it tethers the complex to the dynein cytoskeleton. AMBRA is activated by ULK1 through phosphorylation during autophagy, releasing the PI3K core complex to sites of phagophore nucleation [59]. The Beclin core complex can recruit other subunits, forming protein complexes that regulate autophagy or other membrane trafficking

events differentially. ATG14 is the mammalian homologue of yeast Atg14L, it is also known as BARKOR (Beclin 1-associated autophagy-related key regulator) [60]. [Vps15-Vps34-Beclin]-ATG14 targets the protein complex to sites of phagophore nucleation, now known to be the contact sites between mitochondria and ER. It was recently discovered that the activated Atg13-ULK-FIP200 complex phosphorylates Beclin-1 on Ser14 to enable autophagy induction of Vps15-Vps34-Beclin]-ATG14 complex [61]. The kinase activity of the Vps15-Vps34-Beclin complex converts phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PI3P), leading to an enrichment of PI3P on the inner membrane where the autophagosome is to be formed. PI3P in turn recruits the Atg18−Atg2 complex [62]. UVRAG (UV-irradiation-resistance-associated gene) is another binding partner to the [Vps15-Vps34-Beclin] PI3K complex. It is mutually exclusive to ATG14L and is also a positive regulator of autophagosome formation [63]. These initial events mark the initiation of autophagy.

1.2.3 Elongation of autophagosomes during autophagy

During the elongation stage of autophagosome formation, two molecular conjugation systems are recruited to the PI3P signalling hub on the inner membrane of autophagosomes. They are the Atg12-Atg5-Atg16 complex and the LC3-phosphatidylethanolamine (PE) complex. Autophagy conjugation systems share structural and functional similarities to ubiquitin conjugation pathways during proteasomal degradation. Proteasomal target proteins are covalently tagged with ubiquitin molecules on lysine residues. Three enzymes are involved in the process, namely E1, E2 and E3. E1 is the ubiquitin-activating enzyme, which covalently attaches the ubiquitin molecule onto itself. The ubiquitin on E1 is subsequently transferred to the intermediate protein E2, before it is finally transferred to the E3 ubiquitin ligase. As ubiquitination progresses, specificity increases. E3 protein mediates target substrate protein recognition and the transfer of ubiquitin from E2 to target protein [64].

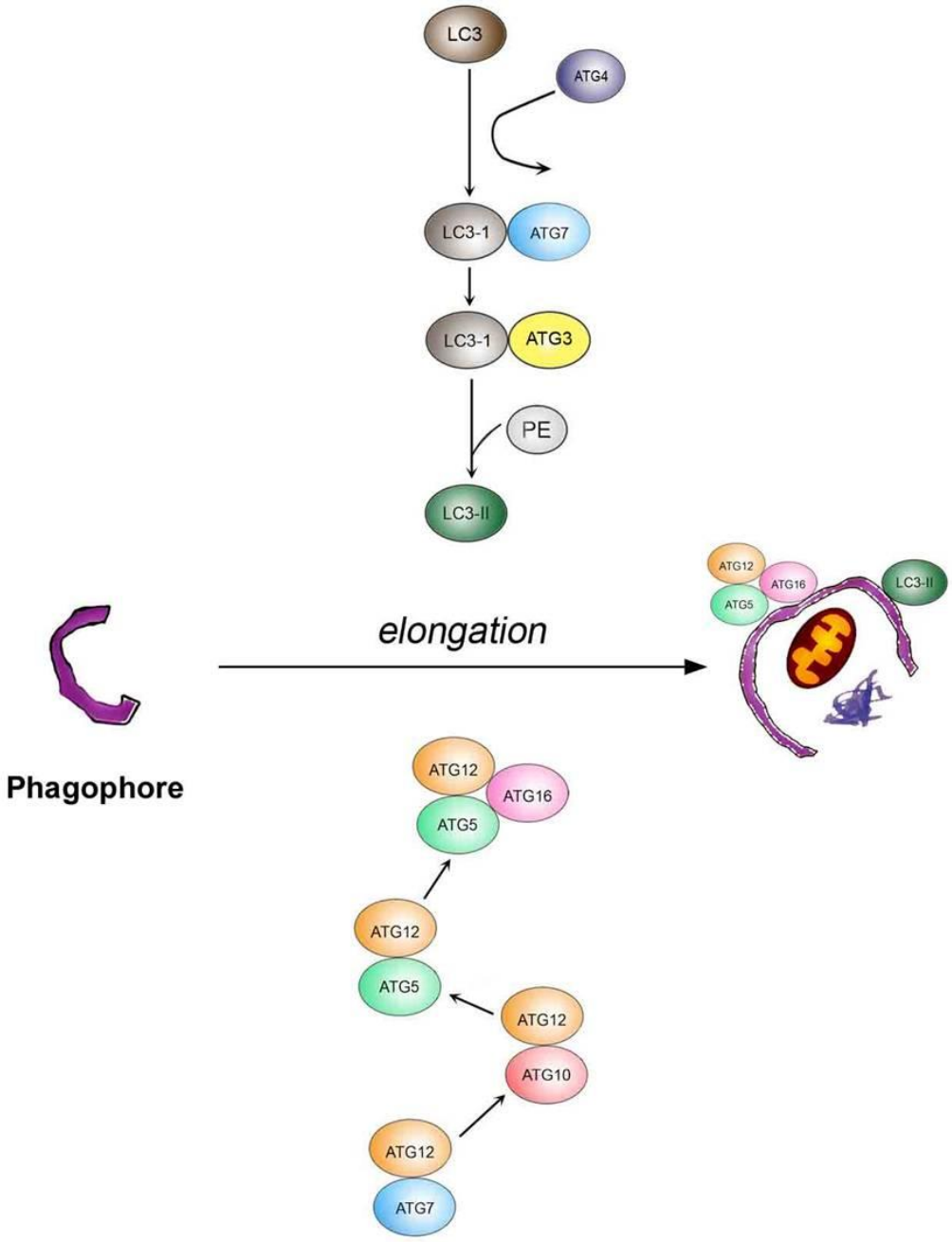


Figure 1.3 Two conjugation systems are required for the formation and elongation of phagophores into an enclosed autophagosome.

A series of conjugation events converts LC3-I to LC3-II and leads to the formation of the Atg12-Atg5-Atg16 complex.

As illustrated in Fig 1.3, Atg7 is an E1-like enzyme which activates and covalently transfers ubiquitin-like protein Atg12 to an E2-like enzyme Atg10. Atg10 in the Atg10-Atg12 complex is then replaced by Atg5. Atg16L (Mammalian homologues of yeast Atg16) is subsequently recruited, forming the Atg12-Atg5-Atg16L complex. Atg16 targets the complex to the membrane of the phagophore [65].

In the other conjugation system, newly synthesised LC3 (Atg8 in yeast) was first processed by cysteine protease Atg4 forming cytosolic LC3-I [66]. Upon autophagy induction, E1 like Atg7 activates LC3-I and transfers it to the E2-like enzyme Atg3, Atg12-Atg5 covalently attaches PE to LC3-I, which leads to the formation of the LC3-II–PE complex. Atg16L in the Atg12-Atg5-Atg16L complex facilitates the localisation of the anchoring LC3-II-PE complex to phagophores. These two conjugation systems are crucial for phagophore elongation and enclosure; phagophores eventually fuse at both ends to form enclosed autophagosomes.

1.2.4 Fusion of autophagosomes with lysosomes

Completed autophagosomes are transported towards endosomes and lysosomes along actin microfilaments and microtubules. Autophagosomes eventually fuse with endosomes and/or lysosomes. The fused vesicles are sometimes referred to as autolysosomes. The molecules involved in this step are part of the membrane-trafficking pathways and are less specific to autophagy. Lysosome-associated membrane protein LAMP2 and the small GTPase Rab7 are essential for the docking and fusion of autophagosomes with lysosomes [67].

Once the cargoes of autophagosomes are degraded in the lysosomes, cellular building blocks such as amino acids, carbohydrates and lipids are released from the vacuoles into the cytoplasm where they can be picked up and re-used. Lysosomes are not exclusive to autophagic pathways; endosome vesicles resulting from endocytosis also fuse with autophagosomes or lysosomes.

Amino acids are actively transported by proton-powered transporters embedded in lysosomal membranes [68] [69], and it is less clear how other building blocks are released and recycled. As mentioned previously, mTORC1, the master regulator of autophagy, is localised to the surface of lysosomes in response to amino acid release. The redistribution of mTORC1 to the lysosomes is essential for mTORC1 activation and subsequent inhibition of autophagy [27] [70]. This feedback mechanism avoids over-activation of autophagy, which can be detrimental to the cells.

During nutrient replete conditions, the cellular pool of amino acids is mainly mediated by the proteasomes [71]. Inhibition of proteasome leads to shortage of amino acids in the cells and an up-regulation of autophagy activities [72]. During starvation, autophagy is up-regulated as an adaptive mechanism and is thought to be the main source of amino acids. It was reported that during starvation, amino acid levels decrease in the absence of autophagy [73] [74].

Macroautophagy involving the formation of autophagosomes can also take place in an Atg5/Atg7 independent way [75]. Atg5/Atg7 independent autophagosomes differ from conventional autophagosomes, and they orginate from the membranes of trans-Golgi and late endosomes [75]. This alternative macroautophagy pathway is activated by metabolic stress and is found to play a role in erythrocyte development [75]. It may have a role in other physiological processes. It is not completely understood how Atg5/Atg7 independent autophagy is regulated or its relative importance in different organs. It remains to be determined what is the relative contribution of bulk protein degradation through this pathway in the cells.

It has been 20 years since the process of autophagy was first characterised in yeast [76]. Since then a wide range of physiological functions of autophagy have been discovered and it has been found to be linked with human aging and disease.

1.3 The importance of autophagy in human aging and disease.

Considering the important role of autophagy in maintaining cellular homeostasis and integrity, it is not surprising that loss of autophagy would perturb this balance and result in human diseases. Autophagy plays a role in a number of physiological aspects and it has been implicated in a spectrum of pathological conditions including neurodegenerative disorders, infections, autoimmune diseases, diabetes, muscular diseases and cancer [50]. It is also found to play a role in human aging. A summary of the role of autophagy is shown in table 1.

Health and diseases	Pro or against diseases	Mechanisms
Aging	Pro	Autophagy plays a role in the clearance of damaged proteins and organelles [77] .
	Against	Exact mechanism unknown. Up-regulation of autophagy has been linked to pre-mature aging in mouse models [78].
Autoimmune diseases	Against	Autophagy is involved in MHCII presentation and it maintains T cell and B cell homeostasis.
Cancer	Pro	Autophagy can be utilised by cancer cells to promote cell survival. See Chapter 1.4
	Against	Autophagy maintains cellular homeostasis through the removal of damaged proteins and organelles. See Chapter 1.4
Diabetes	Pro	Fatty acids can up-regulate autophagy which contributes to pancreatic β-cell death [79].

	Against	Atophagy protects against diabetes-induced cellular stresses in β-cells (As reviewed in [80]).
Infections	Pro	Certain microbes have even evolved mechanisms that utilize components of the autophagic machinery to facilitate viral maturation [81]
	Against	Infectious agents are recognized and engulfed by autophagosomes which are then fused with lysosomes [82].
Muscular diseases	Pro	Excessive levels of autophagy can lead to muscle wasting and myopathies [83].
	Against	Autophagy maintains cellular homeostasis through the removal of damaged proteins and organelles [83].
Neurodegenerative disorders,	Pro	Abnormal clearance of β-amyloid proteins through autophagy generate disease promoting peptides [84].
	Against	Autophagy maintains cellular homeostasis through the removal of damaged proteins, protein aggregates and organelles [83].

Table 1 Summary of the role of autophagy in disease.

Cells have developed precise mechanisms to rapidly remove misfolded or damaged proteins; this is mediated by the proteasome and autophagic pathways. If these proteins are not cleared and accumulate in cells, they can lead to increasing oxidative stress and cellular toxicity. In general, proteasomes degrade smaller proteins which are normally specifically recognised by E3 ligases. Autophagy, on the other hand, degrades bulky proteins and aggregates. In general, autophagy substrates are less specific, though a number of specific autophagy targets have been identified, such as p62 and NBR1. Autophagy is also capable of the

degradation of insoluble protein aggregates which can not access the catalytic cavity buried within the proteasome structures.

In response to unfolded or misfolded proteins in the ER, mammalian cells are able to activate the ER stress response where chaperones are activated to carry out ER-associated degradation. Autophagic pathways are also up-regulated by ER stress activating in parallel with ER stress chaperones [85]. Cells become more sensitive to ER stress and display increased cell death upon inhibition of autophagy [85]. Misfolded proteins result in neuronal inclusions or plaques in the brain, which are responsible for a number of neurodegenerative conditions [86] [87]. Autophagy deletion in mouse models has been linked to protein inclusion bodies in the brain and neurodegeneration [88] and autophagy has been found to be a crucial factor for neuronal development and homeostasis. Autophagy pathways have been linked to Alzheimer's disease [89], Huntington's disease [90], Parkinson's disease [91] and Creutzfeldt-Jakob disease [92]. These conditions often result from inheritable mutations, which lead to protein misfolding and aggregate formation. Autophagy plays a protective role against these orders. A range of small molecule activators of autophagy have shown therapeutic potential in mouse models of Huntington's disease [47].

Ageing is a complex and unavoidable process influenced by a number of signalling mechanisms and environmental factors. In ageing cells, the accumulation of damaged organelles such as mitochondria and proteins is a common feature. Since autophagy is a housekeeping process that mediates the degradation of aberrant cytosolic proteins and organelles, it is not surprising that it plays a part in the ageing process. Genetic studies provide evidence that in *Caenorhabditis elegans* and *Drosophila* models, elevation of basal autophagy decreases the rate of ageing in cells and increases lifespan [93].

Although autophagy is a mechanism of self-digestion, autophagic pathways have been utilized as a defense mechanism by mammalian cells. It takes part in the

removal of invading microorganisms such as bacteria, viruses, protozoa and parasites [94] [95] [96]. Autophagy has been found to be up-regulated during infections. Infectious agents are recognized and engulfed by autophagosomes which are then fused with lysosomes. For example, when inside cells, the bacterium *Legionella pneumophila* which is responsible for Legionnaires' disease forms vacuoles, the vacuoles are recognized and ubiquitinated [82]. The vacuoles then bind to specific autophagy adaptor protein p62/SQSTM1 and are engulfed [82]. However, under selective pressure, certain bacteria and viruses have developed strategies to antagonize autophagy functions to avoid removal. The Bacterium *Shigella flexneri* avoids recognition by autophagic pathways and hence removal by expressing modified surface proteins [97]. Some microbes have even evolved mechanisms that utilize components of the autophagic machinery to facilitate viral maturation [81]. A better understanding of the roles of autophagy in infection would be beneficial to the studies of autophagy in other diseases such as cancer and also to the development of anti-bacterial or anti-viral therapies.

Aberrant autophagy activity has been linked to the pathogenesis of autoimmune diseases such as multiple sclerosis (MS) [98] and lupus [99]. These autoimmune diseases are characterized by deregulated immune responses against the body's own cells or tissues. Rapamycin, which activates autophagy through inhibition of mTORC1, displays immunosuppressant properties. It has shown therapeutic potential to treat lupus in pre-clinical studies [100].

A number of mouse models, especially genetic knockout models, have been established to investigate the roles of autophagy in human pathology. Studies in mouse models have confirmed the links between autophagy and these diseases described above. Targeted deletion of autophagy essential genes
Atg5 or *Atg7* in mice lead to an accumulation of poly-ubiquitinated proteins in neurons and in turn lead to neuro-degeneration. Rapamycin, an autophagy inducer, has been found to prolong the lifespan of mice. Female mice have been found to live longer by 14% and male 9% when rapamycin administration starts at 600 days

of age [101]. *p62* knockout mice also presented accelerated aging, and defective mitochondrial functions and increased oxidative stress were detected in these mice [102]. Studies in mouse models have also confirmed that autophagy is crucial for T cell and B cell survival. Mice with Atg5 or Atg7 deficiency have elavated amount of mitochrondria in T cells which lead to cell death [103]. Targeted deletion of Atg5 in B cells also lead to increased B cell death [104]. Targeted ablation of Atg7 in muscle result in smaller myofiber in mice and the degenerative phenotype is manifested with age [105]. Moreover, GFP-LC3 transgenic mice have been generated by Mizushima and colleagues, the detectable LC3 punctate corresponds to autophagic activities [106].

Since the first genetic link was established between autophagy and cancer in 1999, when *Beclin 1* gene was found to be frequently deleted in human cancers [56], there has been an explosion in autophagy research in the context of cancer. Like most areas of scientific exploration, the more knowledge we gain in the field, the more we realize how much work remains to gain full understanding of the role autophagy plays in cancer.

1.4 The complex role autophagy plays in tumourigenesis

The functions and outcome of autophagy in cancer are highly context specific; paradoxically it can either promote cancer cell survival [107] or cancer cell death [108]. For this reason, reports have referred to autophagy as ‘a double edged sword’ or ‘janus-faced’ [109, 110]. Understanding the exact role of autophagy in cancer under each context has now become a priority.

Cancer development is a highly complicated, multi-step process where normal cells go through a series of aberrant transformations that involve a global change in gene expression, into malignant cells that proliferate uncontrollably and invade into other parts of the body [111]. Cancer itself is a diverse group of diseases, and there are more than 100 distinct types of cancer [7]. The carcinogenesis of each cancer is

varied; only 10% of all cancers are due to germline mutations such as in genes such as TP53 or BRCA1/2 genes, the majority of cancers resulting from somatic mutations [112]. Oncogenesis is a multifactorial process; cancer epidemiology studies have revealed a number of risk factors such as ethnicity, diet, hormones, infections, weight, and radiation. Each type of cancer has a different set of risk factors.

Cancer in general can be divided into three critical stages: initiation, promotion and progression. To become neoplastic, normal cells first acquire insensitivity to growth suppressors and undergo uncontrolled over-growth [7]. Tumour cells are able to evade programmed cell death and achieve replicative immortality. Primary tumours can migrate from the site of origination and invade into a secondary part of the body. Metastatic cancer can establish itself by recruiting blood supply and modify surrounding cells to form a tumour microenvironment [111]. As summarized in Figure 1.4, autophagy, being an important catabolic adaptive mechanism, plays important and complex roles in nearly all aspects of carcinogenesis.

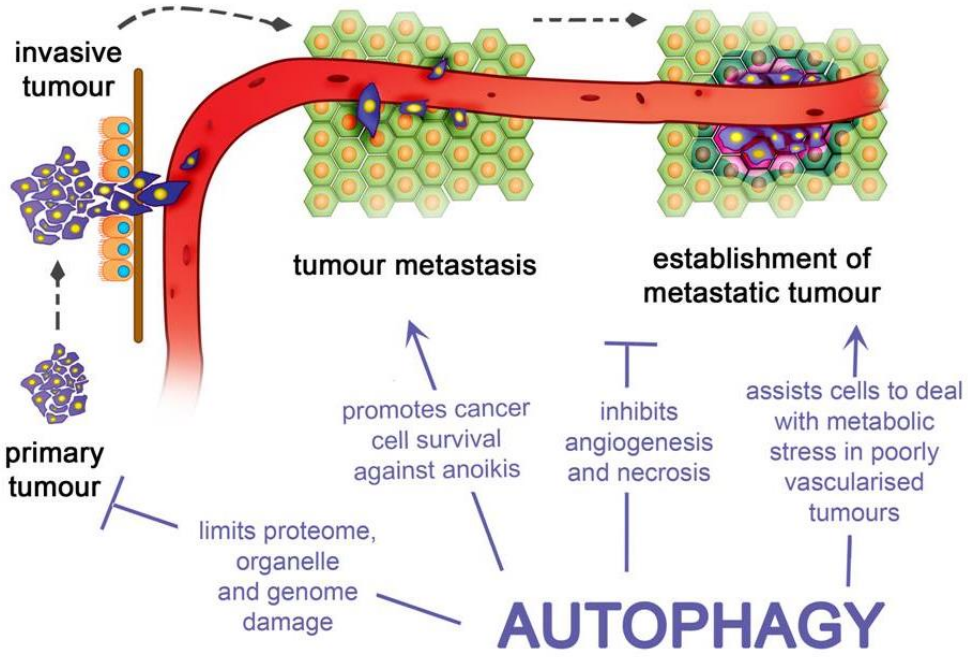


Figure 1.4 Autophagy plays important and paradoxical role in every stage of carcinogenesis

Autophagy is active constitutively at basal levels to maintain cellular homeostasis and limit genomic damage. These autophagic functions are thought to inhibit primary tumour formation. In order for tumour cells to invade into a secondary location, cancer cells need to detach from the primary tumour. Anoikis, which is a type of programmed cell death activated when cells are detached from the extracellular matrix (ECM), can occur. Autophagy has been found to promote cancer cell survival against anoikis. Paradoxically, autophagy inhibits angiogenesis which is needed by a solid tumour to recruit increased blood supply. Finally, autophagy can be a pro-survival mechanism for cancer cells to deal with metabolic stress especially in regions where blood supply is limited.

1.4.1 Autophagy plays a role of tumour suppressor during oncogenesis

Autophagy was first linked with cancer through genetic studies. It was found that a number of autophagy genes are frequently lost in certain types of cancers. For example, human BECN1 (a gene encoding beclin 1) is hemizygously-deleted in around 50% of breast, ovarian and prostate cancers [113] [56]. Autophagy and apoptosis share certain upstream signals and Beclin 1 is thought to be one of the major convergence molecules for the two processes. Monoallelic deletion of beclin 1 in mice increased the incidence of cancer including hepatocellular carcinoma, lung carcinoma and lymphoma [114]. When beclin 1 was stably transfected into MCF-7 cells, autophagy activities were up-regulated [56]. The tumourigenesis of these cells was decreased after injection in nude mice [56].

A number of autophagy executor genes, ATG2B, ATG5, ATG9B and ATG12 have also been linked to carcinogenesis [115]. It was discovered that frame-shift mutations in these ATG genes with mononucleotide repeats are common in gastric and colorectal carcinomas with microsatellite instability [115]. Tumour suppressor genes prevent the cells from progressing to cancer, and pre-malignant cells with genetic instability frequently develop loss-of-function mutations in these genes. The fact that a number of autophagy genes are lost in cancers indicates that autophagy can act as a tumour suppressor.

Several genetic studies in mouse models also show that autophagy can prevent tumour formation. For example, the lack of certain autophagy genes such as *BECN1* [114] can lead to cancer. In contrast, Beclin 1 over-expression, which leads to up-regulation of autophagy, can inhibit tumour development [56]. Also, mice form benign liver adenomas after either mosaic deletion of Atg7 specific in the liver or mosaic deletion of Atg5 [116].

Autophagy can inhibit the initiation of tumourigenesis through limiting genomic instability and inflammation [107]. Defective proteins and mitochondria generate

excessive free radicals that saturate the cells ability to scavenge them and these free radicals lead to reactive oxygen species and oxidative stress. Free radicals can attack DNA molecules, leading to structural lesions. If unrepaired, these DNA lesions result in genetic mutations which may eventually contribute to genetic instability [117]. Normal cells undergo a series of genetic changes to become transformed cancer cells and genetic instability is one of the most important factors for carcinogenesis. For pre-malignant cells, genetic instability enables them to diversify, evolve and finally to acquire mechanisms to reach malignancy [7].

A few mechanisms have been proposed to explain how autophagy acts as a tumour suppressor. Autophagy mediates the degradation of mitochondria in a process referred to as mitophagy, accumulation of defective mitochondria contributes towards increased oxidative stress and carcinogenesis [118]. Autophagy regulates p62 levels in the cells and loss of autophagy leads to p62 accumulation, which contributes to tumourigenesis [119]. Autophagy also limits inflammation and mediates oncogene-induced senescence [119].

Mitochondria in the cells are specifically turned over by autophagy as a way of controlling the amount of mitochondria and also to remove damaged ones. The selective engulfment of mitochondria by autophagy is refered to as mitophagy. Parkin is a ubiquitously expressed E3 ligase that plays a role in Parkinson's disease. It is activated and recruited to damaged mitochondria by PINK1 (PTEN-induced putative kinase protein 1) protein [120]. The outer layer of the mitochondrial membrane is ubiquitinated by Parkin and p62 is also recruited to mitochondria to mediate mitophagy [121]. The expression levels of parkin have been found to be down-regulated or ablated in a number of cancers including ovarian and breast cancers and leukemia [122]. It should be noted that the tumour suppressive role of parkin may also be due to its role in the regulation of glucose metabolism. The loss of parkin up-regulates glycolysis and inhibits mitochondrial respiration, resulting in the Warburg effect [123]. Defective mitophagy is directly linked to tumourigenesis

in mouse models [124]. ROS accumulates in mitophagy deficient cells and in turn this promotes genetic instability and cancer.

p62, also known as sequestosome-1 or SQSTM1 is a cytoplasmic protein with multiple functions. It contains a ubiquitin binding domain and an LC3 binding domain [125]. In general autophagy is a bulk degradation process that non-selectively engulfs organelles and cytoplasm. p62 is a autophagy receptor molecule that can mediate selective autophagy [125]. It can act as a ubiquitin-binding chaperone that recognises and sequesters specific ubiquitinated cargoes destined to be degraded by autophagy. For example, p62 has been found to surround mutant huntingtin aggregates and reduce the cytotoxicity of protein aggregates by promoting their autophagic degradation [126]. p62 is also a regulator of mitochondrial dynamics and functions [127] [128]. The degradation of p62 itself is mediated by autophagy, and the lack of autophagy leads to accumulation of p62 in the cells [129]. dIn healthy cells, p62 serves as a protective scavenger, removing potentially toxic protein waste (e.g. misfolded proteins) from the cells through autophagic degradation; however the beneficial role of p62 is reversed when there are elevated levels of p62 in the cells. Excessive intracellular p62 due to loss of autophagy has been found to have a number of detrimental effects on the cells. It has been found to be responsible for the formation of ubiquitin-positive protein aggregates in the cells, which correlate closely with neurodegeneration, liver injury and hepatocellular carcinoma [130]. Accumulation of p62 has also been linked with increased ROS production which promotes endoplasmic reticulum (ER) stress and accumulation of DNA damage [119].

Nrf2 is (nuclear factor-erythroid 2-related factor-2) is a transcription factor that plays dual roles in tumourigensis. It can reduce ROS levels and protect the cells from oxidative damages, but it also assists cancer cell survival and progression [131] [132] [133]. Nrf2 protein is constitutively turned over in the proteasomes, by binding to an adaptor protein of the ubiquitin ligase complex - Keap1 (kelch-like ECH-associated protein 1) [134]. p62 interacts with Keap1 through Nrf2-binding

site, excessive p62 disrupts the interactions between Keap1 and Nrf2, leading to the stabilization of Nrf2 [131]. Over activation of Nrf2 proteins, through inactivation of Keap1 or over-expression of p62, have been reported in a number of human cancers [135].

Moreover, p62 is often found to be up-regulated in human cancers and this is thought to promote tumorigenesis [119]. In this regard, the link between p62 accumulation from defective autophagy and oncogenesis was also shown in a study where p62 knock-out mice were found to be protected from *Ras* oncogene-induced lung carcinomas relative to Atg7f/f counterparts [136].

The lack of autophagy leads to increased inflammation. Inflammation is a localized protective mechanism in tissues of higher eukaryotes in response to irritation, injury or infection. During inflammation, blood vessels are dilated and the rate of blood flow increased. Leukocytes and macrophages migrate through the blood vessels into areas of inflammation in order to clear away injured tissues or infections. Inflammation is now thought to play important roles during the multiple stages of carcinogenesis [112][111]. Chronic inflammation caused by bacterial infections, viral infections, tobacco smoking [137] and obesity increases cancer risk [138, 139]. Multiple kinds of innate immune cells are often found in tumours. These immune cells produce cytokines, chemokines, growth factors and ROS, which contribute to uncontrolled cell growth and genetic instability and hence promote tumour progression. One of the hallmarks of cancer is its ability to evade recognition by immune cells [7] and it is generally accepted that in more established tumours, the inflammation and immune responses are more pro-tumourigenic. In an inflammatory microenvironment cancer cells have increased growth rate and mutation rates [140].

Autophagy may protect against tumorigenesis by limiting chronic necrosis and inflammation. Hepatocellular tumours due to disruption of the *BECN 1* gene display increased inflammation which is thought to promote tumourigenesis [114]. In the

context of cancer, autophagy plays a role in inflammation through at least two mechanisms.

Firstly, cells undergoing apoptosis are cleared efficiently by phagocytes in normal tissues, without the activation of the immune systems [141]. If these apoptotic corpses remain un-cleared, they can potentially lead to necrosis. Necrotic cells release signals to recruit inflammatory cells of the immune system and this increases tumour-promoting potential [112]. Autophagy plays a role in the engulfment of the apoptotic corpse, thereby limiting inflammation. Secondly, tumour cells often acquire defective apoptotic pathways to evade cell death. If autophagy is down regulated in these cells, it also leads to necrosis [142]. In mice with Atg5 deletions, their retinas and lungs display infiltration of inflammatory cells due to a defect in apoptotic corpse clearance [143]. Autophagy pathways are required for the release of signals by apoptotic cells to attract engulfment, a process which limits inflammation. The autophagy pathway has also been reported to negatively regulate inflammatory signalling, for example, caspase 1 activity and IL-1β production, both of which are pro-inflammatory signals that can be regulated by autophagy [144-146].

Autophagy's role in innate and adaptive immune responses is implicated in immuno-surveillance for pre-malignant cells. Immuno-deficient mice are prone to spontaneous and chemically induced cancers [147]. Patients with prolonged treatment of immunosuppressant drugs after transplant operations have a markedly higher chance of developing cancer [148]. Autophagy is crucial for T cell development, proliferation and differentiation [149], and is important in macrophage functions [150]. Autophagy is also required for antigen presentation on MHC II (major histo-compatibility complex class II) molecules.

Additionally, autophagy contributes towards tumour suppression by playing a role in inducing senescence. Senescence is a phenomenon where diploid cells withdraw from cell cycle progression and cease proliferation. Alongside programmed cell

death, cellular senescence acts as a barrier to oncogenesis [151]. Senescence can be triggered by DNA damage or the activation of oncogenic signals, and autophagy is found to mediate the establishment of oncogene-induced senescence [152].

All studies described above provide both genetic and mechanistic evidence that autophagy can act as a tumour suppressor, especially in the initial stages of tumourigenesis. As mentioned before, constitutively active autophagy functions to provide cells with nutrients and removes defective proteins and organelles, assisting cells to deal with stressful metabolic environments. Therefore it is not surprising that autophagy plays a role in promoting tumour formation. There are studies providing evidence that autophagy pathways can be utilized by cancer cells to promote survival and establish malignancy, as reviewed in the following paragraphs (Chapter 1.4.2).

1.4.2 Autophagy can also promote tumour progression

During the initial stages of oncogenesis, cancer cells undergo uncontrolled proliferation and avoid programmed cell death; solid tumours frequently encounter harsh environments such as hypoxia and the lack of nutrients. Autophagy is frequently up-regulated in solid tumours, especially in the core region of the tumour. In less perfused areas, there is limited availability of nutrients (growth factors, amino acids etc) and oxygen [153]. Autophagy is thought to maintain energy metabolism and help cancer cells deal with the harsh conditions until the environment is improved. In this regard autophagy can act as a survival mechanism promoting cancer cell survival and propagation [154].

Certain cancer cell lines display higher basal autophagic activities to maintain their energy balance. When these cells encounter stressful environments, they often fail to elevate autophagy activities much further. They are described to be addicted to autophagy pathways [155]. For example, pancreatic cancers have constitutively high levels of autophagy and they require autophagy to survive and progress. These pancreatic cancer cells are exquisitely sensitive to autophagy inhibition [156]. RAS

is a well characterised oncogene that promotes cancer growth; RAS signalling also activates autophagy and cancer cells driven by mutant RAS depend on autophagy to promote oncogensis. There is a synthetic lethal situation when autophagy is inhibited in cancer cells with RAS hyper-activation [157].

Cancers do not progress and metastasize in isolation; they require the appropriate tumour microenvrioment which is characterized by the presence of a variety of factors including immune cells, endothelial cells, fibroblasts and ECM (Extra Cellular Matrix) [158]. Tumour microenvironment plays a decisive role in cancer prognosis and responses to therapeutics. Autophagy has been found to influence nearly every aspect of the tumour microenvironment: Autophagy in endothelial cells is found to mediate hypoxia induced angiogenesis [159]. It acts as a mediator in both adaptive and innate immune responses [160]. Autophagy limits inflammatory responses and necrosis (Chapter 1.5.1), however it is found that autophagy can act as an alternative secretary pathway for inflammatory molecules (e.g. chemokines and cytokines) and ECM modulators, in a process termed 'autosecretion' [161] [162]. Autophagy-mediated cancer cell senescence limits oncogenic potential; however, the autophagy-senescence transition in cancer-associated fibroblasts has been found to promote tumour growth [163]. These fibroblasts undergo constitutively active mitophagy and they undergo elevated aerobic glycosis producing and secreting ketone bodies, lactic acid and fatty acids that fuel neighbouring cancer cell growth [164].

The oncogenic role autophagy plays is supported by several genetic studies. For example, depletion of FIP200, a gene essential for autophagy, inhibits RAS oncogene-driven mammary carcinogenesis [165]. As mentioned previously, mice with mosaic deletion of Atg5 or deletion of Atg7 in the liver leads to benign tumours. It should be noted that these tumours do not progress into malignant adenocarcinoma or acquire the capacity to metastasize. This indicates that cancer cells may require autophagy to become more established and achieve malignancy [116].

1.4.3 Autophagy promotes metastasis

Metastatic cancer is the main cause of lethality in cancer patients [166]. A number of studies have demonstrated that autophagy plays a role in metastasis. For example, recent work from our lab using a 3D organotypic model demonstrated that the inhibition of autophagy in cancer cells impairs their invasiveness [167]. DRAM1, a p53 target gene that regulate autophagy and p62, a selective autophagy substrate have been demonstrated to regulate cell motility and invasions in glioblastoma stem cells [168].

TNF-related Apoptosis Inducing Ligand (TRAIL) mediates death-ligand-induced apoptosis in cells and it is found to be crucial for the suppression of metastasis [169]. Autophagy contributes to the resistance to TRAIL mediated cell death in cancer cells [170].

In order to metastasize, cancer cells from primary tumours need to detach from extracellular matrix (ECM), migrate through blood vessels and invade into secondary tissues, where they can settle, expand and colonize. When normal cells are detached from the ECM, they undergo a type of apoptosis termed anoikis [171]. Cancer cells acquire the capacity to evade anoikis and achieve anchorage-independent growth. A number of pathways have been identified to be involved in this critical step of tumourigenesis, including aberrance in Ras and PI3K cell proliferation signal pathways [171]. Autophagy is not only able to assist cancer cell survival in the blood stream by maintaining energy balance; it also has been found to be activated during anoikis and it is thought to be one of the mechanisms that are utilized by cancer cells to promote metastasis [172]. Isolated metastasising cancer cells eventually establish interaction with ECM in a distant organ.

Some cancer patients can develop recurrent metastatic cancer after a dormancy period that can last from years to decades. The cause of this can be explained by tumour cell dormancy [173]. Primary tumour cells can metastasize to distant

secondary sites and enter a dormancy period before resuming malignancy. These cells are difficult to detect and can evade conventional anti-cancer therapies that target rapidly growing cancer cells [174]. Autophagy has been shown as a pro-survival mechanism for cancer cells in dormancy [175].

In summary, autophagy limits tumour initiation by maintaining cellular homeostasis, limiting genetic instability and inflammation; however it promotes tumour establishment and progression by enabling cancer cells to cope with the harsh environment during cancer development and to evade cell death [176].

1.5 The role of autophagy in cancer therapeutics

1.5.1 Autophagy is frequently up-regulated by cancer therapeutic drugs.

The important role autophagy plays in carcinogenesis provides the possibility that autophagy may be targeted for cancer therapy. However, the role of autophagy in cancer therapeutics is also highly context dependent. It may be a pathway induced by cancer therapeutics to achieve drug efficacy or it may be a pro-survival mechanism promoting drug resistance.

A variety of cancer therapeutic treatments have been shown to up-regulate autophagy in cancer cells (Fig. 1.5).

These autophagy inducing treatments include conventional DNA damaging agents such as IR [177], etoposide and camptothecin [178]; novel targeted drugs such as the estrogen receptor inhibitor taxomifen, anti HER2 receptor antibodies, tyrosine kinase inhibtors and proteasome inhibitors [179]. In some cases, autophagy levels are elevated as a secondary adaptive mechanism struggling cancer cells use to promote survivial. Increasing number of novel anti-cancer drugs up-regulates autophagy by targeting the PI3K/Akt/mTOR pathways [180] and this is an undesired effect that enables cancer cells to cope with stressful environment.

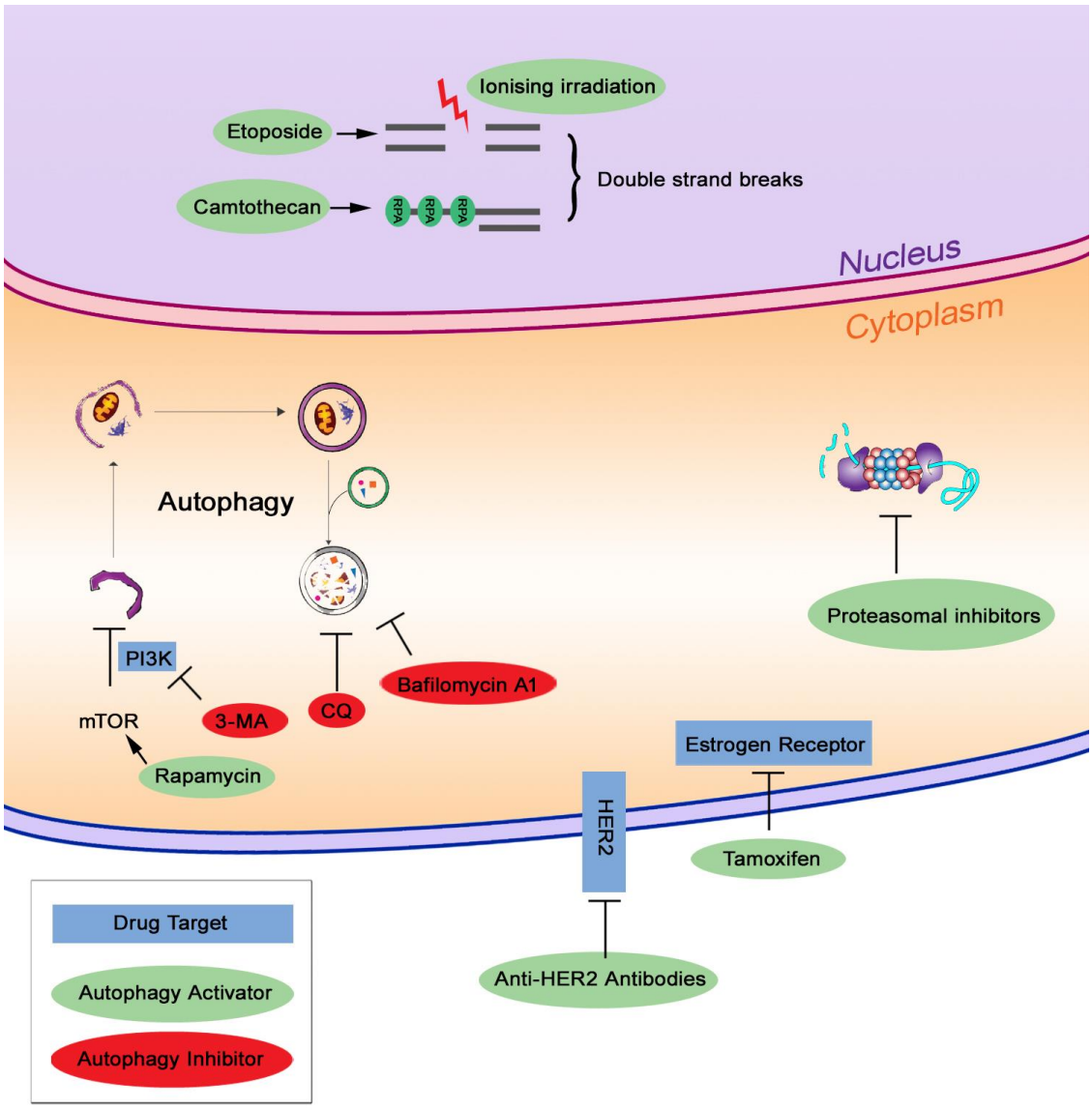


Figure 1.5 Anti-cancer treatments frequently lead to autophagy activation.

(Information from this figure was compiled from references [177-179]). The diagram illustrates that current anti-tumour drugs are developed against a number of pathways. Through various anti-cancer mechanisms, these anti-cancer drugs (In green circles) frequently up-regulate autophagic activities. Generally it is thought that autophagy in this context was induced by cancer cells to deal with the toxicity and promote cell survival. Cancer cells have been found to be hyper-sensitive to autophagy inhibitors such as chloroquine (CQ) and 3-Methyladenine (3-MA) (In

red circles) in combination with other anti-cancer drugs. CQ has been selected to enter clinical trials as part of anti-cancer therapy.

1.5.2 Certain cancer therapies require autophagy to achieve maximal efficacy

Autophagy can assist cells to cope with cytotoxicity until the environment has improved but it does not maintain cell viability in a sustainable way. Autophagy is a catabolic mechanism that recycles energy and cellular building blocks through self-eating. Over stimulation of autophagy can lead to cell death. The pro-death properties of autophagy are thought to play a positive role in anti-cancer treatments. As mentioned previously, many cancer cells develop defects in apoptotic pathways to evade cell death during their transformation. Autophagy induced by anti-cancer treatments can serve as an alternative mechanism to elicit cell death in cancer cells [9]. For example, triptolide, an agent that efficiently inhibits pancreatic cancer cell growth, is found to destroy cancer cells through the induction of autophagy [8].

Cancer therapies induce cell death, and the dying cells may act as signals that recruit the immune system to target residual tumour cells [181]. Certain drugs such as anthracyclines and oxaliplatin are found to induce immunogenic cell death and these agents require the immune system to be effective [182]. ATP is released by dying cancer cells following cancer treatment, and it is critical for chemotherapy induced immune response against cancer cells [183]. Autophagy in cancer cells has been found to be a crucial factor for their immunogenicity [6]. A study by Guido Kroemer and colleagues demonstrated that only autophagy proficient cancer cells can release ATP when they undergo cell death and autophagy deficient cancer cells fail to attract dendritic cells and lymphocytes into the tumour [6].

Rapamycin (and its derivatives) have shown promising therapeutic potential for cancer treatment because of their anti- proliferation and immunosuppressant properties [184]. Rapamycin leads to up-regulation of autophagic activities through mTOR inhibition; a number of recent studies have shown that autophagy is in fact a

critical factor for the anti-tumour effects of rapamycin [185] [186]. Cytotoxicity inflicted by rapamycin in cancer cells is attenuated when autophagy is inhibited.

In summary, autophagy is up-regulated by a number of chemotherapeutic drugs and certain drugs require autophagy to achieve maximal efficacy. A screening study carried out in Kroemer's team tested for autophagy, apoptosis and necrosis induction of chemotherapeutic drugs. 59 out of 1400 drugs potently activate autophagy in cancer cells, however none of these drugs kill cancer cells via autophagy [187]. The study indicates that in most cases, autophagy may be a cyto-protective mechanism. It may be postulated that inhibition of autophagy in combination with these drugs would not reduce drug efficacy during in chemotherapy and actually may enhance synergistic killing of cancer cells.

1.5.3 Autophagy as a cytoprotective mechanism promoting cancer survival during cancer treatment

Cancer cells have a resilient nature and can develop drug resistance through a number of pathways, and autophagy overall is a pro-survival response that assists cells to deal with a range of cytotoxic insults. In some cases autophagy is induced as a pro-cancerous mechanism during cancer treatments, and it can be exploited by tumour cells to promote survival and recovery against cancer therapeutic drugs [188].

There has been compelling evidence that inhibition of autophagy promotes cancer cell sensitivity to certain cancer therapeutic treatments such as IR, tyrosine kinase inhibitors [189] and Src kinase inhibitors [190]. Moreover, although autophagy can inhibit tumour initiation, it promotes tumour progression and metastasis. These observations underscore the use of autophagy inhibitors as adjuvant anti-cancer therapies.

3-MA (3-methyladenine) and wortmannin are PI3K kinase inhibitors which block autophagy initiation. These agents have been evaluated in pre-clinical studies and have been found to potentiate the sensitivity of cancer cell lines to drugs such as 5-FU and cisplatin [191] [192]. However these agents also affect other physiological processes and have a relatively high cytotoxicity at clinically relevant concentrations.

Chloroquine is a drug that has been widely used to treat malaria and it is also used as an anti-inflammatory agent against rheumatoid arthritis and lupus. It is is well tolerated by non-transformed cells. Because autophagy is utilised by cancer cells as a survival mechanism and many current cancer therapies have been found to activate autophagy, the inhibition of autophagy may be beneficial to cancer patients. In fact, a correlation study carried out in the late 80s revealed that the use of anti-malarial chloroquine was associated with lowered incidence of Burkitt lymphoma, which was an endemic in Africa around that time [193].

There is increasing amount of evidence that chloroquitin has potentials as a death-sensitising agent when used in combination with other cancer therapies [194]. A number of clinical trials have been carried out or in progress using the lysosomal inhibitor hydroxychloroquine (a derivative of chloroquine) as a part of cancer treatment (Details available at ClinicalTrials.gov). So far chloroquine is thought to be promising as a novel anti-cancer treatment [195]. For example, a randomised, and double blind trial revealed that chloroquine when used as combination therapy for glioblastoma multiforme, the patients survival time is doubled comparing to placebo control [195]. Since chloroquine inhibits all lysosomal functions and also has other functions in the cells such as activation of ATM and immnosuppression. The exact mechanism of anti-cancer properties of chloroquine is unknown but one likely mechanism is through autophagy inhibition. Whether the central anti-tumour action of chloroquine is due to the inhibition of autophagy or other functions of chloroquine remains to be determined.

In summary (Fig. 1.4), autophagy is frequently up-regulated by anti-cancer therapeutics and can assist drug resistance in cancer cells. Autophagy inhibiting drugs have shown promising effects in clinical trials. However in some scenarios, autophagy facilitates efficient killing by anti-cancer drugs. Therefore it is important to investigate the role autophagy plays during cancer treatment in a context dependent way, and to inhibit autophagy only when it is playing a pro-survival role.

Because of its importance in tumourigenesis as well as cancer therapeutics, a better understanding of the functions of autophagy under specific contexts is of great importance. This is becoming one of the main areas for cancer research. Another critical area of cancer research is the DNA damage response. DNA damage response pathways are frequently altered in cancer cells and a great proportion of current drugs used to treat cancer are DNA damaging agents. Studies of DNA damage response not only helps to understand the carcinogenesis process, but may reveal additional therapeutic approaches in cancer treatment.

Autophagy is a cytoplasmic process, whereas DNA damage and repair take place in the nucleus. There is an increasing amount of evidence that the two processes are closely linked. Under normal circumstances, autophagy is found to play a role in the maintenance of genomic integrity. It has been reported that depletion of autophagy leads to increased DNA damage in the cells. Cells lacking Atg7, a gene essential for autophagy, is reported to accumulate DNA aberrations including increased double strand breaks, polyploid nuclei and gene amplification [1] [2]. When the cells are challenged with DNA damaging agents, such as chemotherapeutic drugs, autophagy is frequently reported to be up-regulated [196]. As mentioned previously, inhibition of autophagy potentiates cancer cell sensitivity to DNA damaging drugs.

The exact mechanisms how loss of certain autophagy genes such as *Beclin1*, *Atg5* and *Atg7* lead to tumours remain to be determined. Because autophagy constitutively removes damaged proteins and organelles to minimise the production

of ROS in the cells, it is speculated that lack of autophagy leads to an increased incidence of DNA damage.

1.6 DNA damage response and cancer genetics

Genomic integrity is crucial for higher life forms, since genetic mutations or chromosomal instabilities can lead to diseases which can be inheritable. One of the most distinctive traits of cancer cells is genetic instability, and the genetic alteration enables tumour cells to acquire a series of functions. These gain of functions are referred to as 'the hallmarks of cancer' [111]. Genetic instability is driven by a number of factors such as DNA damage accumulation, defective DNA repair machinery, and failure to prevent mitotic entry where DNA lesions get passed to daughter cells.

In our body, genomic integrity is constantly being challenged. As each cell acquires 1000 to 1,000,000 DNA lesions every day [197]. These lesions can block replication fork formation during DNA synthesis and can block transcription in the short term. If left unrepaired, these lesions induce genomic mutations and aberrations [198]. Some of the lesions are due to environmental factors such as untra-violet radiation (UV) and heavy metals. Some lesions are caused by physiological processes, for example, DNA replication apparatus such as DNA polymerases occasionally mismatch nucleotides during replication, and reactive oxygen species produced during the oxidative respiration chain can cause damage to the DNA as well. Luckily for most of us, most of this DNA damage is repaired efficiently by our complex DNA repair pathways. For difficult to resolve lesions, the cell can alternatively activate programmed cell death pathways. Occasionally, some DNA lesions escape being identified by DNA repair mechanisms and result in mutations. Humans have a mutation rate of around $1x10^{-4}$ - $1x10^{-6}$ per gamete for a given gene [199].

The links between DNA damage accumulation and carcinogenesis have long been established. Two notable examples are skin cancer from over-exposure to the sun and lung cancer from tobacco smoking. UV radiation from the sun [200] causes mainly two types of lesions - cyclobutane pyrimidine dimers and 6-4 photoproducts.

Both of these are bulky in size and can lead to distortions in the helical DNA structure, hindering transcription and replication [201]. Carcinogens from tobacco such as polycyclic aromatic hydrocarbons and aromatic amines are able to react with DNA molecules directly or through intermediate metabolites [202].

Defects in the DNA damage response (DDR) are responsible for certain human genetic disorders and people with these genetic traits are commonly pre-disposed to cancer. Notable disorders include ataxia-telangiectasia (AT), a neuro-degenerative motor disease due to the cell's failure to resolve oxidative damage in the brain. Patients with AT mutations have defective ATM protein (more details will be covered in the next section). Xeroderma pigmentosum (XP), a condition characterized by extreme sensitivity to sunlight is due to a deficiency in the ability to repair damage caused by ultraviolet (UV) damage in the skin. People with AT have a 25% higher chance of developing cancer in their life [203] and fewer than 40% of people with XP survive beyond the age of 20 [204]. Malignant melanoma and squamous cell carcinoma are the two most common causes of death in XP patients [204]. Besides the genetic disorders outlined above, a number of cancer specific susceptibility genes have been identified. Tumour suppressor gene TP53 which encodes p53 is mutated or lost in a large proportion of human tumours [205]. People with mutations in BRCA genes are predisposed to various forms of cancers, especially breast cancer and ovarian cancer. BRCA 1 and 2 (Breast Cancer 1 and 2) are tumour suppressor proteins involved in the process of homologous recombination DNA repair process [206].

1.6 DNA damage response network

Mammalian cells have evolved a complex set of DNA repair machinery dealing with the high incidence of lesion formations. There are three main aspects of DNA damage response; DNA repair, cell cycle arrest and apoptosis. They are tightly coordinated to minimise the amount of heritable mutations. Defects in these pathways can lead to human diseases including cancer, as discussed in the previous section.

In the event of DNA damage, cells activate their cell cycle checkpoints to prevent DNA replication and also to provide an opportunity for the cells to repair the lesions. If the lesions fail to be repaired, programmed cell death can be activated. Checkpoint pathways share many common components with DNA repair response. Two master kinases, ATM and ATR are responsible for downstream signalling cascades that eventually lead to cell cycle arrest and DNA damage repair.

1.6.1 Cell Cycle Checkpoints

The cell cycle is tightly controlled in mammalian cells to ensure correct cell division and genomic integrity. If disrupted, potentially harmful genetic defects may be passed on to daughter cells. A series of tightly regulated signalling events take place to arrest the cell cycle upon DNA damage, as illustrated in Fig. 1.6.

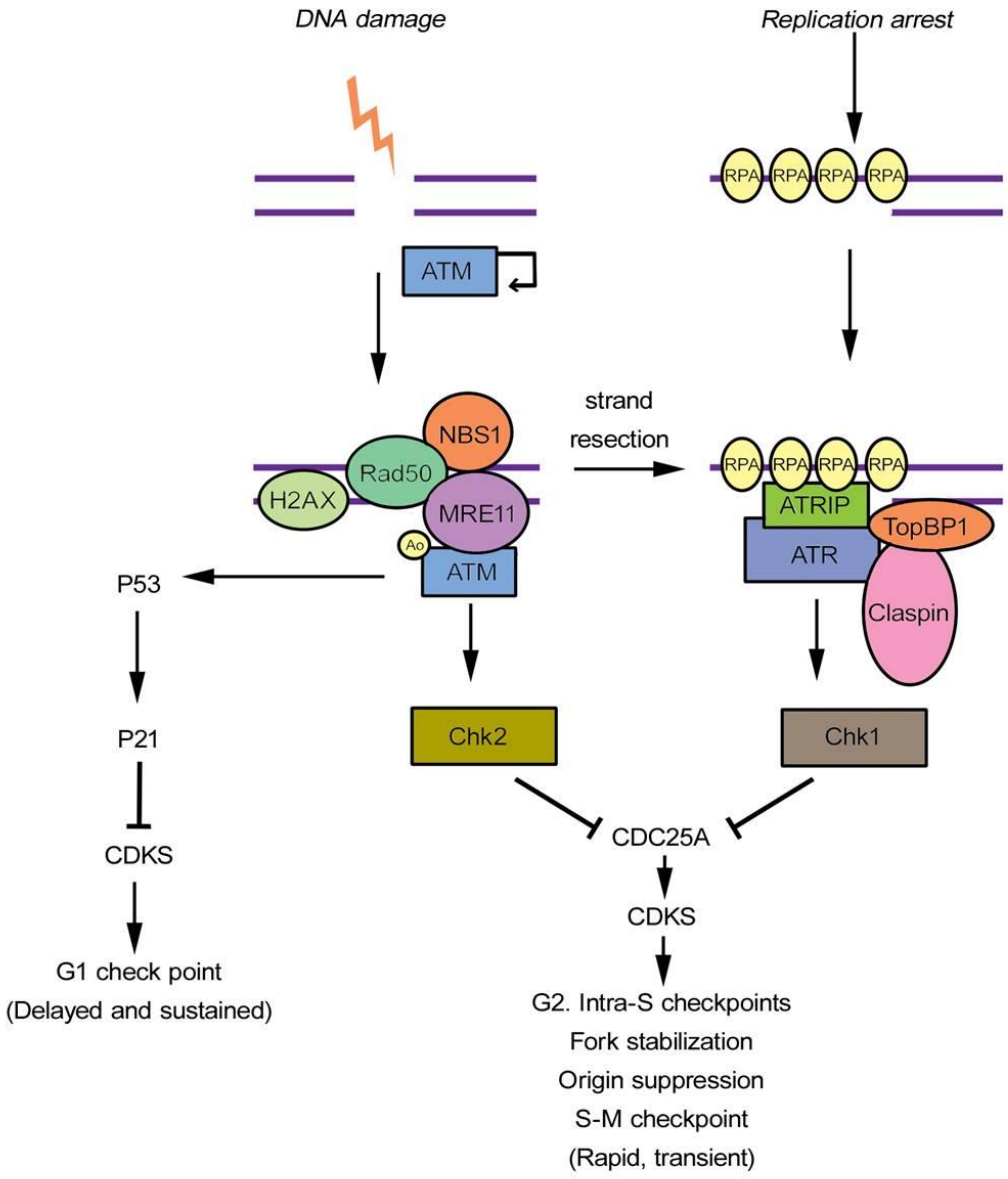


Figure 1.6 Cell cycle checkpoints in response to double strand breaks are mediated by ATM-Chk2, ATR-Chk1 and p53 pathways.

(Adapted from [207]) Double strand breaks on DNA molecules trigger a series of signalling events leading to activation of cell cycle checkpoints.

ATM is one of the first molecules to be activated in response to double strand DNA breaks. Under normal conditions, ATM kinase is in its inactive dimer form. It can rapidly sense double strand breaks and auto-phosphorylate at Ser1981, which causes structural changes and promotes dimer dissociation into active monomeric ATM [208]. Scaffolding complex MRN (MRE11, Rad50 and NBS1) functions as a docking site for ATM to the site of double strand breaks (DSB) [209] . ATM kinase has multiple substrates including histone H2AX, Chk2 and p53. In terms of cell cycle arrest, ATM activates the p53 tumour suppressor protein by phosphorylation at multiple sites (Ser15, Ser46 and Ser9) [210]. p53 protein level is normally kept low in a healthy cell, through constitutive degradation by MDM2 [211]. Phosphorylation of p53 stabilises the protein [212] and p53 level is rapidly elevated in response to ATM activation [213]. p53 transcriptionally activates p21, which arrests cell cycle transition from G1 to S [214, 215]. The cell cycle functions of p53 are of particular importance if DSBs take place during the G1 phase of the cell cycle [216].

When DNA damage occurs after the G1 phase, ATM is able to arrest the cell cycle before mitosis through an alternative mechanism. ATM phosphorylates and activates Chk2 (check point protein 2) which signals to activate G2 and intra-S checkpoints [217]. Moreover, Chk2 is also able to phosphorylate p53, at Ser15 [218]. Besides cell cycle arrest, p53 plays a central role in programmed cell death. The choices between cell cycle arrest or apoptosis depends on a variety of factors including the degree of DNA damage, the types of lesions, the protein levels of p53, and the presence of other pro-death or pro-survival signals [219] .

Both ATM and ATR are serine-threonine kinases containing similar functional domains. The two molecules were historically thought to act in parallel with overlapping functions [220]. They both mediate cell cycle arrest and DNA damage repair. Recent studies have shown that ATM is required for ATR activation in response to double strand breaks [207], with some exceptions.

Once activated and recruited to the site of double strand breaks, ATM kinase phosphorylates and activates a number of substrates including CtIP, which forms a complex with BRCA1 protein. The complex is a crucial component for Exo1 nuclease mediated strand resection [221]. Strand resection is controlled in a cell-cycle dependent manner. Strand resection is mainly observed in S-phase cells where most spontaneous damage occurs [222]. The determination of which repair pathway to undertake depends on the cell cycle (See Chapt. 1.6.2). During strand resection, 3' single strand DNA (ssDNA) overhangs are generated on each end of DNA breaks. ssDNA is readily coated with Replication Protein A (RPA) proteins. ATR protein is recruited, docking to ssDNA region via adaptor protein ATRIP (ATR-Interacting Protein). ATR activates the Chk1 mediator Claspin via phosphorylation. Active Clapsin recruits Chk1 to the site of damage where Chk1 is also phosphorylated by ATR at Ser317 and S345 sites, which are crucial for Chk1 functions [223]. Activated Chk1 is then dissociated from the complex, interacting with its substrates both in the nucleus and cytoplasm [224]. Similar to the function of Chk2 in cell cycle regulation, Chk1 also contributes to G2 and intra-S checkpoint activation.

The ATR-Chk1 pathway is activated in the presence of single strand DNA. Since ATM mediates strand resection during blunt end double strand breaks, ATM is required for ATR activation [225] [226]. ATR can also be activated without ATM, when there are single-stand DNA overhangs caused by replication arrest for example. RPA (Replication protein A) is a small protein that binds to single strand DNA, it stimulates the binding of ATRIP which recruits ATR to the site of DNA damage [227].

Chk1 and Chk2 are able to phosphorylate the Cdc25 protein family [228], and this in turn inhibits CDK (cyclin dependant kinase) functions. 14-3-3 protein promotes CDK1-cyclin B and mitotic entry. Phosphorylated Cdc25 binds and sequesters 14-3-3 protein in the cytoplasm. In summary, ATM-Chk2 and ATR-Chk1 pathways sense and mediate cell cycle arrest upon double strand breaks.

1.6.2 Repair of double strand breaks

During cell cycle arrest, the cells are given the opportunity to repair the damage. The cells have complex mechanisms to deal with different types of DNA damage. There is base excision repair (BER) for damaged bases, single strand break or abasic sites, nucleotide excision repair (NER) pathways remove and repair bulky lesions or dimerisation, and DNA mismatched repair (MMR) for mismatched bases. These lesions are efficiently recognised and regularly repaired in the cells [229]. Among all types of DNA damage, double strand breaks (DSBs) are especially difficult to repair. This type of damage is highly hazardous because it can lead to loss of genetic information; and chromosomal re-arrangements. If two breaks are wrongly joined onto different chromosomes. Tumour suppressor inactivation and oncogene activation can take place in the process. Defects in double strand break repair lead to genetic instability and a higher probability of carcinogenesis [230].

A number of DNA damaging agents cause double strand DNA breaks, such as ionising irradiation (IR), etoposide and doxorubicin. These agents have also been used in cancer therapeutics that target rapidly proliferating cells with defective DNA repair mechanisms. Natural causes of DSBs include free radicals produced by oxidative metabolism, and replication fork collapse when polymerase encounters a single brand break.

DSBs take place in our cells at a frequency of 10 times per cell per day [231]. Cells can repair DNA double-strand breaks by two principle mechanisms - non-homologous end-joining (NHEJ) and homologous recombination (HR). The choice of pathway depends on the cell-cycle phase and the type of DSB ends. ATM and ATR, the two master cell-cycle regulators during DNA damage, are also implicated in DNA damage repair. Both ATM and ATR are required for HR pathways. The key catalytic protein that mediates NHEJ is another serine/threonine kinase called DNA-PKcs (DNA protein kinase catalysing subunit). ATM, ATR and DNA-PKcs all belong to phosphatidylinositol-3 kinase related protein kinases (PIKKs) family.

They are well conserved large proteins and they share similar functional catalytic domains [232].

The process of mammalian NHEJ is illustrated in Fig. 1.7. During NHEJ, the first protein that binds the DNA ends is thought to be KU protein complex, which has high affinity for the ends of DSBs [233]. It is a heterodimer consisting of KU70 and KU80 proteins and provides scaffolding for other NHEJ components. Two KU complexes bind to each broken strand and each interacts with a molecule of DNA-PK protein. The two DNA termini are joined in three steps. DSBs are rarely blunt ended and they frequently contain overhangs and phosphate groups [234]. Firstly, DNA-PK protein forms a complex with nuclease Artemis which mediates broken ends processing. Nucleotides are removed from the broken ends to create blunt ends. Then DNA polymerases are recruited to the complex and fill in the gaps at NHEJ junctions. Finally, Ligase IV/XRCC4 complex joins the ends. Since short sequences are sometimes removed by nucleases and this can lead to loss of genetic information, the NHEJ process is considered to be an error-prone process.

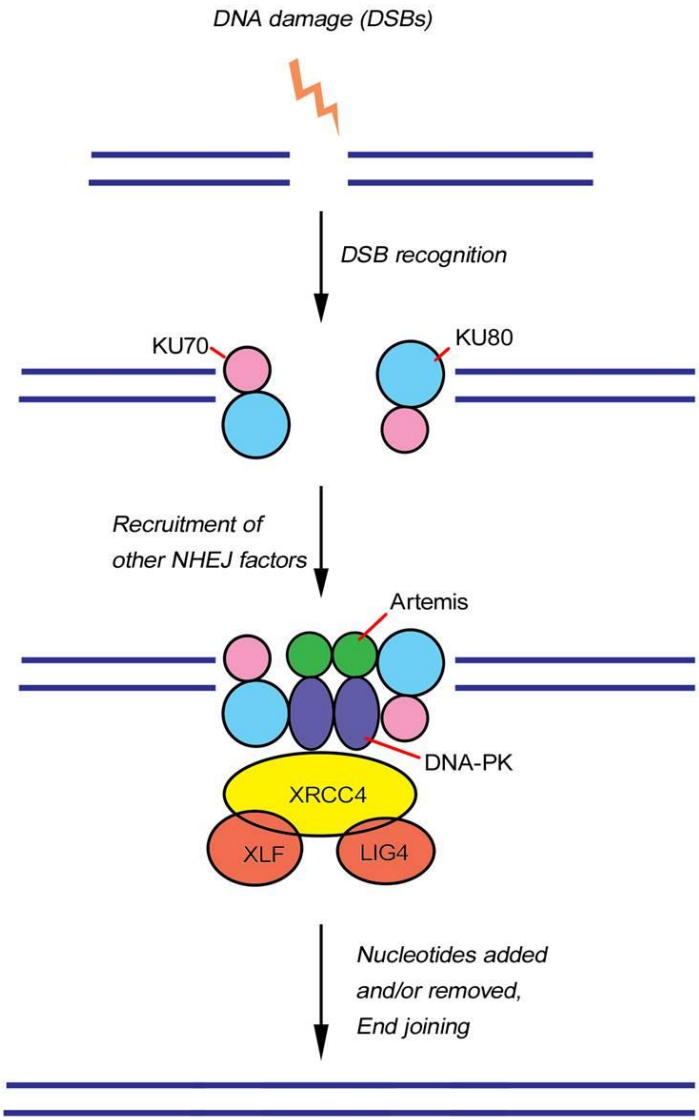


Figure 1.7 Non-Homologous End-Joining (NHEJ) repair mechanism in mammalian cells in response to double strand DNA breaks.

(Adapted from [207]) KU heterodimetric complex recognises the double strand breaks and bind to the ends. Downstream NHEJ factors including Artemis, DNA-PK, LIG4, XRCC4 and XLF are recruited forming a complex. Nucleotides are added and/or removed at the end of breaks and the ends are joined.

In contrast to NHEJ, HR is more or less error-free, as a homologous chromosome is used as a repair template. The process of HR is demonstrated in figure 1.8. During HR, strand resection takes place along the site of damage from 5' to 3'. This process is carried out by Exo1 exonuclease and facilitated by a few protein mediators including BRCA1-CtIP, MRN complex and BRCA2. Nearly all single strand DNA in the cells is coated with RPA molecules [235], which prevents ssDNA forming secondary structures. Rad51 is a crucial factor for HR DNA repair pathways, promoting homology match on a different chromosome and DNA strand invasion – a process where the broken single strand DNA pairs with an intact DNA molecule through homology. Once activated, Rad51 replaces RPA on the ssDNA [236], which is paired with a complementary strand by homology on a different, undamaged chromosome. DNA polymerases fill in the gaps using the partner chromosome as a template. Finally a Holliday junction is formed to resolve the DNA structure, giving rise to two intact DNA molecules.

Mediated by the two master kinases ATM and ATM, cell cycle checkpoints occur in parallel with DNA damage repair pathways. Cell cycle checkpoints are important for two main reasons. When there is DNA damage, the cells cease to grow and proliferate; otherwise it could potentially lead to inheritable mutations. Also, during cell cycle arrest, the cells are given a period of time to repair the DNA lesions.

In the case of heavily damaged DNA where the cells cannot resolve the lesions, activated p53 induces programmed cell death [237].

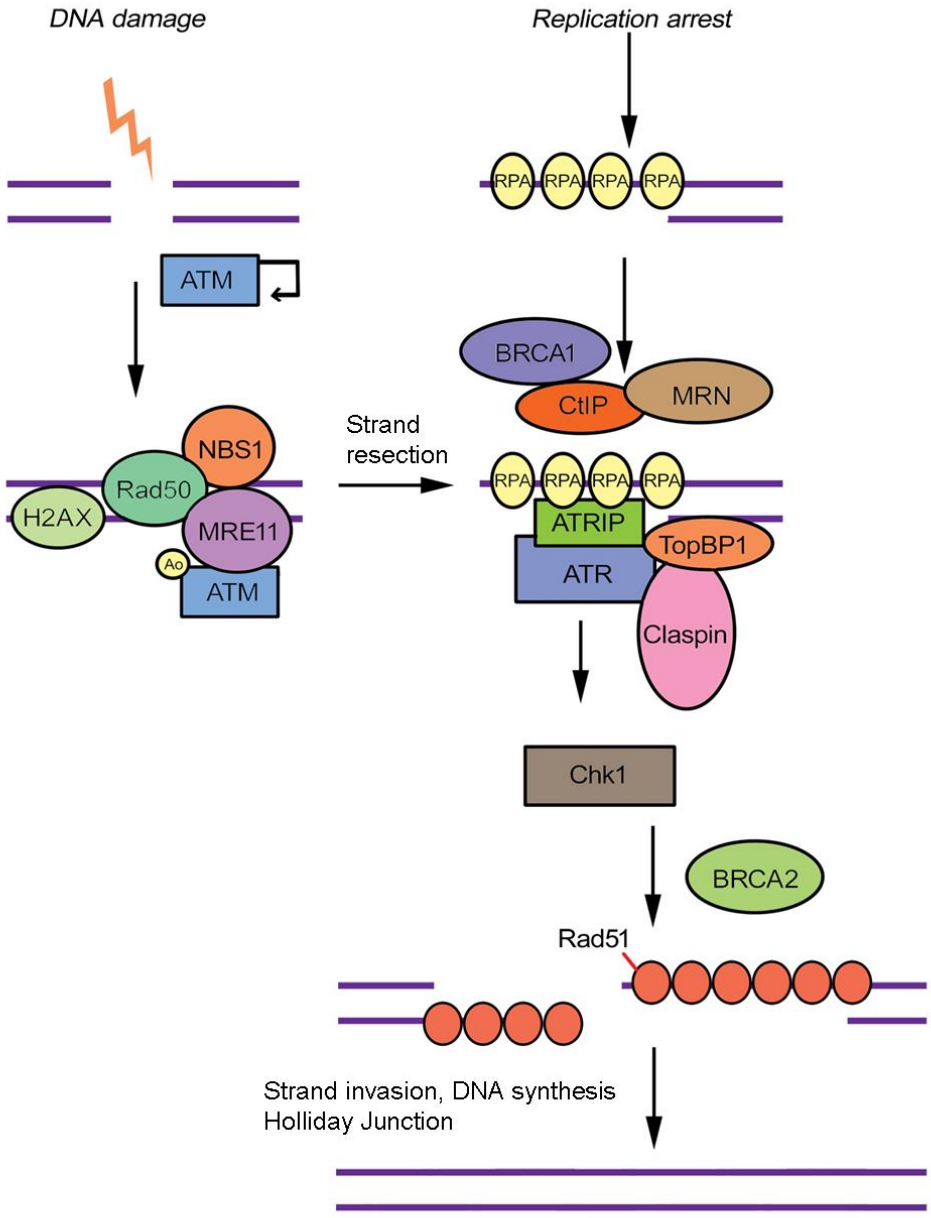


Figure 1.8 Homologous recombination DNA repair is activated when there are double strand DNA breaks or when there is DNA replication fork collapse.

ATM and ATR are the two master kinases that get activated, forming a core for signalling hubs at sites of damage and mediate downstream signalling events that complete the repair.

There is a significant amount of cross-talking in DNA damage response networks. In recent years, it has been discovered that in addition to cell cycle regulations, checkpoint proteins Chk1 and Chk2 can also regulate DNA damage repair. Chk2 is reported to phosphorylate BRCA1 [238] and Chk1 is found to phosphorylate BRCA2 [239] and Rad51 [240]. These molecules are important components of HRR pathways. The cell cycle mediator protein Chk1 has been reported to be a crucial factor for HR DNA repair pathways [240]. In the study lead by Thomas Helleday, Chk1 is found to activate Rad51 through phosphorylation on Thr 309 site, which is a key molecule for homologous recombination. Cells depleted of Chk1 and cells with T309A inactivation mutant fail to form Rad51 nuclear foci in response to DNA damage [240].

Although HR is error-free and NHEJ is error-prone, HR can only take place after DNA replication because of its requirement for a sister chromatid. NHEJ can take place throughout the cell cycle. During S phase, HR is the predominant repair mechanism for DSBs. During G0 and G1 phases, NHEJ is the main pathway. Both processes are thought to be crucial and defects in either pathway lead to diseases and even lethality.

1.7 cancer therapeutics and synthetic lethality

The trait of defective DNA repair mechanisms in cancer cells has been exploited in cancer treatments such as conventional chemotherapy and radiotherapy. Platinum-containing compounds such as carboplatin and cisplatin are DNA damaging agents that intercalate in DNA structure, resulting in distortive DNA lesions. Specific inhibitors of DDR components have been developed and used effectively to treat cancers, for instance, etoposide and irinotecan are agents that inhibit topoisomerase, an enzyme that unwinds DNA by breaking and rejoining the phosphodiester bonds on the DNA backbone in order for transcription or replication to take place. Double strand breaks occur wherever topoisomerase is inhibited. Thanks to the robust DNA repair response in mammalian cells; these lesions are repaired in most of the normal

cells in the body. On the other hand, cancer cells with a defective DNA repair mechanism may not be able to resolve the damage and this leads to cell death. Moreover, cancer cells are often insensitive to signals suppressing growth and they generally proliferate more rapidly than non-transformed cells. DNA molecules are synthesized at a faster rate; therefore cancer cells are more sensitive to these DNA damaging agents. However, one of the major problems of these conventional killing strategies is not tumour specific, these agents frequently lead to cell death in non-transformed cells, especially those with fast proliferation rates. Therefore these drugs have a limited therapeutic window. Patients frequently suffer from a wide range of side effects including hair loss, diarrhea and immuno-suppression.

Clearly, development of targeted therapies is much needed; utilizing synthetic lethality in strategies of cancer therapy is becoming a focus of cancer research. Due to genetic instability, cancer cells develop gain-of-function mutations and they also harbour many non-lethal loss-of-function genetic defects. Novel cancer therapies have been developed to specifically target gain-of-function cancer mutations. For example, anti-HER2 antibodies have been used to treat breast cancers that over-express HER2 receptor [241]. However, loss-of-function defects can not be easily reversed [242]. Synthetic lethality is a situation where two non-lethal genetic defects are not compatible with each other. The combination of the two defects lead to cell death [243].

The multiple pathways in DDR functionally complement each other and there is some redundancy to certain degree. The tumour cells can be viable after loss of certain pathways. A number of DDR synthetic lethal situations have been identified. For example, cancer cells with dysfunctional BRCA are hypersensitive to PARP inhibitors [244]. Cancer cells with loss of BRCA1 or BRCA2 display defect in HR repairs, inhibition of PARP leads to increased single strand DNA breaks that become double strand breaks with overhangs. These damages can only be repaired by HR pathways [245]. A better understanding of synthetic lethal relationships in cancer genetics will potentially lead to breakthroughs in targeted cancer therapies.

1.8 Aim of project - to investigate the role of autophagy in DNA damage repair

The current understanding on the relationships between autophagy and DNA damage is that loss of autophagy leads to accumulation of DNA damage. Eileen White and colleagues discovered that defective autophagy leads to DNA damage response both in vitro and in mammary cancer cells in vivo, and increased γH2AX foci was observed in autophagy deficient cells [1]. Increased DNA damage was also observed. Gene amplification, aneuploidy (A state of cells which have more or less numbers of chromosomes than normal.) and eventually genomic instability take place which contribute towards tumourigenesis [2].

There is also other links recently discovered between autophagy and DNA damage. Protein acetylation and deacetylation are post-translational modifications that are implicated in gene activation/silencing, chromatin dynamics, DNA damage responses [246] and protein stability. Certain acetylated proteins are reported to be degraded through autophagy [247]. Thomas Robert and colleagues showed that yeast sae2 (human CtIP), a recombination protein involved in strand resection is acetylated and degraded by autophagy [248]. The cells possibly use this mechanism to keep levels of HR repair proteins low, especially during cell cycle phases when HR is inactive. FIP200 is a gene essential for autophagy activities, since cells with FIP200 (200 kDa FAK-family interacting protein) depletion are hypersensitive to DNA damaging agents and show deficient DNA damage repair. [249].

The exact mechanism of how autophagy regulates the DNA damage response is still unknown. Since autophagy deficient cells have an accumulation of DNA damage [2], which could be either due to increased incidences of DNA damage occurrences, or due to certain defects in DNA damage response pathways. DNA damage response is a multi-step process and it involves a complex network of molecules. The main aim of this project is to dissect the pathways and investigate the exact role of autophagy in this process.

Chapter 2: Materials and methods

2.1 Composition of routinely used solutions and media

Phosphate Buffered Saline (PBS)	170 mM NaCl, 3.3 mM KCl, 1.8 mM Na2HPO4, 10.6 mM KH2PO4
PBS-Teen (PBT)	0.5% BSA, 0.1% Tween 20 in PBS
5x RIPA	0.75M NaCl, 5% NP40, 2.5% Sodium Deoxycholate, 0.5% SDS, 0.25M Tris pH8.0
SDS Running Buffer:	0.1% SDS, 192 mM glycine, 25 mM Tris pH8.3
Stripping Buffer for Western Blots	0.2M Glycine, 1%SDS and pH adjusted to 2.5 with HCl
Tris-acetate-EDTA (TAE):	40 mM Tris, 0.1% glaciel acetic acid, 1 mM EDTA
Transfer Buffer:	192 mM glycine, 25 mM Tris, 20% methanol, 0.01% SDS
Tris-Buffered Saline (TBS):	25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl
Tris-EDTA (TE):	10 mM Tris-HCl, pH 8.0, 1 mM EDTA
TBST	TBS + 0.1% Tween-20

2 x Western Sample Buffer	100 mM Tris, pH 6.8, 2% SDS, 5% ß- mercaptoethanol, 15% glycerol, bromophenol blue
5 x Western Sample Buffer	60 mM Tris-HCL, pH 6.8, 2% SDS, 4 mM ß-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue

Table 2 Composition of routinely used solutions and media

2.2 Cell culture and treatments

2.2.1 Cell culture

Mouse Embryo Fibroblast (MEF) cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine (Invitrogen), 60 μg/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). Cells were routinely grown in a humidified atmosphere containing 5% CO_2 at 37°C. Cell culture was performed in a Class II hood, using aseptic technique and sterile equipment and reagents.

Cells were passaged at a frequency of every 2-3 days. After media was aspirated from the flask or culture dishes, 10ml of PBS was added to the cells to wash the cells and it was then aspirated. 3ml 0.25% trypsin in PE buffer was added to the flask/dish for approximately 3 minutes at 37°C to detach the cells. Following trypsinisation pre-warmed fresh media was immediately added to the dissociated cells to neutralise trypsin reactions. The cell suspension was then transferred into a fresh flask/dish at a ratio of 1:2 to 1:3.

Cryo-freezing was routinely used for long term deposit of cell lines. Cells were trypsinised and centrifuged at 1000g for 5 minutes at room temperature. The medium/trypsin solution was removed and the cell pellet was resuspended in a solution consist of 90% FCS, 10% dimethylsulphoxide (DMSO). Cells were

aliquotted into cryo-tubes and frozen at -80°C. For longer term storage, the cryotubs were then transferred to liquid Nitrogen tanks which held the cells at -180°C.

Thawing of cells was carried out by placing cryo-tubes in a 37°C water bath at until just thawed. Cells were then mixed with 10ml pre-warmed fresh media, centrifuged at 1000g for 5 minutes and the supernatant was removed to eliminate DMSO from cell freezing medium. The cell pellet was finally resuspened in 10% FCS DMEM.

2.2.2 Cell number counting

The number of cells in suspension was determined using the automated Casy® Cell Counter system (Innovatis). Following trypsinisasion and resuspension in a small amount of medium, the cells were diluted by 50 fold with PBS in a cell counting cup. The cell number was automatically determined by the machine which also provides information about the viability in cell population. For growth curve measurements the cells were counted in triplicate.

2.2.3 Growth Curve construction

Cells from sub-confluent cultured flasks or dishes were trypsinised and diluted in 10% FCS medium. 10^6 cells were plated onto each 10mm dishes and the cells were harvested and counted. For each data point, triplicate of dishes were used and the errors were calculated as standard deviation.

2.2.4 Primary MEF cell isolation

13 – 14 days after female plugging was noticed, the pregnant female was culled and immersed in 500ml deionised water with one Presept tablet dissolved in it. The carcass was sprayed with 70% Ethanol and the uterus containing embryos was excised using sterile dissection tools. The uterus was rinsed in a 15cm tissue culture dish containing 50ml 33ug/ml gentamycin (Invitrogen) in PBS. Once transferred to

a Class II hood, the embryos were freed from the uterus using fine forceps and decapitated using scalpels. Red internal embryonic organs were removed and discarded. The remaining tissues were torn into small pieces using forceps and transferred into a falcon tube with 10ml 0.05% trypsin in PE buffer. The tube was then incubated at 37°C for 30 minutes with gentle shaking every 10 minutes before 800µl of 2.5% trypsin was added to the tube for a further 10 minutes. At the end of the incubation, the cells were seeded into 15cm tissue culture dishes containing 40ml pre-warmed medium at a density of 2-embryo-equivalence of cells per dish.

2.2.5 Transient transfection of plasmid DNA using Calcium Phosphate Precipitation.

Cells to be transfected were seeded onto 100 mm tissue culture plates the day prior to transfection. A total of 20 µg DNA plasmid was diluted in 440 µl distilled H_20 (Invitrogen), the DNA was mixed with 500 µl 2 x HBS thoroughly. Finally 60µl of 2 M $CaCl_2$ solution was rapidly added to the mixture which was then vigorous mixed. DNA calcium phosphate precipitate was allowed to form for 20 minutes at 37°C then added drop-wise to tissue culture plates. The plates were incubated at 37°C in a humidified incubator for 16h – 18h when plasmid DNA was introduced into the cells through the calcium phosphate precipitate. The medium was then was removed after 16 hours before fresh 10% FBS DMEM added.

2.2.6 Eletroporation transfection of primary MEFs.

$1x10^6$ cells MEFs in exponential growth phase were trypsinised centrifuged at room temperature. The supernatant was removed and the pellet was washed with 10ml PBS. The pellet was re-suspended in100µl Nucleofector® Solution (Lonza).The cells were then transferred to an electro-curvette (Lonza) where 10µg DNA plasmid was added. The mixture was electroporated using T20 program. The cells were then rapidly placed in a dish containing 10% FBS DMEM. Transfected cells were incubated in a 37°C/5% CO2 environment until analysis.

2.2.7 Retroviral infections

Retroviral infections were carried out using phoenix-eco retroviral packaging cells which produce retrovirus that targets mouse cells. $1.5x10^6$ phoenix-eco cells were plated in a 100 mm dish the day before, and 20 μg retroviral DNA plasmid was transfected into each plate using Calcium Phosphate Precipitation method as described above. The supernatant containing retrovirus in 20% FBS DMEM was collected and added to target MEF cells three times within two days. 5 μg/ml final concentration of polybrene (hexadimethrine bromide; Sigma) was added to the supernatant. The MEF cells were plated the day before infection at 0.8 $x10^6$ /100mm. The supernatants containing retrovirus were filtered through 0.45 μm filters to remove any phoenix eco cells and it was then added to the MEFs cultures. 24h after the last round of infection, MEF cells were selected for three days in 2.5 μg/ml puromycin (sigma) containing 10% FCS DMEM.

2.2.8 Generation of primary Atg7-/- cell lines.

Primary MEF cell lines with stable deletion of Atg7 were generated using Phoenix-eco as a packaging cell line. Retroviral infection with a control plasmid and a plasmid containing cre-recombinase was performed in primary Atg7flox/flox MEFs.

2.2.9 Establishment of cell lines for detecting HR capacity.

$Atg7^{flox/flox}$ MEFs were transfected with 0.5μg of HR reporter constructs from Kevin Hiom, University of Dundee. Puromycin, at 2.5μg/ml, was added to the media 1 day post-transfection. Colonies were isolated after 8–10 days on selection. Genomic DNA was then extracted and analyzed by Southern blotting to confirm that the cell lines contained a single integrated copy of the reporter cassettes, this was carried out by supervisor Kevin Ryan. Subsequently several colonies were picked and infected with cre recombinase retrovirus to remove Atg7 gene. By following the

steps above, Atg7f/f and Atg7-/- cells with stable genomic insert of HR reporter construct were established.

2.2.10 γ –ray ionizing Irradiation (IR) of cells

IR treatment is a method of damaging DNA by causing double strand breaks. The cells to be irradiated were plated the day before on 10mm dishes. The dishes with adherent cells were irradiated using Alcyon II Cobalt-60 Teletherapy Unit. Depending on the half life of cobalt element, length of treatment was calculated according to dose rates that varied between 2-10 Gymin-1. The dishes were placed underneath the cobalt source and irradiated. Control cells were also brought to the Co-60 source at the same time without being irradiated.

2.3 Molecular cloning

2.3.1 Restriction digests

Restriction Digests were carried out with enzymes and buffers from New England Biolabs (NEB). Five to ten-fold unit excess of enzyme was incubated with 10μg plasmid DNA in the appropriate NEB buffer overnight at for 1 hour 37ºC. After digestion, cleaved vector plasmid was incubated for a further 30 minutes at 37ºC with 5U Shrimp Alkaline Phosphatase (NEB). Finally digested DNA was purified to remove enzymes using Qiagen PCR purification kit and resuspended in Tris-EDTA (TE) buffer.

2.3.2 Ligation

DNA fragments following restriction enzyme treatment were purified before ligation reactions. The fragments were separated by gel electrophoresis followed by excision using a Qiagen Gel Band Purification Kit. Ligations were carried out at room temperature overnight using Rapid DNA Ligation Kit (Roche). An

approximate twice the amount of the insert fragment over the vector plasmid was combined in a final volume of 10µl DNA Dilution Buffer. 10 µl T4 DNA Ligation buffer was then added along with 5U T4 DNA ligase.

2.3.3 Transformation of competent cells

E.Coli DH5α supercompetent cells (Stratagene) were transformed for plasmid preparation. The cells were aliquoted into 50µl volumn and stored at -80ºC. For each plasmid, one aliquot was thawed on ice before 10-20 ng of plasmid DNA (in 1ul TE or water) was gently added into supercompetent cells. The cells were incubated for 15 minutes on ice before heat shock treatment at 42ºC for 30 seconds. The tubes were then rapidly transferred to ice, where 250 µl of 37ºC LB broth was added. The competent cells were incubated in suspension for 45 minutes on an orbital shaker (225-250 rpm). 150 µl of the transformation mixture was then added to and spread on LB agar plates containing 50 µg/ml ampicillin or kanamycin. The plates were incubated at 37ºC overnight in order for colony-formation of the transformed cells.

2.3.4 Screening of transformants – minipreps

Single colonies were isolated from the transformation plates. They were grown overnight at 37ºC in 10ml LB medium with 50 µg/ml ampicillin/kanamycin. Plasmid DNA miniprep were performed using QIAprep Spin Miniprep Kits (Qiagen #27104) (Routinely carried out by Molecular Technology Service, Beatson Laboratories). Analytical restriction digests were performed with appropriate enzymes and buffers and resolved by agarose gel eletrophoresis to validate successful insert of DNA fragments. DNA plasmids with inserts were subsequently sequenced (Carried out by Central Services).

2.3.5 Preparation of plasmid DNA – maxipreps

Maxipreps were carried out for large scale plasmid DNA preparation. After the DNA plasmid was transformed into competent cells, a single bacterial colony was isolated from the bacteria plate and inoculated into 4 ml LB medium containing 50μg/ml ampicillin/kanamycin. The mini culture was incubated for 4 hours with vigorous shaking at 37ºC. The culture was then transferred to a conical flask containing 200 ml LB containing 50μg/ml ampicillin/kanamycin. Following an overnight incubation at 37ºC on an orbital shaker (300 rpm), cells were pelleted by centrifugation at 3000g for 20 minutes at 4ºC and plasmid DNA retrieved using the Qiagen Plasmid Maxi Kit following kit instructions (routinely performed by Molecular Technology Service in the institute).

2.3.6 Agarose gel electrophoresis

1% - 2% agarose gel was prepared by adding agarose (Sigma) to 200ml TAE buffer, before microwave heated to dissolve the agarose. Ethidium Bromide was added to give a final concentration of 0.5μg/ml in the agarose solution. Agarose was poured into a gel tray with combs inserted and allowed to solidify for at least 45 minutes. Samples for electrophoresis were prepared by the addition of 5 x gel loading buffer (30% glycerol, bromophenol blue) and electrophoresed at 120V for approximately 1h in TAE gel running buffer. DNA bands were visualised using a UV transilluminator.

2.4 RNA/cDNA techniques

2.4.1 Preparation of total cellular RNA

Total cellular RNA was isolated from sub-confluent growing cells using Qiagen RNeasy Spin Column kit, in accordance with the manufacturer's instructions. Before the cells were lysed in RLT buffer, Media was aspirated from 6-well plates or 100 mm plates and the cells were rinsed with PBS once. After RNA isolation, the concentration was determined by UV spectrophotometry. A ratio of absorbance

at 260 nm to 280 nm in the range of 1.8-2.0 indicated the RNA samples were relatively free from DNA or protein contamination.

2.4.2 Preparation of cDNAs from RNA using reverse transcriptase

Template RNA was isolated from whole cell lysate as described above. cDNAs were prepared using using DyNAmo™ SYBR® Green 2-Step qRT-PCR kits (Finnzymes), in accordance with manufacturers instructions. 20 µl reaction mix was prepared by mixing 1µg RNA, 10µl RT buffer 1µl Random Hexamers (300 ng/µl) and 2 µl M-MuLV Rnase H+ reverse transcriptase.

Reverse transcriptase reactions were carried out using a Peltier Thermal Cycler (MJ research, Helena Bioscience) under the following parameters: 25ºC for 15min, 37ºC for 30min and 85ºC for 5min. cDNAs were then stored at -20ºC.

2.4.3 Quantitative Real Time Polymerase chain reaction (qRT-PCR)

Prior to analysis, a bulk preparation of serially diluted total RNA from genetically unmodified MEFs was made, aliquoted and stored at -20ºC. The RNA was used as stardards in each qRT-PCR reactions.

qRT-PCR was carried out with 2 µl of cDNA, 10 µl 2x qPCR master mix provided in DyNAmo™ SYBR® Green 2-Step qRT-PCR kits (Finnzymes), 5.5 µl H2O and 2.5 µl designed primers as indicated (QuantiTect Primer Assays – Qiagen) for gene of interest.

The reaction mix was pipetted into 96 well optical plate (BioRad) with an optically clear flat cap strips (BioRad). qPCR was carried out under the following cycling parameters using MJ Opticon Moniter Analysis Software version 3.1, on a Peltier Thermal Cycler (MJ Research) with a Chromo 4 continuous fluorescence detector. Data analysed using MJ Opticon Moniter Analysis Software version 3.1

PCR reaction were carried out using cDNA template prepared as described previously under the following cycling parameters: 95°C for 15min, [94°C 10 sec, 55°C 30 sec, 72°C 30 sec] 40 cycles, 72°C 10 min. Expression levels of genes analysed by qPCR were normalized relative to levels of 18S rRNA.
The following Primers were used

Primers for Atg7:
5′-ATGCCAGGACACCCTGTGAACTTC-3′
5′-ACATCATTGCAGAAGTAGCAGCCA-3′.

Atg5:
5’-AAGTCTGTCCTTCCGCAG-3’
3’-TGAAGAAAGTTATCTGGGTAG-5’

Mouse 18S primers were from Qiagen (QT01036875).

2.5 Protein immunoblotting (Western Blotting)

2.5.1 Cell lysis and protein extraction

Immediately prior to use, 10ml 1x RIPA was prepared and kept on ice by diluting 5x RIPA buffer in deionised water, 1 protease inhibitor tablet and 200µL Na_3VO_4 was added to the buffer to inhibit proteolysis and dephosphorylation after cell lysis.

Plates to be harvested were washed twice with ice cold PBS, 400µL RIPA lysis buffer was added to each 100mm dish. Lysates were collected from plates and transferred to 1.5mL eppendorf tubes, which were incubated on ice for 45 minutes. Subsequently, the whole cell lysates were centrifuged at maximum speed in a desktop minicentrifuge (21,000g) for 10mins at 4°C to pellet DNA and debris. The supernatants were transferred to fresh eppendorf tubes and stored at -20°C until use.

2.5.2 Separation of proteins by polyacrylamide gel electrophoresis (SDS-PAGE)

Protein lysates in RIPA buffer was diluated with 2x western sample buffer. Prior to loading on denaturing polyacrylamide gels, the samples were boiled on a 100°C heat block for 5 minutes. 20µL of protein samples were loaded into a Nupage® Novex® Bis-Tris 4-12% (Invitrogen) gradient gel or a homemade 10% - 15% gel. 8µL of Pre-stained protein standard (Thermo PageRuler®) was loaded into a well so as to determine the molecular weight of the proteins within the samples. Gels were run in 1X NuPAGE® MOPS SDS running buffer for the precast gels, or homemade 1X running buffer for the homemade gels, at 150V in an Invitrogen XCell SureLock^{TM} mini-cell electrophoresis system. The voltage was terminated when the blue dye front had reached the bottom of the gel. For gels to be probes with antibodies recognizing small proteins such as LC3 or H2AX, the voltage was stopped before the dye front had reached the bottom.

Depending on the molecular weight of the protein to be probed and detected, the homemade 10-15% gels were made with from 30% stock acrylamide solution (37.5:1 acrylamide:bis acrylamide) Severn Biotech Ltd) and constituted 375mM Tris-HCl, pH8.8, 0.1% SDS, polymerised with 0.05% ammonium persulphate (APS) (Sigma), 0.1% TEMED (Sigma). Stacking gel was 4% acrylamide and with 125mM Tris-HCl, pH 6.8 , 0.1% SDS, polymerised with 0.05% ammonium persulphate, 0.1% TEMED.

2.5.3 Western blotting

Electrophoretic transfer of separated proteins resolved by SDS-PAGE gels to nitrocellulose (Amersham Biosciences) or PVDF membranes was achieved using Invitrogen XCell SureLockTM mini-cell tanks. An extra step applied for PVDF membranes, they were activated by soaking in methanol for 2min before soacking in transfer buffer for 2 min until uniformly opaque. SDS Gels were blotted onto membranes in a sandwich with sponge and 2 sheets of Whatman 3MM paper, all

pre-soaked in transfer buffer, the gel faces cathode side of the sandwich and the membrane anode. Gel transfer was carried out in Transfer Buffer at 0.4 Amps for 1.5 hours in an XCell IITM blot module. After transfer, ponceau-S (Sigma) staining was performed to assess transfer fidelity. 0.1%. The ponceau S stained membrane was subsequently detained by 2 washes with dH2O and 1 wash in TBST. A list of antibodies used for western blotting analysis and FACs analysis was presented as in table 2.

Antibody	Dilution	Company
actin (clone 1A4)	1:10,000	Sigma
BrdU	1:200	Dako
Claspin H300	1:1000	Santa Cruz
S345 p-Chk1	1:1000	Cell Signalling
total Chk1 G4	1:1000	Santa Cruz
ERK p42	1:1000	Santa Cruz
γ-H2AX	1:1000	millipore
S10-Phospho-Histone-3	1:100	Santa Cruz
LC3B	1:1000	Cell Signalling
Wip1	1:1000	Santa Cruz
Anti mouse IgG HRP linked	1:3000	Cell Signalling
Anti rabbit IgG HRP linked	1:3000	Cell Signalling

Table 3 List of antibodies used for western blotting and flow cytometry analysis.

2.5.4 Probing and signal detection

After electrophoric transfer, membranes were blocked in TBST milk blocking buffer (TBST + 5% skimmed milk powder (Marvel) for 30 min at room temperature on with gentle shaking on a rocker. Membranes were then were incubated with primary antibodies typically a 1:1000 dilution in 5% BSA in TBST with gentle rotation overnight at 4°C.

After incubation in primary antibody, the membranes were washed 3 times with TBST for 15 minutes each time and incubated with for horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature with gentle rotation. The blots were then washed 3 times for 15 minutes with TBST before protein signals were activated using enhanced chemiluminescence reagents (ECL Amersham), in accordance with manufacturer's instructions. X-ray films (Fuji Medical X-ray film) were used to capture the signals and developed in an X-ray processor (AGFA).

2.5.5 Stripping of Western Blots

In order to detect a different protein band on the same membrane, the membrane was incubated in stripping buffer for 30 min at 50°C with constant agitation, to remove bound primary antibodies without removing transferred proteins. The membranes were then washed twice in TBST and incubated in 5% milk in TBST blocking buffer for 30 min at room temperature.

2.6 Flow Cytometry Assays

2.6.1 Sample collection for flow cytometry analysis

Cells were grown on 10mm or 6-well dishes and treated accordingly. At time of sample collection, media that includes floating cells was collected into a 15ml

Falcon tube. The cells were washed with 2ml PBS which was also transferred into the same tube. 2ml of 0.25% trypsin in TE buffer was added to the cells to detach the cells and the cell suspension was combined in the tube. The tubes were centrifuged at 1000g for 5min and the supernatant was aspirated and discarded. Cells were re-suspended in 500 µl PBS by pipetting up and down a few times. 5ml ice cold methanol was added to the cells while vortexting which helped to avoid clumping. The cells were incubated in fixative methanol for a minimum of 2 hours before further sample processing.

2.6.2 Cell Cycle profile assessment with propodium iodide (PI) staining

Prior to FACS analysis, the cells in methanol fixation pelleted by centrifugation and re-suspended in 400 µl PBS, 20 µl 1 mg/ml PI (Sigma) and 0.2 µl RNase (100 mg/ml). The samples were incubated for 30 min at room temperature in the dark to allow for PI incorporation. The cells were then sorted for and analysed for DNA content by measurement of fluorescent signals in the FL2 channel. The percentage of cells with sub-G1 DNA content was taken as a measure of the percentage of apoptotic cells in the cell population. The flow cytometry assays were performed on a Becton Dickinson FACS machine and the acquired data was subsequently analysed using FlowJo software.

2.6.3 S-phase analysis

To determine the percentage of actively proliferating cells in the cell population, the capacity of cells to incorporate either the synthetic thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) into their DNA was tested. Before the cells were harvested and fixed in methanol, 25µM BrdU was added into cell culture medium for the appropriate length of time.

For BrdU flow cytometry analysis, the cells were centrifuged at 1000g for 5 min and the methanol was removed. The cell pellets were re-suspended in 2ml of PBS

before 1ml of 4M Hydrochloric acid (HCl) was added. The samples were incubated at room temperature for 15 min to in order to denature the DNA structures, which allow BrdU epitopes to be exposed and recognized by subsequent antibodies. The cells were washed with 5ml PBS and then with PBT solution. The cells were centrifuged and the pellet was re-suspended in 300µl of PBT containing a 1:200 dilution of the anti-BrdU antibody (Dako). The samples were then incubated at room temperature for 30min in the dark. After the incubation, the cells were washed with 2ml PBT to remove excess primary antibody and re-suspended in 300µl PBT containing a 1:300 dilution of the Alexa 488 conjugated anti-mouse antibody and incubated for 30 minutes in the dark. The excess secondary antibodies were removed by washing with 2ml PBT. Finally the pellet was re-suspended in 400 µl PBS, 20 µl 1 mg/ml PI (Sigma) and 0.2 µl RNase (100 mg/ml). The flow cytometry assays were performed on a Becton Dickinson FACS machine and the acquired data was subsequently analysed using FlowJo software.

2.6.4 Mitosis determinations

The percentage of mitotic cells was determined by detection of cells with phosphorylated Histone H3 protein at ser10. pS10 Histone H3 is a mitotic marker commonly used in flow cytometry analysis or immunofluorescence microscopy. Cell were treated accordingly, fixed and stored as described above.

Prior to analysis, the cells were centrifuged at 1000g for 5min and the fixative was removed. The cells were re-suspended in 2ml of 0.3% Triton-X-100 in PBS and incubated on ice for 15 min. The cells were then pelleted and Triton-X-100 in PBS was aspirated and discarded. The pellet was re-suspended in 200µl of PBT (0.5% BSA, 0.1% Tween 20 in PBS) containing 1:100 dilution of anti-pS10 H3 antibody (Santa Cruz). The samples were incubated for 1 hour at room temperature. The cells were then washed with 5ml PBT and pelleted. The cell pellet was re-suspended in 200µl of PBT containing a 1:100 dilution of the FITC conjugated anti-rabbit antibody for 30 min in the dark. Excess secondary antibodies were removed by

washing with 5ml PBS. The samples were finally pelleted and re-suspended in 400 µl PBS, 20 µl 1 mg/ml PI (Sigma) and 0.2 µl RNase (100 mg/ml). The flow cytometry assays were performed on a Becton Dickinson FACS machine and the acquired data was subsequently analysed using FlowJo software.

2.6.5 Detection of homologous recombination

Plasmid encoding restriction enzyme I-Sce I was transfected into $Atg7^{f/f}$ and $Atg7^{-/-}$ cell lines that contain stable insert of HR plamid as described in Chapter 2.2.9. 48 hours after I-Sce I transfection using electroporation method, $Atg7^{flox/flox}$ and $Atg7^{-/-}$ cells were analysed by flow cytometry in a FL1 versus FL2 dot plot with 20% FL2 – FL1 compensation. GFP positive cell population appears off the autofluorescence diagonal towards FL1. They represented cells that were capable of carrying out HR.

2.6.6 Detection of Non-homologous End-joining (NHEJ)

Plasmids to tests HNEJ activity were kindy provided by Vera Gurbunova and have been previously described [250]. NHEJ reporter plasmid was linearised with HindIII restriction enzyme and purified with Qiagen Spin Column. 10µg of the plasmid was tranfected by electroporation (Amaxa MEF2 Nucleofector Kit by Lonza) together with an RFP-expressing plasmid (2µg) to control for transfection efficiency. 48 hours after transfection, $^{/flox}$ and $Atg7^{-/-}$ cells were analysed by flow cytometry. $Atg7^{flox/flox}$ and $Atg7^{-/-}$ cells were analysed by flow cytometry in 610 (red channel) versus 530 (green channel) dot plot.

2.7 Immunofluorescence microscopy

2.7.1 Cell fixation on coverslips

16mm Coverslips (VWR) were autoclave-treated to achieve sterilization before use. Each coverslip was placed in a well in a six-well plate. 20,000 cells were added to each well in 2ml medium. The plates were agitated slowly to ensure even distribution of cells on the coverlips. The cells were incubated in a tissue culture incubator at 37°C/5%CO_2 overnight. Following treatment, the medium was removed from the six-well plate and the cells were washed once with PBS to remove excess medium. Fixative solution was freshly prepared; it contained 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) in PBS. 2 ml fixation solution was added to each well and incubated at room temperature for 15 min. The solution was aspirated and discarded, 2ml PBS was added to each well and the plates were stored at 4°C before permealibisation step. PFA fixation method was routinely performed for most antibodies. For RPA and CtIP immunocytochemistry, cells were fixed first with PFA and then treated with 70% ethanol in PBS at −20°C. For ATR, pATR and ATRIP immunocytochemistry, cells were fixed in ice cold acetone/methanol (1:1) for 15 min at room temperature.

2.7.2 Permeabilisation and blocking

To permeabilise the cells, the coverslips with adhered cells were incubated in 2ml of 0.3% Triton-X-100 in PBS solution per well for 10min at room temperature. The coverslips were then washed with PBS and blocking solution (3% BSA, 10% FBS, and 5%milk in PBS) was added to the wells. The plates were blocked at room temperature with gentle agitation for 1.5h.

2.7.3 Probing with primary and secondary antibodies

Following blocking, the coverslips were washed 3 times with blocking buffer without milk (3% BSA and 10% FBS in PBS) before incubation in primary antibodies. The antibodies were diluted in blocking buffer without milk as indicated (table 3). For each coverslip, 20µl primary antibody solution was dropped onto parafilm (Pechiney Plastic Packaging Company) which was placed in a light-shielded square dish. The coverslips were placed onto the droplets of primary antibody solutions, with the side with adhered cells covered by the solutions. The dish was placed at 37°C for 1 hour in a tissue culture incubator. Meanwhile, three flasks each containing 100ml of 0.1% Triton-X-100 in PBS solution was prepared. At the end of incubation in primary antibody, the coverlips were washed by dipping three times in each of the three flasks. The coverslips were than incubated in secondary antibodies against the appropriate species in the same manner and washed by the dipping methods.

The coverlips were fixed onto microscope slides using VECTASHIELD® mounting medium with DAPI. 2 drops the mounting medium were placed on the slides and the coverslips were placed onto the droplets, with the side with cells facing the solution. The edge of coverlips was sealed with clear nail varnish.

Antibody	Dilution	Company
S1981 p-ATM	1:500	Cell signaling
ATR	1:100	Santa Cruz
ATRIP	1:100	Bethyl Laboratory
p-ATR	1:100	Santa Cruz
CtIP	1:100	Santa Cruz
H2AX	1:250	Millipore
KU-70	1:100	Santa Cruz
Rad51	1:1000	Calbiochem
γRPA	1:200	EMD
Alexa Fluor® 488	1:200	Invitrogen
Texas Red	1:200	Invitrogen

Table 4 List of antibodies used in immunofluorescent microscopy.

2.7.4 Visualization

Fluorecent microscope images were acquired using a laser scanning confocal microscope (A1R [Nikon]; or FV1000 [Olympus]) using either a Plan-Apochromat VC60× NA 1.40 oil immersion lens or UPLSAPO 60× NA 1.35 oil objective together with NIS-Elements AR (Nikon) or Fluoview version 1.7c (Olympus) software, respectively.

2.7.5 EdU detection with immunofluorescence microscopy

After sedding on glass coverslips MEF cells were treated as required and 1 hour before fixation, 25μM EdU (Invitrogen) was added to cell culture medium. The cells were then fixed with 4% PFA for 15 minutes at room temperature and washed twice with 3% BSA in PBS. The cells were permeabilised with 0.5% triton X-100

in PBS for 20 minutes at RT. Following fixation and peameabilisation, the cells were then washed twice with 3% BSA in PBS. EdU reaction buffer (Click-iT reaction buffer, CuSO4, Alexa Fluor 488 azide and 10x reaction buffer additive) (Invitrogen) was added according to manufacturer's instructions and incubated at for 30 min in the dark at room temperature. The excess reaction buffer was removed and the cells were washed twice with 2ml of 3% BSA in PBS. Fixed cells were then blocked in 3% BSA in PBS for 30 min before incubation in required primary and secondary antibodies as described above.

2.7.6 Statistical Analyses

Image analysis of Rad51 foci was undertaken using an Image J/Fiji Macro to detect enhanced fluorescence (foci) within nuclei. At least 50 cells were examined in each population and the foci density calculated as the foci area of a nucleus relative to total nuclear area (as assessed by DAPI stain).

Statistical analysis of the results obtained using Image J/Fiji Macro assessment of Rad51 foci formation following γ-irradiation was via a Student's t-test (2-sides with unequal variance).

2.8 Analysis of proteasome activity

The proteasome assays were performed in primary $Atg7^{f/f}$ and $Atg7^{-/-}$ cells. The assayw were also performed after treatment of Atg7f/f MEFs with 100nM Bafilomycin A1 for 16 hours, and/or with Lactacystin (10μM) for 3 hours where indicated. The medium was removed from the adherent cells plated in 10mm dishes the day before. The cells were detached with 2ml of 0.25% trypsin in PE buffer and diluted with 8ml room temperature medium, they were then transferred to a 15ml falcon tube. The cells were washed carefully with PBS 3 times to remove traces of trypsin which could affect experimental results. The final pellet was resuspended in room temperature medium and plated into 96 well plates at 10,000 cell per well.

Proteasome-Glo™ Cell-Based Reagents (Promega Bioscience) were prepared in accordance with manufacturer's instructions. The cells were equilibrated at room temperature (approximately 22°C), before 100µl reagents was added to the cells in each well. Luminescence generated from each reaction condition was detected with a Veritas Microplate luminometer according the manufacturer's instruction.

Chapter 3. Investigating whether autophagy deficiency leads to DNA damage accumulation

3.1 Primary $Atg7^{flox/flox}$ and $Atg7^{-/-}$ MEF cell lines were established.

As described in Chapt 1, Atg7 is a gene essential for autophagy. Atg7 protein is required for both ubiquitin-like conjugation systems that form the Atg12-Atg5-Atg16 complex and lipid bound LC3-II, and these two are crucial for phagophore formation.

Mice with whole body Atg7 deletion are born with a normal frequency but they can not survive the neonatal starvation period (48 hours after birth) [74]. Genetically modified mice containing Atg7 gene flanked by loxP sites are commonly used as an experimental model. The loxP sites can be targeted for recombination by Cre recombinase and Atg7 can be deleted in this system [251]. $Atg7^{flox/flox}$ embryos were isolated from pregnant mice at 13 to 14 days after the plug was detected. Primary MEF cell lines were established from the embryos and the cells were pooled. Retroviral vectors with Cre recombinase (pBabe-puro-Cre) were used to induce conditional deletion of Atg7 in these MEFs in vitro. Empty vector with selection marker was used as a control. Comparing to $Atg7^{flox/lox}$ (hereafter referred to as Atg7f/f) and $Atg7^{-/-}$ MEFs isolated from different embryos, the use of cre-mediated in-vitro Atg7 deletion also avoids potential artifacts due to genetic variability between different individuals.

After 3 days of selection in puromycin (2.5μg/ml) for infected cells, Atg7 levels were assessed by qPCR (Fig.3.1). It was revealed that Atg7 mRNA is significantly lower in cre recombinase expressing $Atg7^{flox/flox}$ MEFs. The depletion of Atg7 mRNA occurred within 5 days (Fig. 3.1).

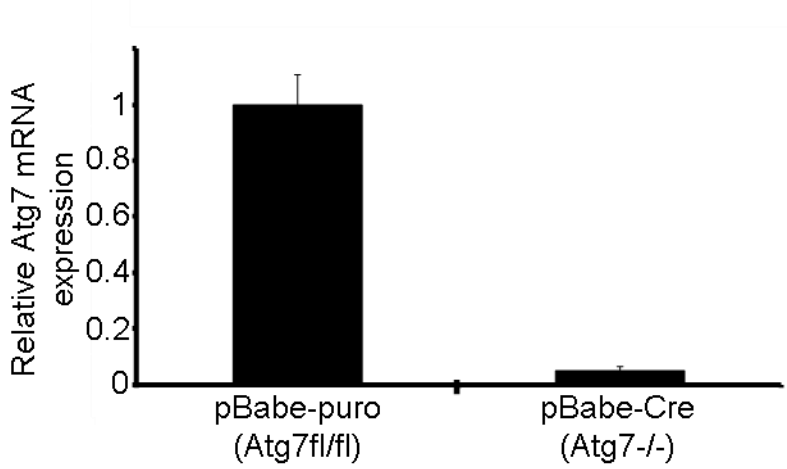


Figure 3.1 Atg7$^{flox/flox}$ locus is efficiently recombined by retroviral Cre recombinase.

Relative mRNA expression of Atg7 was measured by RT-qPCR in Atg7$^{flox/flox}$ cells which were retrovirally infected with either pBabe-Puro or pBabe-Puro-Cre. The experiment was carried out in various batches of MEFs and done at least three times overall.

Atg7 is a long-lived protein that has a half-life of about one week [251]. Three days following the antibiotic selection period, western blotting for Atg7 protein was carried out. Complete Atg7 depletion at the protein level was verified as shown in Fig. 3.2, and the depletion is sustained for at least two weeks after infection.

Atg7 is an E1 like enzyme that is essential for the conversion of LC3-I to LC3-II (Figure 1.3), which is embedded on the inner and outer membrane of the autophagosome. The amount of LC3-II is proportional to the number of autophagosomes [252]. LC3-I (19kD) to LC-II (17kD) conversion on western blots has been used to determine autophagic flux and is a general indicator of autophagy activity. Figure 3.2a shows that both LC3-I and LC3-II were detectable in Atg7f/f cells ($Atg7^{flox/flox}$), indicating that there is constitutively active autophagy in these cells. As expected, LC3-II band is absent in $Atg7^{-/-}$ cells, and there is no significant difference in LC3-I between Atg7f/f and $Atg7^{-/-}$ cells.

Autophagy is generally thought to be a non-selective process that bulk degrades proteins and organelles. However several selective autophagy substrates have been identified. p62/SQSTM1, a well characterized autophagy substrate, is a cytoplasmic chaperone-like protein associated with polyubiquitinated protein cargoes that are destined for degradation via the autophagic machinery. The molecular structure of p62 contains an LC3 binding domain and a ubiquitin binding domain [125]. The LC3 binding domain on p62 allows its association with autophagosomes and therefore selective degradation via autophagy. Under normal conditions, basal autophagy continuously clears p62 and associated cargo (such as toxic aggregate-prone proteins) from the cytoplasm. It is generally accepted that, with a few exceptions, inhibition of autophagy is correlated with increased levels of p62 at protein levels [129]. The levels of p62/SQSTM1 were examined in cre recombinase treated $Atg7^{flox/flox}$ cells. It was revealed that the p62 level was elevated significantly in $Atg7^{-/-}$ cells a week after selection and the up-regulation persisted for at least two weeks (Figure 3.2b).

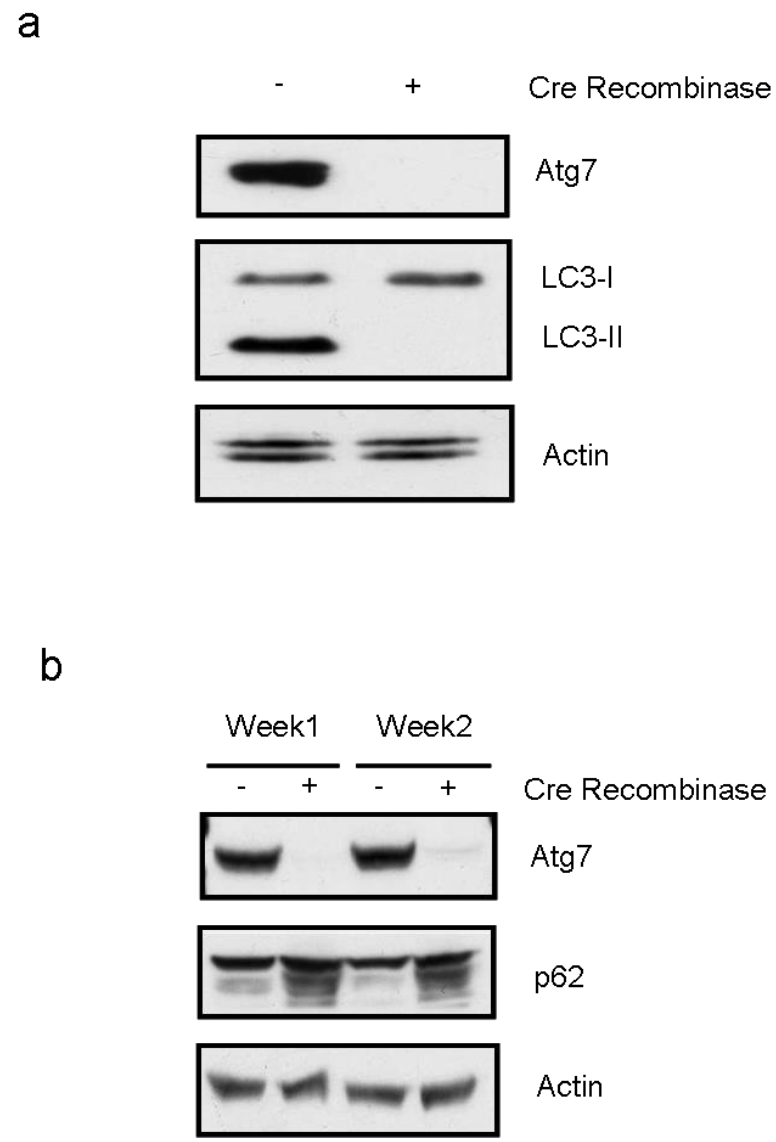


Figure 3.2 Loss of Atg7 depletes LC3-II conversion and leads to p62 up-regulation.

(a) Atg7 level and LC3-I to LC3 conversion were assessed by Western blotting after $Atg7^{flox/flox}$ cells were infected with cre recombinase containing retrovirus. (b) The sustainability of Atg7 depletion and p62 upregulation were examined by western blot analysis. The experiment was carried out at least three times for different batches of MEFs to ensure Atg7 protein was depleted in cre treated Atg7flox/flox cells.

To summarise, Atg7 is completely deleted in cre recombinase infected $Atg7^{flox/flox}$ cells as verified by qPCR and western blotting. Loss of Atg7 leads to failure of LC3-I – LC3-II conversion and accumulation of p62.

3.2 Investigation of the effect of loss of autophagy on DNA damage repair.

Mammalian cells have a complex set of DNA damage responses which are finely coordinated in order to maintain genomic integrity and limit oncogenic transformation. Primary MEF cells were used to dissect the role of autophagy in DNA damage response, because transformed cells frequently harbour genetic defects in DNA damage repair pathways leading to genetic instability and immortal transformation.

A number of molecular components are known to be localised at the sites of DNA damage in the nucleus, forming nuclear foci when visualised by immunofluorescence [253]. Histone protein H2AX is one of the first proteins that become activated via phosphorylation following DNA damage and it is reported that focus formation occurs within 3 minutes [254] [255]. Phospho-H2AX is commonly referred to as γ - H2AX because it was first discovered in cells exposed to γ rays, and can be phosphorylated by ATM, ATR and DNA-PKcs. H2AX facilitates ATM recruitment to the site of DNA breaks [256] and ATM is considered to be the main modulator of H2AX activity [208]. Some of the DNA damage response molecules form foci during certain stages of the cell cycle, whereas γ - H2AX foci formation can take place at any stage of the cell cycle [257]. γ-H2AX focus formation in the nucleus has been used as a cellular marker for DNA damage, especially double strand breaks. Each focus formation covers megabases on the chromatin and represents a repair center for damaged DNA [255].

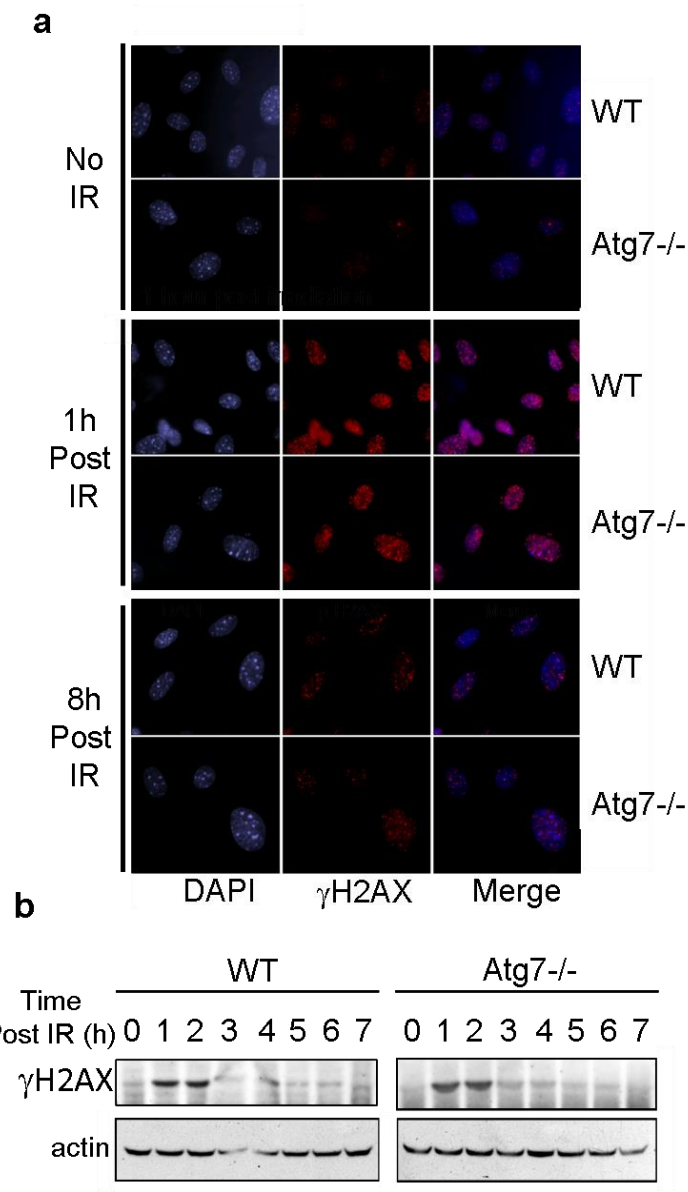


Figure 3.3 Double strand breaks after DNA damage are repaired in Atg7f/f and Atg7-/- cells at similar rates.

Atg7f/f and Atg7-/- cells were irradiated (10Gy) and parameters of DNA repair monitored over time. **(a)** Analysis of the accumulation of γ-H2AX by immunofluorescence microscopy was carried out to detect focus formation 1h and 8h after ionizing radiation (IR, 10Gy). (**b**) γ-H2AX was detected by western blotting at the indicated times following ionizing radiation (IR, 10Gy). *The experiment was carried out at least three times and a representative figure is presented here.

To investigate how the loss of autophagy affects DNA repair, wild-type and Atg7$^{-/-}$ cells were exposed to 10 Gy IR to induce DSBs and γ-H2AX foci formation was monitored (Fig.3.3a).

As shown in Fig. 3.3a, no detectable difference in γ-H2AX nuclear foci was observed at resting state between Atg7f/f and Atg7$^{-/-}$ cells. Comparable levels of focus accumulation were observed in the nucleus for the two cell lines 1 hour after IR, indicating a significant amount of double strand breaks were induced by irradiation. The foci were no longer visible after 8 hours by immunofluorescence in both cell lines. These observations indicate that autophagy deficient cells are able to resolve IR-induced DSBs. Western blotting for γ-H2AX provides a more quantitative assessment of DSB repair (Fig. 3.3b), revealing that Atg7-/- cells are able to repair IR-induced double strand DNA breaks, at a similar rate to Atg7f/f cells. These images also demonstrate the cells can repair damaged DNA fairly efficiently, the repair rate varies across different cell lines and is dependent on the proliferation speed of the cells. For example Fast growing neuroblastoma cells can repair most DNA damage 2 hours after 10Gy IR [258]. We found that in freshly isolated MEFs, most of γ-H2AX foci disappear 8 hours after 10Gy IR.

As described in chapter 1, there are multiple pathways for DNA damage repair; sometimes these mechanisms buffer each other's functions. Double strand breaks are mainly repaired by HR and NHEJ pathways, with less characterized pathways such as microhomology-mediated end joining also able to mediate the repair of DSBs [259]. Assays for γ-H2AX provide evidence that Atg7-/- cells repair DSBs but did not reveal how these lesions were resolved. Further assays examining the activation of downstream parameters the DNA damage response network were performed.

Assessment of the activation of ATM and ATR, the two master kinases for double strand break DNA responses was carried out using immunofluorescence microscopy (Fig. 3.4 and Fig. 3.5). Comparable nuclear foci formation of p-ATM

(S1981), ATR, p-ATR and ATRIP were detected in Atg7f/f and Atg7$^{-/-}$ cells after 10Gy IR. CtIP is an important strand resection protein activated by ATM, and it is also important for ATR activation in response to IR-induced double strand breaks. Equivalent amounts of RPA protein foci, which mark the presence of single strand DNA, were observed in Atg7f/f and Atg7-/- cells. This indicates that there was a comparable extent of strand resection after IR in autophagy deficient cells. It was also revealed that equivalent amounts of CtIP foci were formed in the two cell lines. Immunofluorescence microscopy images for ATR, p-ATR, ATRIP and CtIP were performed by Naihan Xu.

These results (Fig. 3.3 – Fig. 3.5) indicate that autophagy deficient cells are able to sense IR-induced DNA damage, which mainly consists of double strand breaks. ATM and its downstream targets all appear to be efficiently activated upon DNA damage. These DSB ends are efficiently processed and strand resection was able to take place. Strand resection after IR-induced DSB is necessary for the activation of ATR. ATR kinase also was found to be efficiently recruited to the site of damage in the absence of autophagy.

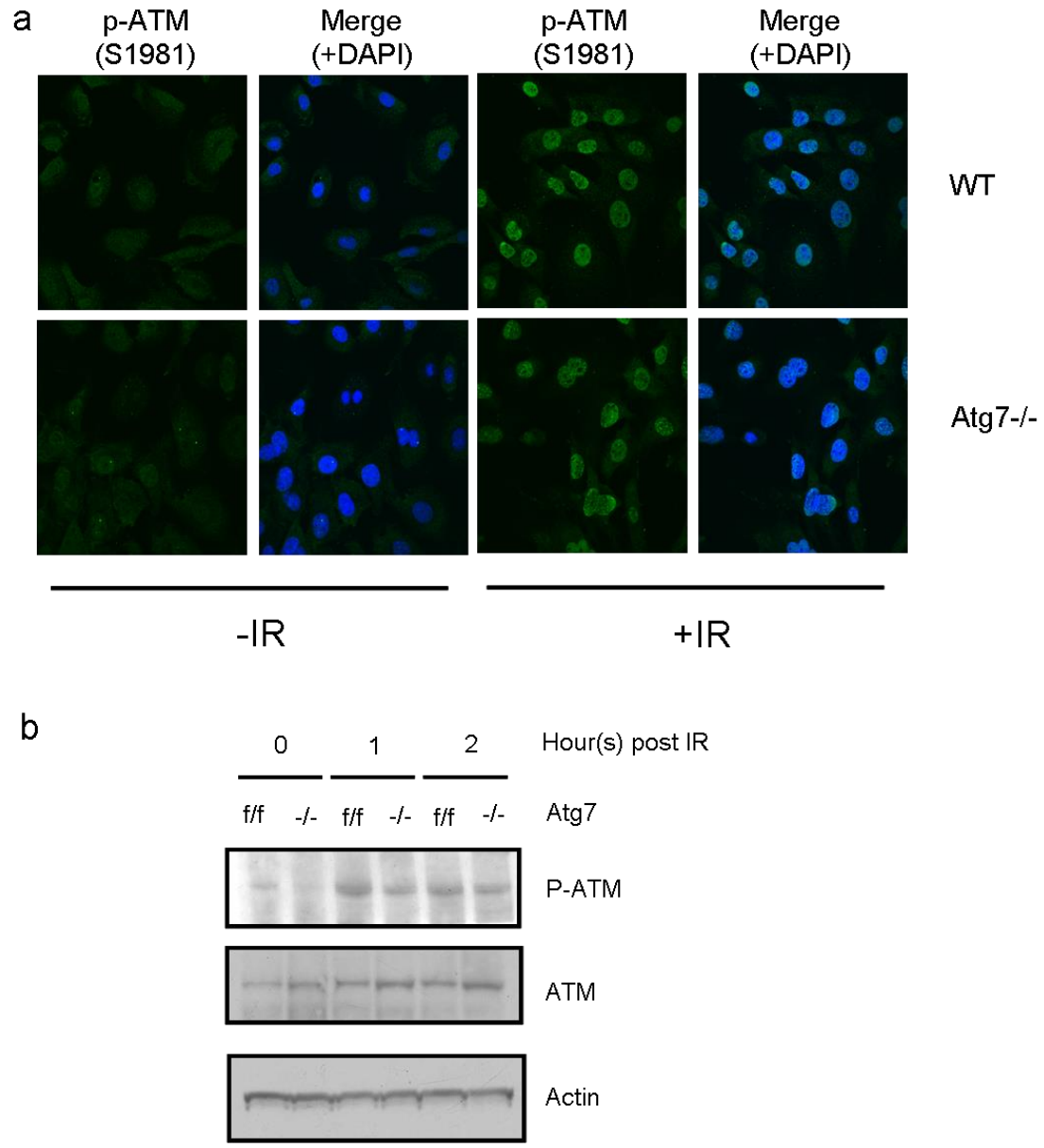


Figure 3.4 Equivalent levels of ATM activation were detected in Atg7f/f and Atg7$^{-/-}$ cells.

Activation of ATM in Atg7f/f and Atg7$^{-/-}$ cells was assessed by the measurement of phospho-ATM (Ser1981) nuclear foci using immunofluoresence microscopy, before and 1h after 10Gy IR. DAPI was used to stain the nucleus.

*The experiment was carried out at least three times and a representative blot is presented here.

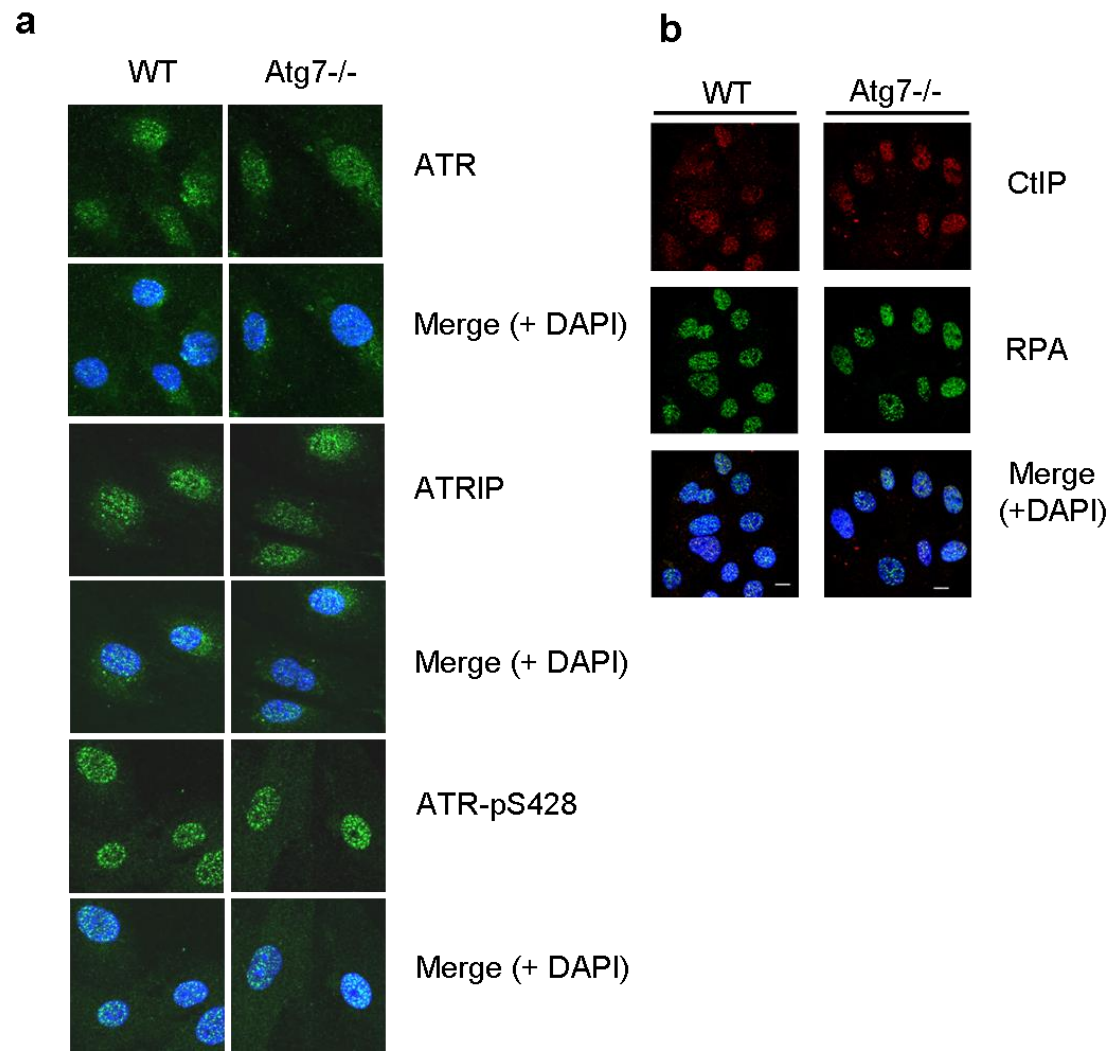


Figure 3.5 Autophagy deficient cells appear to have intact strand resection machinery during DNA damage response.

(Experiments performed by Naihan Xu, a former BICR member). (a) The levels of ATR, ATRIP and phospho-ATR nuclear foci were assessed by immunofluorescence 1h after irradiation (IR, 10Gy) in wild-type and Atg7$^{-/-}$ cells. **(b)** The sub-cellular localization of CtIP and RPA was assessed by immunofluorescence 1h after irradiation (IR, 10Gy) in wild-type and Atg7$^{-/-}$ cells.

Chk1 phosphorylation is considered to be the main functional readout for ATR kinase activities [260]. Chk1 is one of the key molecules in the DNA damage response and it activates different cell cycle checkpoints – G1/S, the intra-S, G2-M and the mitotic spindle checkpoint [207]. In response to genotoxic stress, Chk1 is activated by ATR and initiates a signalling relay that eventually arrests the cell cycle. This provides an opportunity for the cell to repair the DNA damage. Both IR and etoposide are able to induce double strand breaks [261]. Thus we examined Chk1 activation after exposure to IR or etoposide-induced DNA damage. It was revealed that phosphorylation of Chk1 at serine 345, a site phosphorylated by ATR, was greatly impaired in autophagy deficient cells in response to these DNA damaging agents (Fig. 3.6).

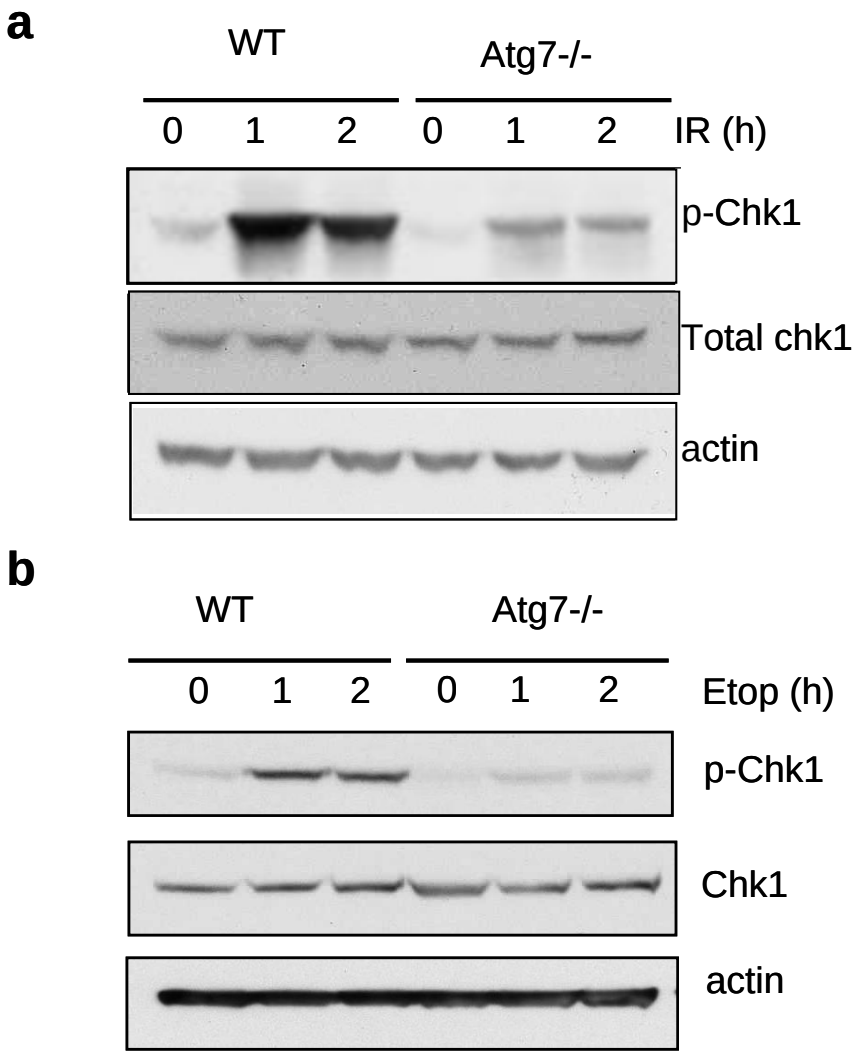


Figure 3.6 Atg7 null cells have a deficiency in Chk1 activation.

(a,b) Phosphorylation of Chk1 at S345 was measured by Western blotting in wild-type and Atg7$^{-/-}$ cells at the indicated times following exposure to 10Gy IR **(a)** or 25μM etoposide **(b).** *The experiment was carried out at least three times and a representative blot is presented here.

3.3 Investigation of the mechanisms through which Chk1 is de-regulated.

Chk1 activation is tightly modulated by a number of pathways, such as Claspin and WIP1. Claspin is an adaptor protein for Chk1 and it is also a target of ATR kinase. The phosphorylation of Claspin in the Chk1 binding domain promotes its association with Chk1 [262]. Claspin facilitates Chk1 phorphorylation by ATR and is required for Chk1 activation. Claspin protein levels are known to be modulated during cell cycle progression and also during the DNA damage response [263]. Claspin is normally degraded by the ubiquitin-proteasome system, and the degradation of Claspin is thought to terminate Chk1 activation and promote cell cycle progression [264]. In order to investigate whether the claspin level was affected in autophagy deficient cells, leading to Chk1 deficiency, western blotting analysis was carried out. The same levels of claspin protein were detected in Atg7f/f and Atg7-/- cells (see Fig. 3.7a).

WIP1/PPM1D is a phosphatase that dephosphorylates Chk1 at Ser345 [265]. Similar to the functions of Claspin, WIP1 is thought to inactivate DNA damage response signalling after the lesion is repaired. WIP1 protein level is also tightly regulated in the cells [266]. If WIP1 for some reason is up-regulated in autophagy deficient cells, this would lead to decreased levels of phosphorylated Chk1. However identical levels of WIP1 were observed in Atg7f/f and autophagy deficient cells (Fig. 3.7b).

In fact, Analysis of Chk1 by western blot showed that both phospho- and total Chk1 levels were diminished at a later time point (e.g. after 2 weeks) following loss of autophagy (Fig. 3.8).

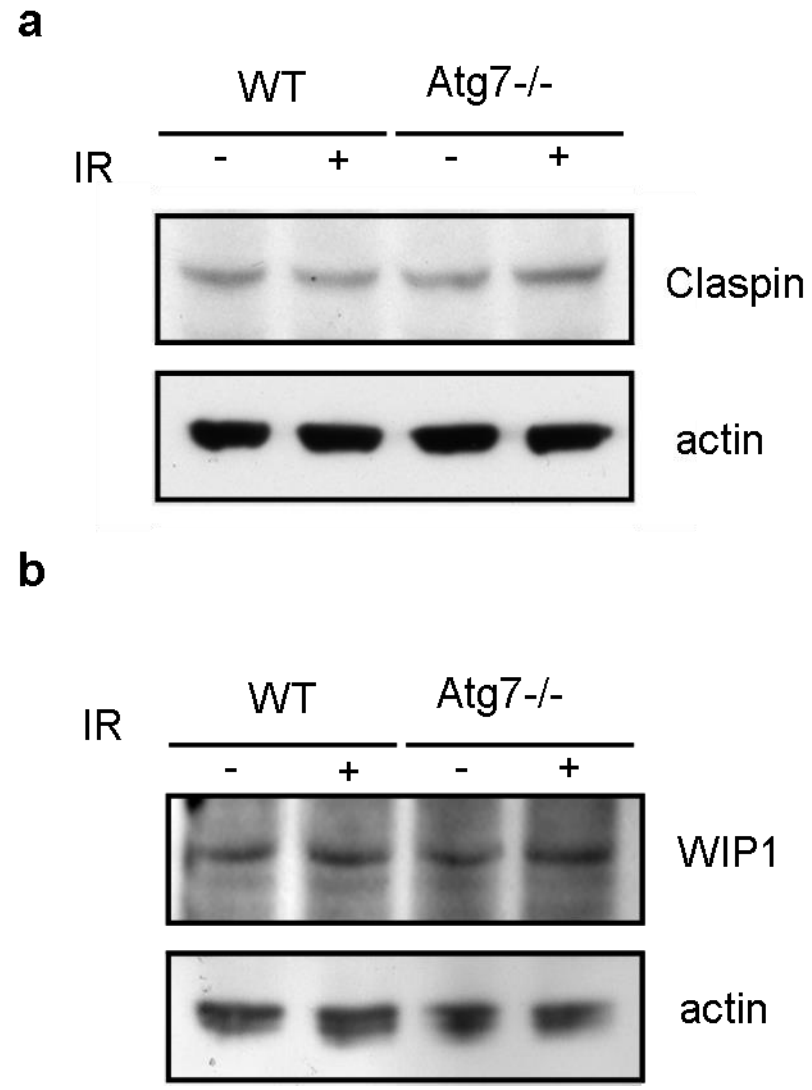


Figure 3.7 Levels of Chk1 modulators Claspin and WIP1 were unchanged after loss of autophagy.

(a,b) Levels of Claspin **(a)** and WIP1 **(b)** were measured by Western blotting in $Atg7^{flox/flox}$ and $Atg7^{-/-}$ cells following treatment with ionising irradiation (10Gy).

*The experiment was carried out at least three times and a representative blot is presented here.

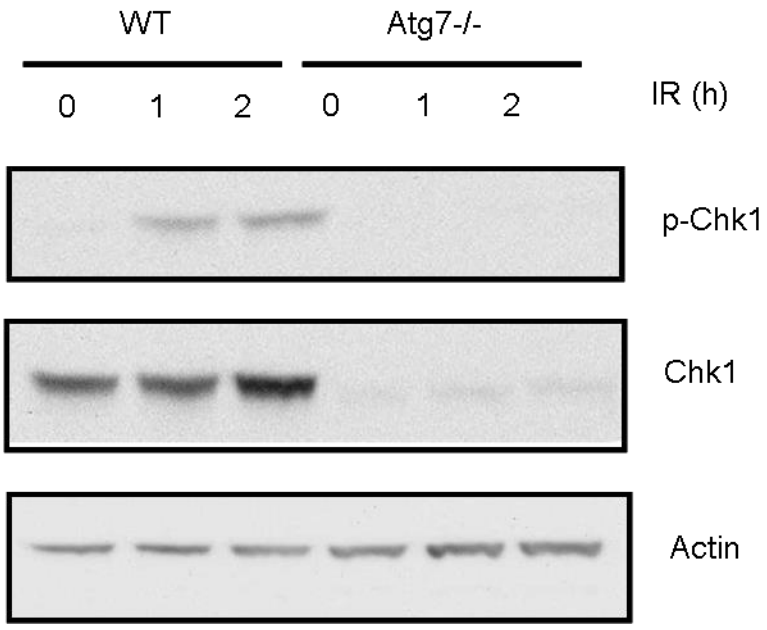


Figure 3.8 Total Chk1 protein level diminishes at later time points in autophagy-deficient cells.

Levels of phoshorylated Chk1 and total Chk1 were assessed by Western blotting in $Atg7^{flox/flox}$ and $Atg7^{-/-}$ cells 2 weeks after recombination following 10Gy of ionising irradiation. *The experiment was carried out at least three times and a representative blot is presented here.

To summarise the findings so far, autophagy deficient cells appeared to be able to resolve IR induced DNA damage. They can activate molecules upstream of Chk1 upon the induction of DSBs and no obvious defects were observed. However cells lacking autophagy displayed defective Chk1 activation. Less phosphorylated Chk1, and at a later time point lower levels of total Chk1 were observed in autophagy deficient cells. The decrease in phosphorylated Chk1 was observed after induction of DSBs; and the decrease in total protein level was observed in the absence or presence of DNA damaging agents. Chk1 activities are not only regulated by adaptor protein Claspin and phosphatase WIP1, the activation of Chk1 is actually coupled to its own degradation [267]. The phosphorylation of Chk1 at Ser345 facilitates its proteasome mediated degradation [267].

Our observations that impaired phosphorylation of Chk1 followed by deceased total protein levels could be explained by activation mediated degradation of Chk1. It is generally accepted that autophagy deficient cells, which cannot efficiently remove damaged mitochondria and proteins, generate more reactive oxygen species (ROS) leading to more DNA damage [268]. ROS can act as signalling molecules at physiological levels in the cells, and they are produced in the mitochondria where oxygen metabolism occurs [269]. At elevated levels, ROS can cause oxidative damage to DNA molecules and accumulation of DNA damage leads to genomic instability and contributes to tumourigenesis. Mitochondria, as the main source of ROS in the cells are dynamic structures that are cleared by autophagy, and the process is referred to as mitophagy. Mitophagy constitutively degrades damaged or unwanted mitochondria. Mitophagy-deficient cells have de-regulated ROS levels which can cause damage to DNA, protein and lipids [270].

Since DNA damage leads to activation of Chk1 and there's increased DNA damage in autophagy deficient cells, it was reasoned that possibly the regulation of Chk1 activation by upstream kinases, adaptor protein Claspsin or phosphotase WIP1 is not perturbed, but that Chk1 is constitutively activated in autophagy deficient cells due to the increased incidences of DNA damage. Activation of Chk1 leads to its

degradation and over time it would then have an impact on the total pool of Chk1. More mechanistic details on how the degradation occurs will be covered in chapter 5.

A different system, without the use of retroviruses was utilized to investigate the role of autophagy in Chk1 activation. $Atg7^{flox/flox}$ mice were crossed with B6 CAG-Cre-ER mice, which express a Cre recombinase targeted deletion system that can be induced by tamoxifen treatment. In contrast to in-vitro infection with cre recombinase, the tamoxifen -ER system does not require three rounds of infections and sequential selection. It was found that 6 days after the cells were first exposed to 4-hydroxytamoxifen (TAM), Atg7 was successfully removed, as assessed by western blotting shown in Fig. 3.9. Total Chk1 was also diminished in the absence of Atg7 (Fig. 3.9). In Atg7f/f cells, Chk1 was activated 1h after IR, as assessed by western blotting for Ser345 p-Chk1; in $Atg7^{-/-}$ cells, less phosphorylation-activation was observed in response to DNA damage. It can also be seen that the basal phosphorylation of Chk1 in $Atg7^{-/-}$ is higher than that of Atg7f/f cells.

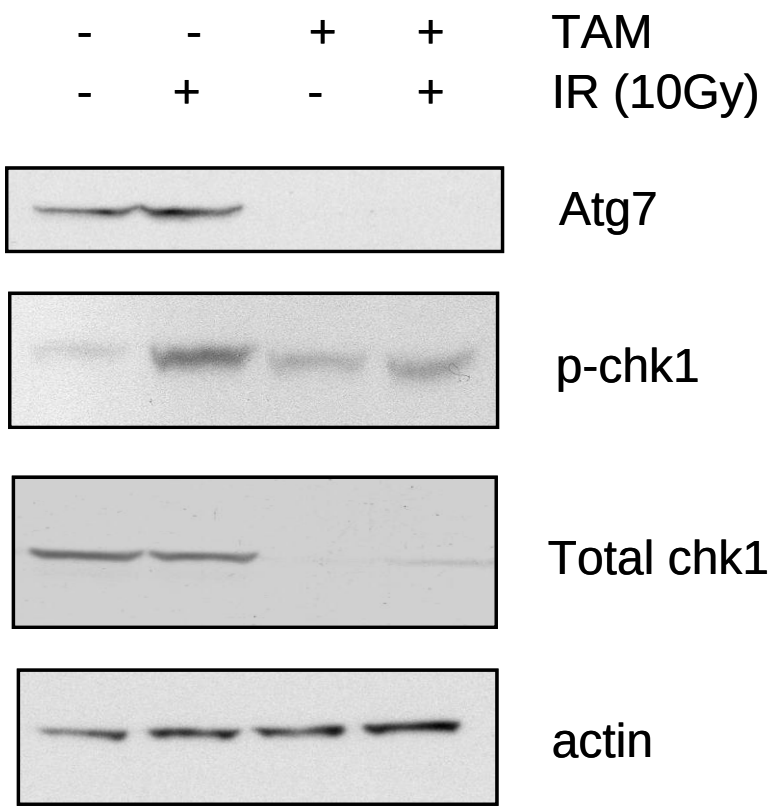


Figure 3.9 Tamoxifen induced deletion of Atg7 in CAG-Cre MEFs also leads to decreased total Chk1 level.

MEFs isolated from Atg7flox/flox – CAG-Cre embryos were treated with 2μM 4-hydroxytamoxifen (TAM) for 48h, and incubated in TAM free medium for a further 96h. The cells were harvested for western blotting 1h after 10Gy IR. *The experiment was carried out at least three times and a representative blot is presented here.

Both in-vitro infection of Atg7flox/flox MEFs and the Tamoxifen mediated Atg7 deletion method involves the use of cre recombinase, which has been reported to have DNA damaging effects in cells without the LoxP site. Cre recombinase is an enzymatic protein originally found in P1 Bacteriophage and it catalyses site specific recombination between its specific recognition sites on the DNA, termed loxP sites [271]. Cre recombinase is a widely used tool in molecular biology to knock in/out specific genes. DNA flanked between two same-orientation loxP sites is excised [272]. Although being a powerful tool, the use of cre recombinase has raised concerns due to its associated toxicity. Naturally occurring sequences with high homology to loxP have been discovered in mammalian cells [273]. These pseudo-LoxP sites can undergo cre-mediated recombination. Cre expression in mammalian cells in the absence of loxP sites have been reported to cause accumulated DNA damage and apoptosis [274] [275]. Therefore it was important to find out whether Chk1 deficiency after loss of autophagy was due to an off-target effect by cre-recombinase, before any further characterization on the effect of autophagy loss on Chk1. To test this, wild type primary MEFs were isolated from day 13-14 embryos with B6 background. These MEF cells were infected with cre-transgene-containing retrovirus and selected with puromycin for 3 days. Western blotting was carried out, and it was revealed that there was no difference in Chk1, either the activated Chk1 or total Chk1 protein level, in cre treated Atg7f/f MEFs (Fig. 3.10).

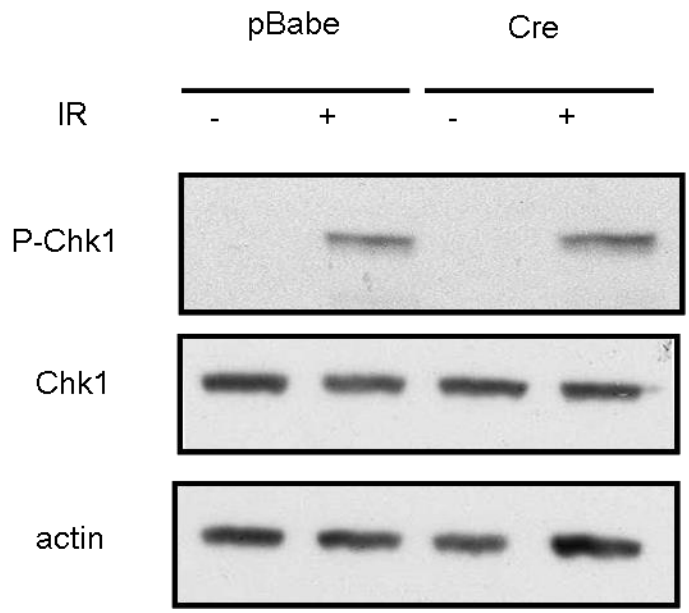


Figure 3.10 WT MEFs treated with cre plasmid do not impede Chk1 activation or decreased Chk1 levels.

Western blotting analysis showing the effect of Cre recombinase on Chk1 phosphorylation (S345) in genetically unmodified (wild-type) primary MEFs. *The experiment was carried out at least three times and a representative blot is presented here.

Chapter 4. Investigating the downstream effect of Chk1 deregulation in autophagy deficient cells

Chk1 is one of the key molecules in the DNA damage response (either DNA strand breaks or blocked replication fork) and it activates different cell cycle checkpoints – G1/S, the intra-S, G2-M and the mitotic spindle checkpoint. Chk1 deletion in mouse models is embryonic lethal [276]. As early as the blastocyst stage before embryo formation, nuclear aberrations and apoptotic cell death can be detected. Chk1 is considered to be essential for cell survival and growth [276]. Chk1 and Chk2 are the two main cell cycle mediators, Chk is from checkpoint kinase, like their name suggests, Chk1 and Chk2 are proteins that play central roles in the crossroads between DNA damage response and cell cycle checkpoints. Once activated by stimuli such as genotoxic stress, the two checkpoint proteins trigger downstream signalling responses leading to cell cycle arrest. Although Chk1 and Chk2 are not structurally related to each other, they are functionally similar and share common substrates including Cdc25A and Cdc25C which are cell cycle checkpoint mediators. In recent years, it was found that the two kinases do not have complete overlapping functions and Chk1 may play a more essential role than Chk2 in the cells. Homozygous Chk1 deletion in mouse is embryonic lethal; however Chk2 knockout mice are viable and fertile without any obvious defects [277], Chk2 deficient mouse cells display normal cell cycle profiles, in contrary to findings in human immortalized cell lines [278], Chk2 was found to be insignificant in damage induced cell cycle checkpoint in mice [279] [280].

As described in the previous chapter, soon after loss of Atg7, Chk1 activation is impaired in autophagy deficient cells; and at later times both total Chk1 protein levels and phosphorylated Chk1 are depleted in $Atg7^{-/-}$ cells. These phenomena lead us to ask the question whether cell cycle regulation is abnormal in autophagy deficient cells, and whether these cells show abrogation in cell survival or proliferation.

4.1 Analysis of cell cycle checkpoints in response to DNA damaging agents in Atg7f/f and Atg7-/- cells.

Both mammalian and chicken somatic cells with complete Chk1 depletion are viable but they display significant checkpoint defects. Chk1 was found to be crucial for the DNA damage induced G2 checkpoint [276] [281]. Cells lacking Chk1 are expected have a de-regulated cell cycle. We therefore examined the cell cycle checkpoints in Atg7-/- cells in response to the genotoxic agent etoposide. Etoposide is a topoisomerase II inhibitor and cells undergo G2/M arrest upon treatment with etoposide [282]. The cell cycle arrest gives the cells some time to repair the double stand breaks caused by etoposide before entry into mitosis.

Cell cycle distribution in Atg7f/f and $Atg7^{-/-}$ MEF cells before and after etoposide treatment was assessed. Equivalent cell cycle distribution was observed in the two cell lines before DNA damage challenge. After etoposide treatment, both cell lines displayed S phase and G2 arrest to a similar extent (Fig. 4.1). The results indicate that autophagy deficient cells, though appearing to have deficiency in Chk1 activity, have an intact G2 checkpoint.

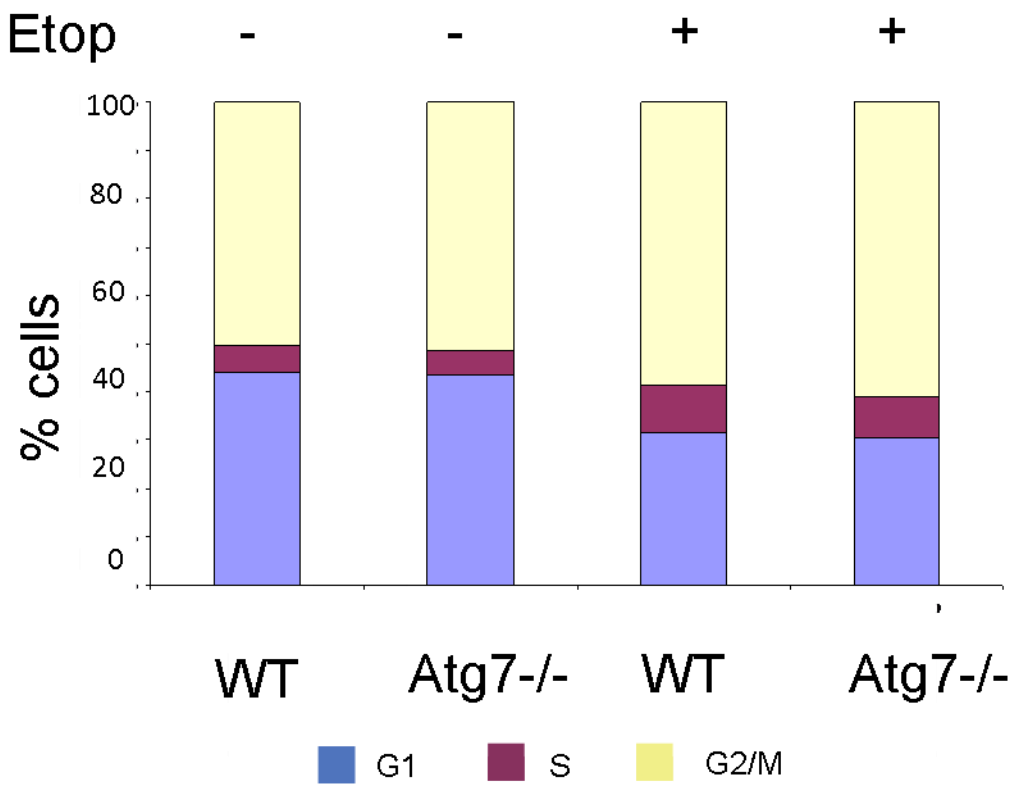


Figure 4.1 Autophagy deficient cells have equivalent cell cycle distribution to Atg7f/f cells prior to and after DNA damage.

$Atg7^{flox/flox}$ and $Atg7^{-/-}$ cells were treated with 25μM etoposide for 8h after which cell-cycle analysis was undertaken by flow cytometry. *The experiment was carried out at least three times and a representative figure is presented here.

During mitosis and meiosis, histone protein H3 becomes phosphorylated at ser10 and this facilitates chromosomal condensation [283]. Mitosis occurs rapidly in the cells and at any given time only a small fraction of cells are pH3 positive. Nocodazole is an agent that inhibits the polymerization of microtubules. Cells treated with nocodazole fail to form metaphase spindles and the cell cycle cannot progress beyond the mitosis stage. The use of nocodazole allows the quantification of the percentage of cells that are going through mitosis.

Checkpoint activities were examined in Atg7f/f and Atg7$^{-/-}$ cells. It was revealed that both cell lines efficiently undergo cell cycle arrest upon treatment with etoposide and neither cell line progresses into mitosis (Fig. 4.2). Results as shown in Fig.4.1 and Fig.4.2 indicate that the G2/M checkpoint is intact in Atg7$^{-/-}$ cells.

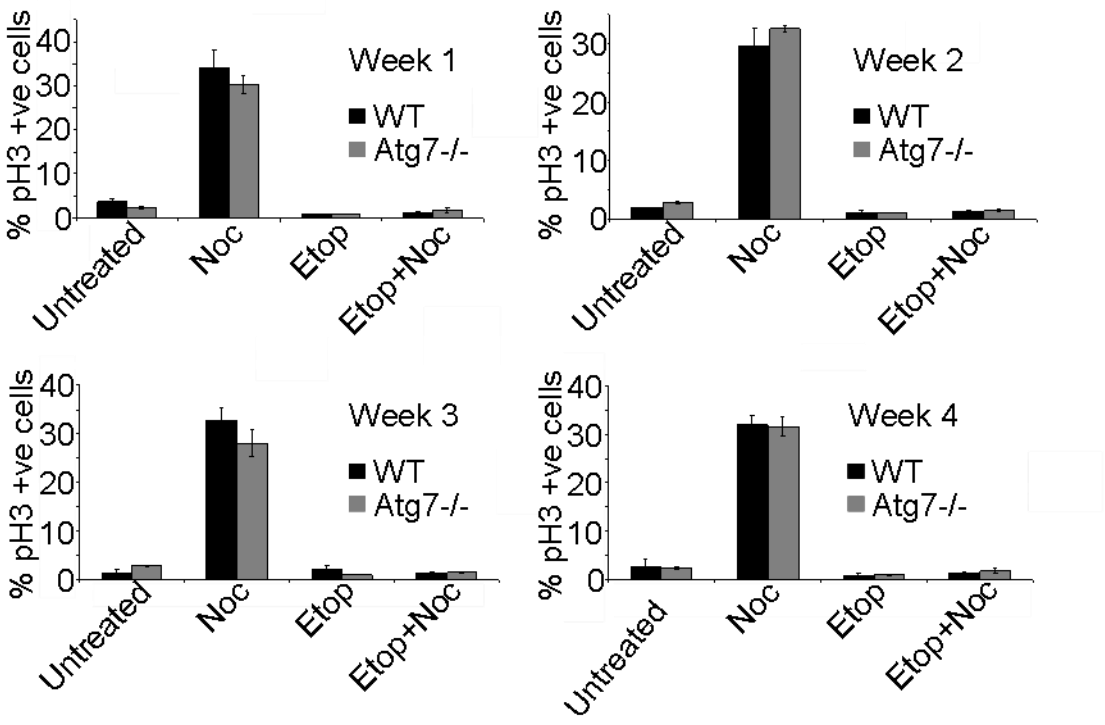


Figure 4.211 deficient cells have intact G2/M checkpoints.

The ability of cells to promote entry into mitosis or undergo G2/M cell cycle arrest was scored by detection of phosphorylated Ser 10 in histone H3 (pH3). Etoposide (25μM) and/or Nocodazole (100nM, to arrest cells in mitosis) was added to the cells for 8 hours where indicated. *The experiment was carried out at least three times and a representative figure is presented here.

Serum starvation induces reversible G1 cell cycle arrest [284, 285]. Atg7f/f and $Atg7^{-/-}$ cells were synchronized in G1 phase by incubation in serum free medium for 16 hours. Final concentrations of 10% serum or/and etoposide (25μM) were added for a further 8 hours before the cells were harvested for flow cytometry analysis. It was found that there were comparable G1 checkpoint activities between the two cells lines (Fig. 4.3). In nutrient replete conditions, around half of the cell population was detected as BrdU positive, indicating that they had been going through S-phase. After starvation, the cells were arrested in G1 phase and did not progress into S-phase. When serum was added back to starved cells, both wild-type and $Atg7^{-/-}$ cells resumed their cell cycle progression and entered S-phase, as indicated by BrdU incorporation. When etoposide was added together with serum, neither of the two cell lines progressed into S-phase, indicating that autophagy deficient cells have an intact DNA-damage mediated G1 checkpoint.

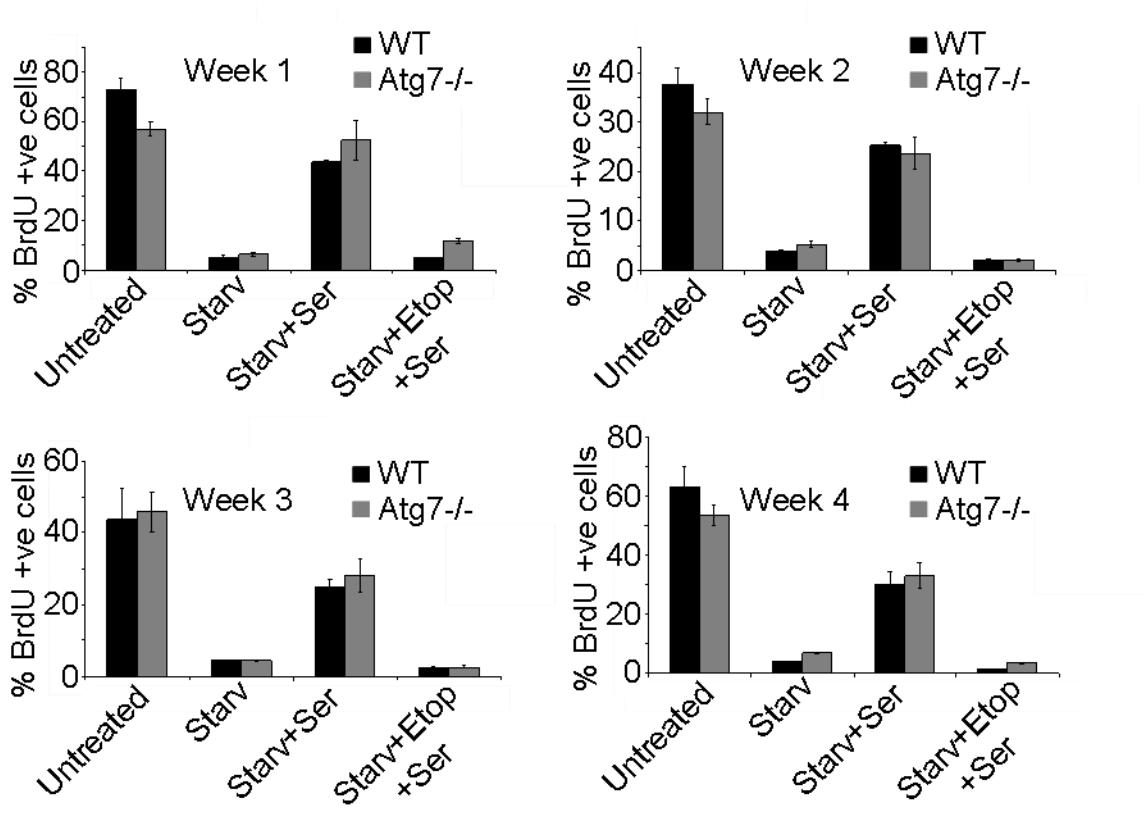


Figure 4.3 Autophagy deficient cells have intact G1 checkpoints.

The ability of $Atg7^{flox/flox}$ and $Atg7^{-/-}$ cells to promote entry into S phase or undergo G1 cell cycle arrest was determined by BrdU incorporation. Where indicated, cells were synchronized via incubation in serum free medium for 16 hours prior to addition of serum or/and etoposide (25μM). *The experiment was carried out at least three times and a representative figure is presented here.

No obvious aberrations in DNA-damage-induced cell cycle controls were observed in Atg7-/- cells. Chk1 mediates cell cycle arrest through downsteam molecules such as Cdc25A and CDK1. Cdc25A is a phosphatase that drives G2/M phase transition by removing the inhibitory phosphorylation (Thr14 and Tyr15) on Cdk1 so Cdk1 can form a complex with cyclin B [286]. Human Cdc25A activity is inhibited by Chk1 via phosophorylation and the protein is rapidly targeted for degradation [287]. Levels of Cdc25A and phosphorylation of CDK1 (Tyr15) in response to IR were examined (Fig. 4.4). Cdc25A levels were found to be constant before or after IR treatment and also there was no difference in the phosphorylation of CDK1. Cdc25A levels were not perturbed in the absence of autophagy, where Chk1 was de-regulated.

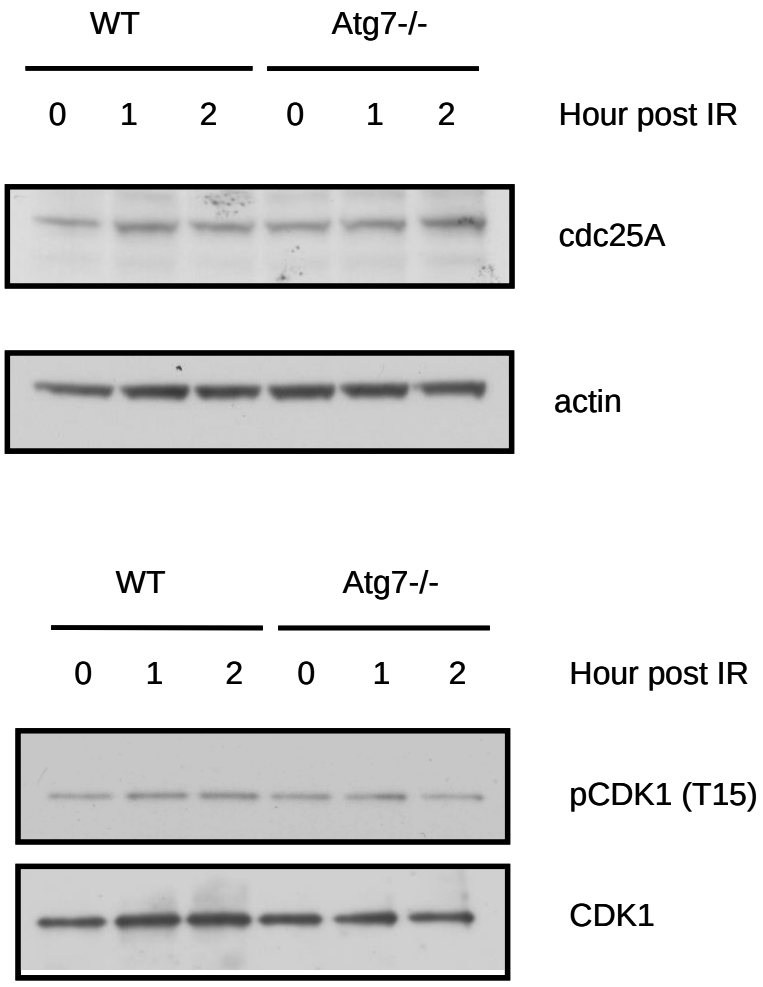


Figure 4.4 Loss of autophagy has no effect on Chk1 downstream molecules Cdc25A and pCDK1.

(a) Levels of Cdc25A were assessed by western blotting prior to, 1h and 2h after 10Gy IR. **(b)** p-CDK1 (Tyr 15) levels were examined in a similar manner. *The experiment was carried out at least three times and a representative blot is presented here.

4.2 Investigation of the effect of autophagy-deficiency on DNA repair of double strand breaks.

Besides cell cycle control, Chk1 is also an important regulator of DNA damage repair [288]. It is found to phosphorylate Rad51 at Thr309, a nuclear protein that mediates homologous recombination during DNA damage repair [240]. Rad51 normally displays a defuse pattern in the nucleus. The phosphorylation of Thr309 is crucial for Rad51 function, and Rad51 fails to form nuclear foci in Chk1 deficient cells (Sorensen et al., 2005). Once activated, Rad51 rapidly replaces RPA on single stranded DNA, and initiates homologous recombination.

Because Chk1 is de-regulated in $Atg7^{-/-}$ cells, we examined Rad51 foci formation in the nucleus after the cells were exposed to IR. Rad51 localises to sites of double strand breaks and forms microscopically detectable foci when cells are challenged with DNA damaging agents such as etoposide or IR [289]. In healthy growing cells, Rad51 foci are also frequently detected during the S-phase of the cell cycle, where most spontaneous double strand breaks occur due to stalled or collapsed replication forks [290]. To differentiate Rad51 focus formation caused by exogenous agents and spontaneous DSBs, proliferation marker EdU (5-ethynyl-2´-deoxyuridine) was used to identify S-phase cells. EdU is a modified nucleoside, an analogue of thymidine that can be incorporated into the DNA. It is similar to BrdU, but detection of EdU does not require DNA denaturation. It was revealed that Rad51 fails to form foci in $Atg7^{-/-}$ cells after DNA damage, whether they are in S-phase or not (Fig 4.5). Nuclear foci formation was further quantified using an Image J/Fiji Macro to detect enhanced fluorescence (foci) within the nuclei. At least 50 cells were examined in each population and the foci density calculated as the foci area of a nucleus relative to total nuclear area (as assessed by DAPI stain). The macro used for data analysis was built by Dave Strachan (BICR).

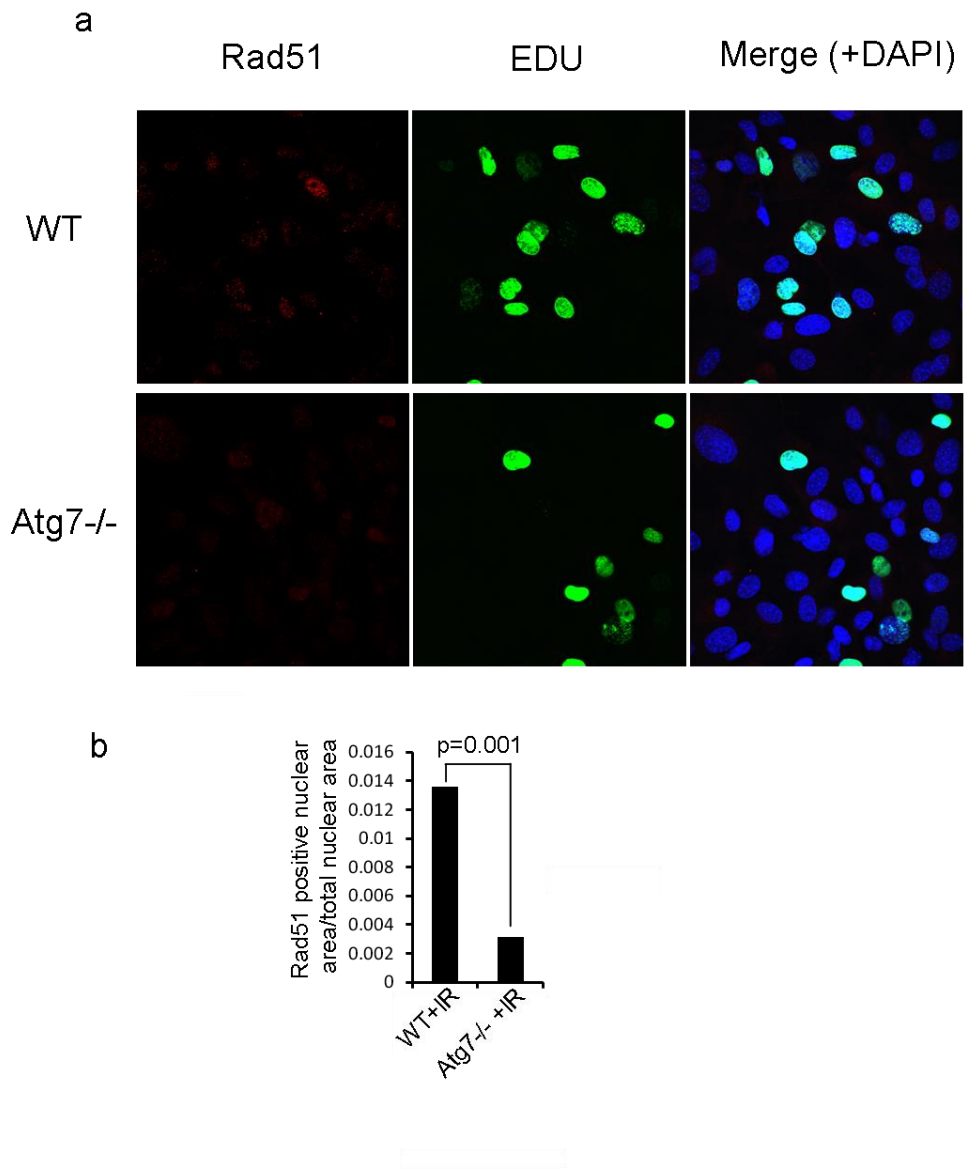


Figure 4.5 Rad51 focus formation does not occur in Atg7$^{-/-}$ cells after DNA damage.

(a) Rad51 nuclear focus formation (IR) was examined by immunofluorescence in wild-type and Atg7$^{-/-}$ cells, 1h after exposure to 10Gy IR. Where indicated in each panel, DAPI was used to stain DNA. **(b)** The graph represents Rad51 positive nuclear area (foci) normalised against total nuclear area in Atg7f/f and Atg7$^{-/-}$ cells.

*The experiment was carried out at least three times and a representative figure is presented here.

The difference in Rad51 foci between Atg7f/f and Atg7-/- cells was visibly small; this was because of the limitations of the antibody available to us. The use of a macro quantifying (Rad51 Foci / Nuclear area) of the cells was able to reveal the difference and its significance, as presented in Fig 4.5(b).

It is important to note that Rad51 expression level is cell cycle dependent, the protein level is lowest during G1 phase, and it increases during S-phase and peaks during G2-M phase [291]. The expression levels coincide with the availability of sister chromatid as a template for HR. As shown in Fig. 4.1, the cell cycle distribution is comparable between Atg7f/f and Atg7-/- cells therefore the difference in foci formation was not due to a difference in cell cycle distribution.

As mentioned previously, Rad51 is a central player in HR DNA repair, Rad51 nucleofilament mediates homology search on an adjacent chromosome and stimulates DNA strand exchange process. To determine the effect of loss of autophagy on HR repair pathways, GFP-plasmid-based flow-cytometry assays were performed to quantify HR capacities in Atg7f/f and Atg7-/- cells.

Atg7f/f and Atg7-/- cells lines that contain stably inserted HR substrate reporter plasmid were established as described in Chapter 2.2.9. The primary structure of the recombination reporter substrate was shown in Fig 4.6a. The reporter has two consecutive copies of modified and inactive GFP (Green Fluorescence Protein) genes. The first copy of GFP gene was modified to contain a restriction enzyme site for I-SceI, an endonuclease which recognises 18bp sequence and cut the sequence leaving 4 base pair overhangs [292]. The second copy of GFP is truncated at 3' end and is also inactive. When I-SceI is introduced into the HR reporter expressing cells, double strand breaks with overhangs were generated by the enzyme, and these lesions can only be fully resolved through HR pathways. NHEJ pathway may be able to repair such lesions; however nucleosides may be added or deleted at breakage ends randomly, resulting in a mutant GFP that is inactive. 48 hours after I-SceI transfection using electroporation method, $Atg7^{flox/flox}$ and $Atg7^{-/-}$ cells were

analysed by flow cytometry in a FL1 versus FL2 dot plot with 20% FL2 – FL1 compensation. GFP positive cell population has a higher FL1 signal relative to that autofluorescence diagonal, as shown in Fig. 4.6b. They represented cells that were capable of carrying out HR.

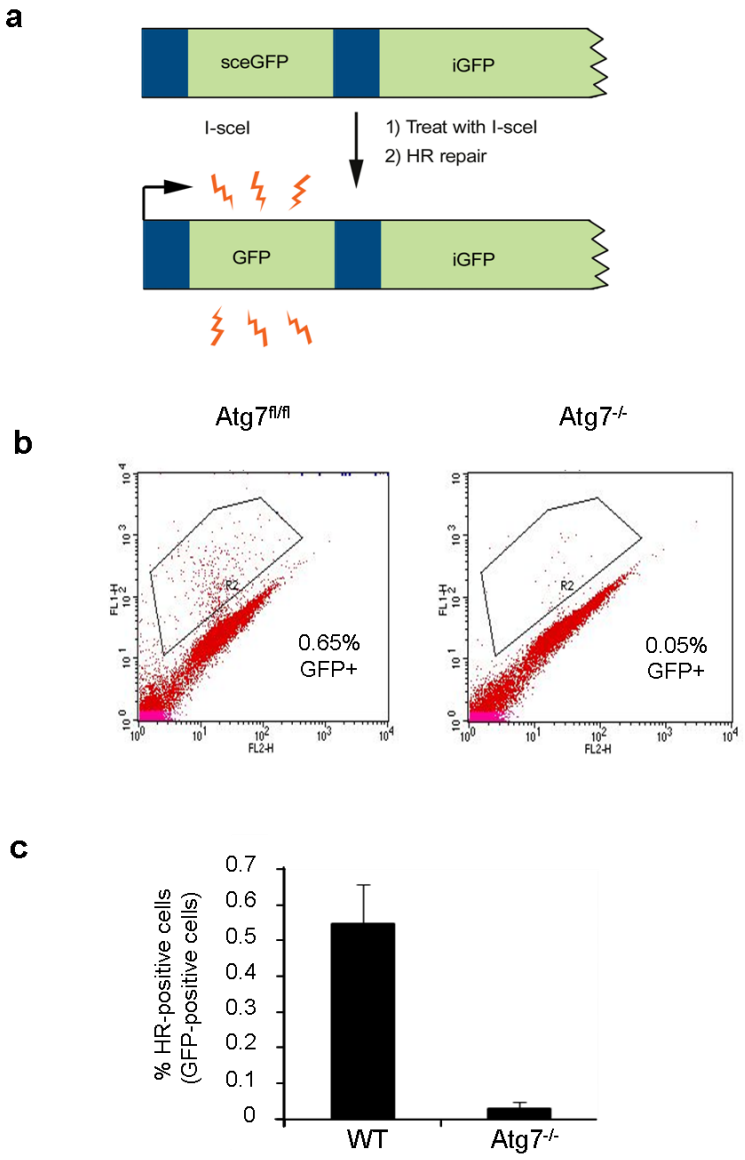


Figure 4.6 recombination is impaired in autophagy-deficient cells.

(a) (Adapted from [293]) The plasmid used in reporter HR assays encodes two copies inactive GFP proteins. When restriction enzyme I-SceI introduces double strand breaks with overhangs, HR takes place to repair the breaks using sequence

information from the second copy of GFP, located on a sister chromatin. (**b**) Plasmid-based assays were used to measure HR activity in wild-type and Atg7-null cells. **(c)** The results of 3 repeated experiments were quantified as represented graph.

*The experiment was carried out at least three times and a representative figure is presented here.

Results from flow cytometry assays as in Fig. 4.6 showed that Atg7-/- cells have diminished capacity to carry out HR relative to Atg7f/f cells. This finding is supported by the fact that Rad51 fails to form foci in autophagy deficient cells.

The two main ways of repairing DSBs are HR and NHEJ. HR appeared to be defective in autophagy deficient cells. Since DNA damaging pathways frequently buffer each other's functions, i.e. NHEJ can be up-regulated when HR is inhibited and vice versa. The next step in this study was to investigate whether there was any irregularity in NHEJ pathways.

The first step of NHEJ involves Ku complex recognizing and assembling at each end of DSBs (Figure in Intro). Ku is a heterodimeric protein complex consisting of two subunits Ku70 and Ku80. Once bound to DNA, it recruits downstream catalytic proteins such as DNA Protein kinase and DNA ligase IV. Identical amounts of Ku70 foci formation were observed in Atg7f/f and Atg7-/- cells in response to IR (Fig 4.7). This would indicate NHEJ is carried out to the same extent in the presence or absence of autophagy.

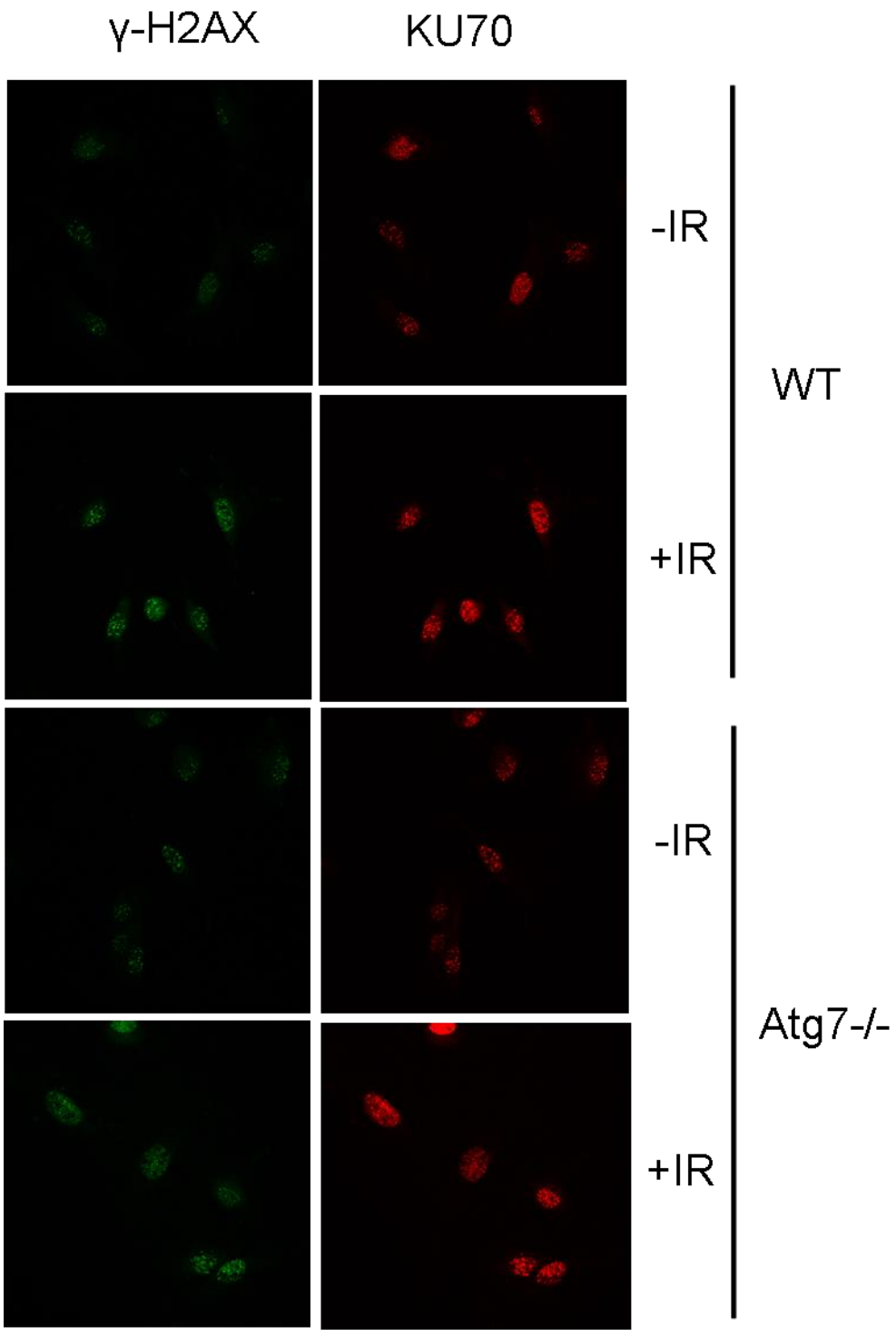


Figure 4.7 Atg7$^{-/-}$ cells have comparable levels of KU positive foci relative to Atg7f/f cells as assessed by immunofluorescence. KU70 nuclear foci formation was monitored in Atg7f/f and Atg7-/- cells 1h after 10Gy IR treatment. DAPI was used to stain the nucleus.

*The experiment was carried out at least three times and a representative figure is presented here.

A more definitive measurement of NHEJ is to use a GFP-readout plasmid-rejoining assay. It was revealed that there are equivalent NHEJ capacities in Atg7f/f and Atg7-/- cells, as measured by the plasmid-based assay (Fig. 4.8).

The use of NHEJ substrate plasmid pEGFP-Peml-Ad2 was first published by Gorbunova lab [250]. The plasmid encodes a GFP sequence which has an inserted adenoviral exon flanked by two introns. The GFP is inactive because of the additional exon. Both introns contain a restriction site for HindIII enzyme, which generates compatible ends for NHEJ. Following treatment with HindIII, NHEJ causes end-joining of the DNA break resulting an active GFP, as illustrated in Fig 4.8 (a).

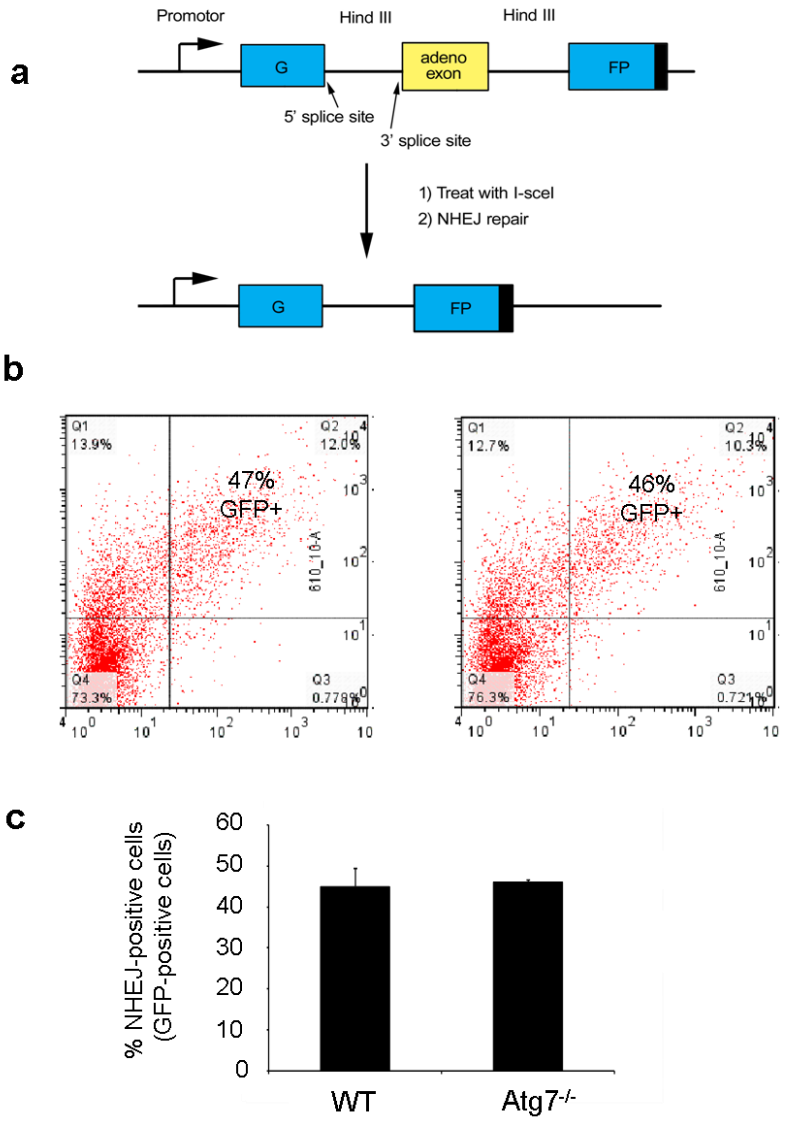


Figure 4.8 Non-Homologous End-Joining is unaffected in autophagy-deficient cells as assessed by flow cytometry assays.

(a) (Adapted from [250]) GFP sequence in reporter substrate (pEGFP-Peml-Ad2) has an artificially inserted adeno-exon flanked by two introns, both of which contain restriction sites for HindIII. The GFP is inactive until the plasmid is digested by HindIII enzyme and rejoined through NHEJ pathways. **(b)** Plasmid-based assays were used to measure NHEJ activity in wild-type and Atg7-null cells. **(c)** The graph represents the quantification of repeated experiments.

*The experiment was carried out at least three times and a representative figure is presented here.

The results above show that autophagy deficient cells have defects in HR pathways but they appear to have intact NHEJ pathways.

It has been reported that defects in HR pathways can lead to an accumulation of chromosomal breaks and promote genomic instability [294]. Cells lacking HR components such as Rad51 or BRCA1 are often viable but they accumulate spontaneous damage and are hypersensitive to DNA damaging agents such as IR or UV [295]. Mouse cells lacking critical HR mediators such as Rad51 or Xrcc2 have chromosomal re-arrangement and aneuploidy and knocking out these genes in mice leads to embryonic lethality [296] [297].

Because of their importance in guarding genomic integrity, it is not surprising that these HR mediator genes are frequently found to be mutated in cancer. Defects in DNA repair pathways promote neoplasmic transformation [298]. Cancer cells lacking HR capacity have been found to be more dependent on alternative repair mechanisms including NHEJ, base excision repair and other less-defined mechanisms [299] [300]. This property of tumour cells has been utilized in synthetic lethal anti-cancer therapeutics. For example, breast cancer cells lacking BRCA1/2 have been found to be hypersensitive to PARP (poly ADP ribose polymerase) inhibitors [301], which is an important mediator for base excision repair pathways, and PARP is frequently found to be up-regulated in cancer cells [302].

Alternative pathways to HR are more error prone because they are not homology-based and they often involve loss of genetic information at the sites of breakage. HR is the predominant repair pathway for cells that have finished DNA synthesis and before they divide into two daughter cells [303]. The lack of HR during these scenarios can potentially contribute to neoplastic transformation. For example, chromosomal re-arrangement can occur if the ends from different chromatids are wrongly joined together [299].

4.3 Characterisation and quantification of genetic instability in autophagy deficient cells.

The profound genetic instability due to loss of HR capacity promotes cancer cell transformation, yet in most cases; cell death is induced when there is an accumulation of chromosomal breakage [294]. Sub-G1 DNA content is a reliable measure of apoptotic cell death. To investigate whether autophagy deficient MEFs have more spontaneous cell deaths, Sub-G1 DNA contents over a period of 3 weeks were monitored in Atg7f/f and Atg7-/- cells. At any given time, Atg7-/- cells displayed increased basal cell death relative to Atg7f/f cells. During the first three weeks after Atg7 recombination, Atg7-/- cells displayed twice the amount of cells going through spontaneous cell death relative to Atg7f/f cells. By the end of 3 weeks, Atg7-/- MEFs entered crisis and had a drastic portion of apoptotic cells (Fig 4.9).

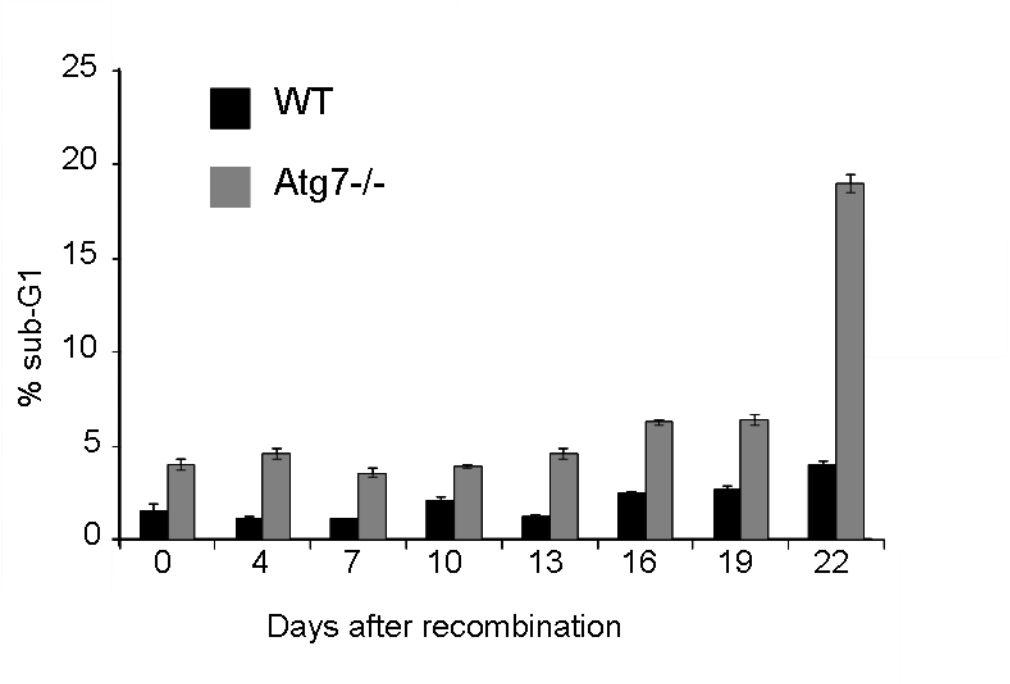


Figure 4.9 Autophagy deficient cells have increased spontaneous cell death.

Sub-G1 DNA content was measured by flow cytometry analysis in Atg7f/f and Atg7$^{-/-}$ cells at the indicated time points after *Atg7* recombination.

*The experiment was carried out twice and a representative figure is presented here.

Before Atg7$^{-/-}$ entered crisis point at the end of the three-weeks after recombination, the absolute percentages of cells going through spontaneous cell death were relatively low (around 2-4% in Atg7f/f cells and 4-8% for Atg7-/- cells). It was expected that increased spontaneous cell death would manifest as defects in cell growth, therefore cell proliferation rates of Atg7f/f and Atg7$^{-/-}$ cells were monitored over a period of 4 weeks. Growth curve analysis revealed that autophagy deficient cells had a significant slower rate of growth (Fig. 4.10). Crystal violet staining assay was also used to detect the difference in growth rates between Atg7f/f and Atg7$^{-/-}$ cells. 10^6 cells were seeded onto 10mm dishes and incubated in a humidified 5% CO2 incubator, after 48h, the dishes were stained with crystal violet solutions. Crystal violet is a chemical that binds sugar containing molecules such as DNA, and it can be easily visualized. The intensity of purple colour is generally correlative to the amount of cells on the dishes. Results as shown in Fig. 4.10b revealed that there was significant less of Atg7$^{-/-}$ cells on the dishes and hence that they grow at a slower rate.

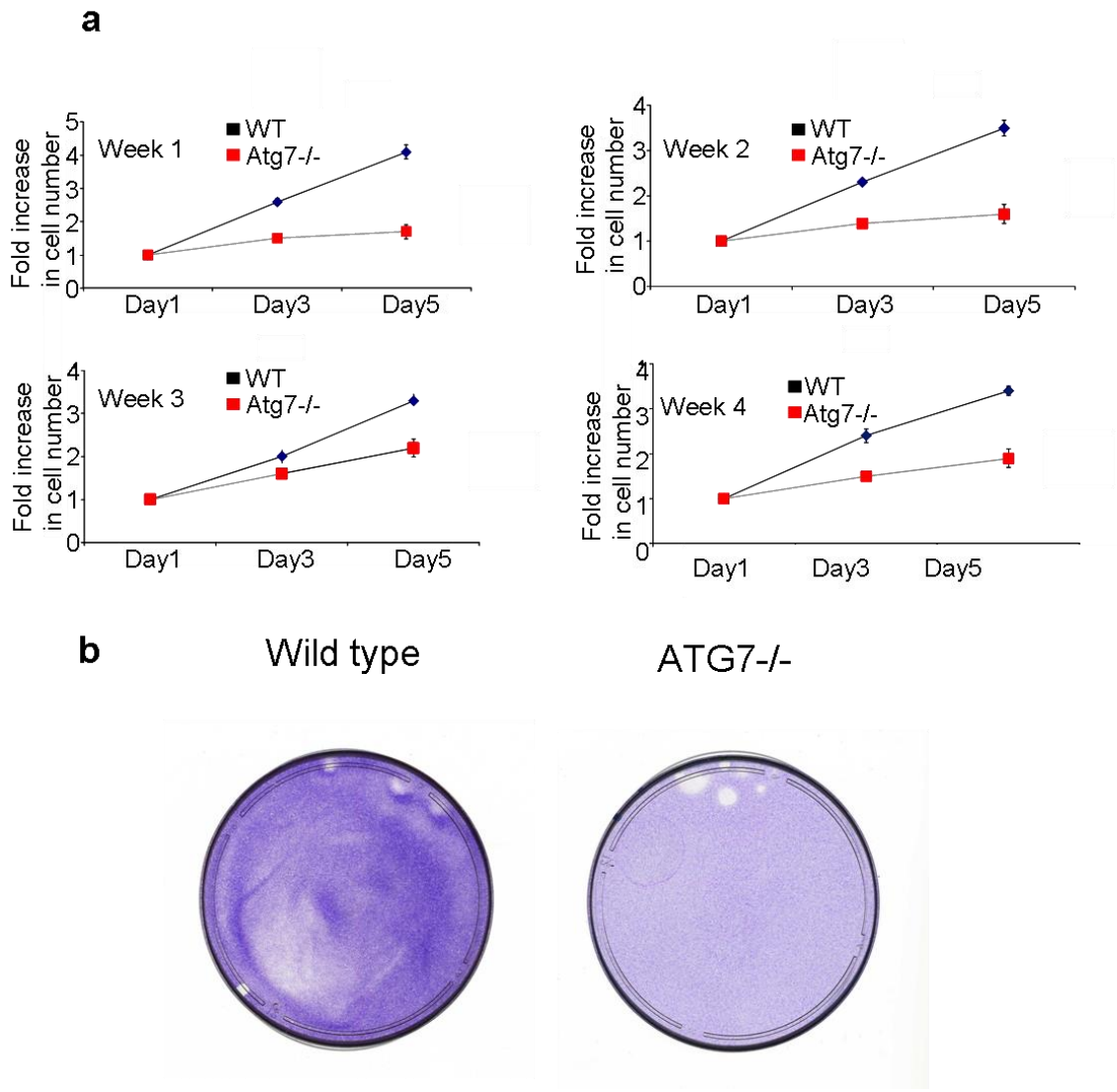


Figure 4.10 Atg7$^{-/-}$ have a significant longer doubling time relative to Atg7f/f cells.

(a) The difference in cell proliferation rate between Atg7$^{flox/flox}$ and Atg7$^{-/-}$ cells was monitored over a time course of 4 weeks. **(b)** 10^6 Cells were plated onto 10mm dishes on day one and the dishes containing adherent cells were stained with crystal violet dye on day 3. *The experiment was carried out twice and a representative figure is presented here.

Micronuclei formation is commonly used as a biomarker to quantify chromosomal damage, especially damage related to double strand breaks [304]. A micronucleus is an irregular small nucleus that is formed during mitosis. Micronuclei are commonly seen in cancer, as a result of chromosomal instability [305]. It contains chromosomal fragments or a whole chromosome that fails to be segregated into a daughter cell. It can be easily visualized using DAPI, which is a dye binding to DNA. Micronuclei can form through multiple mechanisms. Deficiency in HR pathways such as BRCA or Rad51 results in micronuclei accumulation in the cells [306] [307]. It was considered that HR deficient cells become hyper-dependent on NHEJ. NHEJ is an error-prone way of repairing DSBs and is more likely to form dicentric chromosomes (chromosomes attached to two centromeres) and acentric chromosomal fragments (chromosomes unattached to centromere) [305]. These chromosomal aberrations eventually manifest as micronuclei formation.

It was observed that there was increased micronuclei formation in autophagy deficient cells and the difference was quantified (Fig. 4.11). This observation supports that autophagy deficient cells have increased genetic instability, and is possibly due to the fact that HR pathways are defective in autophagy deficient cells.

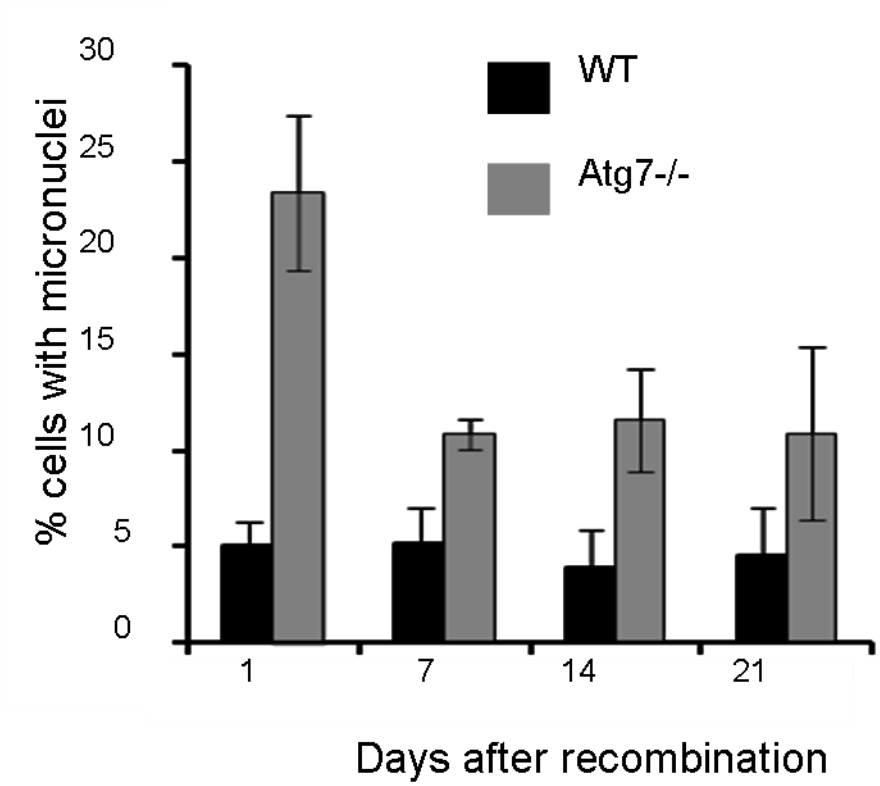


Figure 4.11 Loss of autophagy leads to micronuclei accumulation.

Occurrence of micronuclei was examined and quantified in Atg7f/f and Atg7$^{-/-}$ cells at the indicated time points after Atg7 recombination. *The experiment was carried out at three times and a representative figure is presented here.

4.4 Investigation of synthetic lethal effects due to HR defect in autophagy deficient cells.

Most of DSBs are repaired by NHEJ which is available throughout the cell cycle[303]. Both HR and NHEJ pathways are crucial for the cells to maintain genetic stability; the two pathways can not replace each other and mutations in either pathway leads to diseases including cancer [298]. If one of the two pathways is lacking in the cells, the other pathway can be up-regulated to mediate the repair of DSBs. This plasticity has been demonstrated by several studies. For instance, HR is up-regulated in cells that have defective NHEJ pathways [308]. NU7441 is potent specific DNA-PK inhibitor [309], it has been shown to inhibit NHEJ and increase dependence on HR pathways in cancer cell lines [310].

Autophagy deficient cells have defective HR pathways and intact NHEJ pathways. NHEJ does not appear to be up-regulated by the loss of autophagy. To investigate whether cells became hyper-dependent on NHEJ pathways after the loss of autophagy, Atg7f/f and $Atg7^{-/-}$ cells were treated with DNA-PK inhibitor NU7441 following exposure to 10 Gy IR. Our results, as shown in Fig. 4.12, indicated that Atg7f/f cells were able to repair IR induced DSBs after 30 hours in the absence or presence of DNA-PKi. In Atg7-/- cells where HR was found to be defective, treatment of DNA-PK inhibitor lead to persistence of DNA damage, as marked by γ-H2AX staining, 30 hours following DSB inducing IR treatment (Fig 4.12).

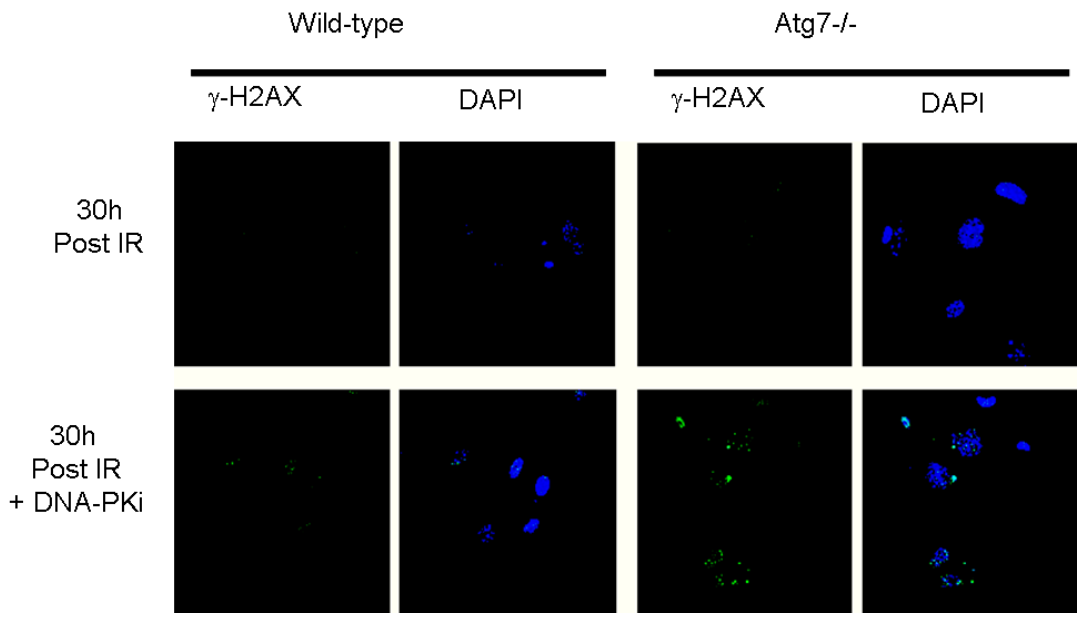


Figure 4.12 DNA double strand breaks persist in autophagy deficient cells following inhibition of DNA-PK.

Immunofluoresent microscopy analysis for the persistence of γ-H2AX foci 30h after 10Gy irradiation (IR) in control and ATG7$^{-/-}$ cells was assessed either in the absence or presence of 10μM DNA-PKcs inhibitor, NU7441 (DNA-PKi). DAPI was used to stain DNA in the nucleus. *The experiment was carried out at twice and a representative figure is presented here.

The accumulation of un-repaired DSBs leads to cell cycle arrest and eventually programmed cell death is activated [311]. As Atg7-/- cells displayed persistent DNA damage after inhibition of NHEJ pathways, cell death analysis was carried out to investigate whether the loss of autophagy leads to increased cell death in response to DSB inducing agents in combination with an NHEJ inhibitor (Fig. 4.13). Atg7f/f and $Atg7^{-/-}$ cells were exposed to 10Gy IR, followed by 48h incubation with or without 10μM NU7441 (DNA-PKi). The cells were harvested for flow cytometry analysis and it was revealed that loss of autophagy leads to hypersensitivity to IR in combination with DNA-PKi.

Higher does of IR (25Gy) led to higher percentage of cell death in autophagy deficient cells (Fig. 4.14). There was limited amount of apoptosis (< 5%) in lower dose IR (10Gy) treated cells, in the absence or presence of autophagy. Inhibition of DNA-PK and therefore NHEJ pathways did not appear to affect cell viability after DNA damaging treatment in Atg7f/f cells. However, in $Atg7^{-/-}$ cells there was a marked increase in cell death in DNA-PKi treated cells after DNA damage induced by 10Gy IR (Fig. 4.13).

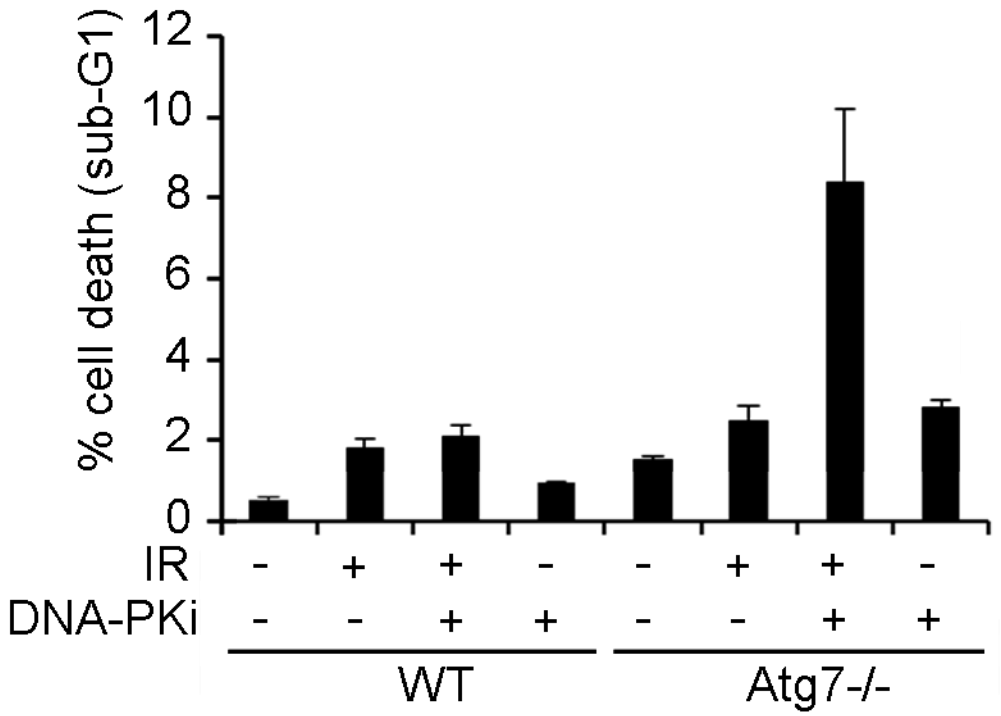


Figure 4.13 Atg7$^{-/-}$ cells are dependent on DNA-PK/NHEJ following treatment with IR.

Sub-G1 DNA contents were analysed by flow cytometry in Atg7f/f and Atg7$^{-/-}$ cells 48h following 10Gy IR in the absence or presence of 10μM DNA-PKcs inhibitor, NU7441 (DNA-PKi). *The experiment was carried out at least three times and a representative figure is presented here.

Numerous studies have shown that the loss of HR capacities in non-transformed or cancer cell lines leads to hypersensitivity to IR, as reviewed in [312]. Results as presented in Fig. 4.13 showed that autophagy deficient cells did not display any radio-sensitivity when exposed to 10Gy IR. However, when cells were treated with 25Gy IR, there was an increase in cell death for cells lacking autophagy (Fig. 4.14). There was increased spontaneous cell death in $Atg7^{-/-}$ cells and upon 25Gy IR, the death was further increased. Atg7f/f primary cells did not display any increase in cell death following IR.

Etoposide is a commonly used chemotherapeutic drug that induces double strand breaks. Etoposide also induces double strand breaks through the inhibition of topoisomerase II [261]. Similar to the synergy in cell death observed with IR and DNA-PKi, $Atg7^{-/-}$ cells were also found to be hypersensitive to etoposide (25μM) in combination with treatment with DNA-PKi (10μM) (4.15).

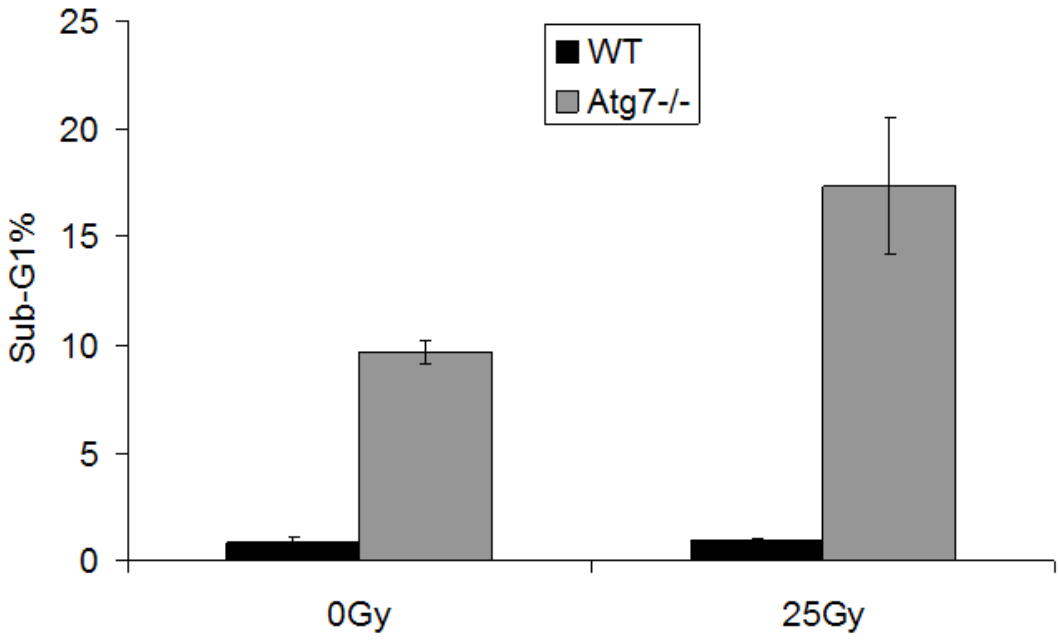


Figure 4.14 Higher dose of IR leads to increased cell death in autophagy deficient cells.

Atg7f/f and Atg7-/- cells were exposed to 25Gy IR and harvested for flow cytometry analysis 48h later. Sub-G1 DNA contents were analysed. *The experiment was carried out at least three times and a representative figure is presented here.

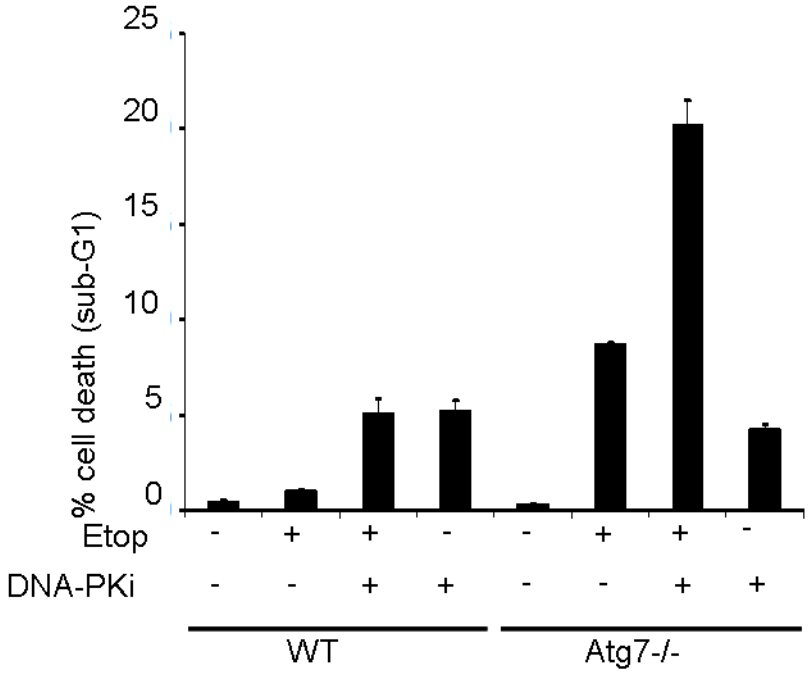


Figure 4.15 Atg7$^{-/-}$ cells are hypersensitive to DNA-PKi (10μM) after treatment with Etoposide.

Sub-G1 DNA contents were analysed by flow cytometry in Atg7f/f and Atg7$^{-/-}$ cells 48h following 25μM Etoposide in the absence or presence of 10μM DNA-PKcs inhibitor, NU7441 (DNA-PKi). *The experiment was carried out at least three times and a representative figure is presented here.

To test whether autophagy deficient cells were intrinsically hypersensitive to etoposide, Atg7f/f and Atg7$^{-/-}$ cells were treated with titrating concentrations of etoposide of up to 60μM. It was found that loss of autophagy had no effect on apoptosis after treatment with etoposide (Fig 4.16).

It has been reported that loss of autophagy is linked to hypersensitivity to chemotherapeutic drug camptothecin (CPT). FIP200 is an autophagy essential gene. Camptothecin does not have a great impact on cell viability in Atg7f/f cells, while FIP200 knockout MEFs have greatly diminished cell viability [249]. In breast cancer cell line MCF-7, inhibition of either Beclin1 or Atg7 leads to hypersensitivity to CPT [313].

In order to test whether autophagy deficient MEFs are hypersensitive to CPT, Atg7f/f and Atg7$^{-/-}$ cells were treated with titrating concentrations of CPT for 16 hours and the cells were harvested for Sub-G1 DNA content analysis using flow cytometry. Autophagy deficient cells displayed marked sensitivity to CPT (Fig. 4.17).

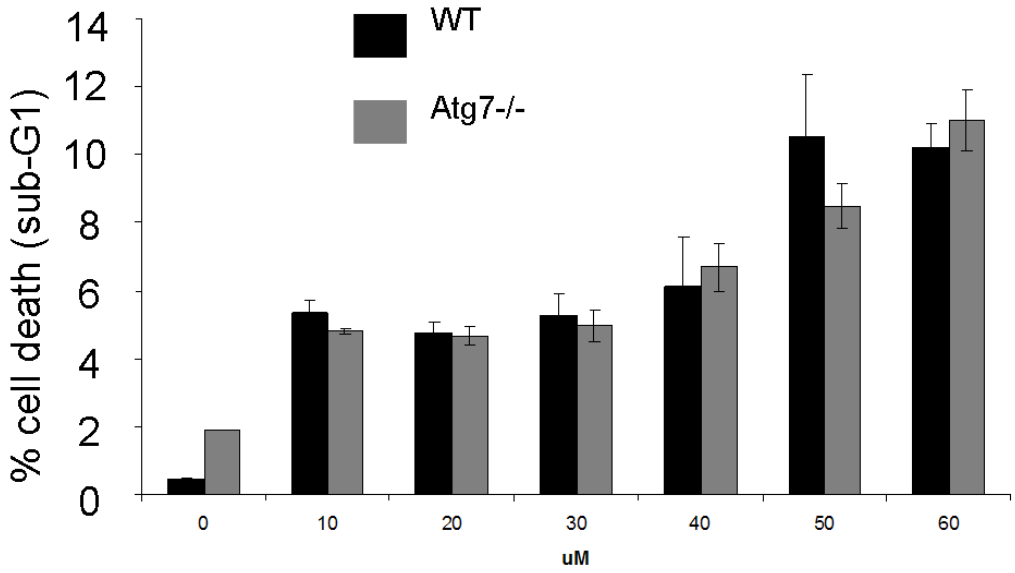


Figure 4.16 Loss of autophagy does not affect etoposide induced apoptosis.

Sub-G1 DNA contents were analysed by flow cytometry in Atg7f/f and Atg7-/- cells 48h following treatment with 25μM etoposide as indicated. *The experiment was carried out at least three times and a representative figure is presented here.

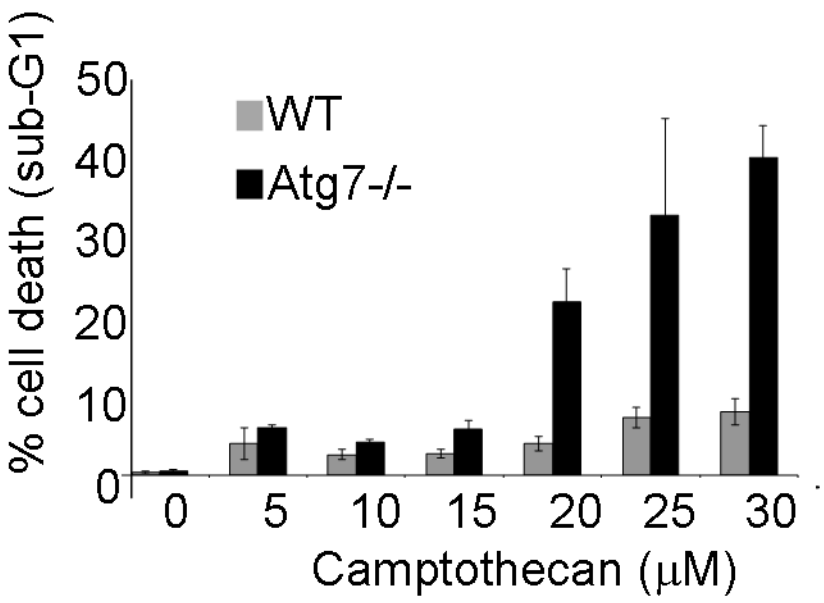


Figure 4.17 Atg7-/- cells are hypersensitive to Camptothecin.

Wild-type and Atg7$^{-/-}$ cells were treated with indicated concentrations of Camptothecin for 16 hours and cell death was accessed by flow cytometry analysis of sub-G1 DNA content. *The experiment was carried out at least three times and a representative figure is presented here.

As shown in Fig 4.16 and 4.17, $Atg7^{-/-}$ MEFs did not display increased sensitivity to etoposide however they are hypersensitive to CPT. Both CPT and etoposide are topoisomerase inhibitors, they form covalent complexes with cleaved DNA molecules and lead to DSBs [314, 315]. CPT is an inhibitor of DNA topoisomerase I, and etoposide of topoisomerase II. The types of DSBs they form are different and they exert different toxicity on cells. CPT leads to DSBs when the replication fork collides with CPT cleavage complex during S-phase [316]. Etoposide forms a cleavage complex with a long half life and eventually the single strand break turns into DSBs. DSBs by etoposide are repaired through HR and NHEJ [317]. The breakages created by CPT have overhangs of ssDNAs and resemble DNA ends after processed by strand resection mediators. DSBs mediated by CPT are primarily repaired with HR pathways [318, 319].

Homologous recombination repair pathways involve DNA synthesis and stable cellular nucleotide levels are required to support DNA biogenesis. The lack of sufficient nucleotide pool in the cells leads to deficiency in DNA damage response and ultimately genetic instability [320]. Autophagy inhibition may limit the size of the cellular nucleotide pool, and so there is a possibility that the intrinsic sensitivity to CPT of $Atg7^{-/-}$ cells was due to the lack of sufficient nucleotide. However, addition of exogenous nucleosides which elevate nucleotide pool did not reverse the effect (Fig 4.18).

Atg7 is an E1-like enzyme crucial for autophagy process; a recent study reported that Atg7 can also form a complex with p53 protein both in the cytoplasm and the nucleus, playing a role in nutrient withdrawal induced cell cycle arrest [268]. It is unclear whether Atg7 plays a role in other pathways of DNA damage response. To ascertain whether the synergetic effects we observed were due to loss of autophagy or specifically Atg7 protein, $Atg5^{flox/flox}$ MEFs were isolated and it was found that loss of Atg5 also leads to hypersensitivity to etoposide when treated with DNA-PKi (Fig 4.19).

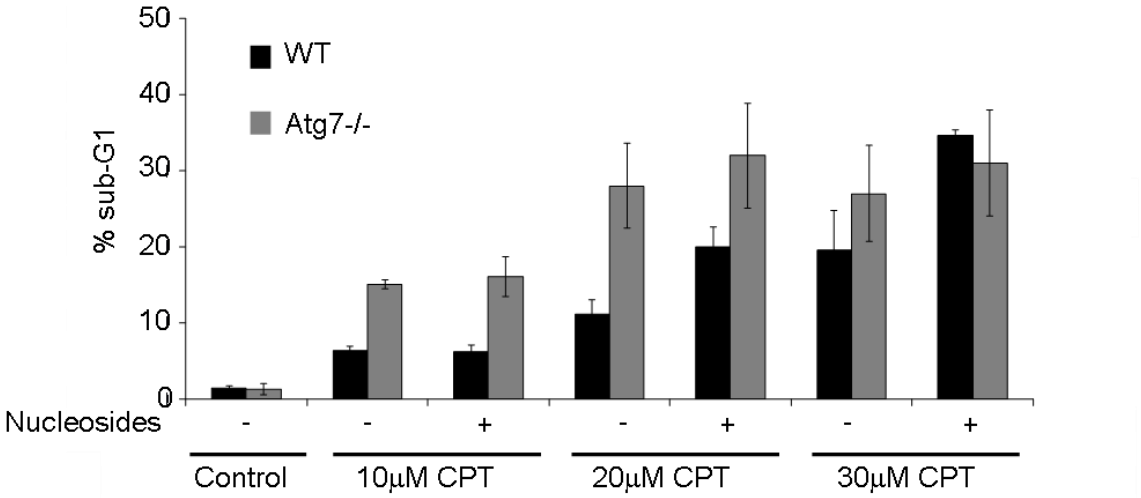


Figure 4.18 Addition of exogenous nucleosides does not rescue CPT sensitivity in autophagy deficient cells.

Apoptotic cell death was assessed 24h after treatment with Camptothecan (CPT) with or without nucleosides (10μM each of adenosine, guanosine, uracil and cytidine) as indicated. Total cell populations were collected and assessed for sub-G1 DNA content by flow cytometry. *The experiment was carried out at least three times and a representative figure is presented here.

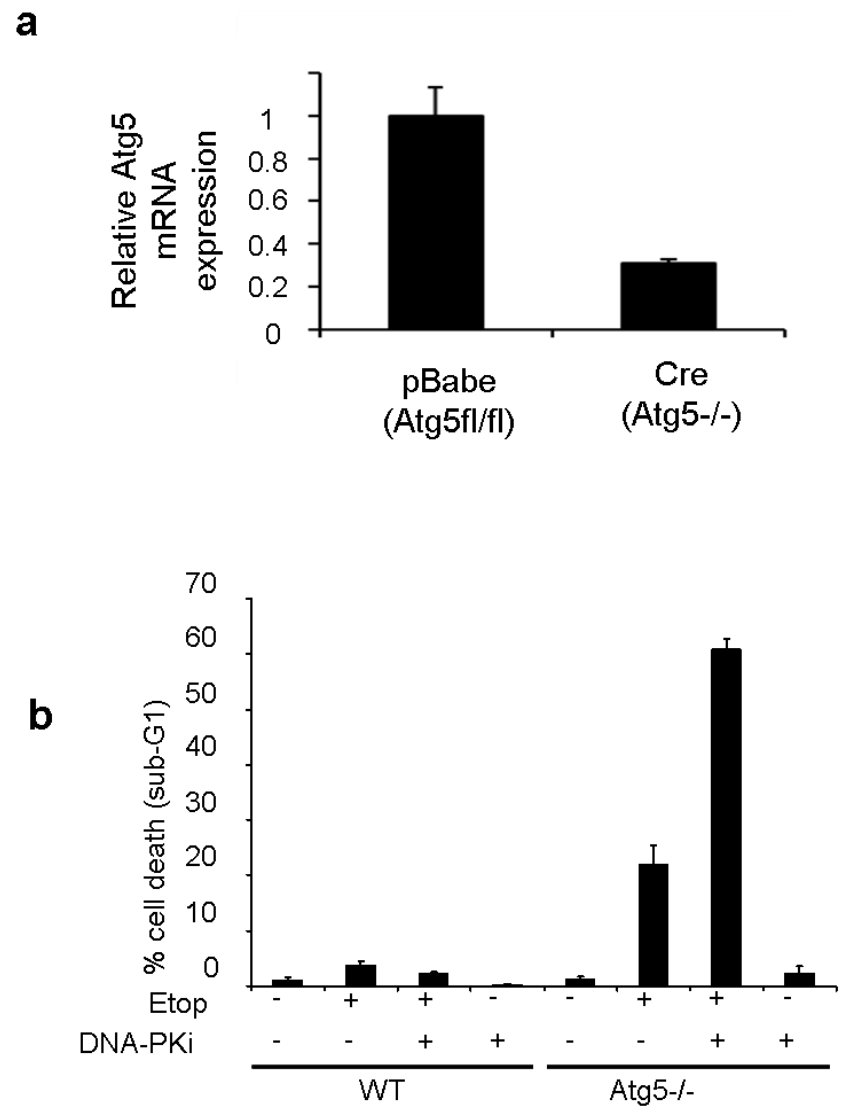


Figure 4.19 Atg5$^{-/-}$ cells display synergy in cell death when treated with DNA PKi after DNA damage.

Atg5$^{flox/flox}$ MEFs were infected with Cre recombinase or empty retroviral vector as contol. **(a)** Following antibiotic selection, cells were, where indicated, exposed to etoposide and/or DNA-PKi for 48h. Total cell populations were collected and assessed for sub-G_1 DNA content by flow cytometry. **(b)** Relative mRNA expression of Atg5 was measured by qRT-PCR in ATG5$^{flox/flox}$ cells which were retrovirally infected with either pBabe-Puro or pBabe-Puro-Cre. *The experiment was carried out at least three times and a representative figure is presented here.

Chapter 5. Investigating the Mechanisms through Which Chk1 Level Is Down Regulated

Protein degradation is a central process in the cells which is crucial for nearly all fundamental cellular activities including cell cycle progression, cell signalling, cell death, DNA transcription and DNA repair [321]. There are two main ways of degrading proteins and organelles in eukaryotic cells; they are the ubiquitin-proteasome pathway and autophagy. The 26S proteasomes are large protein complexes found in all eukaryotic cells. They mainly degrade short-lived nuclear or cytosolic proteins [322]. Target proteins for proteasomal degradation are specifically marked by covalently attached ubiquitin molecules [323]. They are loaded into the catalytic cavity of proteasome complex where they are degraded. On the other hand, autophagy is a bulk degradation process that mainly degrades long-lived proteins. Autophagy can also degrade bulkier proteins that otherwise do not fit in the barrel structure of proteasomes [324]. Both systems are important for the clearance of misfolded proteins and deficiency in either pathway can lead to various neurodegenerative conditions [325].

Most ubiquitinated proteins are targeted for degradation in the proteasome. As described in Chapter 1, certain proteins, such as p62, NBR1 and HDAC6, can be ubiquitinated and specifically degraded via the autophagic machinery. Numerous proteins can be degraded by both proteasome and autophagy pathways, inhibition of either pathway can often lead to the up-regulation of the other [326] [327] [105]. There is also a study showing that the inhibition of autophagy can lead to suppression of proteasome mediated protein degradation through up-regulation of p62 [328]. Accumulation of p62, which contains a ubiquitin binding domain, is thought to delay the delivery of ubiquitinated proteins to the 26S proteasome. p62 itself does not appear to have any effects on the proteasomal activities [328].

Chk1 is a protein degraded by the proteasome. Chk1 protein is rapidly phosphorylated at multiple sites by ATR in response to genotoxic or replicative

stress. The phosphorylation of Ser345 site is thought to relieve the auto-inhibition of Chk1 and promote its kinase activities [329]. DNA damage responses consist of three main aspects: cell cycle arrest, DNA damage repair and programmed cell death. The three processes are finely coordinated and Chk1 is found to play a part in all three processes. The activation of Chk1 promotes cell cycle arrest and DNA repair. When the genotoxic stress is removed and DNA damage is repaired, Chk1 is inactivated and cell cycle resumes. A few mechanisms of Chk1 inactivation have been characterized to promote cell cycle recovery. Chk1 adaptor protein Claspin is regulated to modulate Chk1 activities [330] and phosphatase WIP1 specifically dephosphorylate Chk1 on a Ser345 [331]. Prolonged cellular stress leads to cell death and phosphorylated Chk1 is thought to be one of the intrinsic timers deciding cell fate. Activation of Chk1 facilitates its degradation. [332] [267]. Phosphorylation of Chk1 leads to its exposure to SCF E3 ligase, which mediates the ubiquitination and proteasomal degradation. Eventual depletion of Chk1 leads to permanent S phase arrest and subsequent cell death [333].

5.1 Investigation of proteasomal activities in cells when autophagy is inhibited.

Since p-Chk1 and later total Chk1 were diminished in autophagy deficient cells, it became apparent to test proteasomal activities in Atg7f/f and Atg7$^{-/-}$ cells. The Proteasome-Glo™ Cell-Based Assays (Promega) was used to test proteasomal activities. The assay consists of artificially engineered substrates that can be recognised by the proteasome and cleaved by the chymotrypsin-like protease activities. The cleavage of the substrates leads to production of luciferin, which reacts with luciferase, producing luminescence signal. The strength of signal correlates with proteasomal activities.

Proteasome activity luminescence assays were performed with Atg7f/f and Atg7$^{-/-}$ cells (Fig. 5.1). Where indicated, 10μM lactacyctin was added to the cells 3h prior to harvest. Luminescence generated by proteasomal activities was measured and Atg7$^{-/-}$ cells displayed nearly two fold of activity relative to Atg7f/f cells. The

signals are diminished after treatment with lactacystin, indicating that the signal is truly representative of proteolytic activity of the 26S proteasome complex. Lactacystin is a commonly used specific proteasome inhibitor. It is an compound first identified in bacteria *streptomyces* [334] , it binds covalently to the active site N-terminal threonine residue in beta-subunits of proteasome, inhibiting the catalysis. Lactacycstin was included in all proteasomal activity assays as a negative control.

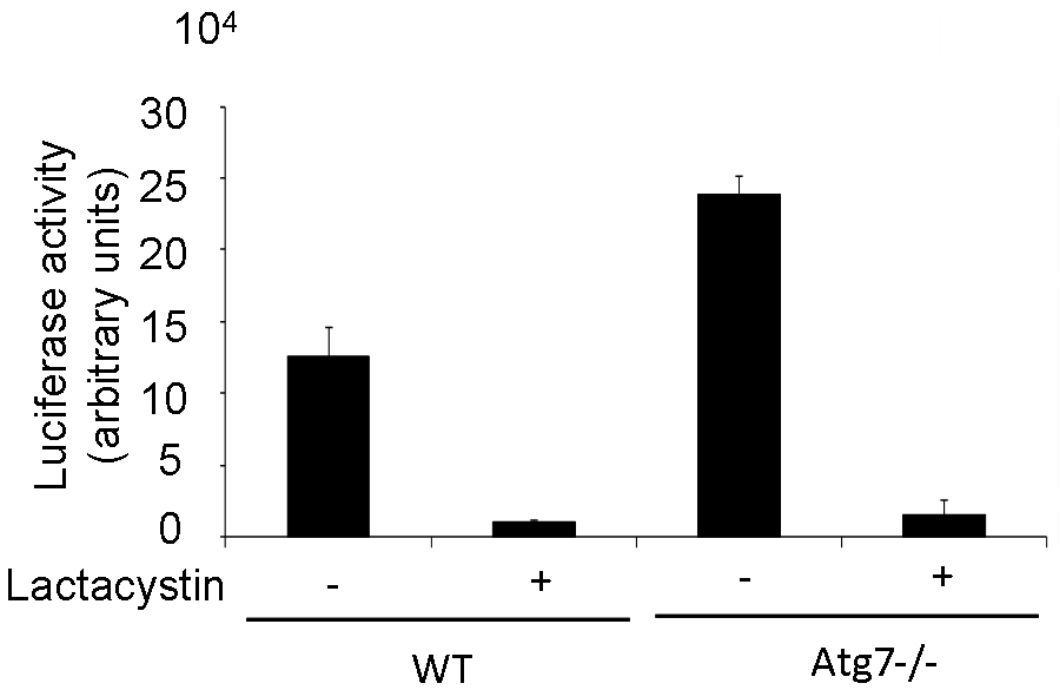


Figure 5.1 Proteasomal activities are up-regulated in Atg7$^{-/-}$ cells.

Proteasomal activity was determined in wild-type and Atg7$^{-/-}$ cells using a luciferase-based assay kit (Promega). Where indicated, lactacyctin (10μM) was added to the cells 3h prior to harvest. *The experiment was carried out at least three times and a representative figure is presented here.

As described previously (Fig. 3.4), there was less phosphorylated Chk1 in response to DNA damage in autophagy deficient cells soon after recombination, and later on there was decreased total Chk1 protein level in autophagy deficient cells (Fig. 3.8). No defects in Chk1 activation pathways were observed. Since activation of Chk1 facilitates its proteasomal mediated degradation, we wanted to find out whether inhibition of proteasomal activities can reverse the effect on Chk1 after loss of autophagy. MG132 (carbobenzoxy-Leu-Leu-leucinal) is another commonly used proteasome inhibitor. While lactacystin inhibition involves covalent modification and is irreversible, MG132 is a potent and reversible peptide inhibitor that inhibits catalytic activities of the 26S proteasome complex [335].

It was found that the proteasomal inhibitor MG132 partially rescued phosphorylation of Chk1 at S345 in $Atg7^{-/-}$ cells after irradiation. This is the case during early stages of recombination when only p-Chk1 is affected in Atg7-/- cells (Fig. 5.2) or when total Chk1 is also diminished at later times (Fig 5.3). Freshly recombined Atg7f/f and Atg7-/- cells were exposed to 10Gy IR and harvested for western blotting an hour later. The cells were treated with MG132 for 6h where indicated. In this case, total Chk1 levels were not noticeably diminished in $Atg7^{-/-}$ cells, however there is decreased level of p-Chk1 (Ser345) after 10Gy IR in autophagy deficient cells (Fig. 5.2). In the presence of MG132, there were increased basal levels of p-Chk1 in both wild-type and Atg7-/- cells. Following treatment with MG132, Chk1 in $Atg7^{-/-}$ cells was activated to a comparable extent as Atg7f/f cells.

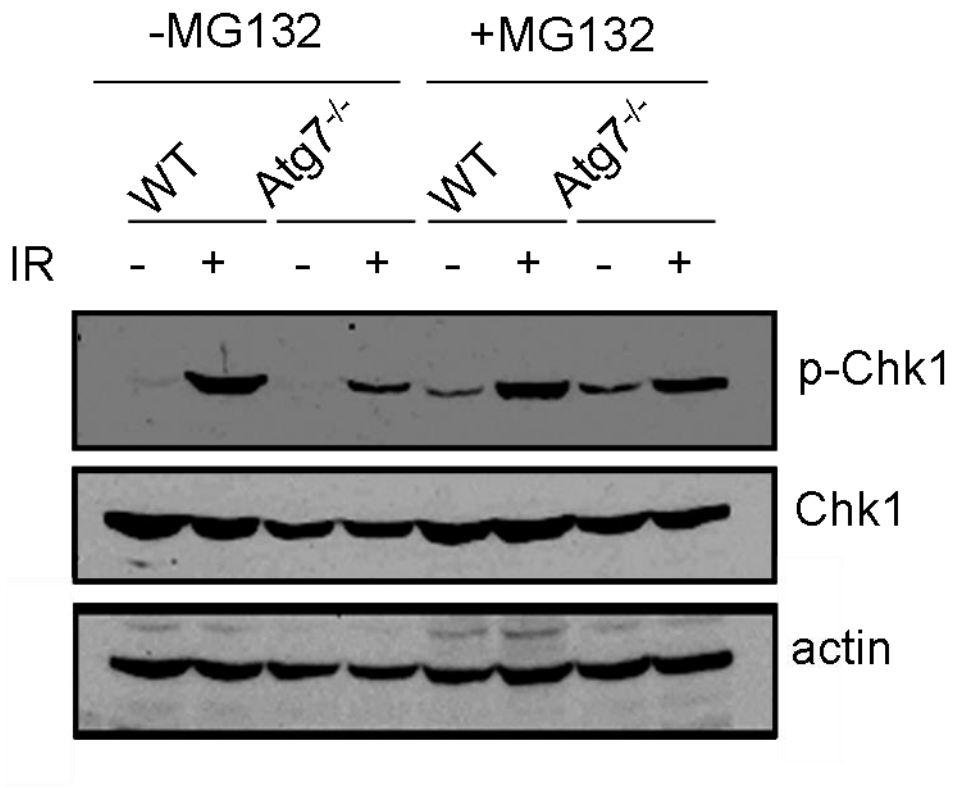


Figure 5.2 Down-regulation of phosphorylated Chk1 in Atg7$^{-/-}$ cells can be rescued by proteasomal inhibitors MG132.

Chk1 phosphorylation at Ser345 and total protein levels in wild-type and Atg7$^{-/-}$ cells were examined 1h post 10Gy IR in either the absence or presence of 10μM MG132 for 6h. Actin was used as a loading control. *The experiment was carried out at least three times and a representative figure is presented here.

We were also interested to find out whether MG132 can rescue diminished total Chk1 protein in Atg7$^{-/-}$ cells. Since Chk1 levels are not elevated after 6h of MG132 treatment (Fig 5.2), in subsequent experiments, the treatment time was extended to 16h (Fig. 5.3). After 16h MG132 treatment, there are higher levels of phosphorylated Chk1 in response to 10Gy IR in Atg7f/f and Atg7$^{-/-}$ cells. Surprisingly, prolonged treatment with MG132 lead to the degradation of total Chk1 in Atg7f/f cells and did not raise the levels of total Chk1 in Atg7$^{-/-}$ either. Since proteasomal activities are elevated in autophagy deficient cells, it remained a possibility that other proteasomal substrates were also degraded faster in these cells. In order to test this, protein levels of Mcl-1, a well characterized proteasomal substrate, were investigated in Atg7f/f and Atg7$^{-/-}$ cells. Mcl-1 (myeloid leukemia cell differentiation protein) is a protein belongs to the Bcl-2 family; it is degraded by the ubiquitin-proteasome pathway and is known to have a very short half-live. Mcl-1 is rapidly turned over in the cells and the half life of Mcl-1 is around 3 hours [336]. Loss of autophagy does not affect levels of Mcl-1 at basal levels, this indicates that the up-regulation of proteasomal activities do not affect all short-lived proteins. After MG132 treatment, levels of Mcl-1 became elevated (Fig 5.3).

A more definitive assay to test whether Chk1 is degraded faster in the absence of autophagy was carried out using Cycloheximide (CHX). Proteins in the cells are constantly recycled and renewed. The half life of a short-lived protein can be determined by western blotting analysis when protein synthesis is inhibited. CHX is a small molecule that blocks eukaryotic translation by binding to the ribosome and inhibits eEF2-mediated translocation [337]. It has been commonly used in assays to determine protein half life in eukaryotic cells. As shown in Fig. 5.4, Autophagy deficient cells were able to degrade Chk1 faster.

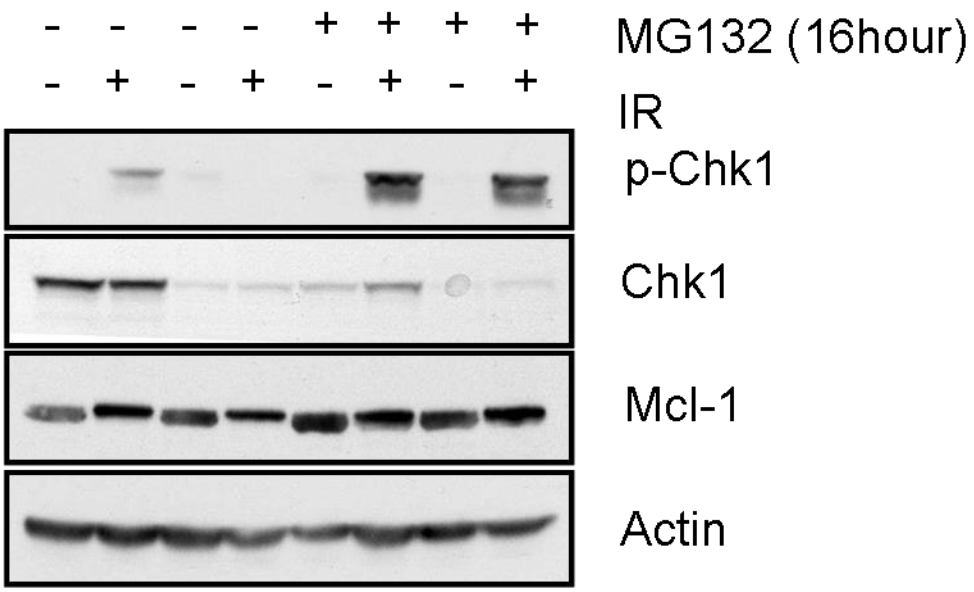
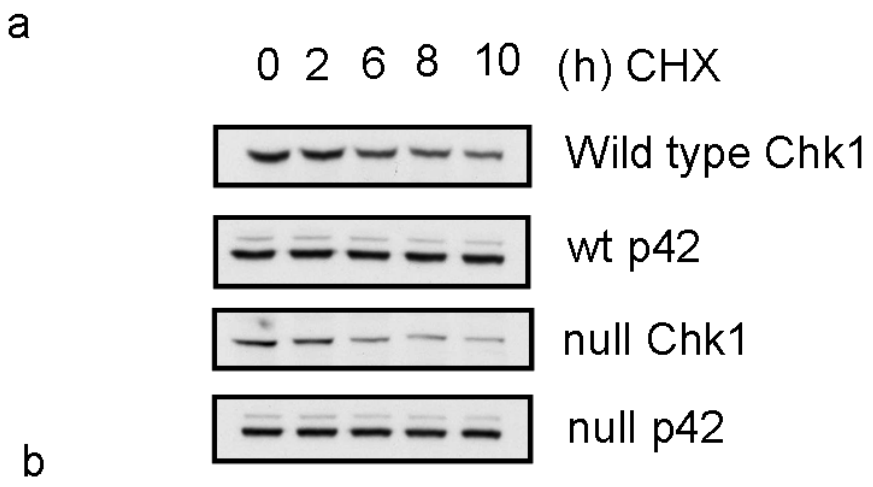


Figure 5.3 Downregulation of p-Chk1 in Atg7-/- cells can be rescued by proteasomal inhibitors MG132 and long term MG132 treatment leads to Chk1 degradation.

Chk1 activation in wild-type and Atg7$^{-/-}$ cells was examined 1h post 10Gy IR in either the absence or presence of 10μM MG132 for 16h. *The experiment was carried out at least three times and a representative figure is presented here.



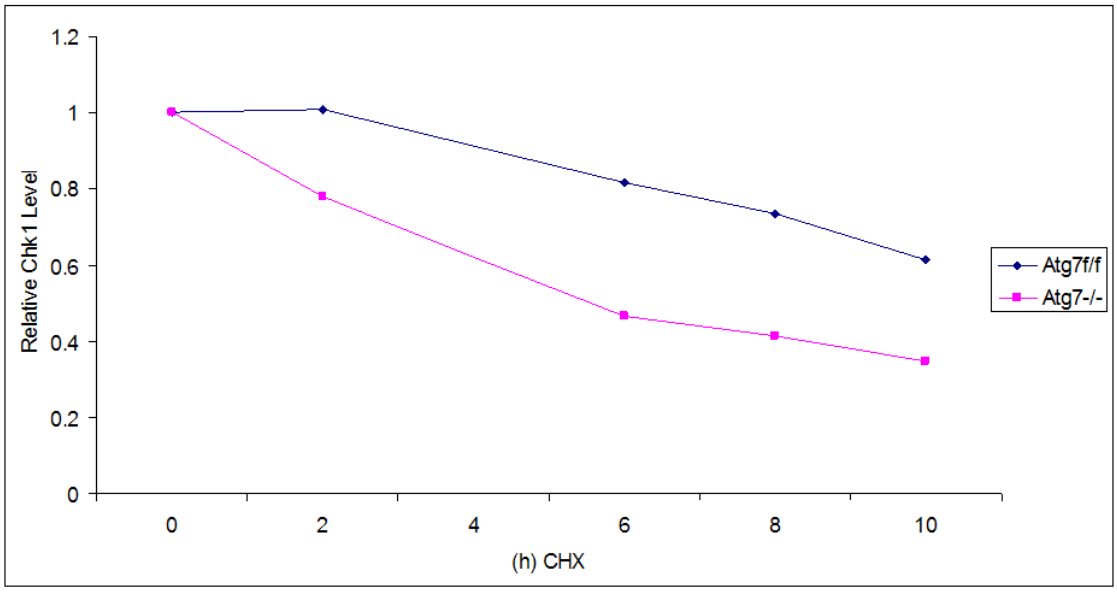


Figure 5.4 Loss of autophagy decreases Chk1 half life.

(a) Endogenous Chk1 levels were determined by western blotting in Atg7$^{flox/flox}$ and Atg7$^{-/-}$ cells, after treated with cycloheximide (10μg) for various length of time as indicated. **(b)** Chk1 levels as shown in **(a)** were quantified using ImageJ software after normalisation to ERK/p42 loading controls. *The experiment was carried out at least three times and a representative figure is presented here.

In Cre mediated recombination system of $Atg7^{flox/flox}$ MEFs where the procedure takes a week including selection with antibiotics, the deletion of autophagy activity is complete and the system is thought to have minimized off-target effects. However, it is difficult to find out how long exactly after infection with cre recombinase containing virus the cells start to lose autophagy. As shown in Fig. 5.1, Atg7-/- cells were found to have elevated levels of proteasomal activities. Cells with Atg7 depletion can not initiate autophagy and it remained a possibility that Atg7 has other functions in the cells independent of its E1 like enzyme activities. The use of a pharmacological inhibitor such as Bafilomycin A1 for autophagy would allow us to find out whether the up-regulation of proteasomal activities only occurred in $Atg7^{-/-}$ cells or the effect is genuinely due to autophagy. Bafilomycin A1 is a natural compound first identified in the bacteria *streptomyces griseus*. It is a specific inhibitor of vacuolar-type hydrogen ATPase and can prevent the re-acidification of lysosomes [338]. Bafilomycin hence inhibits the final step of autophagy when autophagosomes fuse with lysosome and the autophagic cargo is degraded.

Atg7f/f primary MEFs were found to have elevated proteasomal activities after treatment with Bafilomycin A1 in a time-dependent manner. After 12 hours treatment, there was a detectable up-regulation. By 18 hours, the proteasome activity increased by three fold in Bafilomycin treated cells (Fig 5.5). This confirmed our findings in $Atg7^{-/-}$ cells that loss of autophagy leads to increased proteasome activities. In response to dysfunctional lysosomes, the cells respond rapidly by up-regulating proteasomal activities as an adaptive mechanism.

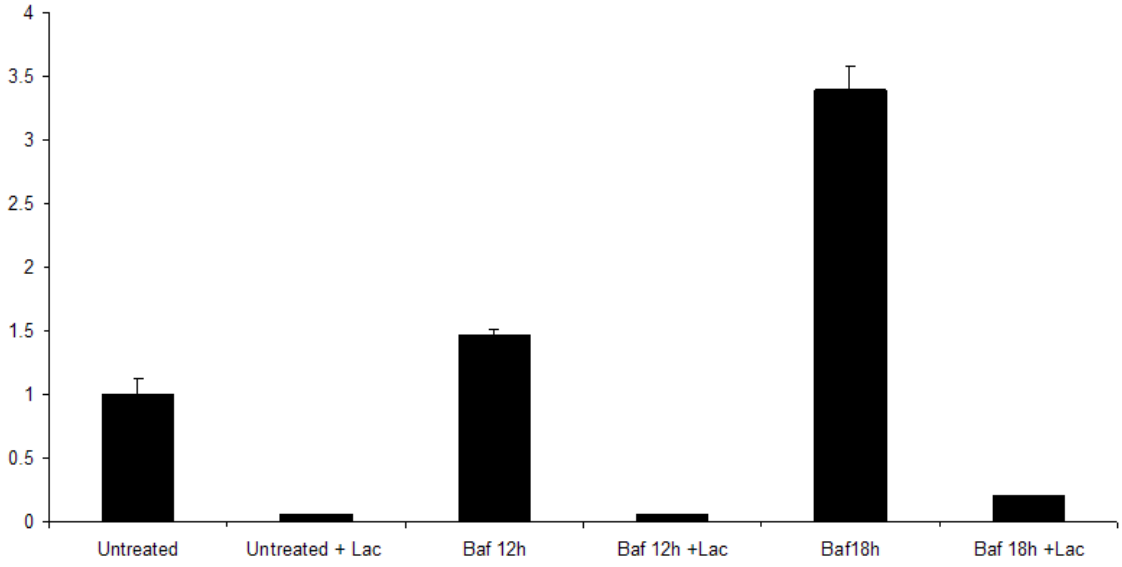


Figure 5.5 Bafilomycin A1 up-regulates proteasomal activities in a time-dependent manner.

Proteasomal activity was determined in cells in the presence of absence of Bafilomycin A1 (100nM) for indicated length using a luciferase-based assay kit (Promega). Where indicated, lactacystin (10μM) was added to the cells 3h prior to harvest. *The experiment was carried out at least three times and a representative figure is presented here.

To investigate whether short term autophagy inhibition has any effects on Chk1 activation or total Chk1, cells were pre-treated with 100nM Bafilomycin A1 for 2 hours where indicated before the addition of 25μM etoposide for indicated length of time. It was revealed that Chk1 is activated as assessed by p-Ser345 after 1 hour etoposide treatment to the same extent in the absence or presence of Bafilomycin. In accordance with published literature [333] [332] [267], Chk1 activation is not sustained in the presence of DNA damage agents, such as etoposide in this case. Comparing to 1h after etoposide treatment, there was decreased p-Chk1 signals in cells after 2h and 4h treatment in etoposide (Fig. 5.6). This is possibly because phosphorylated Chk1 is rapidly targeted for degradation. Total Chk1 protein levels were not affected by short-term Bafilomycin treatment. After 4h exposure to etoposide, Bafilomycin treated cells appeared to have a slightly weaker p-Chk1 signal relative to cells without Bafilomycin.

As shown in Fig 5.6, short term treatment (2 hours) with autophagy inhibitor Bafilomycin did not have any obvious effects on either total Chk1 or Chk1 activation in response to DNA damaging agent etoposide. 2-hour Bafilomycin treatment itself also did not have any effect on Chk1 phosphorylation. However, longer term (12h) treatment of Bafilomycin appeared to have a differential effect on Chk1 activation depending on the presence of DNA damage inducing agent etoposide (Fig. 5.7). Treatment of Bafilomycin for 12 hours caused an increase in Chk1 activation (Fig. 5.7). In the presence of etoposide which creates double strand breaks, Bafilomycin failed to activate Chk1 further. In the presence of proteasome inhibitor MG132, Chk1 is strongly activated after etoposide treatment with or without Bafilomycin. This result indicated that loss of autophagy induced Chk1 activation continuously and phosphorylated Chk1 is readily degraded by the ubiquitin-proteasome pathway. LC3 was probed as an indicator for autophagic activities. As Bafilomycin inhibits lysosomal functions, there was an accumulation of LC3-II in Bafilomycin treated samples.

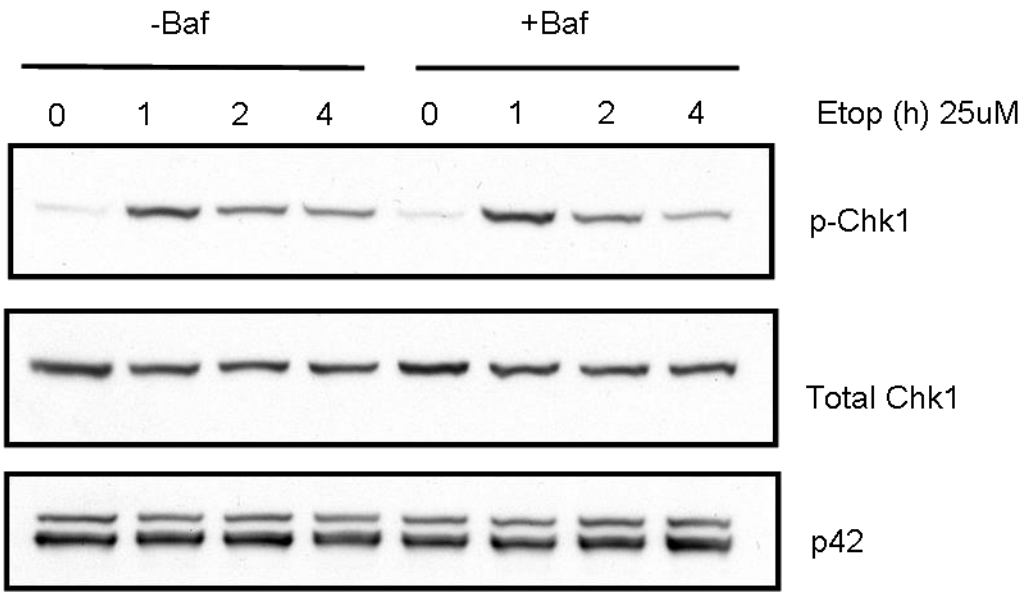


Figure 5.6 Short term treatment of Bafilocymin does not have obvious effects on Chk1 activation in response to DNA damage.

Phopho-Chk1 (S345) activation after Bafilomycin or/and Etoposide treatment was examined by western blotting. Cells were pre-treated with 100nM Bafilomycin for 2h before addition of 25μM Etoposide where indicated. *The experiment was carried out at least three times and a representative figure is presented here.

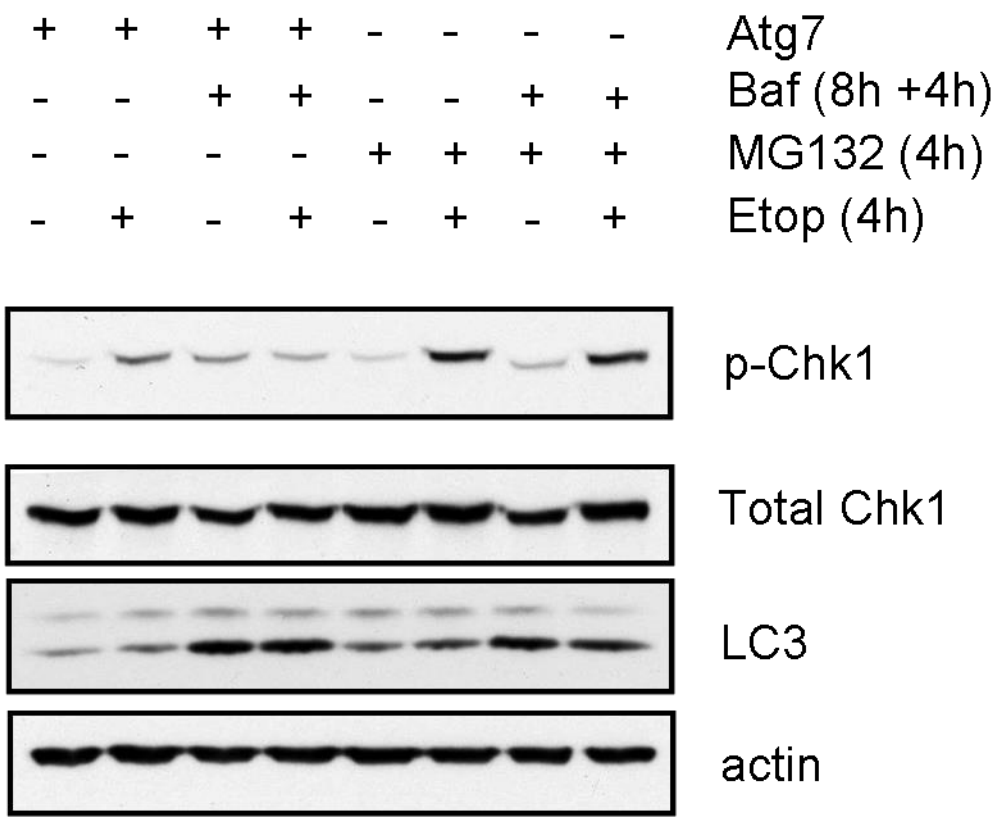


Figure 5.7 Longer period of Bafilomycin treatment has differential impact on Chk1 phosphorylation in the absence or presence of DNA damaging agent.

Levels of p-Chk1 (S345) were examined by Western Blotting in either the absence or presence of Bafilomycin A1 (100nM) for 12h and/or 10μM MG132 for 4h and/or Etoposide (25μM) for 4h where indicated. *The experiment was carried out at least three times and a representative figure is presented here.

5.2 Pharmacological Inhibitors of Autophagy Leads to Chk1 Activation, Followed by Chk1 degradation.

To further explore the relationship between loss of autophagy and Chk1 functions, Atg7f/f primary MEFs were treated with either Bafilomycin or Chloroquine (CQ). Chk1 activations was then monitored over 8 hours (Fig 5.8 and 5.9). It was found that both Bafilomycin and CQ were able to activate Chk1 in a time-dependent manner, CQ also caused total Chk1 level to decrease within 8 hours (Fig 5.9).

Similar to Bafilomycin, chloroquine (CQ) is an autophagy inhibitor that disrupts lysosomal functions. CQ is a basic molecule that enters lysosome and other acidic compartments in the cells where it becomes protonated in the vacuole and leads to less acidic environment. As previously described, Chloroquine has been traditionally used as an anti-malarial drug. In recent years it has shown potential as a promising anti-cancer agent. It has positive anti-tumour effects in a number of clinical trials [195]. The precise mechanism of the anti-tumour effect of CQ is unclear and it is thought that the effects are partially mediated by autophagy inhibition [339]. It should be noted that CQ has also additional effects besides autophagy inhibition [340]. CQ can cause chromatin structural changes that lead to ATM autophosphorylation at serine 1981, which results in the phosphorylation of Chk1 at serine 345 [208]. Therefore the p-Chk1 activation by CQ observed may be largely autophagy-independent. Indeed CQ mediated Chk1 activation (Fig. 5.9) is more pronounced than that of Bafilomycin (Fig. 5.7). CQ also caused Chk1 protein level to decrease within 8 hours, these observation further ties in with the fact that activation of Chk1 facilitates its degradation.

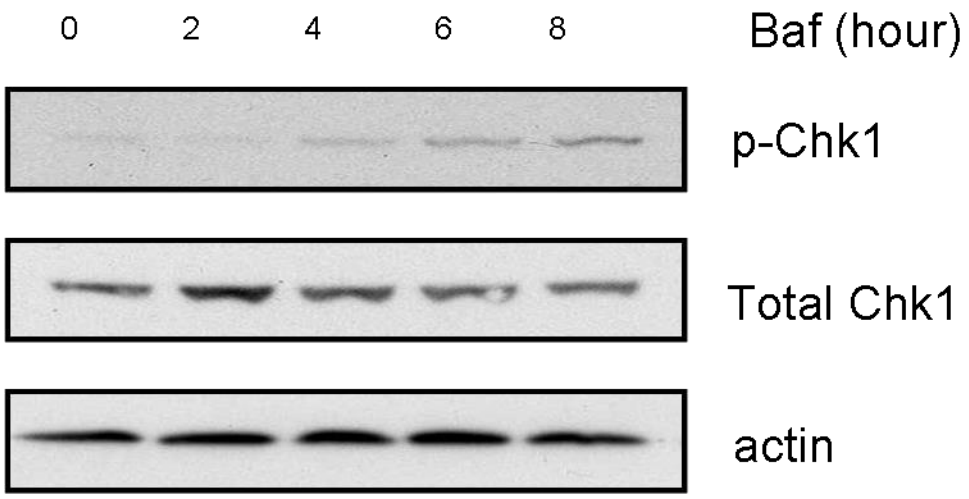


Figure 5.8 Short term Bafilomycin treatment leads to Chk1 activation.

Primary MEFs were treated for the indicated time points with 100nM Bafilomycin A1. Cells were harvested and extracts were subjected to Western blotting with p-Chk1 and total CHk1. Actin was used as a loading control. *The experiment was carried out at least three times and a representative figure is presented here.

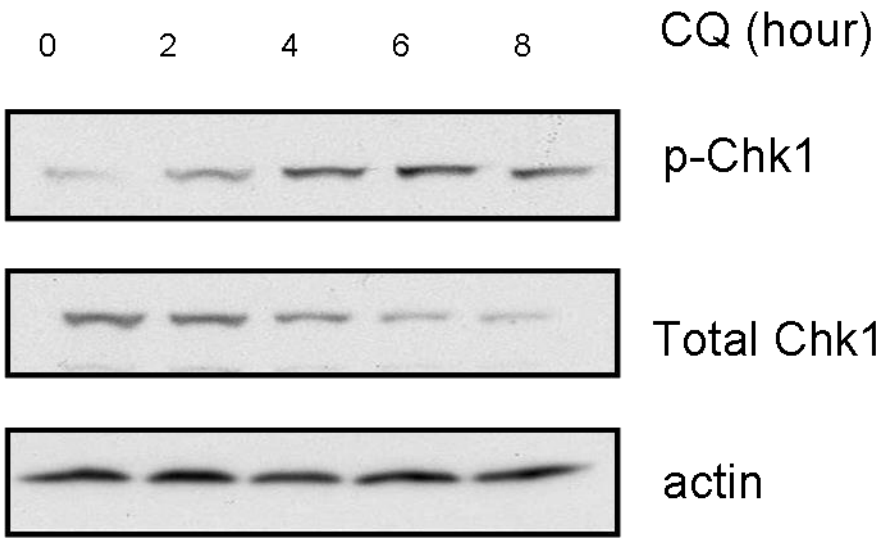


Figure 5.9 Short term Chloroquine (CQ) treatment leads to Chk1 activation.

Primary MEFs were treated for the indicated time points with CQ (100μM). Cells were harvested and extracts were subjected to Western blotting with p-Chk1 and total Chk1. *The experiment was carried out at least three times and a representative figure is presented here.

Since Bafilomycin and CQ activated Chk1 within hours and activation of Chk1 leads to its degradation, this lead us to speculate that treatment with these agents for longer terms may lead to degradation of total Chk1 protein levels. Indeed, when the cells were treated with Bafilomycin for 18 hours, total Chk1 level was diminished (Fig 5.10). Chk1 fails to be activated in Bafilomycin treated cells, very likely due to the decrease of total Chk1 proteins.

The down-regulation of Chk1 mediated by lysosomal inhibitor Bafilomycin can be rescued by MG132 or Lactacystin (Fig 5.11). MG132 treatment for 8h alone does not raise Chk1 level significantly but does appear to reverse the effect of Bafilomycin treatment on Chk1.

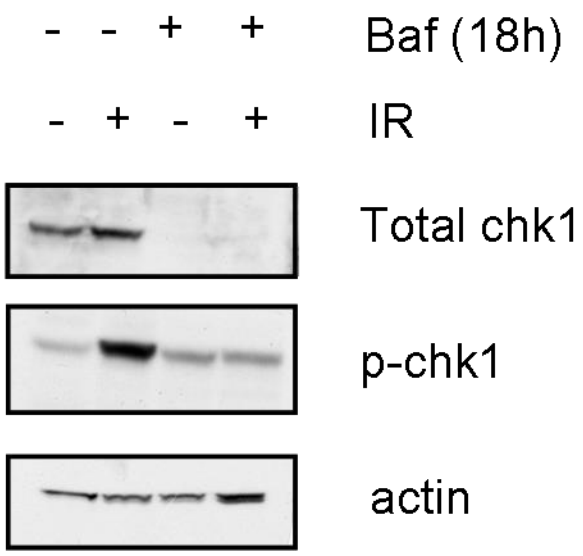


Figure 5.10 Bafilomycin treatment eventually leads to Chk1 degradation.

Where indicated, primary MEFs were treated for 18 hours with Bafilomycin or/and exposed to 10Gy IR 1h before harvested. The cells were harvested and extracts were subjected to Western blotting with p-Chk1 and total Chk1. *The experiment was carried out at least three times and a representative figure is presented here.

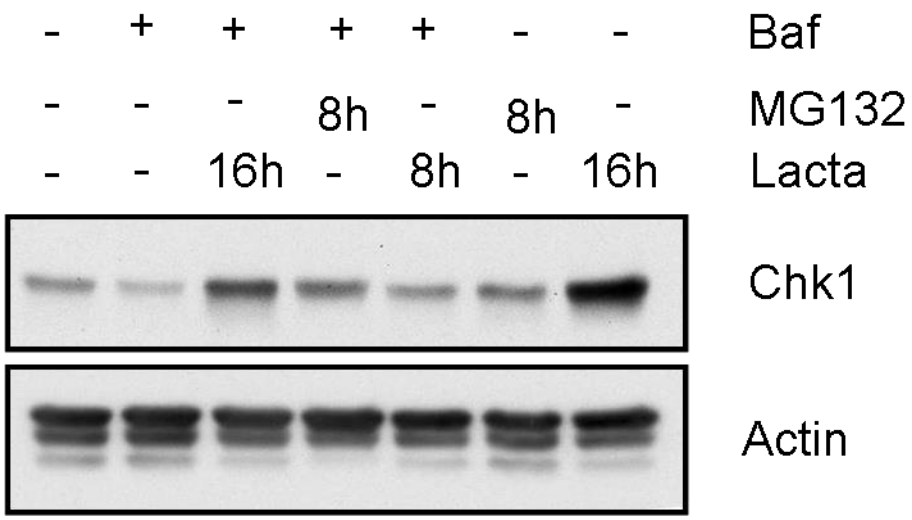


Figure 5.11 The effect of Bafilomycin on total Chk1 can be reversed by treatment of proteasomal inhibitors.

Primary MEFs were treated with Bafilomycin for 16h; and with MG132 or Lactacystin for the indicated time points. Cells were harvested and extracts were subjected to Western blotting. *The experiment was carried out at least three times and a representative figure is presented here.

Chapter 6 Discussion

6.1 Summary

In this study, progress was made in dissecting the roles of autophagy in DNA damage responses. It was found that the loss of autophagy leads to Chk1 de-regulation. Soon following autophagy inhibition, the activation of Chk1 is impaired. Two weeks after Atg7 deletion, the total protein levels of Chk1 were down-regulated. It was also observed that the inhibition of autophagy, with either genetic knockout or pharmacological inhibitors, led to an up-regulation of proteasomal activity in primary MEFs. It is known that phosphorylation and activation of Chk1 facilitates its degradation [267]. Soon after loss of autophagy, Chk1 activation induced by DNA damage was found to be impaired. Our results indicated that this observation may be due to elevated proteasomal activity in $Atg7^{-/-}$ cells.

Prolonged autophagy inhibition leads to a decrease in total Chk1 levels and therefore the amount of Chk1 that could be activated in response to cellular stress. In either case, Chk1 activation was found to be impaired in autophagy deficient cells.

Published studies have shown that Chk1 is a critical factor for HR repair [240] and autophagy deficient cells are more sensitive to CPT, a chemotherapeutic agent that causes double strand breaks that can only be repaired via HR [249]. Autophagy deficient cells accumulate more DNA damage [2] and also display a deficiency in the HR repair pathway as shown in Fig4.6. $Atg7^{-/-}$ cells were however viable and appear to have intact cell cycle checkpoints. As shown in Fig. 6.1, HR and NHEJ are the two main pathways for DNA double strand break repair. The current study shows that Atg7-/- cells were hyper-dependent on the NHEJ pathways, since the inhibition of NHEJ with DNA-PKi increased etoposide or IR induced apoptosis in cells lacking autophagy. In other words, the inhibition of NHEJ pathway gives rise to a synthetic lethal situation when combined with autophagy deficiency.

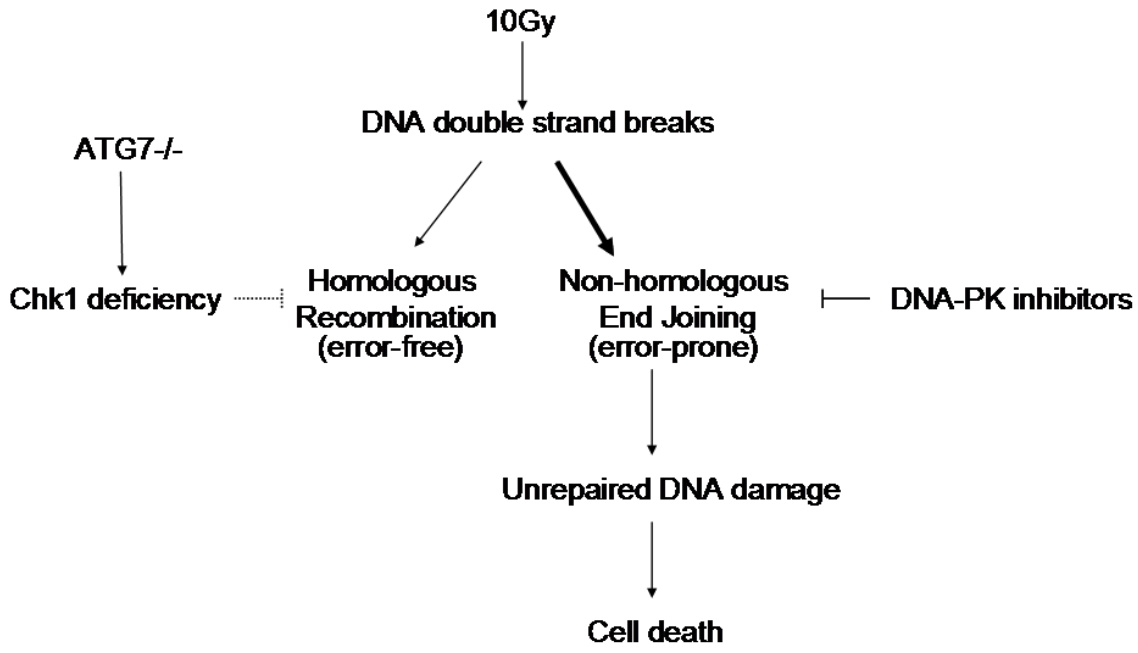


Figure 6.1 Loss of autophagy leads to Chk1 deficiency and defective HR DNA repair pathway.

Atg7$^{-/-}$ cells were found to be hyper-dependent on NHEJ following DNA damaging agents, inhibition of which leads to increased cell death.

6.2 Up-regulation of proteasomal activity following autophagy inhibition

The two pathways have been traditionally thought to function in parallel to maintain cellular homeostasis under normal physiological conditions. In recent years there is emerging evidence that the two processes can cross talk. When one of the two pathways is altered, the other is subsequently perturbed. The interplay between the ubiquitin-proteasomal system and autophagy is complex and is not completely understood [341].

There are several main steps in the ubiquitin-proteasome mediated degradation process – substrate recognition, ubiquitination, substrate delivery and degradation [64]. Autophagy possibly has multiple effects on the proteasomal degradation steps. p62 is frequently found to be up-regulated in autophagy deficient cells, where it has been found to inhibit the delivery of ubiquitinated substrates to the proteasome. As a result, there is an increased level of certain short-lived proteins such as p53 [328]. In contrast, our studies indicate that autophagy may be directly linked to proteasomal activity. The proteasome-Glo^{TM} cell based assay contains specific peptide substrates that can readily access protease sites within the cavity without interaction with E1, E2 and E3 ubiquitin delivery system. The assay is a direct measurement of catalytic activity of the proteasome. It would be interesting to find out which of the two opposing effects – autophagy and proteasome dominates within the cell and whether the effects are cell-type dependent. Korolchuk and colleagues reported an accumulation of p53 as a result of inhibition of substrate protein delivery to the proteasomes [328]. We report here that the half life of Chk1 protein decreases after loss of autophagy. Therefore it would also be interesting to find out whether proteasomal substrates are differentially affected by autophagy inhibition.

Both the proteasome degradation system and autophagy are essential for the maintenence of cellular homeostasis. Proteasomes are thought to be the

predominant organelle that degrades proteins in an un-challenged state and short-lived proteins. Autophagy on the other hand has been found to degrade 40% of all long lived proteins in the cells. [342]. A number of proteins have been identified to be degraded by both systems, such as as α-synuclein [343] and aggregate-prone proteins with polyglutamine and polyalanine expansions [344].

Proteasomes in mammalian cells have relatively long half lives, approximately 1 week in rat liver cells [345]. It is unclear how proteasomes are turned-over but autophagy possibly plays a role in it, since autophagy is thought to be the major mechanism through which large cellular structures are degraded. Proteasomes are largely abundant in the cells and they constitute around 1% of total soluble proteins [346]. When the cells encounter stressful conditions such as starvation, autophagy is up-regulated to degrade cytosolic proteins and organelles as an adaptive mechanism. Proteasomes are large structures and energetically costly for the cells to synthesize or degrade. It has been reported that instead of being degraded during cellular stress, proteasomes are sequestered into storage granules that evades autophagic degradation in yeast cells [347]. It is unclear whether mammalian cells adopt the same mechanism and it remains possible that autophagy inhibition leads to accumulation of proteasomes in MEF cells. One way of testing this is to assess the levels of proteasome subunits through western blotting in Atg7 knockout cells and in Bafilomycin A1 treated cells. In addition, mRNA assessment following autophagy inhibition may reveal any transcriptional regulation of autophagy on proteasome functions.

One important question that remains to be answered is whether autophagy inhibition leads to proteasomal up-regulation in tumour cells and whether it occurs in vivo. Both proteasomal and autophagy inhibitors have shown potential in cancer therapeutics. Autophagy and proteasomes are the two main pathways of protein degradation; if inhibition of one pathway leads to the up-regulation of the other, one may expect that combinational use of the two inhibitors would have synergetic effects in cancer killing. The synergetic concept has been demonstrated in vitro

[348] but the feasibility of using the two inhibitors in combination therapies awaits further clinical investigation.

Apart from cancer, autophagy disruption is implicated in a number of diseases such as autoimmune conditions and diabetes. It is unknown whether proteasomes in those diseased states are also affected.

6.3 Loss of autophagy, Chk1 activities and DNA damage response.

Although Chk1 is generally thought to be a tumour suppressor that is crucial in the maintenance of genetic stability; however Chk1 mutations are very rare occurrences in either sporadic or hereditary cancers [349]. Recent studies have demonstrated that Chk1 inhibition. Cell cycle and cell death are two closely-related processes. A number of cancer treatments target specifically rapidly proliferating cells and some cancer cells can enter temporary growth arrest [350]. Because of the multiple cell cycle checkpoints, cancer cells lacking p53 fail to undergo G1 cell cycle arrest but instead arrest at G2/M mediated by Chk1. Tumour cells are thought to develop drug resistance by undergoing cell cycle arrest and protected from programmed cell death. Besides playing a role in cell cycle arrest, chk1 has also been shown to be required for cell cycle re-entry after stalled replication [351]. Chk1 has been found to be critical for cancer cell survival following anti-metabolite cancer treatments [352]. Chk1 inhibitors have shown promising chemo-sensitising effects in combinational cancer therapies and they are being developed for clinical use (as reviewed in [353]).

Many cancer therapeutic drugs can up-regulate autophagy, which in response to many of those drugs is thought to be an adaptive mechanism that promotes drug resistance in cancer cells. Autophagy inhibitor CQ has shown promising clinical efficacy in cancer treatment, especially in combination therapies. It has not been explored whether autophagy inhibition leads to disruption of Chk1 functions in a tumour setting. It is also not known whether Chk1 is down regulated following CQ treatment in cancer cells. Both Chk1 and autophagy are thought to promote drug resistance in cancer cells. It would be of clinical importance to find out whether the synergy between autophagy inhibition and Chk1 deficiency exist in tumour settings. Further studies are needed to address these questions relevant to cancer therapies.

Autophagy deficiency in primary MEFs leads to hypersensitivity to DNA-PK inhibitor NU7441 in combination with DNA damaging agents, as shown in Fig. 6.2.

NU7441 has shown promising potential as a part of cancer therapeutics in pre-clinical evaluations [309]. DNA-PKcs share structural similarities in its kinase domains with PI3K family proteins and NU7441 is a structurally analogous to LY294002, a PI3K inhibitor. It has been reported that NU7441 also weakly inhibits PI3K [354]. Therefore it is possible that the chemo-sensitization of NU7441 on autophagy deficient cells may also be due to PI3K inhibition. MEFs from DNA-PKcs knockout mice were isolated to address the question. DNA-PKcs knockout mice are more commonly known as SCID (severe combined immunodeficiency) mice because NHEJ pathways are critical for VDJ recombination during antibody maturation. Another member of our lab (Jim O'Prey) has found that SCID MEFs also display synergetic killing following autophagy inhibition with CQ and etoposide (data not shown here), confirming that inhibition of NHEJ pathway leads to increased apoptosis in autophagy deficient cells in response to DNA damage.

Autophagy has been found to inhibit tumour onset, loss of autophagy accelerates tumour formation [116]. It would insightful to find out whether the effect of autophagy inhibition on Chk1 contributes towards the role autophagy plays in tumourigenesis. An initial experiment can be done to see whether loss of autophagy in vivo leads to Chk1 down-regulation. If it does, whether Chk1inhibition or Chk1 knockdown can affect the outcome of tumourigenesis in wild type and autophagy deficient mice should be investigated.

6.4 Loss of autophagy has differential effects on Chk1 functions

It has long been established that complete Chk1 deficiency leads to severe proliferation defects and cell death [276]. Heterozygous Chk1 leads to defective S-phase and mitotic checkpoint as well as DNA damage accumulation [355]. In this study, Chk1 functions are impaired in $Atg7^{-/-}$ primary MEF cells, and these cells displayed growth defects. However they are viable for approximately one month before they enter crisis. Chk1 is important in cell cycle checkpoints [260] and HR DNA damage repair pathways [240]. Our assays demonstrated that $Atg7^{-/-}$ cells

have intact G1 and G2/M cell cycle checkpoints but defective HR pathways. G1 checkpoint is mediated by p53 upon DNA damage [356]. In contrast, Lee and colleagues previously discovered that $Atg7^{-/-}$ cells have defective G1 checkpoint [268]. The differences in conclusions could be due to differed experimental strategies. While cell cycle arrest was assessed 3 hours after amino acid and serum withdrawal in their study; we examined cell cycle progression and found that both cell lines arrest their cell cycles after 16 hour serum starvation. There may be difference in the kinetics of cell cycle arrest between Atg7f/f and Atg7-/- cells and this remained to be determined. The reason we did not observe any defect in cell cycle arrest may be that the extent of Chk1 down-regulation was not sufficient to disturb cell cycles. To test this, Chk1 may be knocked-down to even lower levels in Atg7-/- cells and cell cycle analysis can then be carried out.

Chk1 has been reported to be a haplo-insufficient tumour suppressor [260] and partial Chk1 inhibition is adequate to impair Chk1 functions in cell cycle controls and DNA damage repair [355]. Indeed, our data shows the HR repair pathway to be severely impaired in $Atg7^{-/-}$ cells. It was not expected that $Atg7^{-/-}$ cells also displayed intact G2/M cell cycle checkpoint, as Chk1 activation was found to be down-regulated in $Atg7^{-/-}$ cells and Chk1 has been shown to be a critical factor for G2/M arrest. Since CDC25A promotes cell cycle progression by activating CDKs [357], it was then reasoned that regulation of Cdc25A would provide mechanistic details of Chk1 function in cell cycle control. Studies in a number of human or immortalised MEF cell lines have shown that Cdc25A is degraded rapidly upon DNA damage treatments such as IR [286, 287]. In this report, it was found that Cdc25a levels did not change in either Atg7f/f or Atg7-/- cells following IR. This observation is consistent with Cann and Hick's published study, Cdc25A does not get degraded following DNA damage in primary MEFs [358]. This indicates that MEF cells might have other Chk1 targets similar to Cdc25A regulating CDKs and cell cycle progression.

Though the HR pathways in Atg7-/- cells have been found to be defective, and it is established the defective HR repair is a disinctive trait for BRCA1/2 cells [244]. The phenotype of Atg7-/- cells is different from BRCA1/2 KO cells, for example, Atg7-/- cells are not hyper-sensitive to etoposide or irradation [359]. BRCA1/2 KO cells are embryonic lethal in mice and Atg7-/- mice can survival untill the neonatal starvation period [360]. Again, the difference could be because of the levels of depletion, Chk1 is reduced significantly in Atg7-/- cells and the remaining pool of Chk1 may be sufficient to mediate certain repairs.

In Atg7f/f MEFs, Chk1 is strongly activated in response to DNA damaging agents such as etoposide and IR within 1 hour, and the signal intensity decreases over time, as shown in Fig 5.6 lysosomal inhibition also leads to the phosphorylation of Chk1 at Ser345; but the response was weaker and slower than damage induced activation. It is unclear how lysosomal inhibition leads to Chk1 activation. One potential link may be that autophagy is required for the cells to clear damaged mitochondria. Since mitochondria are considered to be the main source of ROS production, the inhibition of autophagy leads to ROS accumulation and increased DNA damage. Studies from Eileen White's group convincingly showed that autophagy plays an important role in regulating ROS and limiting genetic instability [2, 107, 153]. However it has been reported that lysosomes and/or autophagosomes are another major source of intracellular ROS besides mitochondria [361] [362], and the pharmacological inhibition of lysosome or autophagy actually decreases ROS production [362]. Therefore, more studies need to be carried out to determine the mechanism by which lysosomal inhibition up-regulate Chk1 activity. Assessment of DNA damage, ROS production and p62 levels can be performed to address this question.

In general, cells were harvested one hour after treatment with etoposide or IR in this study, and it has been reported that Chk1 gets activated within 15 minutes following DNA damage [363]. It remains possible that Chk1 activation may be observed in response to IR or etoposide in $Atg7^{-/-}$ cells minutes following treatment. Further

experiments could be carried out to address this, for example, the cells can be harvested 10 minutes after IR or etoposide for western blotting and p-Chk1 and total Chk1 can be probed.

Autophagy deficient cells displayed high levels of spontaneous cell death, it would be insightful to test whether spontaneous and oncogene induced senescence is affected by loss of autophagy.

The loss of autophagy has been reported to give rise to increased DNA damage, which activates Chk1 kinase. Inhibition of autophagy with CQ or Bafilomycin A1 leads to increased phosphorylation of Chk1 (Fig. 5.8 and 5.9), however it is not known that whether cells have increased Chk1 phosphorylation immediately after loss of Atg7 after cre recombination. A direct cause and effect relationship has not been established in this project. The retrovirus-mediated Cre infection procedure and subsequent antibiotic selection takes approximately one week including antibiotics selection. As shown in Fig 3.9, MEF cells from CAG-Cre-ER mice containing $Atg7^{flox/flox}$ transgene were treated with Tamoxifen and there was increased phosphorylated Chk1 6 days after the cells were first exposed to Tamoxifen. One potentially insightful experiment would be to monitor phopho-Chk1 and total Chk1 over a time course of a week following Tamoxifen treatment in these cells. Since CQ and Bafilomycin A1 suppress all lysosomal functions, besides autophagy inhibition, genetic approaches of autophagy inhibition should rule out potential artefacts.

6.5 Modulation of autophagy for therapeutic purposes

Cancer is a group of diseases with divergent pathologies; and unavoidably there were varied responses to anti-tumour treatments. Autophagy is induced by certain cancer drugs, possibly a survival mechanism for cancer cells to countract chemotherapy, whereas other cancer treatments require intact autophagy pathways to achieve sufficient killing. It is therefore important to find out whether autophagy

is beneficial or detrimental for each type of cancer during specific treatments. Cancer cells that up-regulate autophagy as a pro-survival mechanism in response to cancer-killing drugs are referred to as being ‘addicted to autophagy’ [155].

In human patients, autophagosome accumulation has been used as the readout for autophagy activity. This method of monitoring autophagy can be misleading; because autophagosomes can accumulate after activation of autophagy due to increased cargo trafficking but it can also build up due to inhibition of lysosomes, which fuse with and degrade autophagosomes. Better ways of monitoring autophagy activities in cancer patients are needed in order for the appropriate therapies to be applied.

MEF cells which are deficient in Atg7 or treated with CQ or Bafilomycin have been used as autophagy-deficient models. In general, both methods of autophagic inhibition provided similar experimental conclusions. Fundamental differences exist between the two methods and will be discussed here.

Chloroquine (CQ) and Bafilomycin A1 are late stage autophagy inhibitors. They both inhibit lysosomal functions [364] [339]. Hydroxychloroquine (HCQ) is derivative of CQ; in clinic, CQ has been associated with retinal toxicity in patients and is now generally replaced by HCQ, a derivative of CQ, in clinical treatments or trials [365]. At least 16 phase I/II clinical trials are currently in progress [366], and HCQ has shown promising results in a number of clinical trials as part of combination therapy [367]. The current dose of HCQ used for cancer treatment is around 400mg -800mg per day (Clinicaltrials.gov) [366], Amaravadi and colleagues reported that the clinical dose of HCQ may not efficiently inhibit autophagy in all patients [367]. Development of more potent autophagy inhibitors for cancer therapies is currently under way. Among these candidates, compound Lys05 is structurally related to CQ/HCQ, and it has 10 times more efficacy in autophagy inhibition. It has been shown to be a promising anti-cancer drug in mouse studies [368].

CQ has been found to activate ATM, by distorting DNA structures. The activation of ATM in turn activates Chk1. Baf on the other hand has not been found to have similar effects on DNA, and the Chk1 activation from Baf treatment (Fig 5.8) is weaker than that of CQ (Fig 5.9). A potential experiement that can be done is to treat the cells with ATM inhibitors and repeat the experiments as shown in Fig 5.8 and Fig 5.9 and compare between the effect of Baf and CQ on Chk1 levels.

CQ also caused Chk1 protein level to decrease within 8 hours, these observation further ties in with the fact that activation of Chk1 facilitates its degradation.

In this study, most majority of the works has been carried out in primary MEFs, the advantage of the system is that it is a clean system with complete Atg7 removal and DNA damage repair networks are all intact in freshly isolated MEFs. Conclusions from this study apply to untransformed cells. Cancer cells harbour genetic defects especially in DNA repair pathways, therefore it would be interesting to carry out the same experiments in cancer or transformed cell lines and investigate the effect of loss of autophagy on Chk1.

Atg7 knockout leads to complete autophagy inhibition, as Atg7 is an essential protein for the formation of autophagosomes [369]. In terms of therapeutics, knocking down Atg7 in targeted organs is unrealistic due to limitations in the availability of gene therapy techniques. Autophagy is a surveillance process guarding cellular homeostasis and integrity. Complete inhibition of autophagy may contribute towards tumourigenesis and a number of other diseased states. Atg7 is an E1-like enzyme; following the successful development of an inhibitor for NEDD8-activating E1-enzyme, Millennium has undertaken the task of developing Atg7 small molecule inhibitors [367]. Our studies of autophagy in DNA damage response are relevant to the development and potential use of small molecule inhibitors.

p53

The p53 tumour suppressor plays important roles in cell cycle regulation, DNA damage responses and programmed cell death. It is reported that p53 is lost in over 50% of cancers [36]. Chk1 has been found to phosphorylate p53 on multiple sites at both N [370] and C termini [371]. The phosphorylation sites include S20, which is of particular importance to the half life and activity of p53. Future experiments may be carried out to determine the effect of loss of autophagy on p53. It was found that autophagy can inhibit tumour progression in transgenic mice with Kras mutation when p53 is present; however if p53 is lost then loss of autophagy actually accelerates cancer onset. If mice lacking p53 and with Kras mutation are treated with CQ, which is being evaluated for its use as combinational therapy for cancer, these mice form cancer faster comparing to mice with p53.

Chapter 7. List of references

1. Karantza-Wadsworth, V., et al., *Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis.* Genes Dev, 2007. **21**(13): p. 1621-35.
2. Mathew, R., et al., *Autophagy suppresses tumor progression by limiting chromosomal instability.* Genes Dev, 2007. **21**(11): p. 1367-81.
3. Leone, R.D. and R.K. Amaravadi, *Autophagy: a targetable linchpin of cancer cell metabolism.* Trends Endocrinol Metab, 2013. **24**(4): p. 209-17.
4. Amelio, I., G. Melino, and R.A. Knight, *Cell death pathology: cross-talk with autophagy and its clinical implications.* Biochem Biophys Res Commun, 2011. **414**(2): p. 277-81.
5. Kroemer, G. and B. Levine, *Autophagic cell death: the story of a misnomer.* Nat Rev Mol Cell Biol, 2008. **9**(12): p. 1004-10.
6. Michaud, M., et al., *Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice.* Science, 2011. **334**(6062): p. 1573-7.
7. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
8. Mujumdar, N. and A.K. Saluja, *Autophagy in pancreatic cancer: an emerging mechanism of cell death.* Autophagy, 2010. **6**(7): p. 997-8.
9. Shimizu, S., et al., *Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes.* Nat Cell Biol, 2004. **6**(12): p. 1221-8.
10. Scott, R.C., G. Juhasz, and T.P. Neufeld, *Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death.* Curr Biol, 2007. **17**(1): p. 1-11.
11. Cornillon, S., et al., *Programmed cell death in Dictyostelium.* J Cell Sci, 1994. **107 (Pt 10)**: p. 2691-704.
12. Kaushik, S., et al., *Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy.* Mol Biol Cell, 2008. **19**(5): p. 2179-92.
13. Sahu, R., et al., *Microautophagy of cytosolic proteins by late endosomes.* Dev Cell, 2011. **20**(1): p. 131-9.
14. Marzella, L., J. Ahlberg, and H. Glaumann, *Autophagy, heterophagy, microautophagy and crinophagy as the means for intracellular degradation.* Virchows Arch B Cell Pathol Incl Mol Pathol, 1981. **36**(2-3): p. 219-34.
15. Dice, J.F., *Peptide sequences that target cytosolic proteins for lysosomal proteolysis.* Trends Biochem Sci, 1990. **15**(8): p. 305-9.
16. Cuervo, A.M., *Chaperone-mediated autophagy: selectivity pays off.* Trends Endocrinol Metab, 2010. **21**(3): p. 142-50.
17. Fimia, G.M., G. Kroemer, and M. Piacentini, *Molecular mechanisms of selective autophagy.* Cell Death Differ, 2013. **20**(1): p. 1-2.
18. Jung, C.H., et al., *mTOR regulation of autophagy.* FEBS Lett, 2010. **584**(7): p. 1287-95.
19. Schmelzle, T. and M.N. Hall, *TOR, a central controller of cell growth.* Cell, 2000. **103**(2): p. 253-62.
20. Cornu, M., V. Albert, and M.N. Hall, *mTOR in aging, metabolism, and cancer.* Curr Opin Genet Dev, 2013. **23**(1): p. 53-62.

21. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1*. Nat Cell Biol, 2011. **13**(2): p. 132-41.
22. Engelman, J.A., J. Luo, and L.C. Cantley, *The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism.* Nat Rev Genet, 2006. **7**(8): p. 606-19.
23. Dibble, C.C. and B.D. Manning, *Signal integration by mTORC1 coordinates nutrient input with biosynthetic output.* Nat Cell Biol, 2013. **15**(6): p. 555-64.
24. Sancak, Y., et al., *The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1*. Science, 2008. **320**(5882): p. 1496-501.
25. Bar-Peled, L., et al., *Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1*. Cell, 2012. **150**(6): p. 1196-208.
26. Loewith, R. and M.N. Hall, *Target of rapamycin (TOR) in nutrient signaling and growth control.* Genetics, 2011. **189**(4): p. 1177-201.
27. Sancak, Y., et al., *Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids.* Cell, 2010. **141**(2): p. 290-303.
28. Bar-Peled, L., et al., *A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1*. Science, 2013. **340**(6136): p. 1100-6.
29. Settembre, C., et al., *A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB.* EMBO J, 2012. **31**(5): p. 1095-108.
30. Korolchuk, V.I., et al., *Lysosomal positioning coordinates cellular nutrient responses.* Nat Cell Biol, 2011. **13**(4): p. 453-60.
31. Kanazawa, T., et al., *Amino acids and insulin control autophagic proteolysis through different signaling pathways in relation to mTOR in isolated rat hepatocytes.* J Biol Chem, 2004. **279**(9): p. 8452-9.
32. Mordier, S., et al., *Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway.* J Biol Chem, 2000. **275**(38): p. 29900-6.
33. Bell, G.I., et al., *Molecular biology of mammalian glucose transporters.* Diabetes Care, 1990. **13**(3): p. 198-208.
34. Hardie, D.G., *Cell biology. Why starving cells eat themselves.* Science, 2011. **331**(6016): p. 410-1.
35. Djavaheri-Mergny, M., et al., *NF-kappaB activation represses tumor necrosis factor-alpha-induced autophagy.* J Biol Chem, 2006. **281**(41): p. 30373-82.
36. Hollstein, M., et al., *p53 mutations in human cancers.* Science, 1991. **253**(5015): p. 49-53.
37. Tasdemir, E., et al., *A dual role of p53 in the control of autophagy.* Autophagy, 2008. **4**(6): p. 810-4.
38. Ryan, K.M., *p53 and autophagy in cancer: guardian of the genome meets guardian of the proteome.* Eur J Cancer, 2011. **47**(1): p. 44-50.
39. Gao, W., 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): p. 1598-607.

40. Liang, J., 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): p. 218-24.
41. Crighton, D., et al., *DRAM, a p53-induced modulator of autophagy, is critical for apoptosis.* Cell, 2006. **126**(1): p. 121-34.
42. Mah, L.Y., et al., *DRAM-1 encodes multiple isoforms that regulate autophagy.* Autophagy, 2012. **8**(1): p. 18-28.
43. Crighton, D., et al., *p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death.* Cell Death Differ, 2007. **14**(6): p. 1071-9.
44. Pattingre, S., et al., *Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy.* Cell, 2005. **122**(6): p. 927-39.
45. Daido, S., et al., *Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells.* Cancer Res, 2004. **64**(12): p. 4286-93.
46. Bellot, G., et al., *Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains.* Mol Cell Biol, 2009. **29**(10): p. 2570-81.
47. Fleming, A., et al., *Chemical modulators of autophagy as biological probes and potential therapeutics.* Nat Chem Biol, 2011. **7**(1): p. 9-17.
48. Sarkar, S., et al., *Rapamycin and mTOR-independent autophagy inducers ameliorate toxicity of polyglutamine-expanded huntingtin and related proteinopathies.* Cell Death Differ, 2009. **16**(1): p. 46-56.
49. Ravikumar, B., et al., *Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease.* Nat Genet, 2004. **36**(6): p. 585-95.
50. Mizushima, N., *Autophagy: process and function.* Genes Dev, 2007. **21**(22): p. 2861-73.
51. Nakatogawa, H., et al., *Dynamics and diversity in autophagy mechanisms: lessons from yeast.* Nat Rev Mol Cell Biol, 2009. **10**(7): p. 458-67.
52. Jung, C.H., et al., *ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery.* Mol Biol Cell, 2009. **20**(7): p. 1992-2003.
53. Mizushima, N., T. Yoshimori, and Y. Ohsumi, *The role of Atg proteins in autophagosome formation.* Annu Rev Cell Dev Biol, 2011. **27**: p. 107-32.
54. Tooze, S.A. and T. Yoshimori, *The origin of the autophagosomal membrane.* Nat Cell Biol, 2010. **12**(9): p. 831-5.
55. Hamasaki, M., et al., *Autophagosomes form at ER-mitochondria contact sites.* Nature, 2013. **495**(7441): p. 389-93.
56. Liang, X.H., et al., *Induction of autophagy and inhibition of tumorigenesis by beclin 1.* Nature, 1999. **402**(6762): p. 672-6.
57. Sinha, S.C., *Erratum to: Sinha S, Colbert CL, Becker N, Wei Y, Levine B. Molecular basis of the regulation of Beclin 1-dependent autophagy by the gamma-herpesvirus 68 Bcl-2 homolog M11. Autophagy 2008; 4:989-97.* Autophagy, 2009. **5**(2).
58. Cosker, K.E., et al., *Regulation of PI3K signalling by the phosphatidylinositol transfer protein PITPalpha during axonal extension in hippocampal neurons.* J Cell Sci, 2008. **121**(Pt 6): p. 796-803.

59. Di Bartolomeo, S., et al., *The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy.* J Cell Biol, 2010. **191**(1): p. 155-68.
60. Sun, Q., et al., *Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase.* Proc Natl Acad Sci U S A, 2008. **105**(49): p. 19211-6.
61. Russell, R.C., et al., *ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase.* Nat Cell Biol, 2013.
62. Obara, K. and Y. Ohsumi, *PtdIns 3-Kinase Orchestrates Autophagosome Formation in Yeast.* J Lipids, 2011. **2011**: p. 498768.
63. Itakura, E. and N. Mizushima, *Atg14 and UVRAG: mutually exclusive subunits of mammalian Beclin 1-PI3K complexes.* Autophagy, 2009. **5**(4): p. 534-6.
64. Magnani, M., *Ubiquitin/proteasome system.* Nat Biotechnol, 2000. **18**(8): p. 807.
65. Mizushima, N., et al., *Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate.* J Cell Sci, 2003. **116**(Pt 9): p. 1679-88.
66. Kabeya, Y., et al., *LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing.* EMBO J, 2000. **19**(21): p. 5720-8.
67. Jager, S., et al., *Role for Rab7 in maturation of late autophagic vacuoles.* J Cell Sci, 2004. **117**(Pt 20): p. 4837-48.
68. Heublein, S., et al., *Proton-assisted amino-acid transporters are conserved regulators of proliferation and amino-acid-dependent mTORC1 activation.* Oncogene, 2010. **29**(28): p. 4068-79.
69. Ruivo, R., et al., *Mechanism of proton/substrate coupling in the heptahelical lysosomal transporter cystinosin.* Proc Natl Acad Sci U S A, 2012. **109**(5): p. E210-7.
70. Efeyan, A., R. Zoncu, and D.M. Sabatini, *Amino acids and mTORC1: from lysosomes to disease.* Trends Mol Med, 2012. **18**(9): p. 524-33.
71. Vabulas, R.M. and F.U. Hartl, *Protein synthesis upon acute nutrient restriction relies on proteasome function.* Science, 2005. **310**(5756): p. 1960-3.
72. Suraweera, A., et al., *Failure of amino acid homeostasis causes cell death following proteasome inhibition.* Mol Cell, 2012. **48**(2): p. 242-53.
73. Onodera, J. and Y. Ohsumi, *Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation.* J Biol Chem, 2005. **280**(36): p. 31582-6.
74. Kuma, A., et al., *The role of autophagy during the early neonatal starvation period.* Nature, 2004. **432**(7020): p. 1032-6.
75. Nishida, Y., et al., *Discovery of Atg5/Atg7-independent alternative macroautophagy.* Nature, 2009. **461**(7264): p. 654-8.
76. Takeshige, K., et al., *Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction.* J Cell Biol, 1992. **119**(2): p. 301-11.
77. Rubinsztein, D.C., G. Marino, and G. Kroemer, *Autophagy and aging.* Cell, 2011. **146**(5): p. 682-95.
78. Marino, G., A.F. Fernandez, and C. Lopez-Otin, *Autophagy and aging: lessons from progeria models.* Adv Exp Med Biol, 2010. **694**: p. 61-8.

79. Montane, J., L. Cadavez, and A. Novials, *Stress and the inflammatory process: a major cause of pancreatic cell death in type 2 diabetes.* Diabetes Metab Syndr Obes, 2014. **7**: p. 25-34.
80. Las, G. and O.S. Shirihai, *The role of autophagy in beta-cell lipotoxicity and type 2 diabetes.* Diabetes Obes Metab, 2010. **12 Suppl 2**: p. 15-9.
81. Levine, B., N. Mizushima, and H.W. Virgin, *Autophagy in immunity and inflammation.* Nature, 2011. **469**(7330): p. 323-35.
82. Khweek, A.A., et al., *A bacterial protein promotes the recognition of the Legionella pneumophila vacuole by autophagy.* Eur J Immunol, 2013. **43**(5): p. 1333-44.
83. Neel, B.A., Y. Lin, and J.E. Pessin, *Skeletal muscle autophagy: a new metabolic regulator.* Trends Endocrinol Metab, 2013. **24**(12): p. 635-43.
84. Nixon, R.A., *The role of autophagy in neurodegenerative disease.* Nat Med, 2013. **19**(8): p. 983-97.
85. Ogata, M., et al., *Autophagy is activated for cell survival after endoplasmic reticulum stress.* Mol Cell Biol, 2006. **26**(24): p. 9220-31.
86. Taylor, J.P., J. Hardy, and K.H. Fischbeck, *Toxic proteins in neurodegenerative disease.* Science, 2002. **296**(5575): p. 1991-5.
87. Ross, C.A. and M.A. Poirier, *Opinion: What is the role of protein aggregation in neurodegeneration?* Nat Rev Mol Cell Biol, 2005. **6**(11): p. 891-8.
88. Komatsu, M., et al., *Loss of autophagy in the central nervous system causes neurodegeneration in mice.* Nature, 2006. **441**(7095): p. 880-4.
89. Nixon, R.A., et al., *Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study.* J Neuropathol Exp Neurol, 2005. **64**(2): p. 113-22.
90. Sapp, E., et al., *Huntingtin localization in brains of normal and Huntington's disease patients.* Ann Neurol, 1997. **42**(4): p. 604-12.
91. Anglade, P., et al., *Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease.* Histol Histopathol, 1997. **12**(1): p. 25-31.
92. Sikorska, B., et al., *Autophagy is a part of ultrastructural synaptic pathology in Creutzfeldt-Jakob disease: a brain biopsy study.* Int J Biochem Cell Biol, 2004. **36**(12): p. 2563-73.
93. Vellai, T., *Autophagy genes and ageing.* Cell Death Differ, 2009. **16**(1): p. 94-102.
94. Gutierrez, M.G., et al., *Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages.* Cell, 2004. **119**(6): p. 753-66.
95. Nakagawa, I., et al., *Autophagy defends cells against invading group A Streptococcus.* Science, 2004. **306**(5698): p. 1037-40.
96. Talloczy, Z., H.W.t. Virgin, and B. Levine, *PKR-dependent autophagic degradation of herpes simplex virus type 1.* Autophagy, 2006. **2**(1): p. 24-9.
97. Deretic, V., *Autophagy in immunity and cell-autonomous defense against intracellular microbes.* Immunol Rev, 2011. **240**(1): p. 92-104.
98. Mahad, D., H. Lassmann, and D. Turnbull, *Review: Mitochondria and disease progression in multiple sclerosis.* Neuropathol Appl Neurobiol, 2008. **34**(6): p. 577-89.
99. Pierdominici, M., et al., *Role of autophagy in immunity and autoimmunity, with a special focus on systemic lupus erythematosus.* FASEB J, 2012. **26**(4): p. 1400-12.

100. Warner, L.M., L.M. Adams, and S.N. Sehgal, *Rapamycin prolongs survival and arrests pathophysiologic changes in murine systemic lupus erythematosus.* Arthritis Rheum, 1994. **37**(2): p. 289-97.
101. Harrison, D.E., et al., *Rapamycin fed late in life extends lifespan in genetically heterogeneous mice.* Nature, 2009. **460**(7253): p. 392-5.
102. Kwon, J., et al., *Assurance of mitochondrial integrity and mammalian longevity by the p62-Keap1-Nrf2-Nqo1 cascade.* EMBO Rep, 2012. **13**(2): p. 150-6.
103. Stephenson, L.M., et al., *Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes.* Autophagy, 2009. **5**(5): p. 625-35.
104. Miller, B.C., et al., *The autophagy gene ATG5 plays an essential role in B lymphocyte development.* Autophagy, 2008. **4**(3): p. 309-14.
105. Masiero, E., et al., *Autophagy is required to maintain muscle mass.* Cell Metab, 2009. **10**(6): p. 507-15.
106. Mizushima, N., 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**(3): p. 1101-11.
107. Degenhardt, K., et al., *Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis.* Cancer Cell, 2006. **10**(1): p. 51-64.
108. Rosenfeldt, M.T. and K.M. Ryan, *The multiple roles of autophagy in cancer.* Carcinogenesis, 2011. **32**(7): p. 955-63.
109. Vellai, T., M.L. Toth, and A.L. Kovacs, *Janus-faced autophagy: a dual role of cellular self-eating in neurodegeneration?* Autophagy, 2007. **3**(5): p. 461-3.
110. Shintani, T. and D.J. Klionsky, *Autophagy in health and disease: a double-edged sword.* Science, 2004. **306**(5698): p. 990-5.
111. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
112. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
113. Aita, V.M., et al., *Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21.* Genomics, 1999. **59**(1): p. 59-65.
114. Qu, X., et al., *Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene.* J Clin Invest, 2003. **112**(12): p. 1809-20.
115. Kang, M.R., et al., *Frameshift mutations of autophagy-related genes ATG2B, ATG5, ATG9B and ATG12 in gastric and colorectal cancers with microsatellite instability.* J Pathol, 2009. **217**(5): p. 702-6.
116. Takamura, A., et al., *Autophagy-deficient mice develop multiple liver tumors.* Genes Dev, 2011. **25**(8): p. 795-800.
117. Dizdaroglu, M., et al., *Free radical-induced damage to DNA: mechanisms and measurement.* Free Radic Biol Med, 2002. **32**(11): p. 1102-15.
118. Wallace, D.C., *A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine.* Annu Rev Genet, 2005. **39**: p. 359-407.
119. Moscat, J. and M.T. Diaz-Meco, *p62 at the crossroads of autophagy, apoptosis, and cancer.* Cell, 2009. **137**(6): p. 1001-4.

120. Narendra, D.P., et al., *PINK1 is selectively stabilized on impaired mitochondria to activate Parkin.* PLoS Biol, 2010. **8**(1): p. e1000298.
121. Geisler, S., et al., *PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1.* Nat Cell Biol, 2010. **12**(2): p. 119-31.
122. Denison, S.R., et al., *Alterations in the common fragile site gene Parkin in ovarian and other cancers.* Oncogene, 2003. **22**(51): p. 8370-8.
123. Zhang, C., 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): p. 16259-64.
124. Fujiwara, M., et al., *Parkin as a tumor suppressor gene for hepatocellular carcinoma.* Oncogene, 2008. **27**(46): p. 6002-11.
125. Ichimura, Y., et al., *Structural basis for sorting mechanism of p62 in selective autophagy.* J Biol Chem, 2008. **283**(33): p. 22847-57.
126. Bjorkoy, G., et al., *p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death.* J Cell Biol, 2005. **171**(4): p. 603-14.
127. Seibenhener, M.L., et al., *A role for sequestosome 1/p62 in mitochondrial dynamics, import and genome integrity.* Biochim Biophys Acta, 2013. **1833**(3): p. 452-9.
128. Narendra, D., et al., *p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both.* Autophagy, 2010. **6**(8): p. 1090-106.
129. Bjorkoy, G., T. Lamark, and T. Johansen, *p62/SQSTM1: a missing link between protein aggregates and the autophagy machinery.* Autophagy, 2006. **2**(2): p. 138-9.
130. Komatsu, M., et al., *Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice.* Cell, 2007. **131**(6): p. 1149-63.
131. Komatsu, M., et al., *The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1.* Nat Cell Biol, 2010. **12**(3): p. 213-23.
132. Shibata, T., et al., *Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy.* Proc Natl Acad Sci U S A, 2008. **105**(36): p. 13568-73.
133. DeNicola, G.M., et al., *Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis.* Nature, 2011. **475**(7354): p. 106-9.
134. Villeneuve, N.F., A. Lau, and D.D. Zhang, *Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases.* Antioxid Redox Signal, 2010. **13**(11): p. 1699-712.
135. Hayes, J.D. and M. McMahon, *NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer.* Trends Biochem Sci, 2009. **34**(4): p. 176-88.
136. Duran, A., et al., *The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis.* Cancer Cell, 2008. **13**(4): p. 343-54.
137. Takahashi, H., et al., *Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation.* Cancer Cell, 2010. **17**(1): p. 89-97.
138. de Martel, C. and S. Franceschi, *Infections and cancer: established associations and new hypotheses.* Crit Rev Oncol Hematol, 2009. **70**(3): p. 183-94.

139. Khasawneh, J., et al., *Inflammation and mitochondrial fatty acid beta-oxidation link obesity to early tumor promotion.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3354-9.
140. Hussain, S.P., L.J. Hofseth, and C.C. Harris, *Radical causes of cancer.* Nat Rev Cancer, 2003. **3**(4): p. 276-85.
141. Hochreiter-Hufford, A. and K.S. Ravichandran, *Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion.* Cold Spring Harb Perspect Biol, 2013. **5**(1): p. a008748.
142. Kanduc, D., et al., *Cell death: apoptosis versus necrosis (review).* Int J Oncol, 2002. **21**(1): p. 165-70.
143. Qu, X., et al., *Autophagy gene-dependent clearance of apoptotic cells during embryonic development.* Cell, 2007. **128**(5): p. 931-46.
144. Meissner, F., K. Molawi, and A. Zychlinsky, *Mutant superoxide dismutase 1-induced IL-1beta accelerates ALS pathogenesis.* Proc Natl Acad Sci U S A, 2010. **107**(29): p. 13046-50.
145. Harris, J., et al., *Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation.* J Biol Chem, 2011. **286**(11): p. 9587-97.
146. Shi, C.S., et al., *Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction.* Nat Immunol, 2012. **13**(3): p. 255-63.
147. Reiman, J.M., et al., *Tumor immunoediting and immunosculpting pathways to cancer progression.* Semin Cancer Biol, 2007. **17**(4): p. 275-87.
148. Buell, J.F., T.G. Gross, and E.S. Woodle, *Malignancy after transplantation.* Transplantation, 2005. **80**(2 Suppl): p. S254-64.
149. Pua, H.H., et al., *A critical role for the autophagy gene Atg5 in T cell survival and proliferation.* J Exp Med, 2007. **204**(1): p. 25-31.
150. Sanjuan, M.A., et al., *Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis.* Nature, 2007. **450**(7173): p. 1253-7.
151. Kuilman, T., et al., *The essence of senescence.* Genes Dev, 2010. **24**(22): p. 2463-79.
152. Young, A.R., et al., *Autophagy mediates the mitotic senescence transition.* Genes Dev, 2009. **23**(7): p. 798-803.
153. Mathew, R., V. Karantza-Wadsworth, and E. White, *Assessing metabolic stress and autophagy status in epithelial tumors.* Methods Enzymol, 2009. **453**: p. 53-81.
154. Harris, A.L., *Hypoxia--a key regulatory factor in tumour growth.* Nat Rev Cancer, 2002. **2**(1): p. 38-47.
155. Mancias, J.D. and A.C. Kimmelman, *Targeting autophagy addiction in cancer.* Oncotarget, 2011. **2**(12): p. 1302-6.
156. Yang, S., et al., *Pancreatic cancers require autophagy for tumor growth.* Genes Dev, 2011. **25**(7): p. 717-29.
157. Guo, J.Y., et al., *Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis.* Genes Dev, 2011. **25**(5): p. 460-70.
158. Pietras, K. and A. Ostman, *Hallmarks of cancer: interactions with the tumor stroma.* Exp Cell Res, 2010. **316**(8): p. 1324-31.
159. Du, J., et al., *Role of autophagy in angiogenesis in aortic endothelial cells.* Am J Physiol Cell Physiol, 2012. **302**(2): p. C383-91.

160. Kuballa, P., et al., *Autophagy and the immune system.* Annu Rev Immunol, 2012. **30**: p. 611-46.
161. Deretic, V., S. Jiang, and N. Dupont, *Autophagy intersections with conventional and unconventional secretion in tissue development, remodeling and inflammation.* Trends Cell Biol, 2012. **22**(8): p. 397-406.
162. Dupont, N., et al., *Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta.* EMBO J, 2011. **30**(23): p. 4701-11.
163. Capparelli, C., et al., *Autophagy and senescence in cancer-associated fibroblasts metabolically supports tumor growth and metastasis via glycolysis and ketone production.* Cell Cycle, 2012. **11**(12): p. 2285-302.
164. Martinez-Outschoorn, U.E., et al., *Stromal-epithelial metabolic coupling in cancer: integrating autophagy and metabolism in the tumor microenvironment.* Int J Biochem Cell Biol, 2011. **43**(7): p. 1045-51.
165. Liu, F., et al., *Suppression of autophagy by FIP200 deletion leads to osteopenia in mice through the inhibition of osteoblast terminal differentiation.* J Bone Miner Res, 2013.
166. Kenific, C.M., A. Thorburn, and J. Debnath, *Autophagy and metastasis: another double-edged sword.* Curr Opin Cell Biol, 2010. **22**(2): p. 241-5.
167. Macintosh, R.L., et al., *Inhibition of autophagy impairs tumor cell invasion in an organotypic model.* Cell Cycle, 2012. **11**(10): p. 2022-9.
168. Galavotti, S., et al., *The autophagy-associated factors DRAM1 and p62 regulate cell migration and invasion in glioblastoma stem cells.* Oncogene, 2013. **32**(6): p. 699-712.
169. Shin, M.S., et al., *Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers.* Cancer Res, 2001. **61**(13): p. 4942-6.
170. Han, J., et al., *Involvement of protective autophagy in TRAIL resistance of apoptosis-defective tumor cells.* J Biol Chem, 2008. **283**(28): p. 19665-77.
171. Chiarugi, P. and E. Giannoni, *Anoikis: a necessary death program for anchorage-dependent cells.* Biochem Pharmacol, 2008. **76**(11): p. 1352-64.
172. Fung, C., et al., *Induction of autophagy during extracellular matrix detachment promotes cell survival.* Mol Biol Cell, 2008. **19**(3): p. 797-806.
173. Aguirre-Ghiso, J.A., *Models, mechanisms and clinical evidence for cancer dormancy.* Nat Rev Cancer, 2007. **7**(11): p. 834-46.
174. Nguyen, D.X., P.D. Bos, and J. Massague, *Metastasis: from dissemination to organ-specific colonization.* Nat Rev Cancer, 2009. **9**(4): p. 274-84.
175. Lu, Z., et al., *The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells.* J Clin Invest, 2008. **118**(12): p. 3917-29.
176. Koukourakis, M.I., et al., *Beclin 1 over- and underexpression in colorectal cancer: distinct patterns relate to prognosis and tumour hypoxia.* Br J Cancer, 2010. **103**(8): p. 1209-14.
177. Chaachouay, H., et al., *Autophagy contributes to resistance of tumor cells to ionizing radiation.* Radiother Oncol, 2011. **99**(3): p. 287-92.
178. Yang, Z.J., et al., *The role of autophagy in cancer: therapeutic implications.* Mol Cancer Ther, 2011. **10**(9): p. 1533-41.

179. Kondo, Y. and S. Kondo, *Autophagy and cancer therapy.* Autophagy, 2006. **2**(2): p. 85-90.
180. Ciuffreda, L., et al., *The mTOR pathway: a new target in cancer therapy.* Curr Cancer Drug Targets, 2010. **10**(5): p. 484-95.
181. Zitvogel, L., O. Kepp, and G. Kroemer, *Immune parameters affecting the efficacy of chemotherapeutic regimens.* Nat Rev Clin Oncol, 2011. **8**(3): p. 151-60.
182. Tesniere, A., et al., *Immunogenic death of colon cancer cells treated with oxaliplatin.* Oncogene, 2010. **29**(4): p. 482-91.
183. Aymeric, L., et al., *Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity.* Cancer Res, 2010. **70**(3): p. 855-8.
184. Law, B.K., *Rapamycin: an anti-cancer immunosuppressant?* Crit Rev Oncol Hematol, 2005. **56**(1): p. 47-60.
185. Iwamaru, A., et al., *Silencing mammalian target of rapamycin signaling by small interfering RNA enhances rapamycin-induced autophagy in malignant glioma cells.* Oncogene, 2007. **26**(13): p. 1840-51.
186. Cao, C., et al., *Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells.* Cancer Res, 2006. **66**(20): p. 10040-7.
187. Shen, S., et al., *Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study.* Oncogene, 2011. **30**(45): p. 4544-56.
188. Liu, E.Y. and K.M. Ryan, *Autophagy and cancer--issues we need to digest.* J Cell Sci, 2012. **125**(Pt 10): p. 2349-58.
189. Amaravadi, R.K., et al., *Principles and current strategies for targeting autophagy for cancer treatment.* Clin Cancer Res, 2011. **17**(4): p. 654-66.
190. Wu, Z., et al., *Autophagy Blockade Sensitizes Prostate Cancer Cells towards Src Family Kinase Inhibitors.* Genes Cancer, 2010. **1**(1): p. 40-9.
191. Liu, D., et al., *Inhibition of autophagy by 3-MA potentiates cisplatin-induced apoptosis in esophageal squamous cell carcinoma cells.* Med Oncol, 2011. **28**(1): p. 105-11.
192. Li, J., et al., *Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells.* Ann Surg Oncol, 2009. **16**(3): p. 761-71.
193. Geser, A., G. Brubaker, and C.C. Draper, *Effect of a malaria suppression program on the incidence of African Burkitt's lymphoma.* Am J Epidemiol, 1989. **129**(4): p. 740-52.
194. Carew, J.S., K.R. Kelly, and S.T. Nawrocki, *Autophagy as a target for cancer therapy: new developments.* Cancer Manag Res, 2012. **4**: p. 357-65.
195. Sotelo, J., E. Briceno, and M.A. Lopez-Gonzalez, *Adding chloroquine to conventional treatment for glioblastoma multiforme: a randomized, double-blind, placebo-controlled trial.* Ann Intern Med, 2006. **144**(5): p. 337-43.
196. Buchser, W.J., et al., *Cell-mediated autophagy promotes cancer cell survival.* Cancer Res, 2012. **72**(12): p. 2970-9.
197. Lindahl, T. and D.E. Barnes, *Repair of endogenous DNA damage.* Cold Spring Harb Symp Quant Biol, 2000. **65**: p. 127-33.
198. Jackson, S.P. and J. Bartek, *The DNA-damage response in human biology and disease.* Nature, 2009. **461**(7267): p. 1071-8.

199. Scally, A. and R. Durbin, *Revising the human mutation rate: implications for understanding human evolution.* Nat Rev Genet, 2012. **13**(10): p. 745-53.
200. Narayanan, D.L., R.N. Saladi, and J.L. Fox, *Ultraviolet radiation and skin cancer.* Int J Dermatol, 2010. **49**(9): p. 978-86.
201. Young, C., *Solar ultraviolet radiation and skin cancer.* Occup Med (Lond), 2009. **59**(2): p. 82-8.
202. Hoffmann, D., I. Hoffmann, and K. El-Bayoumy, *The less harmful cigarette: a controversial issue. a tribute to Ernst L. Wynder.* Chem Res Toxicol, 2001. **14**(7): p. 767-90.
203. Reiman, A., et al., *Lymphoid tumours and breast cancer in ataxia telangiectasia; substantial protective effect of residual ATM kinase activity against childhood tumours.* Br J Cancer, 2011. **105**(4): p. 586-91.
204. Kraemer, K.H., et al., *The role of sunlight and DNA repair in melanoma and nonmelanoma skin cancer. The xeroderma pigmentosum paradigm.* Arch Dermatol, 1994. **130**(8): p. 1018-21.
205. Wade, M., Y.C. Li, and G.M. Wahl, *MDM2, MDMX and p53 in oncogenesis and cancer therapy.* Nat Rev Cancer, 2013. **13**(2): p. 83-96.
206. Li, M.L. and R.A. Greenberg, *Links between genome integrity and BRCA1 tumor suppression.* Trends Biochem Sci, 2012. **37**(10): p. 418-24.
207. Smith, J., et al., *The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer.* Adv Cancer Res, 2010. **108**: p. 73-112.
208. Bakkenist, C.J. and M.B. Kastan, *DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation.* Nature, 2003. **421**(6922): p. 499-506.
209. Dupre, A., L. Boyer-Chatenet, and J. Gautier, *Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex.* Nat Struct Mol Biol, 2006. **13**(5): p. 451-7.
210. Saito, S., et al., *ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation.* J Biol Chem, 2002. **277**(15): p. 12491-4.
211. Oren, M., *Regulation of the p53 tumor suppressor protein.* J Biol Chem, 1999. **274**(51): p. 36031-4.
212. Ashcroft, M., M.H. Kubbutat, and K.H. Vousden, *Regulation of p53 function and stability by phosphorylation.* Mol Cell Biol, 1999. **19**(3): p. 1751-8.
213. Barlow, C., et al., *Atm selectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways.* Nat Genet, 1997. **17**(4): p. 453-6.
214. Waldman, T., K.W. Kinzler, and B. Vogelstein, *p21 is necessary for the p53-mediated G1 arrest in human cancer cells.* Cancer Res, 1995. **55**(22): p. 5187-90.
215. Li, Y., et al., *Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21.* Oncogene, 1994. **9**(8): p. 2261-8.
216. Di Leonardo, A., et al., *DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts.* Genes Dev, 1994. **8**(21): p. 2540-51.
217. Matsuoka, S., M. Huang, and S.J. Elledge, *Linkage of ATM to cell cycle regulation by the Chk2 protein kinase.* Science, 1998. **282**(5395): p. 1893-7.
218. Hirao, A., et al., *DNA damage-induced activation of p53 by the checkpoint kinase Chk2.* Science, 2000. **287**(5459): p. 1824-7.

219. Liebermann, D.A., B. Hoffman, and D. Vesely, *p53 induced growth arrest versus apoptosis and its modulation by survival cytokines.* Cell Cycle, 2007. **6**(2): p. 166-70.
220. Abraham, R.T., *Cell cycle checkpoint signaling through the ATM and ATR kinases.* Genes Dev, 2001. **15**(17): p. 2177-96.
221. Sartori, A.A., et al., *Human CtIP promotes DNA end resection.* Nature, 2007. **450**(7169): p. 509-14.
222. Chen, X., et al., *Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent Dna2 phosphorylation.* Nat Struct Mol Biol, 2011. **18**(9): p. 1015-9.
223. Kumagai, A. and W.G. Dunphy, *Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1.* Nat Cell Biol, 2003. **5**(2): p. 161-5.
224. Lukas, C., et al., *Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage.* Nat Cell Biol, 2003. **5**(3): p. 255-60.
225. Myers, J.S. and D. Cortez, *Rapid activation of ATR by ionizing radiation requires ATM and Mre11.* J Biol Chem, 2006. **281**(14): p. 9346-50.
226. Jazayeri, A., et al., *ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks.* Nat Cell Biol, 2006. **8**(1): p. 37-45.
227. Zou, L. and S.J. Elledge, *Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes.* Science, 2003. **300**(5625): p. 1542-8.
228. Blasina, A., et al., *A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase.* Curr Biol, 1999. **9**(1): p. 1-10.
229. Casorelli, I., C. Bossa, and M. Bignami, *DNA damage and repair in human cancer: molecular mechanisms and contribution to therapy-related leukemias.* Int J Environ Res Public Health, 2012. **9**(8): p. 2636-57.
230. Ralhan, R., et al., *Links between DNA double strand break repair and breast cancer: accumulating evidence from both familial and nonfamilial cases.* Cancer Lett, 2007. **248**(1): p. 1-17.
231. Lieber, M.R., et al., *Nonhomologous DNA end joining (NHEJ) and chromosomal translocations in humans.* Subcell Biochem, 2010. **50**: p. 279-96.
232. Bosotti, R., A. Isacchi, and E.L. Sonnhammer, *FAT: a novel domain in PIK-related kinases.* Trends Biochem Sci, 2000. **25**(5): p. 225-7.
233. Smith, G.C. and S.P. Jackson, *The DNA-dependent protein kinase.* Genes Dev, 1999. **13**(8): p. 916-34.
234. Mahaney, B.L., K. Meek, and S.P. Lees-Miller, *Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining.* Biochem J, 2009. **417**(3): p. 639-50.
235. Wold, M.S., *Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism.* Annu Rev Biochem, 1997. **66**: p. 61-92.
236. Eggler, A.L., R.B. Inman, and M.M. Cox, *The Rad51-dependent pairing of long DNA substrates is stabilized by replication protein A.* J Biol Chem, 2002. **277**(42): p. 39280-8.
237. Levine, A.J., *p53, the cellular gatekeeper for growth and division.* Cell, 1997. **88**(3): p. 323-31.

238. Lee, J.S., et al., *hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response.* Nature, 2000. **404**(6774): p. 201-4.
239. Bahassi, E.M., et al., *The checkpoint kinases Chk1 and Chk2 regulate the functional associations between hBRCA2 and Rad51 in response to DNA damage.* Oncogene, 2008. **27**(28): p. 3977-85.
240. Sorensen, C.S., et al., *The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair.* Nat Cell Biol, 2005. **7**(2): p. 195-201.
241. Olson, E.M., et al., *Clinical outcomes and treatment practice patterns of patients with HER2-positive metastatic breast cancer in the post-trastuzumab era.* Breast, 2013.
242. Liu, T.C., et al., *Translation of targeted oncolytic virotherapeutics from the lab into the clinic, and back again: a high-value iterative loop.* Mol Ther, 2008. **16**(6): p. 1006-8.
243. Kaelin, W.G., Jr., *Synthetic lethality: a framework for the development of wiser cancer therapeutics.* Genome Med, 2009. **1**(10): p. 99.
244. Issaeva, N., et al., *6-thioguanine selectively kills BRCA2-defective tumors and overcomes PARP inhibitor resistance.* Cancer Res, 2010. **70**(15): p. 6268-76.
245. Dedes, K.J., et al., *Synthetic lethality of PARP inhibition in cancers lacking BRCA1 and BRCA2 mutations.* Cell Cycle, 2011. **10**(8): p. 1192-9.
246. Zhao, S., et al., *Regulation of cellular metabolism by protein lysine acetylation.* Science, 2010. **327**(5968): p. 1000-4.
247. Jeong, H., et al., *Acetylation targets mutant huntingtin to autophagosomes for degradation.* Cell, 2009. **137**(1): p. 60-72.
248. Robert, T., et al., *HDACs link the DNA damage response, processing of double-strand breaks and autophagy.* Nature, 2011. **471**(7336): p. 74-9.
249. Bae, H. and J.L. Guan, *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): p. 1232-41.
250. Seluanov, A., et al., *DNA end joining becomes less efficient and more error-prone during cellular senescence.* Proc Natl Acad Sci U S A, 2004. **101**(20): p. 7624-9.
251. Komatsu, M., et al., *Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice.* J Cell Biol, 2005. **169**(3): p. 425-34.
252. Mizushima, N. and T. Yoshimori, *How to interpret LC3 immunoblotting.* Autophagy, 2007. **3**(6): p. 542-5.
253. Maser, R.S., et al., *hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks.* Mol Cell Biol, 1997. **17**(10): p. 6087-96.
254. Paull, T.T., et al., *A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage.* Curr Biol, 2000. **10**(15): p. 886-95.
255. Nakamura, A.J., et al., *The complexity of phosphorylated H2AX foci formation and DNA repair assembly at DNA double-strand breaks.* Cell Cycle, 2010. **9**(2): p. 389-97.
256. Kobayashi, J., et al., *NBS1 and its functional role in the DNA damage response.* DNA Repair (Amst), 2004. **3**(8-9): p. 855-61.

257. Giunta, S., R. Belotserkovskaya, and S.P. Jackson, *DNA damage signaling in response to double-strand breaks during mitosis.* J Cell Biol, 2010. **190**(2): p. 197-207.
258. Subramanian, C., et al., *CREB-binding protein regulates Ku70 acetylation in response to ionization radiation in neuroblastoma.* Mol Cancer Res, 2013. **11**(2): p. 173-81.
259. Zhong, Q., et al., *BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks.* J Biol Chem, 2002. **277**(32): p. 28641-7.
260. Liu, Q., et al., *Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint.* Genes Dev, 2000. **14**(12): p. 1448-59.
261. Baldwin, E.L. and N. Osheroff, *Etoposide, topoisomerase II and cancer.* Curr Med Chem Anticancer Agents, 2005. **5**(4): p. 363-72.
262. Bennett, L.N., et al., *Claspin is phosphorylated in the Chk1-binding domain by a kinase distinct from Chk1*. Biochem Biophys Res Commun, 2008. **369**(3): p. 973-6.
263. Peschiaroli, A., et al., *SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response.* Molecular Cell, 2006. **23**(3): p. 319-29.
264. Mailand, N., et al., *Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress.* Mol Cell, 2006. **23**(3): p. 307-18.
265. Lu, X., B. Nannenga, and L.A. Donehower, *PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints.* Genes & development, 2005. **19**(10): p. 1162-74.
266. Macurek, L., et al., *Downregulation of Wip1 phosphatase modulates the cellular threshold of DNA damage signaling in mitosis.* Cell Cycle, 2013. **12**(2): p. 251-62.
267. Zhang, Y.W., et al., *Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway.* Mol Cell, 2005. **19**(5): p. 607-18.
268. Lee, I.H., et al., *Atg7 modulates p53 activity to regulate cell cycle and survival during metabolic stress.* Science, 2012. **336**(6078): p. 225-8.
269. Gough, D.R. and T.G. Cotter, *Hydrogen peroxide: a Jekyll and Hyde signalling molecule.* Cell Death Dis, 2011. **2**: p. e213.
270. Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. **160**(1): p. 1-40.
271. Smith, A.J., et al., *A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination.* Nat Genet, 1995. **9**(4): p. 376-85.
272. Metzger, D. and R. Feil, *Engineering the mouse genome by site-specific recombination.* Curr Opin Biotechnol, 1999. **10**(5): p. 470-6.
273. Thyagarajan, B., et al., *Mammalian genomes contain active recombinase recognition sites.* Gene, 2000. **244**(1-2): p. 47-54.
274. Zhu, J., et al., *Cre-mediated recombination can induce apoptosis in vivo by activating the p53 DNA damage-induced pathway.* Genesis, 2012. **50**(2): p. 102-11.
275. Loonstra, A., et al., *Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells.* Proc Natl Acad Sci U S A, 2001. **98**(16): p. 9209-14.
276. Takai, H., et al., *Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice.* Genes Dev, 2000. **14**(12): p. 1439-47.

277. Hirao, A., et al., *Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner.* Mol Cell Biol, 2002. **22**(18): p. 6521-32.
278. Falck, J., et al., *The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis.* Nature, 2001. **410**(6830): p. 842-7.
279. Takai, H., et al., *Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription.* EMBO J, 2002. **21**(19): p. 5195-205.
280. Jack, M.T., et al., *Chk2 is dispensable for p53-mediated G1 arrest but is required for a latent p53-mediated apoptotic response.* Proc Natl Acad Sci U S A, 2002. **99**(15): p. 9825-9.
281. Zachos, G., M.D. Rainey, and D.A. Gillespie, *Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects.* EMBO J, 2003. **22**(3): p. 713-23.
282. Schonn, I., J. Hennesen, and D.C. Dartsch, *Cellular responses to etoposide: cell death despite cell cycle arrest and repair of DNA damage.* Apoptosis, 2010. **15**(2): p. 162-72.
283. Hans, F. and S. Dimitrov, *Histone H3 phosphorylation and cell division.* Oncogene, 2001. **20**(24): p. 3021-7.
284. Shin, J.S., et al., *Serum starvation induces G1 arrest through suppression of Skp2-CDK2 and CDK4 in SK-OV-3 cells.* Int J Oncol, 2008. **32**(2): p. 435-9.
285. Barbet, N.C., et al., *TOR controls translation initiation and early G1 progression in yeast.* Mol Biol Cell, 1996. **7**(1): p. 25-42.
286. Timofeev, O., et al., *Cdc25 phosphatases are required for timely assembly of CDK1-cyclin B at the G2/M transition.* J Biol Chem, 2010. **285**(22): p. 16978-90.
287. Mailand, N., et al., *Rapid destruction of human Cdc25A in response to DNA damage.* Science, 2000. **288**(5470): p. 1425-9.
288. Huang, M., et al., *Chk1 and Chk2 are differentially involved in homologous recombination repair and cell cycle arrest in response to DNA double-strand breaks induced by camptothecins.* Mol Cancer Ther, 2008. **7**(6): p. 1440-9.
289. Haaf, T., et al., *Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes.* Proc Natl Acad Sci U S A, 1995. **92**(6): p. 2298-302.
290. Tashiro, S., et al., *S phase specific formation of the human Rad51 protein nuclear foci in lymphocytes.* Oncogene, 1996. **12**(10): p. 2165-70.
291. Chen, F., et al., *Cell cycle-dependent protein expression of mammalian homologs of yeast DNA double-strand break repair genes Rad51 and Rad52.* Mutat Res, 1997. **384**(3): p. 205-11.
292. Bellaiche, Y., V. Mogila, and N. Perrimon, *I-SceI endonuclease, a new tool for studying DNA double-strand break repair mechanisms in Drosophila.* Genetics, 1999. **152**(3): p. 1037-44.
293. Pierce, A.J., et al., *XRCC3 promotes homology-directed repair of DNA damage in mammalian cells.* Genes Dev, 1999. **13**(20): p. 2633-8.
294. Sonoda, E., et al., *Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death.* EMBO J, 1998. **17**(2): p. 598-608.
295. Moynahan, M.E., et al., *Brca1 controls homology-directed DNA repair.* Mol Cell, 1999. **4**(4): p. 511-8.

296. Lim, D.S. and P. Hasty, *A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53.* Mol Cell Biol, 1996. **16**(12): p. 7133-43.
297. Deans, B., et al., *Xrcc2 is required for genetic stability, embryonic neurogenesis and viability in mice.* EMBO J, 2000. **19**(24): p. 6675-85.
298. Khanna, K.K. and S.P. Jackson, *DNA double-strand breaks: signaling, repair and the cancer connection.* Nat Genet, 2001. **27**(3): p. 247-54.
299. Farmer, H., et al., *Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy.* Nature, 2005. **434**(7035): p. 917-21.
300. Bunting, S.F., et al., *53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks.* Cell, 2010. **141**(2): p. 243-54.
301. Maxwell, K.N. and S.M. Domchek, *Cancer treatment according to BRCA1 and BRCA2 mutations.* Nat Rev Clin Oncol, 2012. **9**(9): p. 520-8.
302. Ossovskaya, V., et al., *Upregulation of Poly (ADP-Ribose) Polymerase-1 (PARP1) in Triple-Negative Breast Cancer and Other Primary Human Tumor Types.* Genes Cancer, 2010. **1**(8): p. 812-21.
303. Rothkamm, K., et al., *Pathways of DNA double-strand break repair during the mammalian cell cycle.* Mol Cell Biol, 2003. **23**(16): p. 5706-15.
304. Terradas, M., et al., *DNA lesions sequestered in micronuclei induce a local defective-damage response.* DNA Repair (Amst), 2009. **8**(10): p. 1225-34.
305. Fenech, M., et al., *Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells.* Mutagenesis, 2011. **26**(1): p. 125-32.
306. O'Donovan, P.J. and D.M. Livingston, *BRCA1 and BRCA2: breast/ovarian cancer susceptibility gene products and participants in DNA double-strand break repair.* Carcinogenesis, 2010. **31**(6): p. 961-7.
307. Kirk, K.E., et al., *Abnormal micronuclear telomeres lead to an unusual cell cycle checkpoint and defects in Tetrahymena oral morphogenesis.* Eukaryot Cell, 2008. **7**(10): p. 1712-23.
308. Shrivastav, M., L.P. De Haro, and J.A. Nickoloff, *Regulation of DNA double-strand break repair pathway choice.* Cell Res, 2008. **18**(1): p. 134-47.
309. Zhao, Y., et al., *Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441.* Cancer Res, 2006. **66**(10): p. 5354-62.
310. Guo, L., et al., *DNA-dependent protein kinase and ataxia telangiectasia mutated (ATM) promote cell survival in response to NK314, a topoisomerase IIalpha inhibitor.* Mol Pharmacol, 2011. **80**(2): p. 321-7.
311. Mladenov, E., et al., *DNA double-strand break repair as determinant of cellular radiosensitivity to killing and target in radiation therapy.* Front Oncol, 2013. **3**: p. 113.
312. Helleday, T., et al., *DNA double-strand break repair: from mechanistic understanding to cancer treatment.* DNA Repair (Amst), 2007. **6**(7): p. 923-35.
313. Abedin, M.J., et al., *Autophagy delays apoptotic death in breast cancer cells following DNA damage.* Cell Death Differ, 2007. **14**(3): p. 500-10.
314. Burden, D.A. and N. Osheroff, *Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme.* Biochim Biophys Acta, 1998. **1400**(1-3): p. 139-54.

315. Pommier, Y., et al., *Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme.* Biochim Biophys Acta, 1998. **1400**(1-3): p. 83-105.
316. Pommier, Y., et al., *Repair of and checkpoint response to topoisomerase I-mediated DNA damage.* Mutat Res, 2003. **532**(1-2): p. 173-203.
317. Schonn, I., J. Hennesen, and D.C. Dartsch, *Ku70 and Rad51 vary in their importance for the repair of doxorubicin- versus etoposide-induced DNA damage.* Apoptosis, 2011. **16**(4): p. 359-69.
318. Jacob, S., et al., *Effects of camptothecin on double-strand break repair by non-homologous end-joining in DNA mismatch repair-deficient human colorectal cancer cell lines.* Nucleic Acids Res, 2005. **33**(1): p. 106-13.
319. Arnaudeau, C., C. Lundin, and T. Helleday, *DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells.* J Mol Biol, 2001. **307**(5): p. 1235-45.
320. Bester, A.C., et al., *Nucleotide deficiency promotes genomic instability in early stages of cancer development.* Cell, 2011. **145**(3): p. 435-46.
321. Goldberg, A.L., *Protein degradation and protection against misfolded or damaged proteins.* Nature, 2003. **426**(6968): p. 895-9.
322. Ciechanover, A., *The ubiquitin proteolytic system: from a vague idea, through basic mechanisms, and onto human diseases and drug targeting.* Neurology, 2006. **66**(2 Suppl 1): p. S7-19.
323. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu Rev Biochem, 1998. **67**: p. 425-79.
324. Korolchuk, V.I., F.M. Menzies, and D.C. Rubinsztein, *A novel link between autophagy and the ubiquitin-proteasome system.* Autophagy, 2009. **5**(6): p. 862-3.
325. Rubinsztein, D.C., *The roles of intracellular protein-degradation pathways in neurodegeneration.* Nature, 2006. **443**(7113): p. 780-6.
326. Ding, W.X., et al., *Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability.* Am J Pathol, 2007. **171**(2): p. 513-24.
327. Fuertes, G., A. Villarroya, and E. Knecht, *Role of proteasomes in the degradation of short-lived proteins in human fibroblasts under various growth conditions.* Int J Biochem Cell Biol, 2003. **35**(5): p. 651-64.
328. Korolchuk, V.I., et al., *Autophagy inhibition compromises degradation of ubiquitin-proteasome pathway substrates.* Mol Cell, 2009. **33**(4): p. 517-27.
329. Katsuragi, Y. and N. Sagata, *Regulation of Chk1 kinase by autoinhibition and ATR-mediated phosphorylation.* Mol Biol Cell, 2004. **15**(4): p. 1680-9.
330. Yoo, H.Y., et al., *Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase.* Cell, 2004. **117**(5): p. 575-88.
331. Lu, X., B. Nannenga, and L.A. Donehower, *PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints.* Genes Dev, 2005. **19**(10): p. 1162-74.
332. Zhang, Y.W., et al., *The F box protein Fbx6 regulates Chk1 stability and cellular sensitivity to replication stress.* Mol Cell, 2009. **35**(4): p. 442-53.
333. Leung-Pineda, V., J. Huh, and H. Piwnica-Worms, *DDB1 targets Chk1 to the Cul4 E3 ligase complex in normal cycling cells and in cells experiencing replication stress.* Cancer Res, 2009. **69**(6): p. 2630-7.

334. Omura, S., et al., *Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells.* J Antibiot (Tokyo), 1991. **44**(1): p. 113-6.
335. Han, Y.H., et al., *The effect of MG132, a proteasome inhibitor on HeLa cells in relation to cell growth, reactive oxygen species and GSH.* Oncol Rep, 2009. **22**(1): p. 215-21.
336. Ding, Q., et al., *Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization.* Mol Cell Biol, 2007. **27**(11): p. 4006-17.
337. Schneider-Poetsch, T., et al., *Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin.* Nat Chem Biol, 2010. **6**(3): p. 209-217.
338. Yamamoto, A., et al., *Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells.* Cell Struct Funct, 1998. **23**(1): p. 33-42.
339. Kimura, T., et al., *Chloroquine in cancer therapy: a double-edged sword of autophagy.* Cancer Res, 2013. **73**(1): p. 3-7.
340. Loehberg, C.R., et al., *Ataxia telangiectasia-mutated and p53 are potential mediators of chloroquine-induced resistance to mammary carcinogenesis.* Cancer Res, 2007. **67**(24): p. 12026-33.
341. Korolchuk, V.I., F.M. Menzies, and D.C. Rubinsztein, *Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems.* FEBS Lett, 2010. **584**(7): p. 1393-8.
342. Clague, M.J. and S. Urbe, *Ubiquitin: same molecule, different degradation pathways.* Cell, 2010. **143**(5): p. 682-5.
343. Engelender, S., *alpha-Synuclein fate: proteasome or autophagy?* Autophagy, 2012. **8**(3): p. 418-20.
344. Ravikumar, B., R. Duden, and D.C. Rubinsztein, *Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy.* Hum Mol Genet, 2002. **11**(9): p. 1107-17.
345. Tanaka, K. and A. Ichihara, *Half-life of proteasomes (multiprotease complexes) in rat liver.* Biochem Biophys Res Commun, 1989. **159**(3): p. 1309-15.
346. Russell, S.J., K.A. Steger, and S.A. Johnston, *Subcellular localization, stoichiometry, and protein levels of 26 S proteasome subunits in yeast.* J Biol Chem, 1999. **274**(31): p. 21943-52.
347. Laporte, D., et al., *Reversible cytoplasmic localization of the proteasome in quiescent yeast cells.* J Cell Biol, 2008. **181**(5): p. 737-45.
348. Hui, B., et al., *Proteasome inhibitor interacts synergistically with autophagy inhibitor to suppress proliferation and induce apoptosis in hepatocellular carcinoma.* Cancer, 2012. **118**(22): p. 5560-71.
349. Bartek, J. and J. Lukas, *Chk1 and Chk2 kinases in checkpoint control and cancer.* Cancer Cell, 2003. **3**(5): p. 421-9.
350. Chambers, A.F., A.C. Groom, and I.C. MacDonald, *Dissemination and growth of cancer cells in metastatic sites.* Nat Rev Cancer, 2002. **2**(8): p. 563-72.
351. Martin, S.A. and T. Ouchi, *Cellular commitment to reentry into the cell cycle after stalled DNA is determined by site-specific phosphorylation of Chk1 and PTEN.* Mol Cancer Ther, 2008. **7**(8): p. 2509-16.

352. Cho, S.H., et al., *Chk1 is essential for tumor cell viability following activation of the replication checkpoint.* Cell Cycle, 2005. **4**(1): p. 131-9.
353. Dent, P., et al., *CHK1 inhibitors in combination chemotherapy: thinking beyond the cell cycle.* Mol Interv, 2011. **11**(2): p. 133-40.
354. Tavecchio, M., et al., *Further characterisation of the cellular activity of the DNA-PK inhibitor, NU7441, reveals potential cross-talk with homologous recombination.* Cancer Chemother Pharmacol, 2012. **69**(1): p. 155-64.
355. Lam, M.H., et al., *Chk1 is haploinsufficient for multiple functions critical to tumor suppression.* Cancer Cell, 2004. **6**(1): p. 45-59.
356. Canman, C.E., et al., *The p53-dependent G1 cell cycle checkpoint pathway and ataxia-telangiectasia.* Cancer Res, 1994. **54**(19): p. 5054-8.
357. Jinno, S., et al., *Cdc25A is a novel phosphatase functioning early in the cell cycle.* EMBO J, 1994. **13**(7): p. 1549-56.
358. Cann, K.L. and G.G. Hicks, *Absence of an immediate G1/S checkpoint in primary MEFs following gamma-irradiation identifies a novel checkpoint switch.* Cell Cycle, 2006. **5**(16): p. 1823-30.
359. Treszezamsky, A.D., et al., *BRCA1- and BRCA2-deficient cells are sensitive to etoposide-induced DNA double-strand breaks via topoisomerase II.* Cancer Res, 2007. **67**(15): p. 7078-81.
360. Gowen, L.C., et al., *Brca1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities.* Nat Genet, 1996. **12**(2): p. 191-4.
361. Nohl, H. and L. Gille, *Lysosomal ROS formation.* Redox Rep, 2005. **10**(4): p. 199-205.
362. Kubota, C., et al., *Constitutive reactive oxygen species generation from autophagosome/lysosome in neuronal oxidative toxicity.* J Biol Chem, 2010. **285**(1): p. 667-74.
363. Leung-Pineda, V., C.E. Ryan, and H. Piwnica-Worms, *Phosphorylation of Chk1 by ATR is antagonized by a Chk1-regulated protein phosphatase 2A circuit.* Mol Cell Biol, 2006. **26**(20): p. 7529-38.
364. Yoshimori, T., et al., *Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells.* J Biol Chem, 1991. **266**(26): p. 17707-12.
365. Wolfe, F. and M.F. Marmor, *Rates and predictors of hydroxychloroquine retinal toxicity in patients with rheumatoid arthritis and systemic lupus erythematosus.* Arthritis Care Res (Hoboken), 2010. **62**(6): p. 775-84.
366. Garber, K., *Inducing indigestion: companies embrace autophagy inhibitors.* J Natl Cancer Inst, 2011. **103**(9): p. 708-10.
367. Gorski, S.M., J. Ries, and J.J. Lum, *Targeting autophagy: the Achilles' heel of cancer.* Autophagy, 2012. **8**(8): p. 1279-80.
368. McAfee, Q., et al., *Autophagy inhibitor Lys05 has single-agent antitumor activity and reproduces the phenotype of a genetic autophagy deficiency.* Proc Natl Acad Sci U S A, 2012. **109**(21): p. 8253-8.
369. !!! INVALID CITATION !!!
370. Shieh, S.Y., et al., *The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites.* Genes Dev, 2000. **14**(3): p. 289-300.

371. Ou, Y.H., et al., *p53 C-terminal phosphorylation by CHK1 and CHK2 participates in the regulation of DNA-damage-induced C-terminal acetylation.* Mol Biol Cell, 2005. **16**(4): p. 1684-95.