

**p62: Linking protein homeostasis  
and the DNA damage response  
during ageing**



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## **Abstract**

The accumulation of DNA damage has long been thought to contribute to both cellular and organismal ageing. The catabolic degradation process autophagy has also been implicated in the ageing process, as an age-dependent functional decline has been reported in many organisms. Moreover, interventions known to delay ageing such as dietary restriction and rapamycin treatment have been shown to require active autophagy. Recent studies have suggested a role for autophagy in the DNA damage response as well as DNA damage repair; however the mechanisms are still poorly understood. In this thesis we set out to understand how autophagy can influence DNA repair. We will also investigate how cross-talk between these processes is relevant to the ageing process. Firstly, we show that cells lacking autophagy have an impaired DNA damage repair kinetic, as measured by the resolution of DNA damage foci (DDF). Importantly, these differences were shown to be dependent on the intracellular levels of autophagy adaptor protein p62. It was recently shown that p62 shuttles rapidly between cytoplasmic and nuclear compartments. However, the role of p62 in the nucleus is still relatively unknown. Mechanistically, we show that both the PB1 and UBA domain of p62 are required for its effect on DNA damage repair. Furthermore we show p62 is recruited to DDF in response to DNA damage induction. Next, we show p62 interacts with the cytoskeletal protein FLNA and DNA damage protein RAD51 within the nucleus facilitating their proteasomal degradation. Both FLNA and RAD51 have previously been suggested to influence DNA repair via the homologous recombination pathway. Cells lacking p62 have higher nuclear levels of RAD51 and FLNA. Importantly these high levels correspond with an increased formation and resolution of RAD51 foci following induction of DNA damage, suggestive of an increase in DNA repair via the homologous recombination pathway. Finally, we observed an increase in co-localisation of p62 with the marker of DDF  $\gamma$ H2A.X in mouse liver during ageing. Additionally, we found that life-long dietary restriction, an intervention known to extend lifespan in mice, prevented the age-dependent increase in frequencies of p62- $\gamma$ H2A.X foci. We propose that p62 plays a novel and important role in DNA damage repair and hypothesise that declining autophagy or dysregulation of p62 can contribute to organismal ageing.

## Awards and Publications

### Awards

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### Publications

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Jodie Birch, Rhys K Anderson, Clara Correia-Melo, Diana Jurk, **Graeme Hewitt** et al: *DNA damage response at telomeres contributes to lung ageing and chronic obstructive pulmonary disease.* **American Journal of Physiology-Lung Cellular and Molecular Physiology** 09/2015, ([Link](#))

Clara Correia-Melo, Francisco DM Marques, Rhys Anderson, **Graeme Hewitt**, et al: *Mitochondria are required for pro-ageing features of the senescent phenotype.* **EMBO J** 04/02/2016 ([link](#))

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**Graeme Hewitt**, et al: *Cell Sorting of Young and Senescent Cells.* Biological Aging edited by Trygve O. Tollefsbol, 08/2013; Humana Press. ISBN: 978-1-62703-555-2 ([link](#))

Bernadette Carroll, **Graeme Hewitt**, et al: *Autophagy and ageing: implications for age-related neurodegenerative diseases.* **Essays in Biochemistry** 09/2013; 55(1):119-31. ([link](#))

**Graeme Hewitt**, et al: *Mechanisms of Cross-talk between Intracellular Protein Degradation Pathways* Hewitt *Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging* 01/2015 Vol:6:103-19 ([link](#))

## Abbreviations List

4EBP1	4E binding protein-1
53BP1	p53 binding protein1
aa	amino acids
AD	Alzheimer's disease
AMP	adenosine monophosphate
aPKC	atypical protein kinase C
ARE	antioxidant-responsive element
ATG	autophagy related
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and rad 3 related
ATRIP	ATR interacting protein
BAG3	Bcl-2-associated athanogene 3
BML	Bloom syndrome, RecQ helicase-like
BRAF	B-Raf proto-oncogene, serine/threonine kinas
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
bZIP	basic leucine zipper
CDC25	cell division cycle 25
CDK1	cyclin-dependent kinase 1
CHK1	checkpoint kinase 1
CHK2	Checkpoint kinase 2
CK2	Casein Kinase II
CMA	chaperone-mediated autophagy
CML	chronic myelogenous leukaemia
COUP-TFII	chicken ovalbumin upstream promoter transcription factor II
CP	core particle
CR	calorie restriction
CtIP RBBP8	retinoblastoma binding protein 8
Cul-3	Cullin 3

D	Diversity
D loop	displacement loop
DAG	diacylglycerol
DDF	DNA damage foci
DDR	DNA damage response
DISC	death-inducing signalling complex
DNA-PK <sub>CS</sub>	DNA-dependent protein kinase
DNA2	DNA replication helicase/nuclease 2
DR	Dietary restriction
<i>DRAM</i>	<i>damage-regulated autophagy modulator</i>
DSBs	double strand breaks
eIF2 $\alpha$	eukaryotic translation Initiation Factor 2alpha
ER	endoplasmic reticulum
ES	Embryonic stem cell
EXO1	exonuclease 1
FIP200	200 kDa FAK-family interacting protein
FKBP12	FK506-binding protein of 12 kDa
GSK-3	glycogen synthase kinase 3
G $\beta$ L	G protein $\beta$ -subunit-like protein
H2A.X	Histone 2AX
HD	Huntington's disease
HR	homologous recombination
hsc70	heat-shock cognate protein of 70 KDa
HspB8	heat shock 22kDa protein 8
HUS1	HUS1 checkpoint clamp component
Ig	Immunoglobulin
IL-1R	interleukin 1 receptor
IL-1 $\beta$ R	interleukin-1 $\beta$ receptor
IL-3	interleukin 3
IP <sub>3</sub>	inositol 1,4,5-trisphosphate
IP <sub>4</sub>	1,3,4,5-tetrakisphosphate
IR	Ionizing radiation
IRAK	interleukin-1 receptor-associated kinase

J	Joining
Keap1	Kelch-like ECH-associated protein
KIR	KEAP1 interacting region
KO	Knock out
LAMP2a	lysosomal-associated membrane protein 2a
LC	Lactacystin
LC3	microtubule-associated protein 1 light chain 3 alpha
LIG4	ligase IV, DNA, ATP-dependent
LIR	LC3-interacting regions
MDC1	mediator of DNA-Damage checkpoint 1
Mre11	MRE11 homolog A, double strand break repair nuclease
MTOC	microtubule organising centre
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NAD <sup>+</sup>	$\beta$ -nicotinamide adenine dinucleotide
NES	nuclear export signal
NF $\kappa$ B	nuclear factor-kappaB
NGF-R	nerve growth factor receptor
NHEJ	non-homologous end-joining
NLS	nuclear localization signals
Nrf2	nuclear factor (erythroid 2)-like 2
NS	Not Significant
NSCLC	non-small-cell lung carcinoma
OIS	Oncogene-induced senescence
p70S6K	p70 S6 Kinase
PARP1	poly [ADP-ribose] polymerase 1
PB1	N-terminal the phox and bem1p
PD	Parkinson's disease
PDK1	phosphoinositide-dependent kinase 1
PE	phosphatidylethanolamine
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIKKs	Phosphoinositide Kinase-Related Kinases

PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	protein kinase C
PLC	G protein coupled receptor activation of phospholipase C
PMA	phorbol 12-myristate 13-acetate
PML	promyelocytic leukemia
PNKP	polynucleotide kinase 3'-phosphatase
PRAS40	proline-rich Akt substrate of 40 kDa
PSMC3	proteasome 26S subunit, ATPase 3
PSME1	proteasome activator subunit 1
RAD17	RAD17 checkpoint clamp loader component
RAD50	RAD50 homolog, double strand break repair protein
RAD51	RAD51 recombinase
RAD9	RAD9 checkpoint clamp component A
raptor	regulatory associated protein of mTOR
RAS	Retrovirus associated DNA sequence
RFC	DNA replication factor C
RHEB	Ras Homolog Enriched In Brain
riCTOR	rapamycin-sensitive companion of mTOR
RIP1	receptor-interacting protein 1
ROS	Reactive oxygen species
ROTOR	protein observed with rictor
RP	regulatory particle
RPA	Replication protein A
Rpt5	proteasome regulatory particle base subunit RPT5
RSSs	Recombination Signal Sequences
S.E.M	standard error of the mean
SCAN1	spinocerebellar ataxia with axonal neuropathy
SCID	severe combined immunodeficiency
SD	Standard deviation
Sen- $\beta$ -Gal	Senescence-associated $\beta$ Galactosidase
SIN1	SAPK-interacting protein 1
SQSTM1	sequestosome 1

ssDNA	Single-stranded DNA
TCR	T Cell Receptor
TDP1	tyrosyl-DNA phosphodiesterase 1
TNF-R	TNF receptor
TOP3a	topoisomerase (DNA) III alpha
TOR	target of rapamycin
TRAF6	TNF $\alpha$ receptor-associated factor 6
TSC1	tuberous sclerosis 1
TSC2	tuberous sclerosis 2
UBA	Ubiquitin-interacting domain
Ub	ubiquitin
ULK1	unc-51 like autophagy activating kinase 1
UPS	ubiquitin-proteasome system
UV	ultra-violet
V	variable
XLJ/NHEJ1	Non-homologous end-joining factor 1
	X-ray repair complementing defective repair in Chinese hamster cells 4
XRCC4	
$\beta$ -ME	beta mercaptoethanol
$\gamma$ H2A.X	Phosphorylated H2A.X

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## Chapter 1. Introduction

### 1.1 DNA Damage Response (DDR)

DNA damage repair is essential for the safeguarding of genomic integrity. The importance of understanding these processes was acknowledged this year by the award of the Nobel Prize in chemistry to Tomas Lindahl, Paul Modrich and Aziz Sancar for their study of DNA damage repair at the molecular level.

DNA can be damaged by a number of extrinsic damaging agents such as; ultra-violet radiation (UV), Ionizing radiation (IR) and chemical compounds as well as intrinsic sources of damage such as mistakes in replication and oxidative damage that occurs due to free radicals generated as part of normal metabolism. It is estimated that on average a cell experiences 19200 DNA lesions per day (Saul and Ames, 1986). In order to maintain genomic integrity cells must possess effective mechanisms for detecting and repairing this DNA damage.

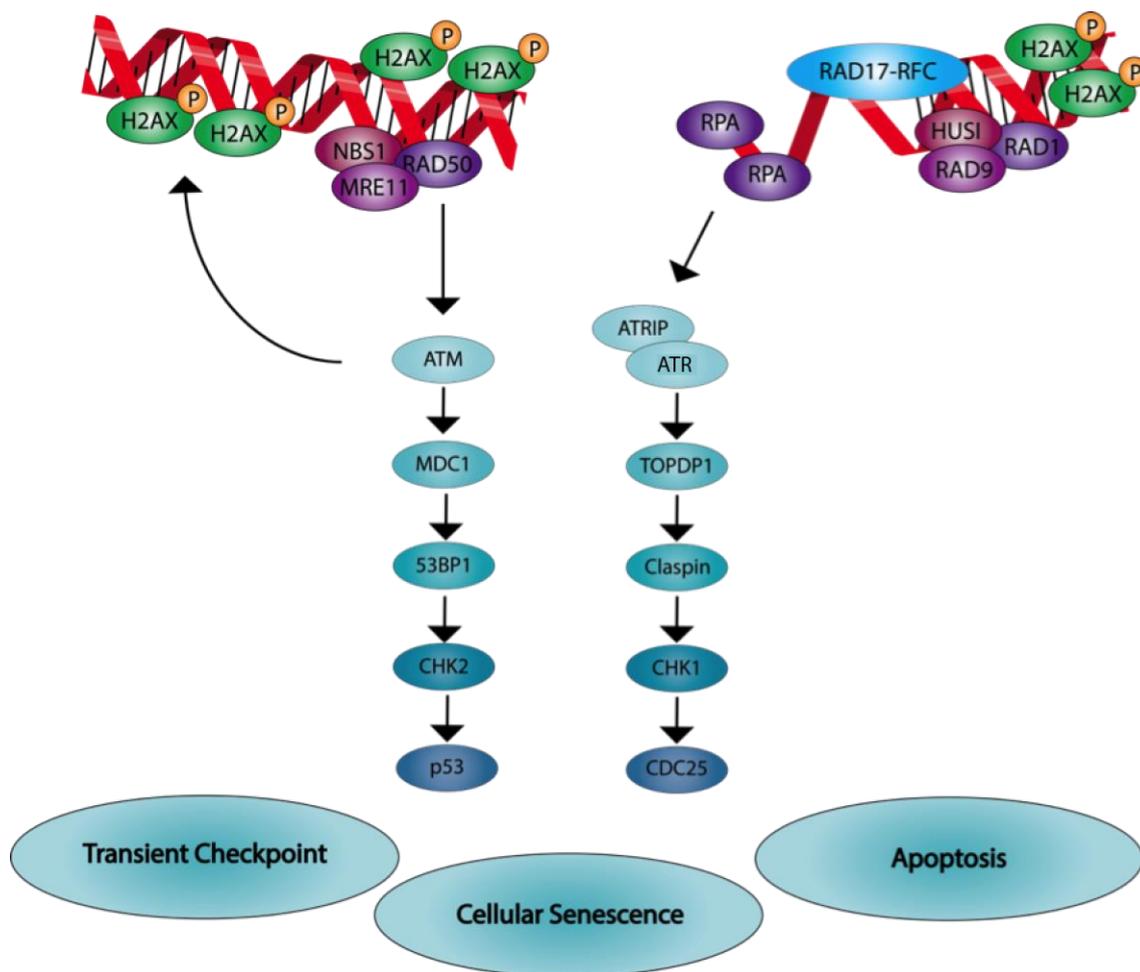
Single stranded DNA (ssDNA), as well as double strand breaks (DSBs) are potent activators of the DDR. Specialised sensing complexes recognize this damage. The Mre11 complex (MRN) made from Mre11/Rad50/Nbs1 in mammals senses and processes DSBs and ataxia-telangiectasia and Rad3 related (ATR), ATR interacting protein (ATRIP), Replication protein A (RPA), Rad9/Rad1/Hus1, Rad17/RSR are all involved in sensing single stranded DNA. These sensors, in turn, recruit large Phosphoinositide Kinase-Related Kinases (PIKKs) ATR and ataxia-telangiectasia (ATM) to the site of damage (Shiloh, 2006). Recruitment of these kinases causes the local phosphorylation of histone H2A.X (Figure 1-1).

At DSBs the ATM mediated phosphorylation of Histone 2AX (H2A.X) to Phosphorylated H2A.X ( $\gamma$ H2A.X) then sets up a positive feedback loop, which recruits more ATM to the DNA damage lesion. This recruitment of yet more ATM causes  $\gamma$ H2A.X to spread along the chromatin 1-2Mb from the site of the lesion (Bewersdorf et al., 2006). The recruitment of ATM to  $\gamma$ H2A.X is facilitated by DDR mediators; mediator of DNA-Damage checkpoint 1 (MDC1) and p53 binding protein1 (53BP1) (Wang et al., 2002). The establishment of this feedback loop and the spreading of  $\gamma$ H2A.X acts to amplify the signal and aids in the recruitment of ATM and other DDR proteins (Figure 1-1).

At SSBs single stranded DNA is bound by replication protein A (RPA) which signals the recruitment of ATR (Cortez et al., 2001). ATR activity is further boosted by the heterotrimeric 9–1–1 complex (RAD9, RAD1 and HUS1) and topoisomerase-II-binding protein 1 (TOPBP1) (Weiss et al., 2002, Kumagai et al., 2006). Downstream of TOPBP1, Claspin leads to activation of Chk1 (Liu et al., 2006).

Activation of ATM/ATR above a certain threshold is required to activate checkpoint kinase 2 (CHK2) (Buscemi et al., 2004). CHK2, once activated, can freely diffuse through the nucleoplasm and phosphorylate further DDR protein substrates (Lukas et al., 2003). Similarly to CHK2, checkpoint kinase 1 (CHK1) is also phosphorylated by ATM and ATR and diffuses through the nucleus also spreading DDR signalling (Bekker-Jensen et al., 2006). Therefore DSBs favour activation of ATM/Chk2 and SSBs favour activation of ATR/Chk1. However, the processing of DSBs during S or G2 phases of the cell cycle can result in the generation of ssDNA and ATR activation (Jazayeri et al., 2006).

Ultimately activation of the DDR leads to the enforcement of cell-cycle checkpoints through multiple signalling pathways such as p53 and cell division cycle 25 (CDC25) phosphatase. DNA damage induces an inactivation of CDC25 that causes rapid cell-cycle arrest as activity of these phosphatases are required for cell division (Mailand et al., 2000). p53 is induced more slowly following activation of a DDR and leads to an increase in transcription of the cyclin dependent kinase inhibitor p21. Increased expression of p21 leads to a stable cell cycle arrest (Deng et al., 1995).



**Figure 1-1: Schematic representation of the DDR.** DSBs are sensed by the MRE11 RAD50-NBS1 (MRN) complex, this leads to the recruitment of the apical protein kinase ATM. ATM is phosphorylated and then goes on to phosphorylate H2A.X to  $\gamma$ H2A.X at the site of damage.  $\gamma$ H2A.X is recognised by MDC1 which leads to further recruitment of the MRN complex and amplification of ATM phosphorylation. This amplification step leads to the spreading of  $\gamma$ H2A.X many base pairs from the site of damage. This causes an increase in the number of DDR factors such as 53BP1 at the site of damage which orchestrate repair. Exposure of single-stranded DNA leads to recruitment of RPA. Following this, ATR and its binding partner ATRIP are recruited and ATR activity can be increased by additional ATR targets, such as the RAD9–HUS1–RAD1 and RAD17–RFC complexes. ATR activity can also be boosted by DNA topoisomerase 2-binding protein (TOPBP1) and Claspin. Activation of ATM and ATR leads to phosphorylation of CHK1 and CHK2, which, through downstream effectors such as p53 and CDC25, leads to cell cycle arrest, senescence or apoptosis. Adapted from (d'Adda di Fagagna, 2008).

The spreading of  $\gamma$ H2A.X many megabases from the initial site of DNA damage acts as a platform to attract and hold a large number of DDR proteins (Downey and Durocher, 2006). This protein recruitment leads to the formation of nuclear foci known as DNA damage foci (DDF). These DDF contain multiple copies of the same proteins and show similarities in both structure and function to DNA replication factories. These replication factories are thought to serve to ensure the optimal use of nuclear space and available replication enzymes to allow accurate replication (Hozák and Cook, 1994). It was previously believed that individual DDF could merge (Aten et al., 2004), however, more recent studies employing live-cell imaging indicate that DNA damage foci are positionally stable (Soutoglou et al., 2007). It is now thought that the combination of multiple DSBs in shared foci could lead to mistakes in ligation and unwanted fusion events and that keeping these lesions positionally separate is in fact advantageous (d'Adda di Fagagna, 2008). Hence, one DNA damage focus is formed per damage lesion and, like replication sites, it is thought to optimise protein interactions and enzymatic activity, which aid in the coordination and amplification of DNA damage signalling. Interestingly, it has even been shown that the artificial colocalisation of DDR proteins alone is sufficient to activate DNA damage checkpoints in the absence of actual DNA damage (Bonilla et al., 2008).

Following the repair of DNA lesions, DDF are disassembled. This is due primarily to the activity of dedicated phosphatases that dephosphorylate  $\gamma$ H2A.X as well as chromatin remodelling (Downey and Durocher, 2006). This means that DNA lesions that are quickly repaired give rise to small transient foci, whereas DNA damage that is less efficiently repaired causes larger long-lived foci (Passos et al., 2010).

Activation of a DDR and cell-cycle arrest can result in a number of different cell fates. This is dependent on the severity, type and location of the DNA damage as well as the cell-type. The primary aim of the DDR is to repair DNA damage and allow cells to progress back into the cell-cycle. If the damage persists then the chronic activation of a DDR can lead to apoptosis (programmed cell death) or cellular senescence. The mechanism that determines the cellular "decision" between these two cell fates is not yet fully understood.

### 1.2.1 Double Strand Break Repair

There are two main pathways in eukaryotic cells that mediate double strand break repair: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is active through the whole cell-cycle whereas HR is only active during the S and G2 phases when sister chromatids can be used as a template for repair.

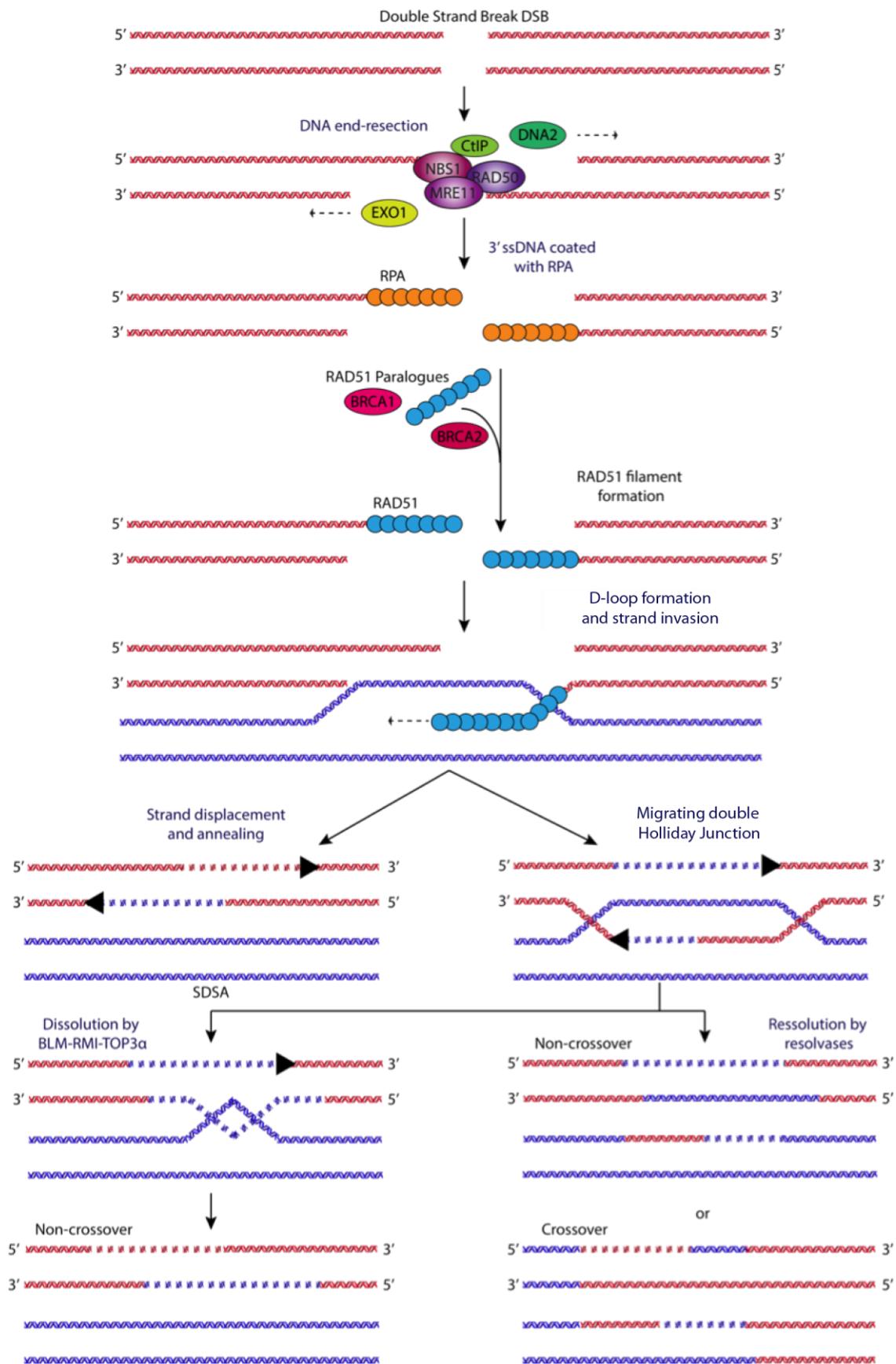
### 1.2.2 Homologous Recombination (HR)

Homologous recombination is an important process that is required for genome maintenance as generally it performs an error-free mode of repair. In the absence of HR, cells must rely on the more error-prone DNA repair pathway NHEJ which can lead to increased mutations and rearrangements potentially leading to genome instability. Key components of HR such as BRCA2 and RAD51C are known tumor suppressors, with inactivating mutations leading to increased genomic instability (Golmard et al., 2013, Wooster et al., 1995). HR is also an important mechanism required for the restart of stalled replication forks during S phase (Budzowska and Kanaar, 2009, Woodbine et al., 2014).

The initial step of HR, DNA resection, involves processing of DSBs to a 3' overhanging tail. In yeast, this processing appears to require the MRX complex (Cao et al., 1990). Defects in this complex lead to sensitivity to IR, but the repair of "blunt" DSBs such as those caused by HO-endonuclease is not affected (Li and Heyer, 2008). MRX and Sae2 initiate HR by performing short range resection (a few hundred nucleotides) (Mimitou and Symington, 2008), then both the 5'-3' exonuclease *Exo1* and DNA2 perform long range resection (two or more kilobases) (Zhu et al., 2008). The mammalian homolog of Sae2, CtIP has been implicated in DSB resection, although the exact mechanisms are still not understood. However, inactivation of CtIP has been shown to be mortal to cells and reduce RAD51 foci formation as well as HR (Nakamura et al., 2010). Following resection, ssDNA is then coated by the single-strand binding protein complex RPA (San Filippo et al., 2008). This is followed by the replacement of RPA with RAD51, a process catalysed by recombination mediator proteins such as BRCA1/2, resulting in RAD51 presynaptic nucleofilament formation (Yang et al., 2005). RAD51 is an ATP-hydrolysing protein

that has a high affinity for ssDNA in the ATP-bound state and is released from DNA following ATP hydrolysis (Petalcorin et al., 2006). BRCA2 not only recruits RAD51 but also stabilises the resulting RAD51 nucleofilament through inhibition of RAD51 ATP hydrolysis (Jensen et al., 2010). The RAD51 filament is responsible for homology search as well as catalysis of strand invasion. This leads to the formation of a displacement loop (D loop) once RAD51 is removed by helicases such as HELQ and RAD54 (Solinger et al., 2002, Ward et al., 2010). The D loop undergoes extension mediated by DNA polymerases such as: DNA polymerase delta (Li et al., 2009), POLN (Moldovan et al., 2010) and eta (McIlwraith et al., 2005). Finally, these HR structures are processed leading to the resolution of the DSB.

# Homologous recombination (HR)



**Figure 1-2: Schematic representation of HR.** HR is initiated by the formation of long 3' single-stranded DNA (ssDNA) stretches. The DNA end-resection is complex and highly regulated and requires the activity of several nucleases such as: CtBP-interacting protein (CtIP), Bloom's syndrome helicase (BLM), exonuclease 1 (EXO1), DNA replication ATP-dependent helicase (DNA2) and the MRN (MRE11–RAD50–NBS1) complex. RPA then binds to ssDNA, a key step in HR is the removal of RPA and its replacement with RAD51 to form a RAD51–ssDNA nucleofilament. This is mediated by breast cancer 1 (BRCA1), BRCA2 and RAD51 paralogues; however the exact mechanisms are not yet fully understood. This RAD51–ssDNA nucleofilament searches for homologous sequence and then undergoes strand invasion and displacement D-loop formation. DNA synthesis is initiated within the D-loop to replace DNA proximal to the break site.

### 1.2.3 Non-Homologous End Joining (NHEJ)

Non-Homologous end-joining (NHEJ) is a pathway responsible for the repair of double strand breaks. It is named “non-homologous” because unlike homologous recombination, described above, NHEJ does not require a homologous template and DNA ends are directly joined by DNA Ligases, although short regions of homology, known as microhomologies, are often used to align DNA ends.

NHEJ begins with the binding of the Ku70/80 heterodimer to an exposed DNA end (Weterings and van Gent, 2004). The Ku70/80 heterodimer forms a ring that encircles DNA, fitting to the spiral structure of DNA, positioning DNA ends in the correct phase to facilitate end-joining (Walker et al., 2001). The Ku70/80 heterodimer then recruits the DNA-dependent protein kinase (DNA-PK<sub>CS</sub>), activating its protein kinase activity (Smith and Jackson, 1999). Upon activation, the major function for this kinase is the regulation of NHEJ by autophosphorylation of DNA-PK<sub>CS</sub> (Chan et al., 2002). This autophosphorylation occurs following juxtaposition of DNA ends and facilitates proper access to DNA ends for other NHEJ proteins (Meek et al., 2007). Compatible ends are now able to be joined directly by the ligase IV/XRCC4 complex. This reaction is mediated by the interaction between XLF and XRCC4 (Ahnesorg et al., 2006).

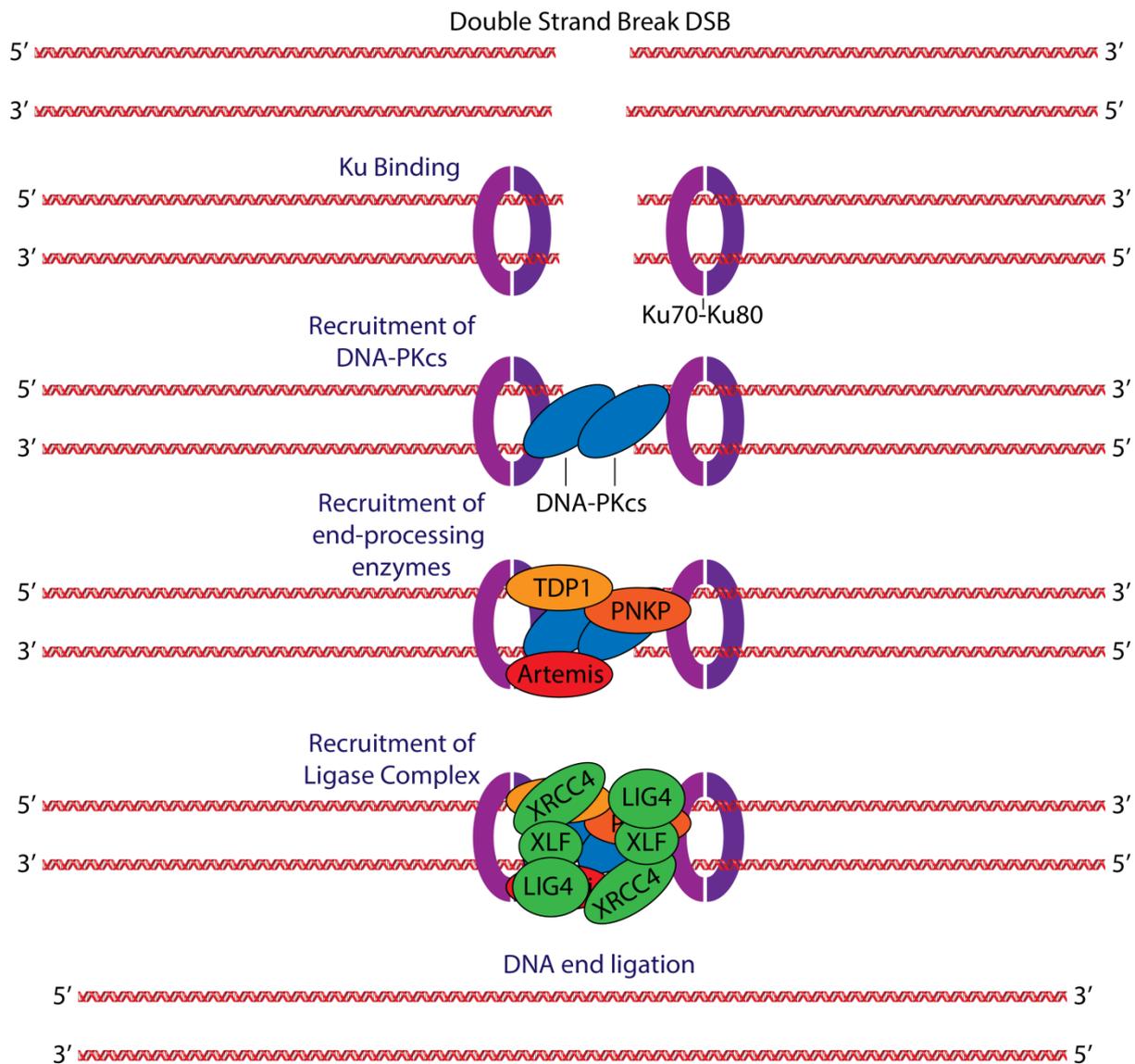
In many cases, DNA ends are not compatible and require processing before they can be ligated. DNA damaging agents such as IR lead to a number of complex lesions containing damaged bases and/or deoxyribose sugars. NHEJ is therefore able to incorporate DNA polymerases, nucleases, polynucleotide kinase and other enzymes to process ends to allow ligation by the ligase IV/XRCC4 complex.

One such nuclease is Artemis, originally discovered as a gene commonly mutated in radiosensitive T<sup>B</sup> severe combined immunodeficiency (SCID) patients (Moshous et al., 2001). Artemis was implicated in V(D)J recombination, a process required for Immunoglobulin (Ig) and T cell receptor (TCR) diversity, via its role in opening hairpin structures that result from RAG1/2 cleavage at recombination signal sequences (Ma et al., 2002). Cells lacking Artemis show considerable radiosensitivity (Riballo et al., 2004, Wang et al., 2005); similarly, cells from Artemis knockout mice show significant chromosomal abnormalities (Rooney et al., 2002). Artemis is unable to process all non-ligatable ends, as such other enzymes have been implicated in DNA-end processing in NHEJ such as PNPKP, an enzyme with

5'-DNA kinase and 3'-DNA phosphatase activities (Karimi-Busheri et al., 1999). PNPKP has been shown to interact with XRCC4 and facilitate end processing and disruption of this interaction has been shown to lead to increased radiosensitivity (Koch et al., 2004). Tyrosyl-DNA phosphodiesterase (TDP1) is another key enzyme involved in end-processing, specifically in response to damage caused by topoisomerase I. This is due to its ability to remove covalently bound tyrosyl-phosphates and polypeptides that can result from a failure of topoisomerases to ligate reaction intermediates (Pommier et al., 2014). TDP1 is able to process a wide range of substrates such as: 3' phosphoglycolate, chain terminating nucleosides and alkylated nucleosides leaving a 3' phosphate. Cells lacking TDP1 are therefore sensitive to damaging agents that produce DSBs with 3' phosphoglycolate such as alkylating agents, and calicheamicin (Pommier et al., 2014, Murai et al., 2012, Inamdar et al., 2002, Interthal et al., 2005). Mutations in TDP1 have been shown to result in the neurodegenerative disorder spinocerebellar ataxia with axonal neuropathy (SCAN1) (Takashima et al., 2002).

NHEJ is not only involved in the repair of DSBs that are generated by exogenous and endogenous stress, it is also involved in the ligation of breaks generated during V(D)J recombination. This process takes place during B and T-cell differentiation and gives rise to antigen specific receptors (Jung et al., 2006). Immunoglobulin (Ig) and T Cell Receptor (TCR) genes contain variable (V), Diversity (D) and Joining (J) segments that are joined by NHEJ to form a mature V(D)J exon in B and T cells, respectively (Schatz, 2004). DSBs are created between Recombination Signal Sequences (RSSs) and coding DNA by RAG1 and RAG2 proteins (McBlane et al., 1995). This creates blunt DNA ends with a hairpin structure that require NHEJ for their successful repair (van Gent et al., 1996). Mutations of genes involved in NHEJ lead to SCID due to an inability to execute V(D)J (Woodbine et al., 2014).

# Non-homologous end-joining (NHEJ)



**Figure 1-3: Schematic representation of NHEJ.** NHEJ is initiated by the rapid recruitment and binding of the Ku70-Ku80 heterodimer to DNA ends at the DSB (de Vries et al., 1989). This is followed by the recruitment of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), an ATM-related kinase, where it is activated by binding and stabilizing DNA ends keeping them in close proximity, as well as recruit end-processing factors such as: polynucleotide kinase/phosphatase (PNKP), Artemis, AP endonuclease 1 (APE1) and tyrosyl–DNA phosphodiesterase 1 (TDP1). These processing factors prepare DNA ends for re-ligation by the X-ray repair cross-complementing protein 4 (XRCCA)–XRCCA-like factor (XLF)–DNA ligase 4 (LIG4) complex.

## 1.3 Ageing and Cellular Senescence

### 1.3.1 Cellular Senescence

Cellular senescence is defined as an irreversible cell-cycle arrest and is associated with a number of phenotypic changes (Campisi and d'Adda di Fagagna, 2007). It was first described in 1961 by Leonard Hayflick and Paul Moorhead (Hayflick and Moorhead, 1961) who demonstrated that human fibroblasts were only able to undergo a finite number of cell divisions before arresting irreversibly when grown in cell culture. This was predicted theoretically by Alexi Olovnikov and James Watson that a progressive shortening of telomeres was responsible for "Hayflick's Limit" (Watson, 1972, Olovnikov, 1971). It took a number of years to confirm experimentally that telomeres did in fact shorten with successive replication (Harley et al., 1990). However, it was still unclear if telomere shortening played a functional role in the induction of senescence, as their shortening could merely be associated with the induction of senescence. This question was answered when it was shown that over-expression of telomerase, an enzyme able to maintain telomere length was able to bypass senescence and resulted in cellular immortalisation (Bodnar et al., 1998).

Telomere shortening is thought to be caused by what is known as the end-replication problem, a phenomenon caused by the DNA replication machinery, specifically DNA polymerase' inability to synthesise in a 3'-5' direction. This means the 5' strand or lagging strand of DNA is replicated in small sections in a 5'-3' direction. These sections are known as Okazaki fragments and are joined together to form a complementary strand by DNA ligase (Ogawa and Okazaki, 1980). Replication of the lagging strand requires the binding of an RNA primer to facilitate the binding of DNA polymerase. The final RNA primer does not have a DNA template and so is unable to be replicated, leading to a loss of around 50-200Bp per replication (de-Lange.T, 2006). The actual rate of attrition seen at telomeres in fibroblasts that reach senescence is a lot greater than what can be accounted for by the end-replication problem alone, suggesting that other factors may contribute to telomere shortening (Levy et al., 1992).

Human skin fibroblasts reach replicative senescence with an average telomere length of around 6-8kb (Allsopp et al., 1992), however senescent cells often contain

one or more extremely short telomeres (Hemann et al., 2001). It is thought that it is these extremely short telomeres that are responsible for the onset of senescence. The exact mechanism by which shortened telomeres induce senescence is not yet fully understood, however it has been suggested that telomere shortening leads to a disruption of the T-Loop (uncapping), exposing the DNA ends (Aubert and Lansdorp, 2008). This then leads to an activation of a DDR and induction of senescence (d'Adda di Fagagna et al., 2003) (Herbig et al., 2004). The threshold of DNA damage required to activate this checkpoint is a matter of intense debate with some groups showing that cells are able to reach senescence with as many as 5 dysfunctional telomeres (Kaul et al., 2012) (Meier et al., 2007) with others showing senescent cells that contain just one DNA damage focus (Herbig et al., 2006).

Originally it was suggested that telomeres could serve as a counting mechanism within cells that would allow a finite number of replications, however a set time or threshold for telomere length to induce senescence has not been found (Von Zglinicki, 2001). This, coupled with the large amount of heterogeneity in telomere length between cells in the same culture (Lansdorp et al., 1996) and the presence of senescent cells in cultures that have undergone a low number of divisions, suggests that telomere length is not just dictated by the number of divisions a cell has undergone (Martin-Ruiz et al., 2004).

It has been shown that telomeric DNA is especially sensitive to SSBs caused by reactive oxygen species (ROS), and that ROS leads to accelerated telomere shortening in replicating cells (von Zglinicki et al., 1995). It was later shown that mitochondrial superoxide levels increase with age and contribute to telomere shortening (Passos et al., 2007). Interestingly, overexpression of TERT (the catalytic subunit of telomerase) reduces mitochondrial superoxide levels independently of telomere length. It was shown that TERT migrates to mitochondria and it is thought to protect mtDNA, however the mechanisms are not entirely understood (Ahmed et al., 2008). It is now thought that the end-replication problem, as well as this stochastic damage, contributes to telomere shortening. It has been suggested that telomeres may act as a sentinel to oxidative stress, becoming shorter and preventing cells that have been exposed to high levels of potentially mutagenic factors from replicating (Martin-Ruiz et al., 2004).

Recently it has been shown in work carried out by our group and Fabrizio d'Adda di Fagagna's group that telomeres are more sensitive to DNA damage and

that this damage is irreparable (Hewitt et al., 2012, Fumagalli et al., 2012). We also have shown that telomeres acquire damage independently of length or telomerase activity showing that it is not only telomere length that is important to the development of senescence. The exact mechanism by which DSB at telomeres are irreparable is unknown however studies have indicated that telomere binding proteins such as TRF2 and RAP1 have inhibitory effects on non-homologous end joining (NHEJ). Thus, telomeres can elicit a permanent DDR resulting in cell cycle arrest and induction of senescence (Hewitt et al., 2012, Cesare et al., 2009, Fumagalli et al., 2012).

As mentioned previously, activation of DDR can often lead to cellular senescence. It has been shown in many cell types that induction of high levels of DNA damage, particularly DSBs, leads to senescence (Di Leonardo et al., 1994). Induction of senescence via the DDR depends heavily on the tumour suppressor p53, which itself activates the cyclin-dependent kinase inhibitor p21 leading to a block in cell-cycle progression (Di Leonardo et al., 1994, Herbig et al., 2004, d'Adda di Fagagna et al., 2003). Additionally the cyclin-dependent kinase inhibitor p16 has been shown to act downstream of the DDR to activate senescence in many cell types (Beausejour et al., 2003, Jacobs and de Lange, 2004, Stein et al., 1999). It has however also been suggested that p16 may also be upregulated independently of DNA damage (Herbig et al., 2004).

Oncogene induced senescence (OIS) has also been shown to associate with the activation of a DDR as a result of hyper-replication (Di Micco et al., 2011, Suram et al., 2012, Di Micco et al., 2006). Oncogenes are genes that when aberrantly expressed have the potential to transform host cells into a tumour cell. The activation of various oncogenes has been shown to induce senescence in a number of different cell types, for example over-expression of RAS, RAF and BRAF have all been shown to induce senescence (Serrano et al., 1997, Zhu et al., 1998, Chrysiis et al., 2005). Interestingly it has been shown that a DDR even in the absence of actual DNA damage is able to lead to induction of cellular senescence, a term the Blagosklonny group name a "Pseudo-DNA damage response" (Pospelova et al., 2009).

While the link between increased levels of DSBs and ageing have been well explored, changes in the activity and efficiency of double strand break repair are less well understood. The importance of DNA damage repair in the ageing process is

highlighted by a number of progeria syndromes where defects in genes involved in repair result in an accelerated ageing phenotype such as: Ataxia telangiectasia (Shiloh, 2001), Werner's syndrome (Epstein et al., 1966), and mice deficient for Ku80 (Didier et al., 2012), DNA-PKcs (Espejel et al., 2004) and ERCC1 (Weeda et al., 1997). Assessing changes in DNA repair pathways is complicated by the changing rate of DSB generation as well as changes that occur in proliferative capacity with age. As mentioned previously, HR is predominantly active during S and G2/M phase where sister chromatids are available (Mao et al., 2008). Senescence is characterised by an abrupt and preeminent loss of proliferative capacity, which was initially thought to be in G1. Recently it was shown that a large percentage of senescent cells up to 60% are arrested in G2 (Mao et al., 2012a). This makes assessing changes in DNA damage repair as a cause or consequence of senescence induction particularly difficult. Early studies looking in normal human lymphocytes indicate an age dependent decline in DNA repair in response to X-ray irradiation as measured by Comet assay (Mayer et al., 1989, Singh et al., 1990). Assessment of damage using this technique does not allow you to assess specific repair mechanisms. Interestingly, it has been observed that senescent cells have a reduced efficiency and accuracy in NHEJ (Seluanov et al., 2004). Importantly, this decline in NHEJ was also observed in pre-senescent cells suggesting that it is not just a consequence of senescence induction. It has also been observed that NHEJ declines with age in mice (Vaidya et al., 2014). Work from the same group has also found a noted decline in HR with progressing population doublings in fibroblasts. This was accompanied by a decline in key components of the HR pathway such as RAD51, RAD51C RAD52 and CtIP. Importantly, this result was not attributed to changes in the cell cycle occurring with progressive PD (Mao et al., 2012b). This study also found that HR could be stimulated by expression of SIRT6 in middle-aged and pre-senescent cells. Taken together these data suggest that age dependent changes in DNA repair could well be a driver of the senescent phenotype and provide a potential therapeutic target to prevent age dependent decline in genome stability that is associated with many aspects of the ageing phenotype.

### 1.3.2 Senescence and Ageing

Despite having been the focus of many studies, reaching back over 5 decades, the existence of cellular senescence *in vivo* and its possible contribution to organismal ageing have been the subject of much debate (Ben-Porath and Weinberg, 2005). However, there have been several studies in the past decade that demonstrate an important role for senescence *in vivo*. Many studies have suggested that senescence plays an important role as a tumour suppressor (Braig et al., 2005, Chen et al., 2005, Collado et al., 2005, Michaloglou et al., 2005). However, there is now mounting evidence suggesting that senescence may also promote tumorigenesis (Krtolica et al., 2001, Dilley et al., 2003, Parrinello et al., 2005, Yang et al., 2006). This is thought to be as a result of the secretion of a number of pro-inflammatory factors known as the senescence associated secretory phenotype (SASP) (Coppe et al., 2008). It is also believed that senescent cells contribute to an age-dependent decline in tissue function (Campisi and d'Adda di Fagagna, 2007).

It was shown in 2006 by Herbig *et al* that the number of cells containing telomere-induced foci (TIF), a well-established senescence marker, increased in the skin of baboons with age (Herbig et al., 2006). Similar observations have been made in mice in a variety of tissues (Krishnamurthy et al., 2004, Wang et al., 2009, Hewitt et al., 2012). Cells bearing senescent markers have also been observed in the context of age-related diseases such as diabetes (Sone and Kagawa, 2005) and atherosclerosis (Minamino and Komuro, 2007). Although interesting, these data only demonstrate a correlation between the accumulation of senescence and age and age-related disease. They do not shown how causally senescence is involved in ageing.

Recently the group led by Jan van Deursen attempted to address whether senescence was playing a causal role in the ageing process. To do this they created a mouse strain where the removal p16Ink4a-positive cells could be induced with drug treatment. This was done in the BubR1 progeroid background, a mouse model where improper chromosomal segregation leads to a progeroid phenotype. Using this model the group was able to show a delay in age-related pathology in the eye, adipose and skeletal tissues when p16Ink4a-positive cells were cleared (Baker et al., 2011). This is an exciting observation and suggests a causal role of senescent cells

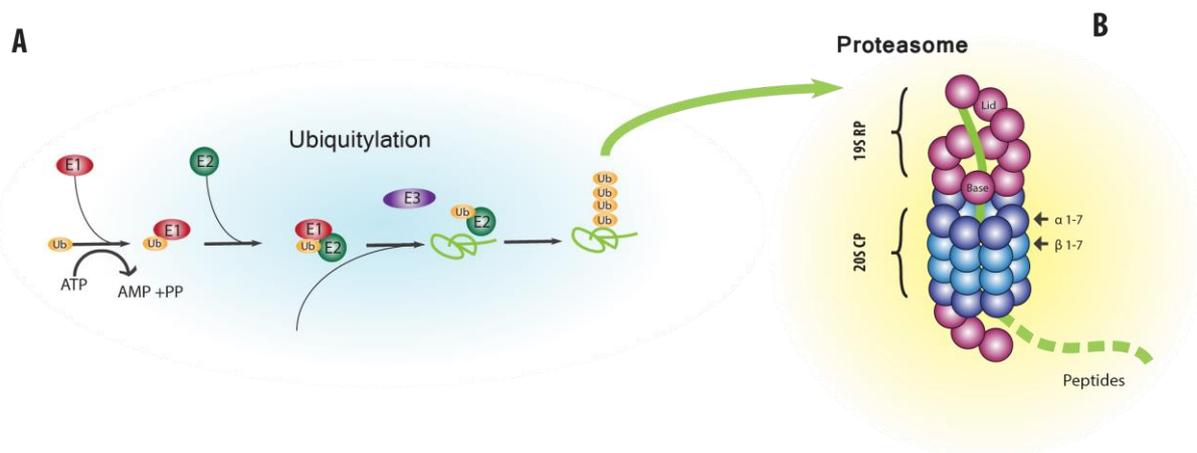
in the progression of ageing as well as age related disease. Next it is important to confirm these results in the context of normal ageing.

#### **1.4 The Ubiquitin Proteasome System**

Regulation of protein hemostasis has been heavily implicated in both DNA repair and ageing. The ubiquitin proteasome system (UPS) is the principle mechanism of protein degradation in the nucleus and cytosol. This chapter will introduce the key processes involved in protein degradation via the UPS as well as describe its known function in DNA repair pathways and the ageing process.

In 2004 Avram Hershko, Aaron Ciechanover and Irwin Rose received the Nobel prize in chemistry for their work in discovering the ubiquitin-proteasome system (UPS) reviewed in (Herrmann et al., 2007). The exact mechanisms by which proteolysis was occurring remained unknown until key experiments revealed the majority of protein degradation was occurring independently of the lysosomal compartment and required adenosine triphosphate (ATP) reviewed in (Ciechanover, 2009). Interestingly, it was shown that proteolysis needs at least two constituents: one with protease activity and the other a 8.5-kDa heat-stable protein. These components were later recognized as the proteasome and ubiquitin, respectively (Arrigo et al., 1988, Waxman et al., 1987).

The UPS is active in both the cytoplasm and nucleus and is responsible for the degradation of short-lived, soluble proteins. The UPS is thought to be responsible for the degradation of around 90% of nuclear and cytoplasmic proteins. Tagging of target proteins with ubiquitin serves to make the protein degradation via the UPS highly selective. Through the tight regulation of protein levels, the UPS has been shown to regulate a plethora of cellular processes such as: protein homeostasis, cell-cycle, signal transduction, DNA repair and many more (Glickman and Ciechanover, 2002).



**Figure 1-4: Schematic representation of the ubiquitination cascade and the proteasome.** (A) The proteasome: composed of the catalytic 20S core particle (CP) and the 19S regulatory particle (RP). (B) The ubiquitination cascade: involving enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), E3 (ubiquitin-ligase). A series of ubiquitination events tag substrates with polyubiquitin chains, selectively targeting them for degradation via the UPS. Figure adapted from (Hewitt et al., 2015).

### 1.4.1 Ubiquitin-dependent protein targeting

Proteins are targeted for degradation via the proteasome by the tagging of ubiquitin to lysine residues. This process is mediated by a series of enzymatic reactions involving E1, E2 and E3 enzymes that activate, transfer and conjugate ubiquitin respectively (Jentsch, 1992). It has been shown that the labelling of a protein substrate with one ubiquitin molecule at one or many lysine residues (mono-ubiquitylation) is insufficient to mediate targeting to the UPS for degradation. Instead, multiple rounds of ubiquitylation of the same lysine residue occur, leading to a poly-ubiquitylated substrate and successful targeting of the substrate to the UPS for degradation (Thrower et al., 2000).

This process of polyubiquitination gives the UPS an externally high degree of specificity. The numerous E1 enzymes present in mammalian cells can interact with all known E2 enzymes. E2 enzymes, however, can only act with a subset of E3 enzymes. Moreover, E3 enzymes have to interact directly with protein substrates in order to perform ubiquitin conjugation, a step which confers further specificity as each E3 enzyme is only able to interact with a limited number of substrates (David et al., 2011). Ubiquitin contains numerous lysine residues on positions 6, 11, 27, 31, 33, 48 and 63 that are susceptible to self-ubiquitylation reactions. The ability of ubiquitin to self-oligomerise via different linkages further contributes to the complexity and diversity conferred by tagging of protein substrates with polyubiquitin chains. Polyubiquitin chains comprised of four or more ubiquitin molecules have been identified as a robust signal to target substrates to the proteasome (Thrower et al., 2000). Poly-ubiquitylation mediated by K11, K29 and K63 linkages have all been shown to mediate proteasomal targeting of protein substrates (Thrower et al., 2000). Interestingly, K11 ubiquitin linkage appears to be particularly important in regulating the turnover of proteins that regulate the cell cycle (Jin et al., 2008).

### 1.4.2 The molecular architecture of the proteasome

The transport of polyubiquitinated substrates to the proteasome are as yet, poorly understood. The 26S proteasome is a large ATP-dependent protease complex made from two main components, the catalytic 20S core particle (CP) and the 19S regulatory particle (RP). Entry of ubiquitinated substrates to the CP is controlled by the RP. Upon entry to the CP proteins are subjected to cleavage reactions that result in the degradation of substrates to oligopeptides that are released into the cyto or nucleoplasm (Peters et al., 1993).

The CP comprises a total of 28 subunits that are arranged in a barrel- like structure that is made from four ring structures, containing seven subunits per ring. The two outermost rings are made up from  $\alpha$ -subunits while the two innermost rings are made from  $\beta$  subunits (Figure 1-4B). These outer  $\alpha$ -rings are thought to serve as a gate controlling the entry of protein substrates into the inner chamber formed by the  $\beta$ -rings. It is within this catalytic chamber that trypsin, caspase and chymotrypsin-like catalytic reactions degrade proteins (Heinemeyer et al., 1997). Different subunit compositions of the  $\beta$ -ring inner chamber confer further specificity of the proteolytic activity of the UPS (Nandi et al., 2006).

Entry of ubiquitylated proteins into the CP is controlled by the RP which regulates the opening and closing of the  $\alpha$ -ring structures, acting as a gate to the CP. The RP is formed from 19 subunits arranged to form lid and base structures. The base is formed from 4 non-ATPase and 6 ATPase subunits (referred to as Rpn 1-2, 10 and 12 and Rpt 1-6, respectively) . This ATPase activity is required to provide energy required for the de-ubiquitylation and protein unfolding required to facilitate entry of protein substrates into the 20S CP (Nandi et al., 2006).

### 1.4.3 DNA damage and the UPS

The controlled recruitment, retention and disassembly of proteins at the site of damaged DNA are essential for the correct execution of DNA repair pathways. The link between DNA damage repair and the UPS was first suggested when Jentsch et al showed that the DNA repair gene Rad6 in *Saccharomyces cerevisiae* encoded a ubiquitin-conjugating enzyme (Jentsch et al., 1987). Since then the study of the UPS in the context of many DNA repair pathways has expanded massively. In keeping with the focus of this thesis, the role of the UPS in DSB repair will be reviewed below.

Crosslinking and CHIP analysis have shown the presences of subunits of the 26s proteasome at DSBs, suggesting that proteolysis is occurring alongside DSB repair (Krogan et al., 2004). Components of both NHEJ and HR have been shown to interact with the proteasome, such as DNA polymerase IV (Pol4) and Rad52 (Tseng and Tomkinson, 2002, Krogan et al., 2004). In yeast, HR protein Rad52 has been shown to associate with Sem, a component of the 19s proteasome. Following knockout of Rad52 the recruitment of Sem1 to DSBs is reduced. Furthermore, knockout of *Sem1* in yeast capable of only HR or NHEJ, but not both, conferred impaired cell growth suggesting that *Sem1* and the proteasome are required for the successful repair of DSBs by HR (Krogan et al., 2004). Interestingly, the human homolog of yeast Sem1 Deleted in Split hand/Split foot 1 (DSS1), is also part of the human 19s proteasome and implicated in HR through its interaction with Brca2 (Marston et al., 1999). Similar to the knockout of BRCA2 (Yuan et al., 1999b), knockdown of DSS1 significantly reduces HR in human cells, inhibition of the proteasome result in a smaller reduction on HR, suggesting DSS1 has a role in HR beyond proteolysis (Kristensen et al., 2010).

HR appears to be more reliant on proteasome activity than NHEJ. Gudmundsdottir et al observed that treatment with the proteasome inhibitor Epoxomicin in an ES cell line lead to a shift towards the error-prone single-strand annealing pathway from the error-free gene conversion pathway in the repair of repetitive elements (Gudmundsdottir et al., 2007). Similarly it has been observed that inhibition of the proteasome with MG132 and LC caused a reduction in HR-dependent DSB repair and only had a modest effect on NHEJ (Murakawa et al., 2007). Moreover Ku-70 deficient cells that have impaired NHEJ show reduced repair

kinetics in response to X-Ray induced DSBs when the proteasome is inhibited with MG132 treatment. Treatment of HeLa cells with MG132 inhibits the formation of RAD51 foci and Brca1 foci in response to X-ray irradiation suggesting that the proteasome is involved in the early stages of HR, prior to the formation of the Brca2-DSS1 complex (Murakawa et al., 2007).

Due to the need for the controlled assembly and degradation of protein complexes in order to execute successful DNA damage repair programs it is unsurprising that proteasomal degradation has been shown to be integral to many stages of this process.

### 1.4.3 UPS and Ageing

The accumulation of damaged and misfolded proteins is well documented during the ageing process. This loss of protein homeostasis has been suggested to effect the ageing process directly, as well as influence many age associated diseases (Lopez-Otin et al., 2013). The UPS is a key component of the proteostasis network that has been shown to decline in function with age. This dysfunction has been shown to occur at a number of levels such as a decreased expression of proteasomal subunits(Wooten et al., 2000), aberrant proteasome composition(Ferrington et al., 2005), proteasome disassembly (Ferrington et al., 2005) or inactivation via interaction with protein aggregates (Grune et al., 2004). This can set up a catastrophic cycle where reduced proteostasis results in increased aggregate formation which in turn can inhibit proteasomal degradation further. This cycle was highlighted recently in a study by Andersson *et al* where they showed by enhancing protein disaggregation they could restore functional proteasome activity in aged yeast (Andersson et al., 2013).

A decline in proteasome function has been observed in many mammalian cell types and tissues (Bulteau et al., 2000, Carrard et al., 2003, Chondrogianni et al., 2003, Petropoulos et al., 2000, Wagner and Margolis, 1995, Bardag-Gorce et al., 1999, Bulteau et al., 2002, Conconi et al., 1996, Husom et al., 2004, Keller et al., 2000, Shibatani et al., 1996). Interestingly, a decline in proteasome function is also a common feature of cellular senescence (Chondrogianni et al., 2003). Furthermore, treatment of fibroblasts with proteasome inhibitors leads to a shortened replicative life span and the induction of a senescent like phenotype (Torres et al., 2006). In addition, transgenic mice with reduced proteasomal chymotrypsin-like activity have a reduced lifespan and exhibit a premature ageing phenotype as well as enhanced age-related metabolic disorders such as obesity and fat accumulation in hepatic cells (Tomaru et al., 2012).

Notably, there have been several reports that proteasome activity correlates with longevity in long lived animals such as the naked mole rat (Perez et al., 2009) and the giant clam (Ungvari et al., 2013). Interestingly, proteasome activity is also increased in particularly long lived humans. A study by Chondrogianni *et al* found that the levels of several proteasomal subunits, as well as proteasomal activity in

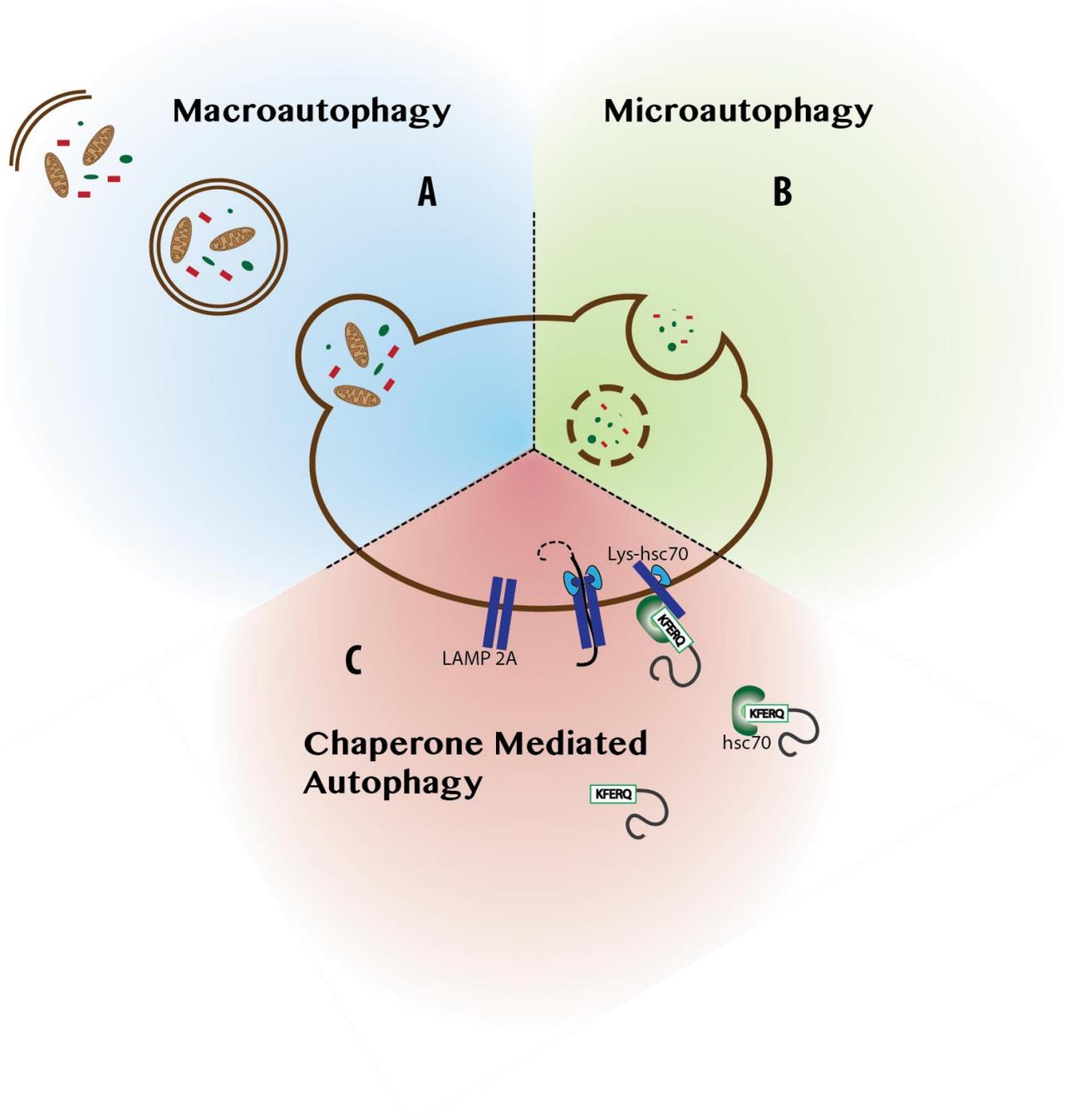
fibroblasts derived from centenarians was comparable to younger, rather than older control donors (Chondrogianni et al., 2000).

The link between proteasome activity and longevity has been further strengthened by studies taking a genetic approach. For example, ectopic expression of 19S proteasome subunits has been shown to extend lifespan in model organisms such as *D. melanogaster* and *C. elegans*. Overexpression of Rpn11 reduces the age-dependent reduction of 26S/30S proteasome activity and leads to an extension of lifespan in *D. melanogaster*. Additionally, increased Rpn11 levels are able to suppress expanded PolyQ-induced progressive neurodegeneration (Tonoki et al., 2009). Similarly, In *C. elegans* overexpression of Rpn6 confers protection against toxic aggregates in PolyQ-disease models and extends lifespan under conditions of proteotoxic stress (Vilchez et al., 2012). Interestingly, overexpression of the 20S proteasome subunit  $\beta 5$  in human fibroblasts leads to increased numbers of proteasomes and confers resistance to oxidative stress, delaying the onset of cellular senescence (Chondrogianni et al., 2005). Similarly, overexpression of another protein involved in proteasome assembly, the proteasome chaperone POMP, increases proteasome activity and protects against oxidative stress in human fibroblasts (Chondrogianni and Gonos, 2007).

Increasing evidence suggests that the functional decline of the proteasome and accumulation of damaged proteins are indeed determinants of the ageing process. Long-lived individuals (centenarians), as well as long lived model organisms positively correlate with increased functional proteasome activity. Moreover, genetic manipulations that enhance proteasome activity not only confer resistance to proteotoxicity and oxidative stress, they also extend lifespan. These data, taken together, indicate that proteasomal activity plays a key role in stress resistance, DNA damage repair, cellular senescence and organismal ageing.

## 1.5 Autophagy

Like the UPS, autophagy is a cellular degradation pathway that has been closely linked to the maintenance of cellular homeostasis. Autophagy has also been implicated in stress resistance, DNA damage repair, cellular and organismal ageing. Autophagy translates from the Greek “Auto” meaning oneself and “Phagy” meaning to eat and describes catabolic cellular degradation pathways involving delivery of cargo to lysosomes. There are three main described autophagic pathways present in eukaryotic cells shown in Figure 1-5. These include: microautophagy (direct engulfment and degradation of portions of the cytoplasm via invagination of the lysosome (Mijaljica et al., 2011), chaperone-mediated autophagy (direct translocation of targeted proteins containing the KFERQ motif into the lysosome via the LAMP-2A receptor) and macroautophagy (formation of a double membrane around cytoplasmic proteins and organelles which seals to form organelles known as autophagosomes). These autophagosomes are then trafficked to lysosomes where they fuse to deliver their cargo for degradation by lysosomal enzymes (Bejarano and Cuervo, 2010). Since the discovery of autophagy-related (ATG) proteins in yeast, the machinery of autophagy has been dissected in great detail. A key breakthrough in understanding of the molecular machinery came from genetic studies in yeast in which 35 ATG genes have been identified. These are genes that are essential canonical autophagosome formation (Nakatogawa et al., 2009).



**Figure 1-5: Schematic diagram of the three main forms of autophagy in mammalian cells.** (A) Formation of double membrane vesicles that then travel along microtubules and fuse with the lysosome (Macroautophagy). (B) Invagination of the lysosomal membrane to engulf regions of the cytoplasm (Microautophagy). (C) Selective uptake of proteins containing KFERQ motif across the lysosomal membrane via LAMP-2A mediated by chaperones Hsc70 and LysHsc-70 (Chaperone Mediated Autophagy) Abbreviations: hsc70: heat-shock cognate protein of 70 KDa, LAMP-2A: lysosome associate membrane protein type 2A. Figure adapted from (Hewitt et al., 2015).

### 1.5.1 Chaperone-mediated autophagy

Unlike the other forms of autophagy, CMA is an exclusively selective degradation process. As the name may suggest, CMA is aided by a protein chaperone called heat shock cognate protein (hsc70). The interaction of hsc70 with cytosolic protein substrates is mediated by a consensus pentapeptide motif KFERQ found in all CMA-targeted proteins (Figure 1C). Hsc70 alone or in complex with a protein substrate is able to bind to a plethora of co-chaperones through interactions that are thought to participate in specific substrate recognition, substrate delivery, protein unfolding and the final protein translocation across the lysosomal membrane. Delivery of the protein substrate to the lysosome is facilitated by binding to the cytosolic tail of the transmembrane protein, lysosomal-associated membrane protein 2A (Lamp-2A). The substrate protein is then unfolded and transported into the lumen of the lysosome via a poorly understood mechanism. Following translocation the substrate is rapidly degraded by hydrolytic enzymes (Ravikumar et al., 2010).

The mechanisms that regulate CMA activation, substrate recognition, transport and translocation are not well understood. Evidence suggests, however, that regulation of LAMP-2A expression levels is particularly important for efficient CMA and indeed the level of lysosome-associated LAMP-2A directly correlates with CMA activity. Thus, induction of CMA by oxidative stress has been shown to induce LAMP-2A transcription and in CMA-activating conditions the protein half-life of LAMP-2A increases, thus enhancing the CMA response. In addition, during prolonged CMA activation, LAMP-2A can be transported from the lysosomal membrane into the matrix but can seemingly be retrieved from an intact pool of LAMP-2A and reinserted back into the lysosomal membrane, again ensuring a robust maintenance of CMA response (Cuervo and Dice, 2000b).

Interestingly, while only approximately 25 proteins have been identified as substrates for CMA, the targeting motif, KFERQ is relatively common in cytosolic proteins (approximately 30%). Proteins that have been confirmed to be CMA substrates participate in a range of cellular processes including glycolysis, transcription and proteasome-based protein degradation. CMA is therefore an important participant in the general turnover of proteins required to maintain cellular homeostasis.

### 1.5.2 Microautophagy

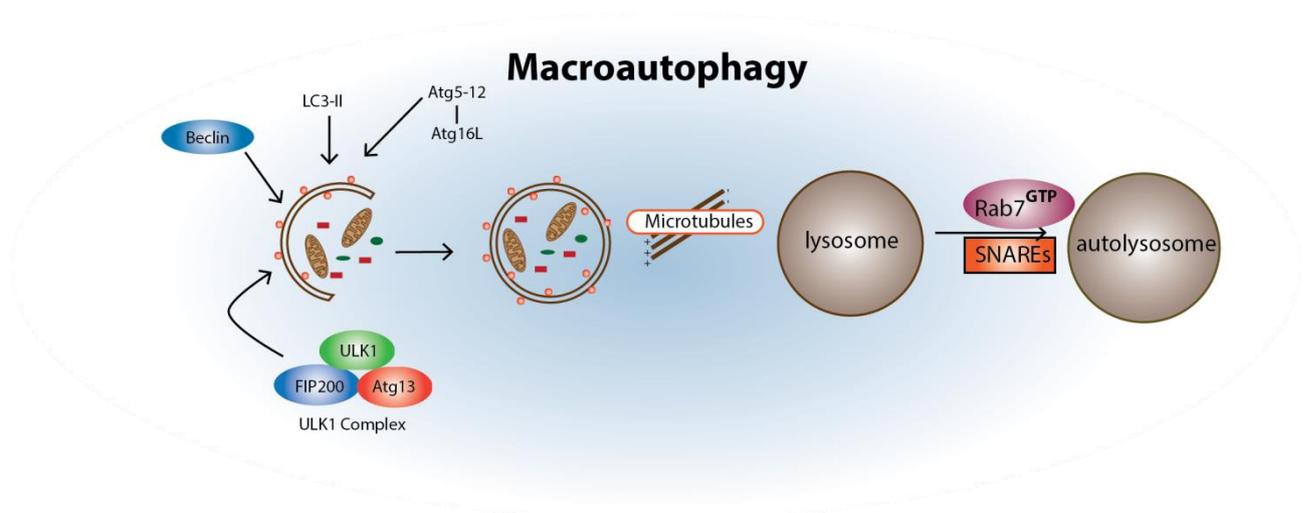
Direct delivery of cytoplasmic contents, either by lysosomal membrane invagination or protrusion is referred to as microautophagy (Figure 1-5B). The specific regulatory mechanisms are poorly understood in mammalian cells but seminal work in yeast indicates that microautophagy can mediate degradation of cytoplasmic contents via both non-selective and selective mechanisms, reviewed in (Mijaljica et al., 2011). Indeed, direct lysosomal-engulfment of mitochondria and nuclear fragments have been observed in yeast and are referred to as micromitophagy (Li et al., 2012b) and micronucleophagy (Boya and Codogno, 2012), respectively. Since little is known about any specific regulators of microautophagy, the primary tool for investigating this process is electron microscopy, limiting the scope for experimentation. Despite this, the process of microautophagy was characterised to occur via five main steps: 1) invagination of the lysosomal membrane, 2) vesicle formation, 3) vesicle expansion, 4) vesicle scission and 5) vesicle degradation (Li et al., 2012b). Due to the poorly defined molecular mechanisms in mammalian cells microautophagy will not be discussed further in this chapter except to say that, in yeast models at least, it shares some key upstream regulators with macroautophagy including the autophagy-related Atg proteins and the potent negative regulator, target of rapamycin (TOR) (Li et al., 2012b). Future work will undoubtedly unravel the current mystery that is microautophagy and of particular interest will be to identify how common upstream regulators are able to influence multiple protein degradation pathways.

### 1.5.3 Macroautophagy

Macroautophagy is an evolutionarily conserved catabolic process involving the formation of double-membrane vesicles (autophagosomes) that engulf cellular macromolecules and organelles, which are finally transported along microtubules leading to fusion with lysosomes and degradation of their contents (Figure 1-6). It is responsible for what is often referred to as bulk degradation; it has the broadest range of substrates and is by far the best studied of all three autophagic pathways, often being referred to simply as autophagy. Macroautophagy occurs in all cells at

basal albeit varying levels and these basal levels are often reflective of cellular function and energy demands. The most well-known regulator of macroautophagy is the serine/threonine kinase mTOR as part of the macromolecular complex (in mammals called mTORC1), which is activated in nutrient-rich, low cellular stress conditions and promotes protein translation and cell growth. In these growth-promoting conditions macroautophagy is largely suppressed. Cellular starvation (amino acids and growth factors), in addition to many other cellular stressors such as hypoxia and DNA damage, can lead to the inactivation of mTORC1 and subsequently activate macroautophagy. This up-regulation of autophagy facilitates the removal of unwanted or damaged cellular components and proteins freeing molecular building blocks and energy for other cellular processes.

### 1.5.4 Autophagic Machinery



**Figure 1-6: Schematic diagram of macroautophagy:** Macroautophagy involves the sequestration of cytoplasmic contents, such as lipids, organelles and proteins into a double-membrane-bound organelle. These structures are called autophagosomes and they are transported along the microtubule network towards the perinuclear, lysosome-rich microtubule organising centre (MTOC). Autophagosomes fuse with lysosomes in a Rab7- and SNARE-dependent manner and deliver their contents for hydrolytic degradation. Macroautophagy induction is regulated by a number of autophagy related proteins and protein complexes which interact sequentially orchestrating the proper initiation, elongation and maturation of autophagosomes. Figure adapted from (Carroll et al., 2013).

The formation of phagophores (the precursor to autophagosomes) requires two protein complexes the Beclin1/Vps34 complex and the ATG1 (or ULK1) complex. The ULK1 complex is negatively regulated by mTOR (Jung et al., 2009), this is the major regulatory signal for autophagosome formation and will be discussed in further detail later in this chapter. The origin of the autophagosomal membrane component is not fully known, however it is likely to come from either endoplasmic reticulum, mitochondria, Golgi or the cell membrane (Tooze and Yoshimori, 2010).

The elongation of the double membrane structure, a crucial stage in autophagosome formation, involves two ubiquitination like reactions. Firstly, Atg7 conjugates Atg12 to Atg5, these conjugates then interact non-covalently with Atg16 to form a complex. The Atg12-Atg5-Atg16 complex associates with phagophores during autophagosome formation but dissociates at its completion. The role of this protein complex is not yet fully understood, however it is necessary for normal autophagosome formation (Lin et al., 2007). Genetic knockout of *Atg5* leads to a complete abolition of autophagy, however the autophagic machinery is able to function even when Atg5-Atg12 conjugate levels are reduced by 90% (Hosokawa et al., 2007) indicating that only very low levels of Atg5-Atg12 are required for autophagy. Secondly, microtubule-associated protein 1 light chain 3 (LC3) is conjugated to the lipid phosphatidylethanolamine (PE) in the phagophore membrane by Atg7 and Atg3. Here it is converted from its soluble form LC3-I to its autophagosome associated form LC3-II (Yang and Klionsky, 2010). Detection of LC3-II by microscopy or western blotting is often used to measure cellular autophagy (Mizushima et al., 2010). Phagophores then elongate and engulf cytoplasmic cargo. Following the formation of these LC3-II-positive vesicles (autophagosomes) they are trafficked along microtubules towards the late endosome or lysosome. Upon meeting the lysosome the two structures fuse and the autophagic cargo is delivered for degradation (Mizushima, 2007).

### 1.5.5 Selective Autophagy

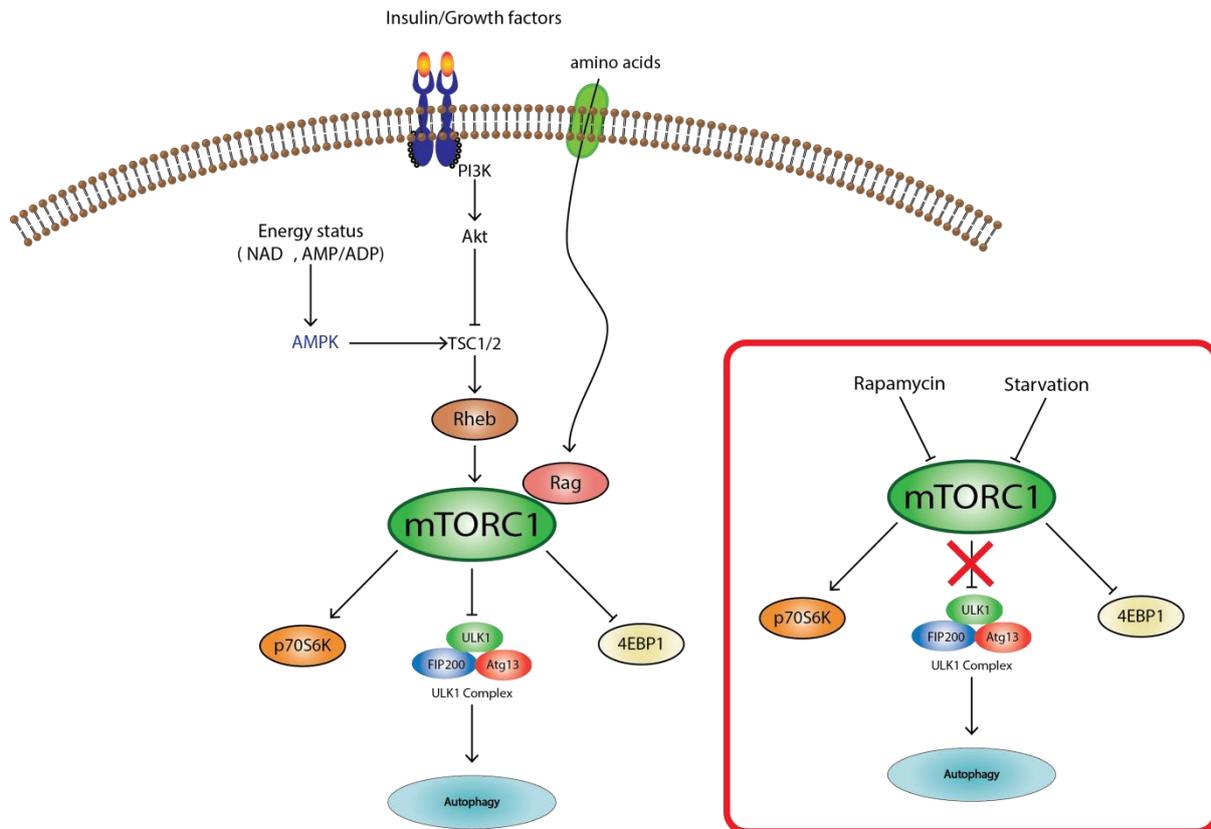
Macroautophagy was originally thought to be a non-specific bulk degradation pathway, however there is now strong evidence which suggests that autophagy can, in fact, be highly specific (reviewed in (Reggiori et al., 2012)). Autophagy has been shown to be involved in the selective turnover of many organelles including the endoplasmic reticulum (ER) (reticulophagy), peroxisomes (pexophagy), parts of the nucleus (nucleophagy), pathogens (xenophagy), protein aggregates (aggrephagy), ribosomes (ribophagy) and mitochondria (mitophagy) (Klionsky et al., 2007). This quality control role of autophagy requires that it is able to distinguish between functional and dysfunctional substrates. The molecular mechanisms underlying this substrate selection is relatively unknown. Similarly to the UPS, Ubiquitylation has been shown to play a role in this specificity by acting as a signal for selective autophagy (Kraft et al., 2010).

### 1.5.6 Regulation of Autophagy

Given the large number of stimuli able to modulate autophagy it is unsurprising that numerous signalling pathways are involved in its regulation. One of the most important regulators of autophagy is the mTOR pathway.

#### 1.5.6 mTOR pathway

The mTOR pathway regulates a number of cellular functions such as cell growth, protein translation, lipid biogenesis, DNA synthesis, mitochondrial biogenesis and autophagy (Wullschleger et al., 2006). mTOR is an atypical serine/threonine kinase that exists in functional complexes: mTOR complex 1 (mTORC1) made up from the mTOR catalytic domain, regulatory associated protein of mTOR (raptor), G protein  $\beta$ -subunit-like protein (G $\beta$ L), proline-rich Akt substrate of 40 kDa (PRAS40). mTORC1 is rapamycin sensitive and is the branch of the mTOR pathway responsible for the regulation of autophagy (Levine and Kroemer, 2009). mTOR complex 2 (mTORC2) is composed of mTOR, rapamycin-sensitive companion of mTOR (rictor), G $\beta$ L, SAPK-interacting protein 1(SIN1) and protein observed with rictor (PROTOR). mTORC2 is not believed to be a direct regulator of autophagy (Jung et al., 2010).



**Figure 1-7: mTOR regulation of autophagy.** The phosphatidylinositol 3-kinase (PI3K)/mTOR pathway is activated by the binding of insulin or growth factors to cell surface receptors. Activated PI3K converts Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>), which recruits and activates Akt. Akt then inactivates TSC1/2 leading to activation of Rheb and mTORC1. AMPK senses changes in the adenosine triphosphate (ATP)/ adenosine monophosphate (AMP) ratio and directly phosphorylates tuberous sclerosis 1 (TSC1) tuberous sclerosis 2 (TSC2) activating it, which results in inactivation of mTORC1. Amino acids can activate mTORC1 via Rag GTPases and Rheb and suppress autophagy. mTORC1 suppresses autophagy via phosphorylation-dependent inhibition of ULK1 and Atg13. Inhibition of mTORC1, such as that caused by rapamycin or starvation, leads to dissociation of mTORC1 from the ULK1-Atg13-200 kDa FAK-family interacting protein (FIP200) complex resulting in a dephosphorylation-dependent activation of autophagy.

There are many different cellular signals that converge to modulate the mTORC1 pathway such as glucose, energy status, amino acids, growth factors and different forms of stress (Sarbassov et al., 2005). Amino acids and growth factors activate mTORC1 via Rag GTPases, these bind raptor and target mTOR to cellular compartments that contain its activator, small GTPase Rheb (Sancak et al., 2008). Further light was shone on this process when it was shown that amino acids induce recruitment of mTORC1 to lysosomal membranes via a trimetric complex known as Ragulator. This recruitment places mTORC1 in close proximity to membrane bound Rag GTPases with the Ragulator acting as a docking site, allowing the activation of mTOR via Rheb (Sancak et al., 2010). It has also been suggested that constitutive targeting mTORC1 to the lysosomal membrane eliminates sensitivity to amino acids (Sancak et al., 2010). This highlights this translocation event as an essential part in the regulation of mTORC1 by amino acids.

Recently the autophagy adaptor protein p62 has been shown to be an integral part of the mTORC1 complex and is necessary to mediate amino acid signaling. p62 interacts with mTOR and raptor in an amino acid dependent manner. Additionally, p62 is able to bind Rag proteins favoring the formation of the active Rag heterodimer. p62 co-localises with Rags at the lysosome and has been shown to be required for targeting mTORC1 to the lysosome membrane (Duran et al., 2011).

The Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway is a major signalling cascade that feeds into mTORC1. Class 1a PI3K is activated by the binding of insulin and growth factors to cell surface receptors. Following this, the regulatory subunit facilitates activation of the p110 catalytic subunit by direct interaction with phosphotyrosine residues of activated receptors. Once activated, PI3K converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>), wherein pleckstrin homology (PH) domain proteins such as AKT and phosphoinositide-dependent kinase 1 (PDK1) are recruited to the plasma membrane (Cantley, 2002). Activation of PI3K leads to the recruitment and activation of AKT which in turn phosphorylates and deactivates TSC2. TSC2 inhibits mTOR through Rheb (Ras homolog enriched in brain). Once TSC2 is inactivated, Rheb is maintained in its GTP-bound state leading to the activation of mTOR (LoPiccolo et al., 2008).

mTORC1 can also act as a sensor for changes in cellular energy via AMP-activated kinase (AMPK) (Meijer and Codogno, 2006). AMPK senses changes in the

ratio between ATP and AMP and directly phosphorylates TSC2 facilitating the subsequent additional phosphorylation by glycogen synthase kinase 3 (GSK-3) resulting in an inhibition of mTORC1 signaling (Inoki et al., 2006).

Activation of mTORC1 leads to phosphorylation of two downstream effectors: p70 S6 Kinase (p70S6K) and translation initiation factor 4E binding protein-1 (4EBP1) at Thr389/Thr421/Ser424 and Thr37/Thr46, respectively (Han et al., 1995). This phosphorylation leads to activation of cell growth and protein translation. The activity of mTORC1 can be inhibited by rapamycin, an antibiotic originating from *Streptomyces hygroscopicus*. In mammalian cells rapamycin exerts its effect by forming a complex with the immunophilin FK506-binding protein of 12 kDa (FKBP12). This stabilises the mTOR-raptor interaction and inhibits mTOR kinase activity (Kim et al., 2002). Neither p70s6K or 4EBP1 are directly involved in the regulation of autophagy, which will be expanded upon on the following section.

### 1.5.7 mTOR regulation of autophagy

Activation of mTORC1 is the main negative regulator of autophagy (Sarbasov et al., 2005) (Figure 1-7). Inhibition of mTORC1 by rapamycin is a potent inducer of autophagy. Nutrient deprivation also activates autophagy via stabilisation of the mTOR-raptor complex. This activation is thought to serve to recycle intracellular components and provide an alternative source of amino acids (Ravikumar et al., 2010). The importance of the up-regulation of autophagy as an alternative means of supplying cellular energy is highlighted in *Atg5<sup>-/-</sup>* mice. Mice lacking *Atg5* are autophagy deficient and are unable to survive the neonatal starvation period and die shortly after birth (Kuma et al., 2004). *Atg5<sup>-/-</sup>* mice are only able to survive early embryogenesis due to maternally inherited *Atg5*. Eliminating maternal *Atg5* protein with oocyte-specific *Atg5*-knockout results in embryonic lethality (Tsukamoto et al., 2008). The exact mechanism for which autophagy is required is not yet understood, however it has been suggested that it could be needed to meet the high demand for amino acids required for protein synthesis at this early stage in embryogenesis (Tsukamoto et al., 2008).

Experiments initially carried out in yeast began to shine light onto the mechanisms linking TOR signalling to autophagy. It is thought that *Atg1* and the

Atg1-Atg13-Atg17 complex act downstream of TOR to regulate autophagosome formation (Kamada et al., 2000). More recently studies have identified some of the key molecular components acting downstream of mTORC1 in the regulation of autophagy in mammalian cells. Similar to yeast, the ULK1/2-Atg13-FIP200 complex is involved in the initiation of autophagy downstream of mTORC1 (Jung et al., 2009). Under nutrient rich conditions autophagy is inhibited by direct interaction of mTOR with the ULK1/2-Atg13-FIP200 complex and mTORC1 dependent phosphorylation of Atg13 and ULK1, which results in a reduction in kinase activity. Conversely, following starvation, mTOR dissociates from the complex resulting in a reduction of mTOR dependent phosphorylation of Atg13 and ULK1. This causes a dephosphorylation-dependent increase in kinase activity of ULK1 resulting in ULK1 mediated phosphorylation of Atg13 and FIP200 leading to an induction of autophagy (Jung et al., 2010, Kim et al., 2011, Egan et al., 2011).

### 1.5.8 mTOR-independent regulation of autophagy

The first study to show mTOR independent regulation of autophagy was published by Sarkar *et al* in 2005, where they show that autophagy is negatively regulated by intracellular inositol and inositol 1,4,5-trisphosphate (IP<sub>3</sub>) levels (Sarkar et al., 2005). This pathway is initiated by G protein coupled receptor activation of phospholipase C (PLC), this hydrolyzes PIP<sub>2</sub> to form IP<sub>3</sub> and diacylglycerol (DAG) (Berridge, 1987). IP<sub>3</sub> is then able to bind to its receptors (IP<sub>3</sub>R) on the endoplasmic reticulum (ER), causing the release of stored calcium, activating a large number of cellular responses (Berridge et al., 2003). Autophagy can be induced by a number of inositol-lowering agents, this activation is dependent on downstream levels of IP<sub>3</sub> as pharmacological interventions that raise levels of IP<sub>3</sub> such as *myo*-inositol or an inhibitor of prolyl oligopeptidase prevent the induction of autophagy (Sarkar et al., 2005). Moreover, direct reduction of IP<sub>3</sub> such as that caused by over-expression of cytosolic IP<sub>3</sub> kinase A, which phosphorylates IP<sub>3</sub> to inositol 1,3,4,5-tetrakisphosphate (IP<sub>4</sub>), induces autophagy (Williams et al., 2008). IP<sub>3</sub>R has been shown to regulate the Beclin 1 complex and Xestospongine B, an IP<sub>3</sub>R antagonist, disrupts the interaction of Beclin 1 and IP<sub>3</sub>R/Bcl-2 complex causing the induction of autophagy (Vicencio et al., 2009)

### 1.5.9 Regulation of autophagy by ROS

Aberrant levels of reactive oxygen species (ROS) are a common intracellular stress that leads to the induction of autophagy. Starvation, a potent inducer of autophagy, has been shown to increase ROS in an PI3K-dependent manner, whilst treatment with antioxidants reduces the ability of starvation to induce autophagy (Scherz-Shouval et al., 2007). ROS has been suggested to affect autophagy directly through interaction with Atg4. It has been shown that oxidation of a conserved cysteine residue in position 81 of Atg4 inhibits its catalytic activity preventing the cleavage of LC3 from the autophagosomal membrane thus promoting lipidation of LC3 and inducing autophagy (Scherz-Shouval et al., 2007). Interestingly the down regulation of ROS with antioxidants causes a down regulation of basal autophagy, consistent with ROS playing a positive regulatory role on autophagy (Scherz-Shouval and Elazar, 2011)

### 1.5.10 Autophagy Adaptor Proteins

Targeting of substrates for selective degradation via autophagy usually requires specific cargo-recognising adaptor molecules. These adaptor molecules serve to couple substrates with LC3 in pre-autophagosomal membranes. There are a number of different adaptor molecules involved in the targeting of different substrates, however common to all is the ability to interact with ubiquitinated substrates and LC3 (Johansen and Lamark, 2011).

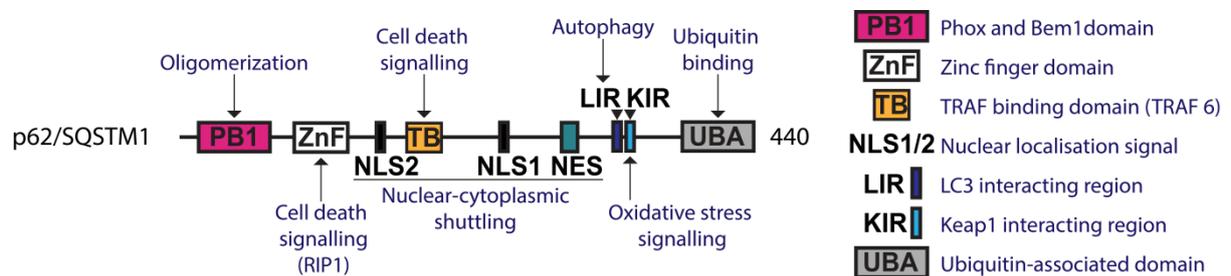
#### 1.5.10.1 p62

p62 also known as sequestosome 1 (SQSTM1), A170 or ZIP is a multifunctional protein induced in response to a number of cellular stresses. It serves as an autophagy adaptor selectively targeting polyubiquitinated proteins for degradation (Pankiv et al., 2007). It has also been shown to shuttle ubiquitinated substrates for degradation via the proteasome (Seibenhener et al., 2004), thus, playing a central role in cellular protein homeostasis. p62 has also been shown to act as part of many signalling pathways (Sanz et al., 1999, Sanz et al., 2000a, Joung et al., 1996). Mutations, the *sqtsm1* (p62) gene have been linked to a number of diseases, such as: Paget's disease of bone (Laurin et al., 2002, Hocking et al., 2002), familial and sporadic ALS (Teyssou et al., 2013). Interestingly recent research has shown dysregulation of p62 is common in many age-related pathologies such as neurodegeneration and cancer.

#### **Structure**

*sqtsm1* (p62) gene is highly conserved among vertebrates. The human *sqtsm1* (p62) gene is located on chromosome 5q35 and is comprised of 16Kb of genomic DNA. *Sqtsm1* contains 8 exons that encode a 440 amino acid protein. There are two protein isoforms of p62 arising from three mRNA variants. Protein isoform1 (440aa) is encoded by transcript variant 1 while protein isoform2 (356aa) is encoded by transcripts variant 2 and 3 which have the same coding sequence which lacks a portion of the 5'coding region compared to transcript variant 1 resulting in a slightly shorter N terminus in isoform2 compared to isoform1. Transcript variants 2 and 3 only differ slightly in their 5'UTR. Differences in the function and expression of these isoforms are not yet fully understood but are beginning to be investigated. A

recent paper from the Handa Laboratory suggests that Isoform1 is protective against oxidative stress while Isoform2 is transcriptionally active it is translationally inactive. (Wang et al., 2014). p62 has many conserved domains that mediate its multiple cellular functions (NCBI GeneID:8878) (Figure 1-1-8).



**Figure 1-1-8: Schematic representation of domain structure of p62:** p62 consists of: an N-terminal Phox and Bem1 (PB1) domain (which mediates interaction with protein kinases (aPKC, MEK5, MEKK3), NBR1 and oligomerisation), a zinc finger (ZnF) domain (which mediates interaction with RIP1), a (TRAF6) binding (TB) domain, two nuclear localization signals (NLS) and one Nuclear Export Signal (NES) (which mediate shuttling of p62 in and out of the nucleus), LC3 interacting region (LIR), KEAP1-interacting region (KIR) and a Ubiquitin Association (UBA) domain responsible for binding to ubiquitin.

The N-terminal Phox and Bem1 (PB1) domain (aa 20–102) allows p62 to interact with other PB1 containing proteins as well as to oligomerise (Lamark et al., 2003). The PB1 domain of p62 mediates interactions of p62 with several protein kinases such as atypical protein kinase C (aPKC) (Joung et al., 1996) and the MAPK kinase MEK5 (Lamark et al., 2003)-one of the ways in which p62 participates in signal transduction cascades. Moreover, PB1-mediated oligomerisation has been shown to be central to the role of p62 in the selective degradation of protein substrates via autophagy (Itakura and Mizushima, 2011, Pankiv et al., 2007). p62 has also been shown to interact with the autophagy adaptor NBR1 via its PB1 domain (Kirkin et al., 2009).

The C-terminal Ubiquitin Association (UBA) domain (aa 389–434) is necessary for p62 to bind to ubiquitinated proteins and organelles (Seibenhener et al., 2004, Vadlamudi et al., 1996, Geisler et al., 2010). The UBA functional domain is a motif of 45 amino acids which is conserved among proteins with the ability to bind to ubiquitin (Ub) (Hofmann and Bucher, 1996). The structure of the UBA has been elucidated using nuclear magnetic resonance and shows a compact three-helix bundle, with a hydrophobic surface on one side which is the proposed site for its interaction with Ub (Dieckmann et al., 1998, Bertolaet et al., 2001). p62 has an increased affinity for lysine 63 (K63) poly-ubiquitin chains over other forms of ubiquitination (Seibenhener et al., 2004). K63 linkage is believed to promote the formation of protein inclusions as well as target them for degradation predominantly via autophagy (Tan et al., 2008). This view however has been challenged by the Layfield laboratory where K63 affinity was not observed. Instead they observe equivalent affinity of p62 for K63 and K48 poly-ubiquitin chains (Long et al., 2008). The affinity of p62 for ubiquitin can be modulated by the phosphorylation of the UBA on Ser-403 by Casein Kinase II (CK2), increasing its binding to poly-ubiquitin and promoting the formation of p62 inclusion bodies. These p62-rich inclusion bodies or “sequestosomes” have been proposed to serve as signalling hubs and can be cleared by autophagy (Matsumoto et al., 2011).

p62 contains a zinc finger (ZnF) domain (aa 122–167) that primarily facilitates the involvement of p62 in a number of cytoplasmic signalling cascades. The ZZ domain is necessary for the binding of p62 to the receptor-interacting protein 1 (RIP1) kinase in the TNF receptor (TNF-R) complex. This binding allows the signalling of TNF-R through protein kinase C (PKC) and the activation of nuclear

factor-kappaB (NF $\kappa$ B) (Sanz et al., 1999). Aside from mediating the role of p62 in a number of cytoplasmic signalling cascades the ZZ domain has also been implicated in the association of p62 with a number of transcription factors in the nucleus such as the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) (Marcus et al., 1996) and has been suggested to stimulate transcription through the SV40 enhancer (Rachubinski et al., 1999)

p62 binds to TRAF6 via the TNF $\alpha$  receptor-associated factor 6 (TRAF6) binding (TB) domain. This p62-TRAF6 interaction is required for NF $\kappa$ B signalling from the interleukin 1 receptor (IL-1R) (Sanz et al., 2000b) and nerve growth factor receptor (NGF-R) (Wooten et al., 2000). Following stimulation, p62 binds TRAF6 via its TB domain and aPKC via its PB1 domain, serving as a scaffold that brings these proteins into close spatial proximity which facilitates kinase activation.

The presence of two nuclear localization signals (NLS) and one Nuclear Export Signal (NES) was first reported by Pankiv *et al.* (Pankiv *et al.*, 2010). They observed that p62 shuttles rapidly between the cytoplasm and the nucleus and that upon inhibition of nuclear export p62 lead to an accumulation of polyubiquitinated proteins in promyelocytic leukemia (PML) bodies. They also observed that nuclear p62 contributed to the formation of proteasome-containing degradative compartments (Pankiv et al., 2010). It was also previously suggested that p62 may enter the nucleus by binding other NLS containing proteins such as aPKC (Geetha and Wooten, 2002), The authors infer the presence of a NES signal as p62 accumulates in the nucleus in response to inhibition of exportin 1 with Leptomycin B treatment. They also claim that p62 does not contain its own NLS. In light of more recent work by Pankiv *et al.* it may be necessary further study whether p62 is able to enter the nucleus bound to another NLS containing protein, independently of its own NLS domain.

The LC3 interacting region (LIR) (aa 321–342) facilitates the interaction between p62 and LC3, a key component of the autophagosome membrane. This domain is key to the function of p62 as an autophagy adaptor protein, and is required for the degradation of p62, by autophagy (Pankiv et al., 2007). Interestingly, it has been shown that p62 is still able to localise to the nucleation point of autophagosomal membranes in the absence of the LIR domain. This interaction has instead been shown to be dependent on the PB1 domain and oligomerisation

(Itakura and Mizushima, 2011). It is however possible that this occurs due to PB1 dependent interactions with other LIR domains containing proteins such as NRB1.

The Kelch-like ECH-associated protein (Keap1) interacting region (KIR) (aa 346–355) mediates the association of p62 with Keap1 (Jain et al., 2010, Komatsu et al., 2010, Lau et al., 2010). The binding of p62 to Keap1 occurs on a site essential for Keap1 to bind nuclear factor (erythroid 2)-like 2 (Nrf2). This binding interrupts the repressor function of Keap1 on Nrf2 leading to its activation. Although the affinity of p62 for Keap1 is lower than that of Nrf2 (Komatsu et al., 2010), inhibition of autophagy and over expression of p62 have both been shown to sequester Keap1 leading to the activation of Nrf2 (Jain et al., 2010, Komatsu et al., 2010). The ability of p62 to bind and sequester Keap1 also requires the PB1 domain and the ability of p62 to form oligomers (Jain et al., 2010).

#### **1.5.10.2 Regulation of intracellular levels of p62**

##### ***Transcriptional regulation***

Nrf2 is one of the main players in the transcriptional regulation of p62. Nrf2 is a member of basic leucine zipper (bZIP) family of transcription factors. Nrf2 is a key regulator of the antioxidant response and upon increased oxidative stress translocates to the nucleus where it binds to the antioxidant-responsive element (ARE motif) located in the p62 promoter to induce expression of p62 mRNA (Jain et al., 2010). p62 is a positive regulator of Nrf2 and this sets up a positive feedback loop in which p62, through activation of Nrf2, is able to drive its own transcription (Jain et al., 2010). Additionally, analysis of the 5'-flanking region of the p62 promoter has revealed binding sites for numerous transcription factors, such as NF- $\kappa$ B, Ets-1 AP-1, and SP-1 (Vadlamudi and Shin, 1998). Expression of p62 has indeed been shown in response to transcription other than that induced from Nrf2 with p62 expression increasing very rapidly (30 mins - 2 hours) in response to stimuli such as calcium, interleukin 3 (IL-3) and phorbol 12-myristate 13-acetate (PMA) (Lee et al., 1998). Interestingly, RAS-transformed fibroblasts have been shown to have high levels of p62 mRNA expression, which is reduced upon invalidation of the AP-1

binding site present upstream on the p62 promoter. These findings suggest that p62 is regulated at the transcriptional level by the Ras/MEK/ERK1/2 pathway via the AP-1 domain in its promoter (Duran et al., 2008). p62 has also been shown to be transcriptionally regulated downstream on JNK/c-Jun pathway in chronic myelogenous leukemia (CML) cells (Puissant and Auberger, 2010, Puissant et al., 2010). The exact molecular mechanism, however, by which the JNK/c-Jun pathway elicits this effect on p62 transcription is still unknown.

### ***Post translational regulation***

As mentioned previously, p62 is a substrate for degradation by autophagy via its LIR domain-mediated interaction with the autophagosomal membrane (Pankiv et al., 2007). As a result, the intracellular levels of p62 can be greatly influenced by changes in autophagic flux. Thus, pro-autophagic stimuli such as starvation, hypoxia and treatment with autophagy inducing drugs lead to a decrease in intracellular p62. Similarly, inhibition of autophagy leads to an accumulation of p62. As such, intracellular levels of p62 are often used in the assessment of autophagic flux (Klionsky et al., 2012). Similarly, p62 has been shown to be a proteasomal substrate (Seibenhener et al., 2004) and so stimuli that modulate proteasomal activity could also influence intracellular levels of p62.

### **1.5.10.3 p62 as a Signalling Molecule**

p62 was initially identified as an interacting partner of Lck-tyrosine kinase and atypical protein kinase C ( $\alpha$ PKC) (Joung et al., 1996, Puls et al., 1997). Following its identification, p62 has been shown to participate in a number of signalling pathways. p62 has been shown to serve as a scaffold where its function is to bring together two or more components of a signaling pathway for example the ZZ domain of p62 binds active RIP1 kinase and PB1 domain binds protein kinase C  $\lambda/1$  (PKC  $\lambda/1$ ) in TNF-R signalling. Thus, upon stimulation of TNF-R p62, PKC  $\lambda/1$  and RIP1 form a complex facilitating NF $\kappa$ B activation. Disruption of the ZZ domain or reduced p62 expression is sufficient to impair TNF $\alpha$ -mediated NF $\kappa$ B activation (Sanz et al., 1999). p62 plays

a similar scaffold role in activation of NF $\kappa$ B downstream of interleukin-1 $\beta$  receptor (IL-1 $\beta$ R) (Sanz et al., 2000b) and the nerve growth factor receptors TrkA and p75<sup>NTR</sup> (Wooten et al., 2000). Here p62 binds to the ubiquitin ligase TRAF6 via its TB domain along with interleukin-1 receptor-associated kinase (IRAK). PKC  $\lambda$ /I is then recruited to this complex where it binds to the PB1 domain of p62 and is phosphorylation by IRAK leading to its activation (Sanz et al., 2000b, Mamidipudi et al., 2002). p62 plays further roles in IL-1 $\beta$  and NGF signal transduction aside from acting as a scaffold, with the UBA domain and PB1 domain of p62 having been shown to be necessary for the oligomerisation and self-ubiquitination of TRAF6 (Wooten et al., 2005). Moreover, p62 has been shown to act as a link between two NGF receptors, TrkA and p75<sup>NTR</sup>. The formation of this bridge facilitates the ubiquitination of TrkA via TRAF6, a process required for both receptor internalisation (Geetha et al., 2005) as well as turnover via the proteasome (Geetha et al., 2008). Unsurprisingly, it has been shown that in IL-1 signalling intracellular levels of p62 can modulate signal intensity through IL-1 $\beta$ R. As a result, increased degradation of p62 via the proteasome or autophagy lead to a dampening in IL-1 signalling causing an anti-inflammatory effect. This mechanism was first identified in *Atg16L1*<sup>-/-</sup> cells. Lee *et al* discovered that Atg16L1 is able to modulate levels of p62 via the proteasome and autophagy. Atg16L1 is essential for neddylation of Cul-3 (Cullin 3) and its activation. The Cul-3 complex has E3 ubiquitin ligase activity responsible for targeting p62 for proteasomal degradation. Therefore knock-out of Atg16L1 leads to a build up of p62 as a result of decreased autophagy and reduced degradation via the proteasome (Lee et al., 2012b). As well as impacting on IL-1 signalling the interaction of p62 with Cul-3 has been shown to stimulate the extrinsic apoptosis pathway. Here active Cul-3 is recruited to the death-inducing signalling complex (DISC) where it facilitates that addition of poly-Ub chains to caspase 8. Next, p62 facilitates aggregation of Ub-tagged caspase-8 leading to its auto-catalytic cleavage and activation (Jin et al., 2009).

p62 has also been implicated in amino acid sensing through mTOR. This is mediated through interaction of p62 with raptor, a key component of the mTORC1 complex, enabling its translocation to the lysosome (Duran et al., 2011). This is thought to facilitate interaction of mTOR with the Rag family of small GTPases (Sancak et al., 2010). It has been shown that TRAF6 is also important to this process. TRAF6 is involved in recruiting mTORC1 to the lysosome and mediates the

K63-linked ubiquitination of the mTOR subunit of the complex allowing optimal activation by amino acids (Linares et al., 2013). Knockdown of TRAF6, similar to p62 reduced proliferation and transformation-rate in cancer cells as well as increasing autophagic flux. This could be rescued by the expression of a constitutively active RagB mutant. Thus, p62 acts as a scaffold upstream of Rag proteins specifically regulating components involved in mTORC1 signalling (Linares et al., 2013, Duran et al., 2011). Recently, it was shown that phosphorylation of p62 at T269/S272 is critical to amino acid sensing through mTORC1 (Linares et al., 2015). The authors report that p62 is phosphorylated via a cascade that includes MEK3/6 and p38 and requires interaction with MEKK3 via its PB1 domain. This phosphorylation proceeds the recruitment of TRAF6 and the ubiquitination and activation of mTOR described previously (Linares et al., 2013). Importantly, mTORC1 can be activated through a number of different inputs. Amino acid sensing is the only known input to require p62 and so activation via insulin for instance can occur independently of p62 status (Duran et al., 2011).

p62 has been shown to activate an anti-oxidant response via its interaction with the Keap1-Nrf2 pathway (Komatsu et al., 2010). The Keap1-Nrf2 pathway is one of the major cellular antioxidant pathways (Itoh et al., 1999). Keap1 is a negative regulator of the antioxidant response element through its interaction with transcription factor Nrf2 (Itoh et al., 1999). In physiological conditions Nrf2 is sequestered in the cytoplasm via a strong noncovalent interaction with Keap1 homodimers. Keap1 then recruits Cul-3 leading to the ubiquitination of Nrf2 and its degradation by the proteasome (Cullinan et al., 2004, Furukawa and Xiong, 2005). Under oxidative conditions Keap1 is oxidised and undergoes a conformational change inhibiting its interaction with Nrf2, thereby inhibiting its degradation (Wakabayashi et al., 2004). This allows free Nrf2 to enter the nucleus where it is able to activate the transcription of a number of genes involved in ROS scavenging, DNA repair and mitochondrial function (Motohashi and Yamamoto, 2004).

p62 can lead to the activation of Nrf2 in the absence of oxidative stress. Here, p62 binds to Keap1 via its KIR domain preventing its inhibitory effect on Nrf2 (Lau et al., 2010). This interaction with p62 has also been shown to lead to the degradation of Keap1 via autophagy (Fan et al., 2010). The interaction between p62 and Keap1 is much weaker than that between Keap1 and Nrf2, therefore it has been suggested that p62 will only affect this pathway when it reaches supraphysiological levels, such

as those that occur due to inhibition of autophagy. Interestingly, there is a small portion of Keap1 that is constitutively bound by p62 (Copples et al., 2010) suggesting that there may be other influences such as post-translational changes to either p62 or Keap1 that may regulate this process (Bitto et al., 2014).

All of these findings show that p62 plays a role in multiple signalling pathways placing it at the heart of the maintenance of cellular homeostasis.

#### **1.5.10.4 p62 and Protein Homeostasis**

One of the most studied cellular functions of p62 is its role in protein homeostasis. This is a function that came to light with the discovery of its UBA domain (Seibenhener et al., 2004, Vadlamudi et al., 1996). p62 has been shown to facilitate the selective autophagy of proteins (Pankiv et al., 2007), organelles (Geisler et al., 2010) and bacteria (Zheng et al., 2009). These substrates are usually targeted for autophagy through tagging with poly-ubiquitin chains (K63 or K27 linkages) and association with the UBA domain of p62. These p62 bound substrates then form aggregates mediated by oligomerisation of p62 with other molecules of p62, or other PB1 domain-containing proteins. These p62-substrate aggregates are referred to as sequestosomes and are targeted to autophagosomes via the LIR domain of p62, where they are degraded (Pankiv et al., 2007). As well as facilitating the formation of aggregates, p62 is also able to bind to pre-existing protein inclusions such as mutant huntingtin aggregates (Bjorkoy et al., 2005). The formation of these inclusions is greatly enhanced by phosphorylation of UBA domain on serine 403, a modification that increases the affinity of p62 for polyubiquitin linkages (Matsumoto et al., 2011). The ability of p62 to form oligomers is essential for its ability to target aggregates to the initiation point of the autophagosomal membranes, a process essential for efficient engulfment of these substrates (Itakura and Mizushima, 2011). These data suggest that p62 plays more than just an adaptor function, linking substrates to the autophagosomal membrane and places it as an active player in the autophagy process (Komatsu et al., 2007). As well as being able to target ubiquitinated substrates for degradation via autophagy, p62 has also been shown to facilitate the autophagic clearance of non ubiquitinated aggregates via interaction with its PB1 domain (Watanabe and Tanaka, 2011).

As well as its role in autophagy p62 has been implicated in targeting ubiquitinated proteins to the proteasomal degradation pathway. Selection of which degradation pathway is chosen is thought to depend on posttranslational modification such as ubiquitin linkage. Interestingly, p62 is able to interact with the proteasome via its PB1 domain and knockdown of p62 can impair the degradation of proteasomal substrates (Seibenhener et al., 2004, Pankiv et al., 2010, Geetha et al., 2008). Furthermore, p62 contains two PEST sequences and is itself target for proteasomal degradation via Cul-3 mediated ubiquitination (Lee et al., 2012b). Interestingly, levels of p62 appear to play an important role in regulation of proteasomal degradation with reduction (Seibenhener et al., 2004) or an accumulation (Korolchuk et al., 2009) of p62 leading to an impairment of proteasomal degradation. This apparent dual role in the regulation of proteasomal degradation is dependent on intracellular levels of p62, while single molecules of p62 are able to bind to proteasomal substrates and target them for degradation, when levels of p62 increase beyond a certain threshold, large oligomers begin to form and proteasomal substrates become sequestered within them. These aggregates are themselves too large to be degraded via the proteasome, however, it has been suggested that the formation of aggregates comprised of p62 and proteasomal substrates, may be a route by which proteins classically regarded as substrates of the proteasome become targets for degradation by autophagy (Bitto et al., 2014).

#### **1.5.10.5 p62 and the DDR**

As discussed above, autophagy has been shown to affect DNA damage response and repair. One of the hypothesized mechanisms is through its regulation of cellular levels of p62. Thus, Bae and Guan have shown that in addition to a marked increase in p62 levels inhibition of autophagy (through the knock-out of FIP200) results in impaired repair of DNA damage induced by IR, Camptothecin and Etoposide (Bae and Guan, 2011). Knockdown of p62 in cells lacking FIP200 with shRNA restored DNA damage repair in response to CPT as measured by  $\gamma$ H2A.x foci. Moreover, p62 knock-down also rescued cell survival. Similarly, p62 knock-down partially rescued cell viability and DNA repair in response to IR- and Etoposide-induced damage (Bae and Guan, 2011). It has also been suggested that the increased levels of ROS seen in autophagy deficient cells may mediate

increased levels of DNA damage (Mathew et al., 2007b). However, treatment with the antioxidant NAC did not rescue deficient DNA damage repair in FIP200 KO MEFs compared to wild-type (Bae and Guan, 2011). These results suggest that the effect of autophagy knockdown on DNA damage repair is at least in part due to increased levels of p62 and can occur independently of ROS. However, the exact mechanism by which p62 can affect DNA damage repair is not yet understood. Although p62 is mainly located in the cytoplasm, the discovery of nuclear-cytoplasmic shuttling along with interaction between p62 and PML bodies within the nucleus (Pankiv et al., 2010) could provide an interesting avenue of research when trying to elucidate its role in the DDR. PML bodies have been implicated in DNA damage especially the orchestration of homologous recombination (Yeung et al., 2012).

#### **1.5.10.6 p62 in Disease**

##### **Cancer**

As described above p62 is involved in several signalling pathways including NF $\kappa$ B and Ras/Raf/MAPK and mTOR. These are signalling pathways that are commonly modified during tumour transformation and help promote proliferation migration and invasion of tumour cells. This, combined with the role of p62 in the regulation of autophagy (Duran et al., 2011), place p62 as a potential player in carcinogenesis. However, the exact contribution of p62 in tumour progression is still poorly understood, this is in part due to its wide range on cellular functions and depends on its involvement in pro-oncogenic signalling coupled with its role in both pro and anti-tumorigenic autophagy.

The transformation of cells requires them to reprogram metabolic pathways to escape cell death and acquire new invasive properties. Autophagy has long been known to be dysregulated during carcinogenesis (Mathew et al., 2007a). Yet, the exact role for autophagy in this process is still poorly understood with autophagy having been reported to have both pro and anti-tumorigenic activity. During the initiation of tumour growth autophagy helps cancer cells overcome metabolic stress, protecting against low O<sub>2</sub> and nutrients that occur due to reduced perfusion in the absence of vascularization (Degenhardt et al., 2006). Therefore, in the early stages of tumour development it is thought autophagy plays a pro-tumorigenic role.

However, during the later stages of tumour development loss of autophagy is thought to lead to increased levels of ROS as well as damaged organelles which, in turn, results in an increase in the rate of DNA mutations and loss of genome stability (Mathew et al., 2007b). Furthermore, this decreased autophagy leads to an increase in intracellular p62.

Accumulation of p62 has been seen in many different cancer types such as non-small-cell lung carcinoma (NSCLC) (Inoue et al., 2012) and breast cancer (Thompson et al., 2003). Levels of p62 have also been shown to correlate with a poor prognosis (Inoue et al., 2012) or increased severity (Rolland et al., 2007) of cancer in a clinical context. p62 is able to influence tumour progression independently from its role in autophagy. As described previously, p62 plays a role in a number of signalling pathways. Many of these interactions have been shown to be key to the role of p62 in cancer development and progression. Increased synthesis of p62, as a result of NF- $\kappa$ B activation downstream of constitutive Ras activation, has been observed in ductal pancreatic adenocarcinoma cells. Here, p62 through its interaction with TRAF6, serves to amplify Ras activation setting up a positive feedback loop (Ling et al., 2012). Similar p62-dependent activation of NF- $\kappa$ B signalling has been observed in lung cancer, Here this activation is thought to elicit an antioxidant response leading to inhibition of deleterious ROS (Duran et al., 2008). Interestingly, high levels of p62 and related MAPK activation have been observed in a subset of aggressive glioblastoma, with inhibition of p62 leading to a reduction in mortality and invasiveness of tumour cells (Galavotti et al., 2013).

p62 has also been observed to play a pro-tumorigenic role through its activation of the mTOR pathway (Duran et al., 2011). Knock-down of p62 using shRNA has been shown to inhibit the growth of tumour cells with hyper-activated mTOR (Duran et al., 2011). p62 has also been shown to be necessary for the development of tumours in *Tsc2*<sup>+/-</sup> mice (Parkhitko et al., 2011). Similarly, knockdown of p62 lead to increased survival in mice injected with (*TSC2*<sup>-/-</sup>) MEFs (Parkhitko et al., 2011). These results highlight the activation of mTOR via p62 as a key pro-tumorigenic event.

There are also data suggesting that p62 may play a tumour suppressive function in some contexts, particularly during mitosis (Linares et al., 2011). Here, they show that phosphorylation of p62 by cyclin-dependent kinase 1 (CDK1) at T269

and S272 is an important regulator of levels cyclin B1 and CDK1 activity which are necessary to properly control entry and exit from Mitosis. Lack of this phosphorylation leads to a more rapid exit from mitosis, resulting in increased proliferation and tumorigenesis following Ras-induced transformation (Linares et al., 2011).

Taken together these studies highlight the importance of p62 during cancer development, with p62 playing an apparent dual role in tumorigenesis, both dependent and independent of its role in autophagy. Further understanding of the involvement of both p62 and autophagy in the context of tumorigenesis will no doubt provide novel avenues for drug development and cancer treatment.

### **Neurodegeneration**

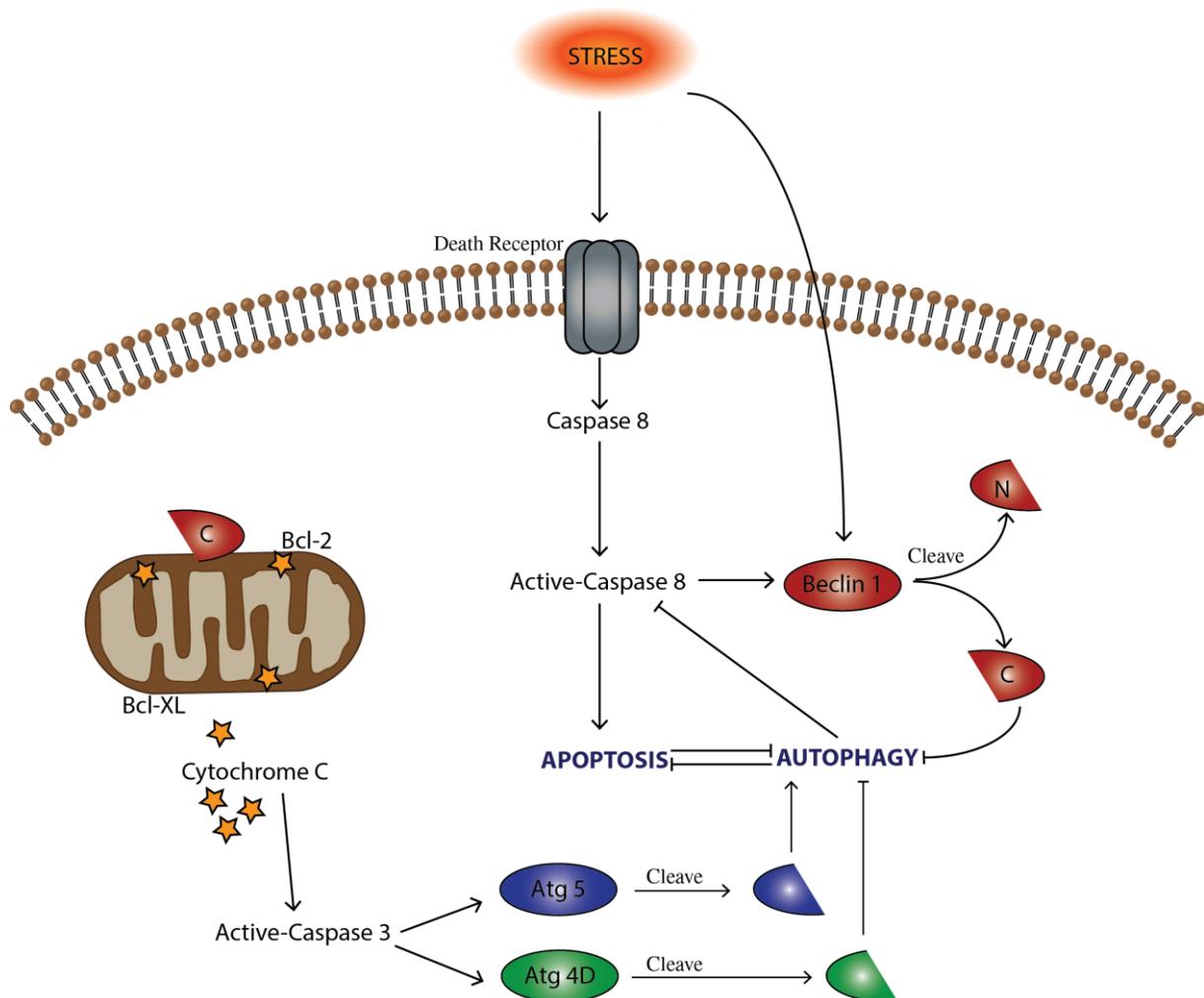
Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) are characterized by the buildup of protein aggregates in the brain. Different proteins are responsible for the aggregates seen in each disease but the presence of p62 associated with these aggregates is a common feature of many proteinopathies (Kuusisto et al., 2001). The presence of p62 in these aggregates was originally believed to be required for their formation (Kuusisto et al., 2002). However, more recently, p62 has been shown to associate with protein inclusions following their formation (Bjorkoy et al., 2005) , in the case of AD, p62 is responsible for the degradation of Tau via the proteasome (Babu et al., 2005). In agreement with p62 playing a protective role in AD, mice lacking p62 show many AD associated characteristics such as age-associated accumulation of K63-tagged tau, neuronal cell death, increased anxiety and reduced short term memory (Ramesh Babu et al., 2008). Interestingly, levels of p62 are reduced in AD brains compared to control. This is thought to occur due to oxidative damage to the promoter region of p62 (Du et al., 2009a), a process which has been shown to increase with age and is a common feature of many neurodegenerative diseases (Du et al., 2009b).

Both oxidative stress and mitochondrial dysfunction are common features of neurodegenerative diseases (Lin and Beal, 2006) particularly in PD (Geisler et al., 2010, Narendra et al., 2008). p62 has been suggested to play a role in the

maintenance of mitochondrial homeostasis, (Geisler et al., 2010, Narendra et al., 2010) as well as antioxidant defense through its role in the Keap1-NRF2 pathway (Lau et al., 2010). A loss of p62 has been shown to correlate with a reduction in Nrf2 activity in neurons of those affected by neurodegeneration (Ramsey et al., 2007). Interestingly, an *Nfe2l2* haplotype that confers increased transcriptional activity of Nrf2 appears to have a protective effect against the development of PD (von Otter et al., 2010). Similarly, expression of Nrf2 in a *Drosophila melanogaster* model of PD protects against neurodegeneration (Barone et al., 2011). These data show that p62 can have a protective effect on neurodegeneration both through its role in the clearance of pathogenic protein aggregates as well as its role in maintenance of mitochondrial homeostasis and antioxidant defence.

### 1.5.11 Autophagy and Apoptosis

The connection between autophagy and apoptosis and other forms of cell death is a growing area of research. Autophagy and apoptosis are regulated by common stimuli and signalling pathways (Figure 1-9).



**Figure 1-9: Crosstalk between apoptosis and autophagy.** Autophagy and apoptosis are controlled by overlapping signalling pathways and show a degree of mutual inhibition. Sustained induction of apoptosis leads to a caspase 8 mediated cleavage of Beclin 1 generation N and C fragments inhibiting their ability to induce autophagy. The C-terminal fragment translocates to mitochondria sensitizing the cell to pro-apoptotic signals. Although caspase-mediated cleavage of Atg5 and Beclin 1 has an inhibitory effect on autophagy, caspase 3-mediated cleavage of Atg 4D generates a fragment with increased autophagic activity. Autophagy can also inhibit apoptosis by degrading active caspase 8.

As described earlier, Beclin1 is essential for the formation of autophagic vesicles. Beclin1 is known to interact with Bcl-2, a key regulator of apoptosis as well as another anti-apoptotic protein from the Bcl family, Bcl-xL. Bcl-2/Bcl-xL not only inhibits apoptosis by binding pro-apoptotic proteins Bax and Bak, it also plays a role in the inhibition of autophagy through the binding of Beclin 1 (Pattingre et al., 2005). This interaction was shown to occur via the Bcl-2 homology 3 (BH3-only) domain on Beclin 1 (Feng et al., 2007). Conversely it has been shown that disruption of the interaction between Beclin 1 at the BH3 domain and Bcl-2 leads to an induction of autophagy (Maiuri et al., 2007). Activation of c-Jun N-terminal kinase 1 (JNK1) has been shown to phosphorylate Bcl-2 and cause it to dissociate from Beclin1, highlighting another mechanism by which stress can activate autophagy (Wei et al., 2008). Despite BH3-only proteins of the Bcl-2 family being well known inducers of autophagy and apoptosis, Beclin 1, which contains this domain, does not activate apoptosis even when overexpressed and autophagy is active (Boya and Kroemer, 2009). Instead, Beclin 1 has been shown to offer cytoprotection to several apoptotic stimuli including starvation, suggesting an anti-apoptotic role for Beclin 1 (Boya and Kroemer, 2009).

Caspases are cysteine aspartyl proteases that play a pivotal role in the execution of apoptotic cell death. Activated caspases cleave a large number of proteins usually resulting in inactivation through removal of regulatory domains (Yi and Yuan, 2009). Interestingly, caspases can cleave Beclin 1 during sustained exposure to pro-apoptotic stimuli, resulting in a loss of its pro-autophagic capacity (Wirawan et al., 2010). Atg5 and Atg4D can also be cleaved by caspases in response to apoptosis, the cleavage product of Atg5 is thought to interact with Bcl-2 proteins at mitochondria and help to induce cell death by apoptosis (Yousefi et al., 2006). Unlike cleavage of Atg5 and Beclin 1 that inhibits autophagy, cleavage of Atg4D generates a fragment that increases autophagic activity. This fragment has, however, been shown to have a cytotoxic effect independent from activation of autophagy thought to correlate with its recruitment to mitochondria (Betin and Lane, 2009). Thus, caspase-mediated cleavage of the autophagy components Atg5, Atg4D and Beclin 1 may serve to amplify the apoptotic programme through the mitochondrial pathway.

Recent work has identified Atg12, an essential autophagy protein, as a potential point of cross-talk between autophagy and apoptosis. In work by Rubinstein et al it was shown that Atg12 is a positive mediator of apoptosis, with knockdown of Atg12 leading to an inhibition of Bax activation and cytochrome c release. Additionally, it was shown that over-expression of Atg12 reduced the anti-apoptotic activity of Mcl-1 (Rubinstein et al., 2011). The cross-talk between autophagy and apoptosis highlights the importance of autophagy in determining cell-fate in response to stress.

### **1.5.12 Autophagy and the DDR**

Despite autophagy being a cytoplasmic process and DNA damage occurring in the nucleus, there is a growing body of evidence suggesting a link between the two processes. It has been observed that activation of autophagy occurs following the induction of DNA damage by a number of different of genotoxic agents (Katayama et al., 2007, Abedin et al., 2007, Yao et al., 2003). Autophagy has been shown to play a cytoprotective role in response to DNA damage, with inhibition leading to increased cell death (Liu et al., 2015, Elliott and Reiners, 2008, Apel et al., 2008, Amaravadi et al., 2007). Furthermore, inhibition of autophagy has been shown to lead to increased genome instability (Zhao et al., 2012, Katayama et al., 2007) suggesting a cytoprotective role for autophagy in response to DNA damage. Conversely, the activation of autophagy in response to DNA damage has also been shown to lead to cell death when canonical apoptosis pathways are impaired (Kim et al., 2005, Assuncao Guimaraes and Linden, 2004). The exact role of autophagy in the DDR is still not fully understood however has been a great area of interest in recent years.

#### ***Activation of autophagy following DNA damage***

The exact mechanisms by which autophagy is activated in response to DNA damage are still being debated. It has been shown that autophagy is activated in a PARP-1 dependent manner following DNA damage induced by doxorubicin (Munoz-Gamez et al., 2009). The enzyme PARP-1 (poly [ADP-ribose] polymerase 1) is activated in response to DNA damage. This enzyme converts  $\beta$ -nicotinamide

adenine dinucleotide (NAD<sup>+</sup>) into polymers of poly(ADP ribose) and plays a role in nuclear homeostasis. Once hyper-activated in response to DNA damage, PARP-1 causes depletion in NAD<sup>+</sup> and ATP, eventually leading to energy crisis and cell death (Ha and Snyder, 1999). Here it has been suggested that autophagy is activated in response to reduced intracellular ATP and serves a protective function against energy stress. Indeed, inhibition of autophagy in this context has been shown to lead to increased cell death (Munoz-Gamez et al., 2009, Huang and Shen, 2009).

Another means by which autophagy has been shown to be activated downstream of a DDR is through p53 (Crighton et al., 2006, Kang et al., 2009, Feng et al., 2005). The exact mechanism of p53-dependent activation of autophagy is not fully understood. It has been shown to be independent of the transcriptional activity of p53: through AMPK and TSC1/2 activation leading to long term inhibition of mTORC1 (Feng et al., 2005). As well as dependent on the transcriptional activity of p53: through p53 mediated expression damage-regulated autophagy modulator (*DRAM*) encoded lysosomal protein that leads to increased autophagy (Crighton et al., 2006). Additionally, autophagy has also been shown to be induced in response to expression of tumour suppressor ARF in both a p53-dependent and p53-independent manner (Abida and Gu, 2008). Although activation of p53 can induce autophagy it has also been shown that p53 can also have an inhibitory effect on autophagy (Tasdemir et al., 2008). Autophagy has also been shown to be activated downstream of phosphorylated ATM, a key step in the initiation of the DDR. Here ATM activates TSC2 via AMPK to inhibit mTORC1 and activates autophagy (Alexander et al., 2010). These results highlight p53 as a bilateral regulator of autophagy. It has been suggested that activation of autophagy in response to low levels of DNA damage may assist in DNA repair through the degradation of damaged proteins and organelles by freeing cellular energy to be used in the repair process. However, on the flip side of this if the damage is too severe or is unable to be repaired then the prolonged activation of autophagy may help to accelerate cell death in a p53-dependent manner (Crighton et al., 2006).

### **Autophagy and DNA repair**

More recently it has been suggested that autophagy may have a more direct effect on DNA damage repair. There have been results showing that inhibition of autophagy leads to a decreased repair capacity in response to DNA damage agents (Bae and Guan, 2011, Liu et al., 2015). In the paper by Bae and Guan, autophagy is inhibited by the genetic knock out of 200 kDa FAK-family interacting protein (FIP200), an essential component of the mammalian autophagy machinery leading to a reduced DNA damage repair capacity. The exact mechanism is not explored however they do suggest that it is in part due to increased levels of p62 found in autophagy compromised cells and independent of increased levels of ROS (Bae and Guan, 2011). More recently a paper from Kevin Ryan's group in Glasgow showed that loss of autophagy through an inducible knock-out of *Atg7* leads specifically to an inhibition of homologous recombination through the enhanced proteasomal degradation of checkpoint kinase 1 (Chk1) (Liu et al., 2015). They report that cells lacking autophagy have a higher proteasome activity that results in a reduction in both phosphorylated and total levels of Chk1. Downstream analysis of homologous recombination via quantification of RAD51 foci or using the reporter plasmid described in (Pierce et al., 1999) show a reduction in this repair pathway in cells lacking autophagy.

#### **1.5.13 Autophagy and Senescence**

Autophagy and senescence are both activated in response to various cell stressors such as radiation, chemotherapy and oxidative stress (Katayama et al., 2007, Yao et al., 2003, von Zglinicki et al., 2005). The relationship between the two, however, is poorly understood with some reports suggesting a direct involvement of autophagy in the progression of senescence (Young et al., 2009) and others suggesting that inhibition of autophagy is a key driver of senescence (Kang et al., 2011).

A number of papers have provided data to suggest a collateral induction of senescence and autophagy. An increase in autophagosomes and senescence-associated  $\beta$ -galactosidase (Sen- $\beta$ -Gal) activity has been observed in ageing fibroblasts (Gerland et al., 2003). Markers for both autophagy and senescence have been observed in the bile ducts of patients with primary biliary cirrhosis, along with

biliary epithelial cells in mice treated with DNA damaging agents such as hydrogen peroxide and Etoposide, senescent endothelial cells as well as senescent human dental pulp (Sasaki et al., 2012, Sasaki et al., 2010, Patschan et al., 2008, Li et al., 2012a). These observations however are merely correlative and do not address the question whether autophagy and senescence are linked or interdependent responses.

One of the first studies carried out to test a potential direct relationship between autophagy and senescence was carried out by Young *et al* in human fibroblasts (Young et al., 2009). Here the authors suggest that oncogene-induced senescence could be dependent on the activation of autophagy as pharmacological and genetic interference with autophagy caused suppression in senescence. However, the conclusion from this work is that inhibition of autophagy delays but does not abrogate senescence, with senescence eventually achieving identical levels in cells with and without autophagy. Moreover, the authors also showed that inhibition of autophagy could not rescue the development of senescence supporting the idea that autophagy can accelerate oncogene induced senescence in fibroblasts, but once initiated the development of senescence is independent of autophagy. The mechanism by which increased autophagy could accelerate senescence is not yet understood. It has been suggested that the up-regulation of autophagy could provide the cell with a supply of energy in anticipation of the permanent cell cycle arrest seen in senescence (Gewirtz, 2013).

Other studies have also reported a decrease in senescence with the inhibition of autophagy (Singh et al., 2012, Mosieniak et al., 2012). Similarly, others report an increase in autophagy with the induction of senescence (Maddodi et al., 2010, Capparelli et al., 2012), however it has yet to be conclusively proven that the two are functionally connected.

Both autophagy and senescence are believed to serve cytoprotective functions in response to stress. Autophagy and senescence are regulated by overlapping signalling pathways such as p53 and mTOR. This makes the study of the role of autophagy in senescence difficult as interventions intended to modulate autophagy could have a direct effect on senescence itself. One challenge is that the suppression of autophagy in cells undergoing senescence causes an increase in the population of apoptotic cells and so what appears to be an increase in the

percentage of senescent cells is actually due to cell loss through apoptosis (Thorburn, 2008).

#### **1.5.14 Autophagy and Ageing**

It has been well documented that there are age-associated changes in autophagy pathways. This is due to many factors, such as an increased load of damaged and misfolded proteins, as well as dysfunctional organelles (Martinez-Vicente et al., 2005). These changes are believed to occur due to an increase in oxidative stress, as well as changes in regulatory factors. Age-related decline in macroautophagy and CMA activity has been observed (Cuervo and Dice, 2000a, Vittorini et al., 1999). It is thought that this decreased activity in both of these degradation pathways could contribute, at least in part, to the accumulation of damaged proteins and organelles and help contribute to a loss of homeostasis and aberrant stress responses in ageing cells.

#### ***Age-dependent Changes in Autophagy Pathways***

Changes in macroautophagy with age are accompanied by a number of morphological changes to the lysosomal system. These include an expansion of the cellular acidic compartment, an accumulation of autophagic vacuoles and a build-up of undegraded material in the lumen of the lysosome known as lipofuscin (Cuervo and Dice, 2000b). A decline in autophagy with age has been previously observed and has been suggested to contribute to cellular ageing (Terman, 1995, Vittorini et al., 1999, Lipinski et al., 2010).

In contrast to macroautophagy, there have been no age-associated morphological changes in the subset of lysosomes able to perform CMA (Cuervo and Dice, 2000c). Functional analyses, however, have shown that both substrate binding and translocation across the lysosomal membrane is greatly impaired in lysosomes taken both from organs from aged animals and senescent cells. These functional changes in the CMA pathway have been attributed to an age-dependent decrease in LAMP-2A on lysosomal membranes (Cuervo and Dice, 2000a). This gradual decline in LAMP-2A levels begins at middle age, although the decline in

function is temporarily offset by an increase in the number of lysosomes recruited to perform CMA. This compensatory increase in the number of lysosomes containing Hsc70 is only transient and, eventually, a functional decline in CMA becomes apparent (Cuervo and Dice, 2000a).

This age related reduction on LAMP-2A is not caused by a reduction in transcription, synthesis or the lysosomal trafficking of LAMP-2A. Instead it is thought to be caused by a reduction of the stability of LAMP-2A in the lipid membrane of the lysosome. The exact mechanisms underlying this drop in stability are unclear, however, changes in the lipid composition of the lysosomal membrane have been observed with age (Rodriguez-Navarro et al., 2012). It is possible that this disrupts the dynamics of LAMP-2A within this system and could contribute to the reduced levels of this receptor seen in old lysosomes.

Additional work is required in order to fully elucidate the changes that occur in autophagy with age. There is increasing evidence that manipulation of autophagy can have an impact on longevity, as well as have beneficial effects on many age associate pathologies.

### ***Manipulation of Autophagy Impacts on Lifespan***

Many studies designed to investigate mechanisms which impact on ageing and longevity, have identified pathways that modulate autophagy. One of the first identified and reproducible interventions able to extend lifespan was calorie restriction (CR). The life-span promoting effects of CR are evolutionary conserved and have been observed in yeast, worms (*C. elegans*), flies (*D. melanogaster*) and rodents (Fontana et al., 2010). The increase in lifespan seen with CR is, at least, in part, due to autophagy as deletion or mutation of autophagy proteins inhibits the life span extension seen in CR (Rubinsztein et al., 2011, Hansen et al., 2008). Treatments that activate autophagy, such as rapamycin and resveratrol, have also been shown to increase lifespan (Wood et al., 2004, Harrison et al., 2009). Similarly, interventions that down-regulate autophagy have been shown to have a negative effect on longevity and lead to an accelerated ageing phenotype (Hansen et al., 2008). Interestingly, it has been shown that short term DR, an intervention associated with life span extension, causes an increase in the turnover of mitochondria (Miwa et al., 2008). It was recently shown that overexpression of Atg5 in mice leads to an increase in autophagy and extension of lifespan. Moreover,

MEFs isolated from these Atg5 transgenic mice showed a marked resistance to oxidative stress-induced cell death. This resistance was reduced when autophagy was inhibited with either 3-MA or Baf (Pyo et al., 2013). These data suggest that an up-regulation of autophagy or prevention of its decline could decrease the accumulation of damaged macromolecules and organelles and improve longevity.

## 1.6 Dietary Restriction as a modulator of longevity

Dietary restriction (DR), reduced calorie intake without causing malnutrition, was first shown to extend lifespan almost 80 years ago (McCay et al., 1989, McDonald and Ramsey, 2010) since then it has been shown to extend lifespan in many model organisms (Guarente and Kenyon, 2000) as well as reduce the progression of age-associated diseases, reviewed in (Masoro, 2002) . These factors led DR to be one of the most commonly studied interventions when trying to uncover the mechanisms of organismal ageing. The exact mechanisms by which DR elicits this effect on lifespan are not yet fully understood, however, it is well known that DR reduces the accumulation of oxidatively damaged molecules that occurs with age in rodents reviewed in (Walsh et al., 2014). This effect could be down to a reduction in the rate at which ROS is generated in animals under DR (Miwa et al., 2014, Sanz et al., 2005) or an increase in the activity of protective pathways such as antioxidant defenses (Walsh et al., 2014) and repair pathways (Haley-Zitlin and Richardson, 1993). Interestingly, even animals subjected to short term DR show lower levels of DNA damage as measured by  $\gamma$ H2A.X foci compared to AL (*Ad-Libitum*) (Wang et al., 2010). DR has also been shown to reduce the levels of damaged proteins in mice and rats (Sohal et al., 1994, Aksenova et al., 1998, Youngman et al., 1992). Moreover, life extension by DR has been shown to require autophagy (Rubinsztein et al., 2011, Jia and Levine, 2007). In mice DR has been shown to activate autophagy (Bergamini et al., 2003) through the activation of either AMPK or SIRT1 (Kim et al., 2011) as well as increase expression of proteasome 26S subunit, ATPase 3 (Psmc3) proteasome regulatory particle base subunit (Rpt5), a subunit of the 19s proteasome, as well as proteasome activator proteasome activator subunit 1 (PSME1) (Lee et al., 1999). Taken together, these data show correlations between DR and a reduction in various forms of cellular damage, however we are still yet to fully understand the exact contribution of these changes to increasing longevity.

## **1.7 Aims**

Although autophagy has been shown to be an important determinant of longevity, the mechanisms by which autophagy is involved in the ageing process have not yet been fully identified. Recent studies have suggested a role for autophagy in the DNA damage response and repair; however the underlying processes are not yet understood. The general aim of this thesis is to understand the role of autophagy in DNA damage repair and to test if crosstalk between these two processes is a feature of normal ageing.

### ***Specific aims***

- 1.To investigate the role of autophagy in DNA damage repair.
- 2.To investigate the role of the autophagy adaptor p62 in DNA damage repair.
- 3.To investigate the contribution of interactions between p62 and DNA damage during ageing.

## Chapter 2. Material and Methods

### 2.1 Materials

#### 2.1.1 Cell lines

##### ***Prokaryotic cell lines***

$\alpha$ -select GOLD Efficiency chemically competent cells (Bioline #BIO-85027) were used for non-lentivirus constructs. NEB Stable competent *E.coli* (High Efficiency) (New England BioLabs #C3040) were used for lentivirus constructs.

##### ***Eukaryotic cell lines***

###### *Human cell lines*

Human embryonic lung MRC5 fibroblasts and HeLa cells were obtained from ECACC (European Collection of Cell Cultures) (Salisbury, UK) and HEK 293FT were purchased from Life Technologies #R700-07).

###### *Mouse cell lines*

p62 knock-out  $p62^{-/-}$  and wild type  $p62^{+/+}$  mouse embryonic fibroblasts (MEFs) (Komatsu et al., 2007) were kindly provided by Dr. Eiji Warabi of the University of Tsukuba. Autophagy-related protein 5 (Atg5)-deficient  $Atg5^{-/-}$  and wild-type  $Atg5^{+/+}$  MEFs (Kuma et al., 2004) and M5-7 MEFs (Hosokawa et al., 2007) were kindly provided by Dr Noboru Mizushima (Tokyo Medical and Dental University). The M5-7 cell line is a cell line derived from  $Atg5^{-/-}$  MEFs, here  $Atg5^{-/-}$  MEFs have been coupled with a Tet-off system, allowing an inducible knock out of Atg5 when Dox is present. M5-7s have the benefit over  $Atg5^{-/-}$  MEFs as they allow the study of acute autophagy knock out. This is advantageous as cells have less time to adapt to the lack of autophagy and as the cells are the same at the beginning of an experiment there is less impact of clonal differences between cells with and without autophagy.

## Cloning

For lentiviral expression full length wild type FLAG-tagged p62 was subcloned into the pLENTI6/V5-DEST vector using EcoRI and XhoI (NEB). Briefly, FLAG-p62 and pLENTI6/V5-DEST vector were digested with EcoRI and XhoI prior to gel purification (QIAquick GEL Extraction Kit (Qiagen)). The vector was dephosphorylated by calf intestinal alkaline phosphatase (Fermentas) and the ligation with FLAG-p62 was carried out using T4 DNA Ligase (New England BioLabs). Cloning was performed by Gisela Otten (Newcastle University).

mCherry-53BP1c for lentiviral expression was carried out as follows: a 2.7 kb C-terminal portion of 53BP1 (53BP1c), was excised from pAcGFP-53BP1c (Nelson et al., 2009) using BamHI and XhoI and ligated into pENTR2B (Invitrogen) to create pENTR2B-53BP1c. The sequence for mCherry fluorescent protein was amplified via PCR from pRSETB-mCherry (Shaner et al., 2004), incorporating Sall sites at both ends and a 5 amino acid linker at the 3' end. This product was ligated into pENTR2B-53BP1c Sall site in frame 5' of 53BP1c. A correct, sequence verified clone was then recombined into pLenti6-UbC/V5-DEST using LR Clonase following the manufacturer's instructions (Invitrogen) to produce pLenti6-mCherry-53BP1c. Cloning was performed by Dr James Wordsworth (Newcastle University).

## 2.1.2 Antibodies

Table 2.1: Primary antibodies for Immunofluorescence on cells

Primary antibodies			
Protein	Host	Dilution	Reference/ <u>Manufacturer</u>
p62	Guinea pig	1:200	Progen #GP-62-C
53BP1	Rabbit	1:200	CST #4937
p62	Rabbit	1:1000	MBL #PM045
Rad51	Rabbit	1:1000	Millipore #ABE257
p62	Mouse	1:1000	BD Laboratories #610832

**Table 2.2: Secondary antibodies for Immunofluorescence on cells**

Secondary antibodies				
Protein	Species	Host	Dilution	Reference/ <u>Manufacturer</u>
Anti-mouse Fluorescein- conjugated AlexaFluor 488	Mouse	Goat	1:4000	A21042 -Invitrogen
Anti-mouse Fluorescein- conjugated AlexaFluor 594	Mouse	Goat	1:4000	A21044 - Invitrogen
Anti-mouse Fluorescein- conjugated AlexaFluor 647	Mouse	Goat	1:4000	A21238 - Invitrogen
Anti-rabbit Fluorescein- conjugated secondary antibody AlexaFluor 488	Rabbit	Goat	1:4000	A21212 - Invitrogen
Anti-rabbit Fluorescein- conjugated AlexaFluor 594	Rabbit	Goat	1:4000	A21213 - Invitrogen
Anti-rabbit Fluorescein- conjugated AlexaFluor 647	Rabbit	Goat	1:2000	A21244 - Invitrogen

**Table 2.3: Primary antibodies for Western Blotting**

Primary antibodies for Western Blotting			
Protein	Host	Dilution	Reference/ <u>Manufacturer</u>
Atg5	Rabbit	1:1000	Sigma #A0856
CHD4	Rabbit	1:1000	Active Motif #39290
Chk1	Rabbit	1:500	CST #2360
FLAG M2	Mouse	1:2000	Sigma #F1804
GAPDH	Rabbit	1:10000	CST #5174
GFP	Chicken	1:2000	Abcam #ab13970
LC3B	Rabbit	1:1000	CST #3868
p21	Rabbit	1:1000	CST #2947
p53	Rabbit	1:1000	CST #9282
p62	Guinea pig	1:1000	Progen #GP-62-C
PARP	Rabbit	1:1000	CST #9284
p-Chk1 (Ser345)	Rabbit	1:1000	CST #2348
p-p53 (Ser15)	Rabbit	1:1000	CST #9284
Filamin A	Rabbit	1:1000	CST #4762
RAD51	Rabbit	1:1000	Millipore #ABE257

**Table 2.4: Secondary antibodies for Western Blotting**

<b>Secondary antibodies for Western Blotting</b>				
<b>Protein</b>	<b>Species</b>	<b>Host</b>	<b>Dilution</b>	<b>Reference/<u>Manufacturer</u></b>
Goat anti-rabbit IgG - HRP conjugated	Rabbit	Goat	1:5000	A0545 - Sigma
Goat anti-mouse IgG - HRP conjugated	Mouse	Goat	1:5000	A2554 - Sigma
Rabbit anti-Guinea Pig IgG -HRP conjugated	Guinea Pig	Rabbit	1:2500	P 0141 - Dako
Goat anti-chicken IgG - HRP conjugated	Chicken	Goat	1:5000	ab97135 Abcam

**Table 2.5: Primary antibodies for Immunostainings on Mouse Tissues**

Primary antibodies				
Protein	Species	Host	Dilution	Reference/ <u>Manufacturer</u>
γ-H2A.X(Ser139)	Human Mouse	Rabbit	1:200	#9718 - CST
p62	Human Mouse	Guinea Pig	1:100	#GP-62-C – Progen

**Table 2.6: Secondary antibodies for Immunostainings on Mouse Tissues**

Secondary antibodies				
Protein	Species	Host	Dilution	Reference/ <u>Manufacturer</u>
Anti-guinea pig Fluorescein- conjugated secondary antibody AlexaFluor 594	Guinea pig	Goat	1:2000	A11076 - Invitrogen
Anti-rabbit Vectastain (biotinylated)	Rabbit	Goat	1:4000	PK4001 –Vector lab
Fluorescein Avidin DCS			1:500	A-2011 - Vector Laboratories

## 2.1.3 Cell Culture

Table 2.7: Reagents for Cell Culture

Reagent	Manufacturer	Reference
Trypsin-EDTA Solution	Sigma	T3924
Penicillin-Streptomycin	Sigma	P4333
Mycoalert Detection Kit	Lonza	LT07-218
L-Glutamine Solution	Sigma	G7513
Dulbecco'S Modified Eagle'S Medium (DMEM)	Sigma	D5796
Dimethyl Sulfoxide (DMSO)	Sigma	D2650
20 X PBS	New England Bio	9808
10% Heat Inactivated Foetal Bovine Serum	BioSera	NA

**Table 2.8: Cell Treatments**

<b>Reagent</b>	<b>Manufacturer</b>	<b>Reference</b>
Bafilomycin A1	Enzo	BML-CM110-0100
Blasticidine	Sigma	15202
Etoposide	Sigma	E1383
G418	Sigma	A1720
Leptomycin B	Cell Signalling	9676
Polybrene	Sigma	107689
Puromycin	ThermoFisher Scientific	A11138
Rapamycin	Sigma	R8781
Tetracycline hydrochloride	Sigma	079K1498
Z-Leu-Leu-Leu-al (MG132)	Sigma	C2211

**Table 2.9: Cell Culture Consumables**

Reagent	Manufacturer	Reference
CryoTube Vials	ThermoFisher Scientific	377267
0.6ml 'Crystal Clear' Microcentrifuge Tube, 2 x 500	Star labs	E1405-0600
1.5 microcentrifuge tubes	Star labs	S1615-5500
12-well plate	Fisher	TKB-100-110R
15ml Centrifuge Tube, Conical (Sterile), Loose	Star labs	E1415-0200
175cm <sup>2</sup> TC treated flask with filter cap	Greiner-Bio one	661175
2.0ml 'Crystal Clear' Microcentrifuge Tube, 1 x 500	Star labs	E1420-2000
24 well plates	Fisher	TKB-100-115H
50ml Centrifuge Tube, Conical (Sterile), Loose	Star labs	E1450-0200
6-well plates	Fisher	11825275
75cm <sup>2</sup> TC treated flask with filter cap	Greiner-Bio one	658175
Acrodisc <sup>®</sup> Minispikes syringe filters	Sigma	Z260444-1PAK
Acryl Aquaclean	WAK-Chemie Medical GmbH	WAK-AQA-250-50L
Bijou sample container, plain label	Sigma	Z645346-700EA
Cell culture dish treated with vents sterile polystyrene non-pyrogenic	ThermoFisher Scientific	10075371
Coverglass 13mm/0.16mm	VWR	631-0150
glass pasteur pipettes 230mm	VWR	612-1702
serological pipettes 10ml	Sarstedt	86.1254.001
serological pipettes 25ml	Sarstedt	86.1685.001
serological pipettes 5ml	Sarstedt	86.1253.001

## 2.1.4 Plasmid Prep

Table 2.10: Plasmid Prep Reagents

Reagent	Manufacturer	Reference
Agarose	ThermoFisher Scientific	BP1356-500
Alpha-Select Bronze Efficiency	Bioline	BIO-85025
Ampicillin	Sigma	A5354
Burner Bunsen Natural Gas 13mm	SLS	BUR3000
Cell culture dish treated with vents sterile polystyrene non-pyrogenic	ThermoFisher Scientific	10075371
Glass spreaders	Sigma	S4522-6EA
Kanamycin	Sigma	K0254
LB Agar Miller	ThermoFisher Scientific	10734724
LB Broth Miller Powder	ThermoFisher Scientific	10638013
Molecular Grade RNase-free water	ThermoFisher Scientific	B-003000-WB-100
NEB stable competent <i>E.coli</i> (high efficiency)	New England Bio	C3040H
peqGREEN	Peqlab	37-5000
PureYield™ Plasmid Midiprep System	Promega	A2492
S.O.C. Medium	ThermoFisher Scientific	15544-034

**2.1.5 Transfections****Table 2.11: Transfection Reagents**

<b>Reagent</b>	<b>Manufacturer</b>	<b>Reference</b>
Lipofectamine™ 2000 Transfection Reagent	ThermoFisher Scientific	11668019
OptiMEM	ThermoFisher Scientific	11058021

## 2.1.6 Lentiviral Vectors

Table 2.12: Plasmid Lentiviral Vectors

Insert	Expression Vector	Packaging Vectors	Packaging Cells	Selection	Selection conc	Maintenance conc	Target Cells
FLAG-p62	pLenti6	3rd Gen (GAG pLP1, VSV- pLP/VSVG, REV pLP2)	HEK293FT	blasticidin	8 µg/ml	4 µg/ml	<i>p62</i> <sup>-/-</sup> MEFs
mCherry- 53BP1	pLenti6	3rd Gen (GAG pLP1, VSV- pLP/VSVG, REV pLP2)	HEK293FT	blasticidin	none	none	<i>p62</i> <sup>+/+</sup> <i>p62</i> <sup>-/-</sup> MEFs
Empty	pLenti6	3rd Gen (GAG pLP1, VSV- pLP/VSVG, REV pLP2)	HEK293FT	blasticidin	8 µg/ml	4 µg/ml	<i>p62</i> <sup>-/-</sup> MEFs
Atg5 shRNA 2	pLKO-puro	2nd Gen (VSV-G HIV-1)	HEK293FT	puromycin	none	none	MRC5
Atg5 shRNA 3	pLKO-puro	2nd Gen (VSV-G HIV-1)	HEK293FT	puromycin	none	none	MRC5
Atg5 shRNA 4	pLKO-puro	2nd Gen (VSV-G HIV-1)	HEK293FT	puromycin	none	none	MRC5
Atg5 shRNA 5	pLKO-puro	2nd Gen (VSV-G HIV-1)	HEK293FT	puromycin	none	none	MRC5
GFP shRNA	pLKO-puro	2nd Gen (VSV-G HIV-1)	HEK293FT	puromycin	2µg/ml	0.5µg/ml	MRC5
p62 shRNA 3	pLKO-puro	2nd Gen (VSV-G HIV-1)	HEK293FT	puromycin	2µg/ml	0.5µg/ml	MRC5
p62 shRNA 4	pLKO-puro	2nd Gen (VSV-G HIV-1)	HEK293FT	puromycin	2µg/ml	0.5µg/ml	MRC5
p62 shRNA 5	pLKO-puro	2nd Gen (VSV-G HIV-1)	HEK293FT	puromycin	2µg/ml	0.5µg/ml	MRC5

### 2.1.7 Plasmids

pEGFP-p62, pEGFP- $\Delta$ PB1p62, pEGFP- $\square$ NESp62 and pEGFP- $\Delta$ UBAp62 constructs were previously published (Bjorkoy et al., 2005, Pankiv et al., 2010, Lamark et al., 2003). FLAG-p62 was kindly provided by Robert Layfield (University of Nottingham, Nottingham, UK) (Najat et al., 2009). pLKO-puro GFP was kindly provided by Dr Simon Wilkinson (Edinburgh Cancer Research Centre, University of Edinburgh). mCherry-53BP1 was kindly provided by Dr Glyn Nelson (Newcastle University, Newcastle) pG-AcGFP-53BP1c has been described previously (Nelson et al., 2009). Filamin A-GFP was kindly provided Dr Aragay (Institut de Biologia Molecular de Barcelona)

### 2.1.8 siRNA

**Table 2.13: siRNA**

<b>ON-TARGETplus SMARTpool siRNA from Dharmacon</b>		
<b>Protein</b>	<b>siRNA</b>	<b>Reference/<u>Manufacturer</u></b>
Non-targeting	ON-TARGETplus Non-Targeting control pool siRNA	# D-001810-10 Dharmacon
p62	ON-TARGETplusSqstm1 siRNA	L-047628-01-0005-Dharmacon
FLNA	ON-TARGETplusFlna siRNA	L-058520-01-0005 - Dharmacon

## 2.1.9 Cell fractionation

Table 2.14: Cell Fractionation Reagents

Reagent	Manufacturer	Reference
20 x PBS	New England Bio	9808
Calcium chloride 1M solution	Sigma	21115
IGEPAL® CA-630 (NP40)	Sigma	I3021
Magnesium chloride 1M solution	ThermoFisher Scientific	AM9530G
Phosphatase inhibitor cocktail 100X	ThermoFisher Scientific	1861280
polyoxyethylene sorbitan (Tween-20)	Sigma	93774
Sodium chloride	Sigma	S7653
Trisma base	Sigma	T1503
Triton X-100	Sigma	T8787
<b><u>4x Laemmli Buffer</u></b>		
20% SDS solution	ThermoFisher Scientific	AM9820
Bromophenol Blue	Sigma	B0126
Glycerol	Sigma	G5516
$\beta$ -mercaptoethanol (BME)	Sigma	M3148

## 2.1.10 Immunofluorescence

**Table 2.15: immunofluorescence in Cells Reagents**

Reagent	Manufacturer	Reference
20 x PBS	New England Bio	9808
Bovine Serum Albumin	Sigma	5482
Gelatin from cold water fish skin	Sigma	G7041
Microscope slide ground edges, twin frosted	ThermoFisher Scientific	FB58628
Prolong Gold	ThermoFisher Scientific	P36935

**Table 2.16: immunofluorescence in Tissues Reagents**

Reagent	Manufacturer	Reference
CyStain UV Ploidy (DAPI)	Partec	05-5001
Ethanol	ThermoFisher Scientific	E/0650DF/17
Histoclear	National Diagnostics	HS-200
Microscope slide ground edges, twin frosted	ThermoFisher Scientific	FB58628
Normal Goat Serum Blocking Solution	Vector Lab	S-1000
Trisodium citrate	Sigma	S1804
Vectashield mounting medium	Vector Lab	H-1200

## 2.1.11 Immunoblotting

Table 2.16: Materials for Western Blotting

Reagent	Manufacturer	Reference
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma	T9281
20x PBS	New England Bio	9808
20% SDS solution	ThermoFisher Scientific	AM9820
2xLaemmli buffer	Biorad	1610737
Acrylamide-Bis acrilamide	Severn Biotech	20-2100-10
Ammonium persulphate (APS)	Sigma	A3678
Bovine serum albumin (BSA)	Sigma	A2153
Clarity western ECL substrate	Biorad	170-5061
DC Protein Assay Kit	Biorad	500-0112
Gel loading tips	Starlabs	1022 0600
Glycine	Sigma	G8898
Immobilon-P polyvinylidene difluoride (PVDF) 0.45 $\mu$ M membrane	Milipore	IPVH00010
Marvel non-fat dry milk powder	Asda	NA
Methanol	Sigma	32213
Phosphatase inhibitor cocktail 100X	ThermoFisher Scientific	1861280
Polyoxyethylene sorbitan (Tween-20)	Sigma	93774
Ponceaux	Sigma	P2395
Precision Plus Protein™ Dual Color Standards	Biorad	610374

Restore# PLUS Western Blot Stripping Buffer	ThermoFisher Scientific	46430
Thick blotting paper	VWR	732-0594
Trisma base	Sigma	T1503
$\beta$ -mercaptoethanol (BME)	Sigma	M3148
Triton X-100	Sigma	T8787
<b><u>4x Laemmli Buffer</u></b>		
20% SDS solution	ThermoFisher Scientific	AM9820
$\beta$ -mercaptoethanol (BME)	Sigma	M3148
Glycerol	Sigma	G5516
Bromophenol Blue	Sigma	B0126

## 2.1.12 Comet Assay

Table 2.17: Reagents for Comet Assay

Reagent	Manufacturer	Reference
10x TBE	ThermoFisher Scientific	AM9863
20x PBS	New England Bio	9808
Agarose	Sigma	A0169
Agarose, low gelling temperature	Sigma	A9414
Dimethyl sulfoxide	Sigma	D8418
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E6758
Sodium chloride	Sigma	S7653
Sodium hydroxide	Sigma	795429
Superfrost™ Plus Adhesion Slides	ThermoFisher Scientific	10143352
SYBR® Gold Nucleic Acid Gel Stain	ThermoFisher Scientific	S-11494
Trisma base	Sigma	T1503
Triton X-100	Sigma	T8787

## 2.1.13 Immunoprecipitation

Table 2.18: Reagents for immunoprecipitation

Reagent	Manufacturer	Reference
<u>Buffer</u>		
Triton X-100	Sigma	T8787
Trisma base	Sigma	T1503
Sodium chloride	Sigma	S7653
Polyoxyethylene sorbitan (Tween-20)	Sigma	93774
Magnesium chloride 1M solution	ThermoFisher Scientific	AM9530G
Calcium chloride 1M solution	Sigma	21115
<u>Beads</u>		
ANTI-FLAG M2 Affinity Gel	Sigma	A2220
Anti-FLAG M2 Magnetic Beads	Sigma	M8823
Recombinant protein A Sepharose	Generon	PC-A5
<u>Gel-stain</u>		
Sodium thiosulfate	Sigma	13481
Sodium carbonate	Sigma	451614
Silver nitrate	Sigma	209139
GelCode Blue stain reagent	ThermoFisher Scientific	10608494
Formaldehyde solution	Sigma	F8775
Acetic acid	Sigma	320099

## 2.2 Methods

### 2.2.1 Cell Culture

All cells were grown in DMEM (Dulbecco's Modified Eagle's Medium, Sigma) supplemented with 10% heat-inactivated FCS (Foetal Calf Serum, Biosera), 5% penicillin/streptomycin (Invitrogen) and 2 mM L-glutamine (Sigma) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Autophagy was abolished in M5-7 MEFs by treating with 1 µg/ml tetracycline for at least four days. HEK293FT were maintained in 500 µg/ml G418 (Sigma #A1720) prior to transfection.

#### 2.2.1.2 Cryogenic storage

Exponentially growing adherent cells were trypsinised (when at ~70% confluence) with Trypsin-EDTA (Sigma). Trypsin was neutralized with the addition of pre-warmed media and cells centrifuged at 150g for 3 minutes at room temperature. The supernatant was removed and cells were re-suspended in Foetal Calf Serum (FCS) containing 10% (v/v) dimethyl sulfoxide (DMSO) at a density of 1x10<sup>6</sup> cells/ml. Cell suspension was immediately transferred to cryo-vials in one ml aliquots (containing 1x10<sup>6</sup> cells) and placed in a Mr. Frosty™ Cryo freezing container filled with isopropanol (Thermo Scientific). Cells were placed @ -80°C for 24h to allow slow freezing, before being transferred to liquid nitrogen for long term storage.

#### 2.2.1.3 Resuscitation of frozen cells

Cryo-vials containing cell suspension were removed from liquid nitrogen and placed in a water bath @ 37°C to thaw (1-2min). Thawed cell suspension was immediately added to pre-warmed media and seeded. Cell culture media was replaced 24h following to remove DMSO and cell debris.

#### 2.2.1.4 Calculating cell density and population doublings

Cell concentration was calculated using a Fuchs Rosenthal haemocytometer (VWR International, UK) where 20 µL of trypsinised cells suspension was analysed under a standard microscope (DMIL, Leica Microsystems, UK). The average of three counts of 8 squares is equivalent to the number of cells x10<sup>4</sup>/mL. This allowed the

calculation of the total number of cells by multiplying the volume of cell suspension (mL) with the cell concentration (cells/mL). For human primary cell lines the population doubling (PD) was calculated using the following equation:  $PD = X + \ln(N1/N2)/\ln 2$

where, PD = population doublings  
X = previous PD  
N1 = number of cells harvested  
N2 = number of cells seeded

For immortalised cell lines cells were tracked by recording the dilution factor and the number of passages.

## 2.2.2 Over-expression Vectors

### 2.2.2.1 Bacterial Transformation

All bacterial transformations for transiently expressed plasmids were performed using  $\alpha$ -select GOLD Efficiency chemically competent cells (Bioline, #BIO-85027) according to supplier's instructions. Briefly, 10  $\mu$ L of cells were thawed on wet ice. 2  $\mu$ L of DNA solution was added and cells were mixed and incubated for 30 min on wet ice. Tubes were placed at 37°C for 30 seconds and then incubated on wet ice for a further 2 min. Transformation reactions were then diluted in 500  $\mu$ L SOC medium (Invitrogen, 15544-034). SOC Medium: 2% Tryptone, 0.5% Yeast Extract, 0.4% glucose, 10mM NaCl, 2.5mM KCl, 10mM MgCl<sub>2</sub> & 10mM MgSO<sub>4</sub>. Tubes were then incubated at 37°C for 1h while shaking. Cells were then spread on agar selection plates containing lysogeny broth (LB) medium plus ampicillin/kanamycin. Plates were incubated overnight at 37°C. Individual colonies were then grown overnight in 100mL selective LB. DNA was purified using PureYield Plasmid Midiprep System (Promega, #A2492) as described by manufacturer.

Bacteria transformations for lentiviral plasmids was performed in NEB stable Competent *E. coli* (High Efficiency)(New England BioLabs, #C3040H) Transformation process was as above although overnight incubation at 37°C was replaced with incubation at 30°C for 24 hours. DNA was purified using PureYeild Plasmid Midiprep System (Promega, #A2492).

#### Transformed bacteria stocks

Glycerol stocks were made from 500 $\mu$ L LB containing transformed bacteria mixed with 500 $\mu$ l 80% glycerol, then snap frozen in liquid nitrogen and stored at -80°C.

### 2.2.2.2 Transfection and Transduction Protocols

#### Transfection

Cells were transfected using polyethylenimine (PEI) as described in (Segura et al., 2010) or Lipofectamine 2000 (Life Technologies #11668) according to the manufacturer's protocols for 24h prior to lysis or fixation.

### Transduction

Lentivirus particles were generated in HEK293FT following the manufacturer's protocol (Life Technologies). HEK293FT cells were seeded in antibiotic-free medium supplemented with 0.1 mM MEM non-essential amino acids (Invitrogen) and then co-transfected with lentiviral expression vectors and 2<sup>nd</sup> or 3<sup>rd</sup> generation packaging system plasmids. After 24 hours, media was replaced with fresh media without antibiotics. 48 hours after transfection, viral transduction was performed by transferring media from HEK293FT cells 70% confluent in the presence of 6 µg/ml Polybrene (Sigma). Media containing virus was replaced after 24 hours with fresh media containing antibiotic for selection of transduced cells. Media was replaced every 2-3 days for 10-12 days by keeping the antibiotic selection. Transduced MEFs were then maintained in lower levels of selection antibiotic until seeding for experimental purposes.

## **2.2.3 Induction of DNA Damage**

### **2.2.3.1 X-Ray Irradiation**

DNA damage was induced using X-ray irradiation (X-Rad 225, Precision X-Ray INC, N-BRANFORD, CT USA) with doses as indicated (0.25-20Gy). Media was changed immediately after irradiation to prevent further damage from residual free radical generated by IR.

### **2.2.3.2 Etoposide**

DNA damage was also induced using the topoisomerase inhibitor Etoposide. Cells were treated with doses and times as indicated (0.01-1 $\mu$ M). Media containing Etoposide was then removed, cells were washed with warm PBS and media was replenished. Cells were then left to recover for time indicated.

## **2.2.4 Treatments of cells in tissue culture:**

### **2.2.4.1 Rapamycin**

mTORC1 inhibition was used as a means to stimulate autophagy. mTORC1 was inhibited by treating cells with 100nM Rapamycin (Sigma, #R8681) immediately after irradiation.

### **2.2.4.2 Bafilomycin A1**

Autophagic flux was inhibited by treatment with Bafilomycin A1 (Enzo, #BML-CM110-0100) at 100-400nM as indicated, for 440 min, or the duration of the experiment as indicated.

### **2.2.4.3 Leptomycin B**

Cells were incubated with 20nM Leptomycin B (CST, #9676) for 1-4 hours as indicated to inhibit nuclear protein export.

### **2.2.4.4 MG132**

Proteasomal inhibition was achieved by treatment of 10 $\mu$ M MG132 (Sigma, #C2211) for 4 hours.

### **2.2.4.5 N-Acetyl-Cysteine (NAC)**

Cell were treated with 5 $\mu$ M NAC immediately after X-Ray Irradiation

## **2.2.5 Knock down by small interfering RNA**

Cells were transiently transfected with ON-TARGETplus SMARTpool siRNA against mouse p62 (#18412), FLNA (L-012579-02) and non-targeting SMARTpool siRNA (D-001810-04) purchased from Dharmacon. Final siRNA concentrations of 100nM were used for 96 hours for silencing and transfections were carried out using Lipofectamine 2000 (Life Technologies, #11668) as per company instructions. Knockdown efficiency was measured by western blot analysis.

## 2.2.6 Mice

### 2.2.6.1 Mice Groups, Treatments and Housing

All mice were inbred C57BL/6 (Harlan, Blackthorn UK). Mice were housed in same-sex cages in groups of 4 to 6 (56 × 38 × 18 cm, North Kent Plastics, Kent, UK) and individually identified by an ear notch. Mice were housed at 20 ± 2°C under a 12 h light/12 h dark photoperiod with lights on at 7.00 am. The diet used was standard rodent pelleted chow (CRM (P); Special Diets Services, Witham, UK) for *ad libitum* (AL)-fed mice and the same diet, but as smaller pellets were offered to dietary restricted (DR) mice. DR mice were offered 60% of AL intake (calculated based on average food intake in 90 control AL mice between 5 and 12 months of age) as one ration at 9.30 am daily. All mice were fed AL until 3 months of age and then split into AL or DR groups, matched for body mass and food intake.

### 2.2.6.2 Mouse tissue collection and preparation

Tissues were collected during necropsy and fixed with 4% formaldehyde aqueous solution (VWR; Cat. Number 9713.9010) and paraffin embedded for histochemical analysis. Parts of the tissues were also snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

### 2.2.6.3 Tissues provided by collaborators

Paraffin embedded tissue sections of the small intestine from AhCre; APC f/wt mice ages 27weeks were kindly provided by Masashi Narita (Cancer research UK Cambridge Institute)

### 2.2.7 Cell fractionation

Cellular fractionation was carried out as in (Suzuki et al., 2010). Briefly,  $6 \times 10^5$  cells were seeded on 10 cm dishes 48 hours prior to collection. Cells were washed in ice-cold PBS, scrapped in 1 ml ice-cold PBS and centrifuged for 10 seconds at 13,000 rpm at 4°C. The supernatant was aspirated and cells were resuspended (triturated 5 times) in 1 ml ice-cold 0.1% NP40 in PBS. 200  $\mu$ L was collected in a fresh tube (whole cell sample). Samples were centrifuged again for 10 seconds at 13,000 rpm at 4°C and supernatant, which represents the cytoplasmic fraction, was collected in a fresh tube. The pellet was resuspended (triturated once) in 1 ml ice-cold 0.1% NP40 in PBS. Samples were centrifuged for 10 seconds at 13,000 rpm at 4°C, supernatant was discarded and the nuclear pellet was processed as described below.

For Immunoblot: Whole cell samples and cytoplasmic fractions were mixed 3:1 with 4x Laemmli sample buffer, sonicated using a microprobe for five seconds on ice and boiled at 100°C for 5 minutes in the presence of 2.5%  $\beta$ -ME (beta mercaptoethanol). Nuclear pellets were resuspended in 200  $\mu$ L 1x Laemmli sample buffer, sonicated using a microprobe for 5 seconds on ice and boiled at 100°C for 5 minutes in the presence of 2.5%  $\beta$ -ME then centrifuged for 10 minutes at 13,000 rpm at 4°C and transferred to a new tube.

For immunoprecipitation: The nuclear pellets were resuspended in 200  $\mu$ L IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.1% Tween 20, 0.5% Triton-X100 and 2x Halt Protease & Phosphatase inhibitor cocktail (Thermo Scientific, #1861280)). The samples were sonicated using a microprobe for 5 seconds centrifuged for 10 minutes at 13,000 rpm at 4°C and transferred to a new tube. Immunoprecipitation was performed as described below.

### 2.2.8 Live cell imaging

$p62^{-/-}$  and  $p62^{+/+}$  MEFs, stably expressing mCherry-53BP1c were seeded on a 35 mm glass bottomed dish (IWAKI) 48 hours prior to treatment. Cells were irradiated with 0.25 Gy X-Ray irradiation and immediately transferred to the heated, XLmulti S1 humidified stage (95% air, 5% CO<sub>2</sub>) of a Zeiss CellObserver spinning disk confocal for imaging. Images were captured using a 561 nm laser and 40 × 1.3NA objective (Zeiss) driven by Axiovision software (v4.8.1, Zeiss, Cambridge, UK). Z-stacks encompassing the entire cell were taken every 10 minutes for 8 hours. Foci were tracked using Cell/Vesicle function in Imaris (Bitplane, Oxford Instruments).

## 2.2.9 Immunostainings

### 2.2.9.1 Immunofluorescence staining on fixed cells

#### Fixation

Cells grown in coverslips were fixed in 500µL of 2% formaldehyde in PBS (VWR, 9713.9010) for 8 minutes at room temperature. Paraformaldehyde was removed and cells were washed twice with PBS. Coverslips were stored in PBS at 4°C (short term) or -80°C (long term). Cells were defrosted/washed once in PBS before beginning staining procedure.

#### Permeabilisation

Cells were incubated for 45 minutes at room temperature with 500µL PBG-Triton (0.2% cold water fish gelatine, 0.5% BSA and 0.5% Triton in PBS).

#### Immunofluorescence staining

Cells were incubated for 45 minutes in primary antibody (diluted in PBG – Triton) with gentle agitation or overnight at 4°C without gentle agitation. Cells were washed twice with PBG-Triton for 5 minutes. Cells were incubated for 45 minutes with fluorophore-conjugated secondary antibody (1/2000) diluted in PBG–Triton and then washed three times with PBS for 5 minutes. Cells were washed 3 times in PBS before mounting cells on slides using ProLong® Gold Antifade Mountant with DAP (ThermoFisher Scientific, P36935)

### 2.2.9.2 Immunostainings on paraffin embedded tissues

#### Dewax and Hydration

Paraffin tissue sections of 3µm thickness were deparaffinised by incubating for 10 minutes in HistoClear (National Diagnostics; Cat. Number HS-200) X2 and rehydrated in graded concentration of ethanol solutions: 100% (2x 5 minutes), 90% (5 minutes), 70% (5 minutes) and H<sub>2</sub>O (10 minutes).

### Antigen Retrieval

Antigens were retrieved by incubating tissues sections in 0.01M citrate buffer pH6.0 (29.41g of trisodium citrate in 1L of distilled water, pH6.0) in the microwave: high power (800W) for 5 minutes until boiling followed by 10 minutes at medium power (400W). Tissue sections were cooled by incubating for 20 minutes on wet ice. Tissue sections were then washed twice in H<sub>2</sub>O for 10 minutes.

### Immunofluorescence staining

Tissue sections were incubated in blocking buffer (goat IgG, #S1000, Vector Lab) for 30-60 minutes at room temperature. Tissue sections were further blocked with Avidin/Biotin (Vector Lab, # SP-2001) for 15 minutes. Primary antibody (rabbit  $\gamma$ H2A.X 1:200 in blocking buffer, CST, #9718) was applied overnight at 4°C. Slides were washed three times with PBS and incubated for 30 minutes with secondary antibody (Vector Lab, #PK-4001). Sections were washed three times with PBS and incubated with Fluorescein Avidin DCS (1:500 in PBS, Vector Lab, #A-2011) for 20 minutes. Sections were washed three times with PBS and incubated for 30 minutes with blocking buffer. Second primary antibody (guinea pig p62 1:100 in blocking buffer, Progen, #GP-62-C) was applied overnight at 4°C (note antibodies were incubated sequentially). Slides were washed three times with PBS and incubated for 30 minutes with secondary antibody (anti guinea pig Alexa Fluor® 594 1:2000, Molecular Probes #A-11076 ). Sections were stained with DAPI for 5-10 minutes and mounted in Vectashield mounting medium (Vector Lab, # H-1200).

### **2.2.9.3 Microscopy**

Cells were imaged with a Leica DM 5500B Widefield Microscope through an HCX PL APO 100x/1.40-0.70 or oil HCX PL APO 40x/1.25 oil objective using a Leica DFC 360 FX camera. Alternatively, for co-localisation and live cell imaging analyses, images were captured using a Zeiss CellObserver spinning disk confocal microscope equipped with: CSUX1 spinning disk confocal head (Yokogawa), and Quant EM CCD (Photometrics), using a 405, 488 and 561 nm lasers and 63x 1.4NA objective (Zeiss) driven by Axiovision software (v4.8.1, Zeiss, Cambridge, UK).

## 2.2.10 Protein expression analysis

### 2.2.10.1 Protein Extraction

Cells were washed with ice cold PBS and then lysed with ice cold Radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP40, 0.5% NaDoC, 0.1% SDS, 50 mM Tris pH 7.4 and 1x phosphatase and protease inhibitors cocktail (Thermo Scientific, 78442) by scraping using a rubber cell scraper. Samples were then collected into 1.5 mL microcentrifuge tubes and immediately stored at -80°C (alternatively samples could be immediately used for protein quantification and further analysis).

### 2.2.10.2 Protein quantification

Cell lysates were defrosted on wet ice before being centrifuged for 10 minutes at 16,000g at 4°C. Protein quantification was performed using a colorimetric Bio-Rad DC Protein Assay (Bio-Rad; Reagent A Cat. Number 500-0113, Reagent B Cat. Number 500-0114, Reagent S Cat. Number 500-0115) according to the manufacturer's instructions. Absorbance was measured using a Fluostar Omega plate reader (BMG Labtech) Protein concentration of each sample was calculated and normalised by mixing adjusted volumes of protein lysate and loading buffer [950 µL of 2xLaemmli buffer (Bio-Rad; Cat. Number 161-0737) plus 50 µL of β-mercaptoethanol (Sigma, M6250) or (4x Laemmli 8% SDS, 40% glycerol, 240mM Tris pH6.8, 10% β-mercaptoethanol). Protein denaturation was achieved by incubating samples at 100°C for 5 minutes. Samples were immediately placed on ice after denaturation. At this stage denatured samples were either stored at -20°C or used immediately for western blotting.

### 2.2.10.3 Western blotting

#### Electrophoresis

Acrylamide gels were prepared as following:

1. A resolving gel was prepared according to size of the proteins of interest and poured into a cassette (Invitrogen, NC2015 or NC2010). 1ml of water was added on top of gel to insure a straight edge (see Table 3.6 for gel preparation)
  - 5% gels: > 250 kDa
  - 8% gels: 250-120 kDa
  - 10% gels: 120-40 kDa
  - 12% gels: 40-15 kDa
  - 15% gels: < 20 kDa
2. After the resolving gel has polymerised, a 5% acrylamide staking gel was prepared, poured into the cassette and allowed to polymerize (see Table 2.19 for gel preparation).
3. Gels were placed in a XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen) and Tris-Glycine running buffer (250µM Tris, 1.92mM Glycine and 0.1% SDS) was added. Samples were loaded into wells along with a Protein standard (Bio-Rad, 161-0374) and electrophoresis was performed at 120V, 35mA for 90 minutes.

**Table 2.19: Acrylamide gels for Western Blotting analysis**

1Gel	5%	8%	10%	12%	15%	Stacking
Sterile H <sub>2</sub> O	6.8ml	5.1ml	4ml	3.3ml	2.3ml	3.4ml
30% Acrylamide	1.7ml	2.6ml	3.3ml	4ml	5ml	830ul
1.5M Tris pH 8.8 (1M Tris pH 6.8 (Stacking))	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml	630ul
10% SDS	100ul	100ul	100ul	100ul	100ul	50ul
10% Ammonium Persulphate	100ul	100ul	100ul	100ul	100ul	50ul
TEMED	8ul	4ul	4ul	4ul	4ul	5ul

### Protein transfer to membrane

Following electrophoresis gels were removed from cassettes and Proteins were transferred to a 0.45  $\mu\text{m}$  polyvinylidene difluoride (PVDF) membrane (Millipore, IPVH00010). Between transfer pads (VWR, 732-0594) soaked in transfer buffer (250 $\mu\text{M}$  Tris, 1.92mM Glycine). Transfer was performed using the Trans-Blot® SD Semi-Dry Transfer Cell (BioRad) at 17 volts for 45-60min. The membrane was then stained with Ponceaux (Sigma, P2395) solution (0.5% Ponceaux and 5 % Acetic Acid in H<sub>2</sub>O) for detection of protein bands to check transfer and allow trimming and cutting of the membrane prior to antibody incubation.

### Immunoblotting

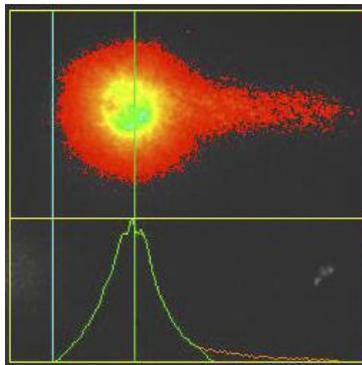
Following transfer membranes were washed one in PBS then incubated for 1 hour in blocking buffer (5% Fat free dry Milk (Marvel) in 0.05% PBS-Tween) at room temperature on a shaker. The membrane was then incubated overnight at 4°C while shaking gently in primary antibody diluted in 5% Milk in 0.05% PBS-Tween (5% BSA in 0.05% PBS-Tween for antibodies against phosphorylated proteins). Membrane was washed 3 times in PBS before incubation with the secondary antibody diluted in 5% Milk in 0.05% PBS-Tween for 1 hour at room temperature while shaking gently. Membranes were washed 3 times with PBS followed by a 3 minutes wash in 0.05% PBS-Tween at room temperature while shaking gently, before a final 3 washes in PBS.

### Chemiluminescence and evaluation

Blots were incubated in Clarity™ Western ECL substrate (Bio-Rad, 170-5060) for 5 minutes. The blot was visualised using Fuji film Intelligent Dark box II and Image Reader Las-4000 Software. Protein standard was used to confirm protein of interest by size comparison. ImageJ analysis software was used to quantify the integrated density of signal on the blot. Densitometry quantification of the protein of interest of calculated after Background subtraction and normalisation to a loading control.

### 2.2.11 Comet Assay

Comet assay (single cell gel electrophoresis) is a method for analysing DNA strand breaks in eukaryotic cells. Cells are embedded in an agarose gel on top of microscope slides. These gels are then lysed in buffer containing detergent and a high salt concentration. This results in the formation of nucleoids of supercoiled DNA. Slides are then subjected to electrophoresis and DNA is stained and visualised by fluorescent microscopy. DNA Loops that contain breaks are able to migrate from the nucleoid toward the anode giving rise to a comet like appearance. The intensity of the comet “tail” relative the “head” (nucleoid body) reflects the number of breaks.



**Figure 2-1: Example of a Comet assay image.** Area under the graph (green) is the comet head, orange is the comet tail

Cells were trypsinised and centrifuged at 1600rpm, supernatant was removed and cells were resuspended in 500µl 10% DMSO in FBS. Cells were then frozen at -80°C in in a Mr. Frosty™ Cryo freezing container filled with isopropanol (Thermo Scientific, #5100-0001). Cells were placed at -80°C and allowed to slow freeze, cells were then removed and stored at -80°C until required. Cells were defrosted on wet ice then washed in cold PBS and centrifuged at 4°C at 1600rpm for 5 mins. Supernatant was removed and cells were resuspended in 0.7% LMP agarose at 37°C to a concentration of  $2 \times 10^5$  cells/ml. 70µl of cell/agarose mix was placed on slides coated in 1% agarose between a cover-slip, care was taken to avoid bubbles. Slides were then placed at 4°C for 10 mins to allow the gel to set. Cover slips were removed and the slides were placed in lysis buffer (2.5M NaCl, 100nM EDTA, 10nM Tris, 250nM NaOH 10% DMSO, 1% Triton X-100) for 1h at 4°C. Slides were then washed twice in cold PBS.

### Alkaline Comet

Slides were placed for 40 mins in alkaline buffer (300mM NaOH, 1mM EDTA) at 4°C to denature DNA. Samples were subjected to electrophoresis for 30 minutes at 25V at 4°C in alkaline buffer.

### Neutral Comet

Samples were subjected to electrophoresis for 30 minutes at 25V at 4°C in Tris-borate EDTA (TBE) buffer.

### Visualisation

After slides were washed 2x in cold PBS, 500µl of 1000x Sybr Gold (Invitrogen) in TBE buffer was added to each gel and slides were incubated for 40 mins in a dark humid chamber. Slides were washed 2x in MilliQ water and allowed to dry. Samples were imaged using an Olympus BX51 widefield microscope with Olympus UPlanFL 20x/0.50 air objective. Comets were scored using Comet assay IV (Perceptive Instruments Ltd., Haverhill, Suffolk, United Kingdom). Each experiment was run on 2 separate gels and an average reading for the two gels was quantified.

## **2.2.12 Immunoprecipitation**

Cells were seeded  $6 \times 10^5$  in a 10 cm dish and transfected as described above 24 hours later. Following another 24 hours, nuclear fractions were prepared as described above.

For FLAG-tagged protein: lysates were incubated with pre-washed and equilibrated anti-FLAG M2 magnetic beads (Sigma Aldrich, #M8823) for two hours at 4°C with constant rotation. Beads were washed twice with lysis buffer and the pulled-down protein was eluted from the beads by incubation with 25 µl 0.2 M glycine-HCl, pH 2.5, for 10 minutes at room temperature. Eluent was neutralised by the addition of 2.5 µl Tris-HCl, pH 8.8. The samples were then mixed with sample buffer and boiled at 100°C for five minutes in the presence of 2.5% β-ME before being subjected to SDS-PAGE and immunoblot.

For GFP-tagged protein: lysates were incubated with 3 µl anti-GFP rabbit serum (Life Technologies, #A-6455) for one hour at 4°C with constant rotation. Lysates were then incubated with 20 µl pre-washed Protein A Sepharose beads (Generon, #PC-

A5) for 1 hour at 4°C with constant rotation. Beads were then washed twice in IP buffer, mixed with sample buffer and boiled at 100°C for 5 minutes in the presence of 2.5%  $\beta$ -ME. Samples were centrifuged for 10 minutes at 13,000 rpm at 4°C and transferred to a new tube. Samples were then subjected to SDS-PAGE and immunoblot.

### **2.2.13 Mass Spectrometry**

Immunoprecipitation was carried out as described above. Gel was then stained using Coomassie and bands present in IP and absent in control were excised. Excised bands were digested in-gel and the resulting tryptic peptides analysed by LC-MSMS using an Orbitrap XL (Thermo Scientific) coupled to a nanoAcquity (Waters). MSMS data was acquired in a top 6 DDA fashion and raw files were processed in Proteome Discover v1.4 using the Sequest search engine against a Uniprot human database (downloaded 030314, 68,710 entries). CAM cysteine was set as a fixed modification with oxidised methionine and deamidated asparagine/glutamine as potential variable modifications. FDR calculations were performed using Percolator with peptides filtered to 0.01 FDR. Mass spectrometry analyses were performed by Dr. Robin Antrobus at Cambridge Institute for Medical Research.

### **2.2.14 Statistical analyses**

Two-tailed, paired or unpaired Student's t-tests were carried out on experimental data from at least three individual experiments using Excel. A one-way Anova was used for multiple comparisons between groups using Sigma Plot.

### **2.2.15 Ethics statement**

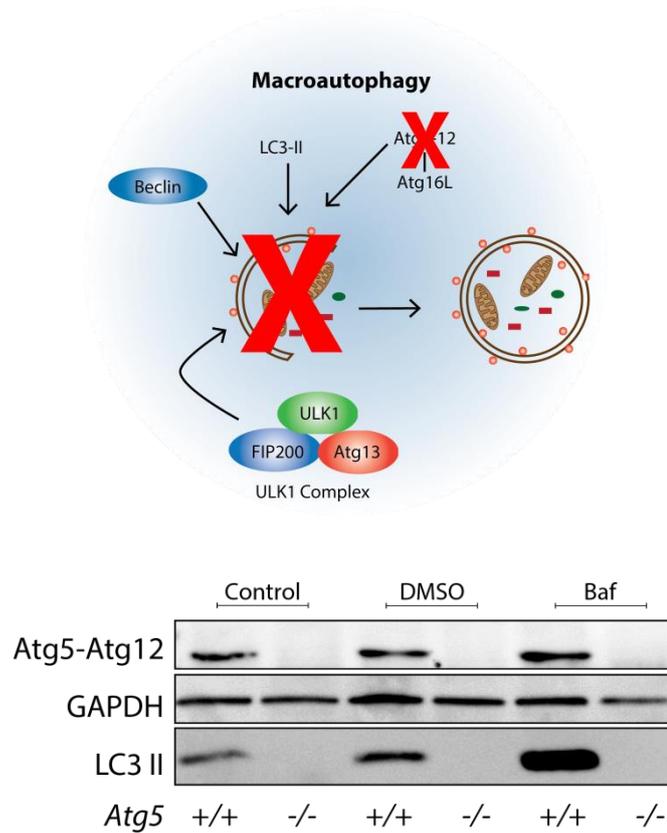
Ethical approval was granted by the LERC Newcastle University, UK. The work was licensed by the UK Home Office (PPL 60/3864) and complied with the guiding principles for the care and use of laboratory animals.

### **Chapter 3. The Effects of Autophagy on DNA Damage Repair**

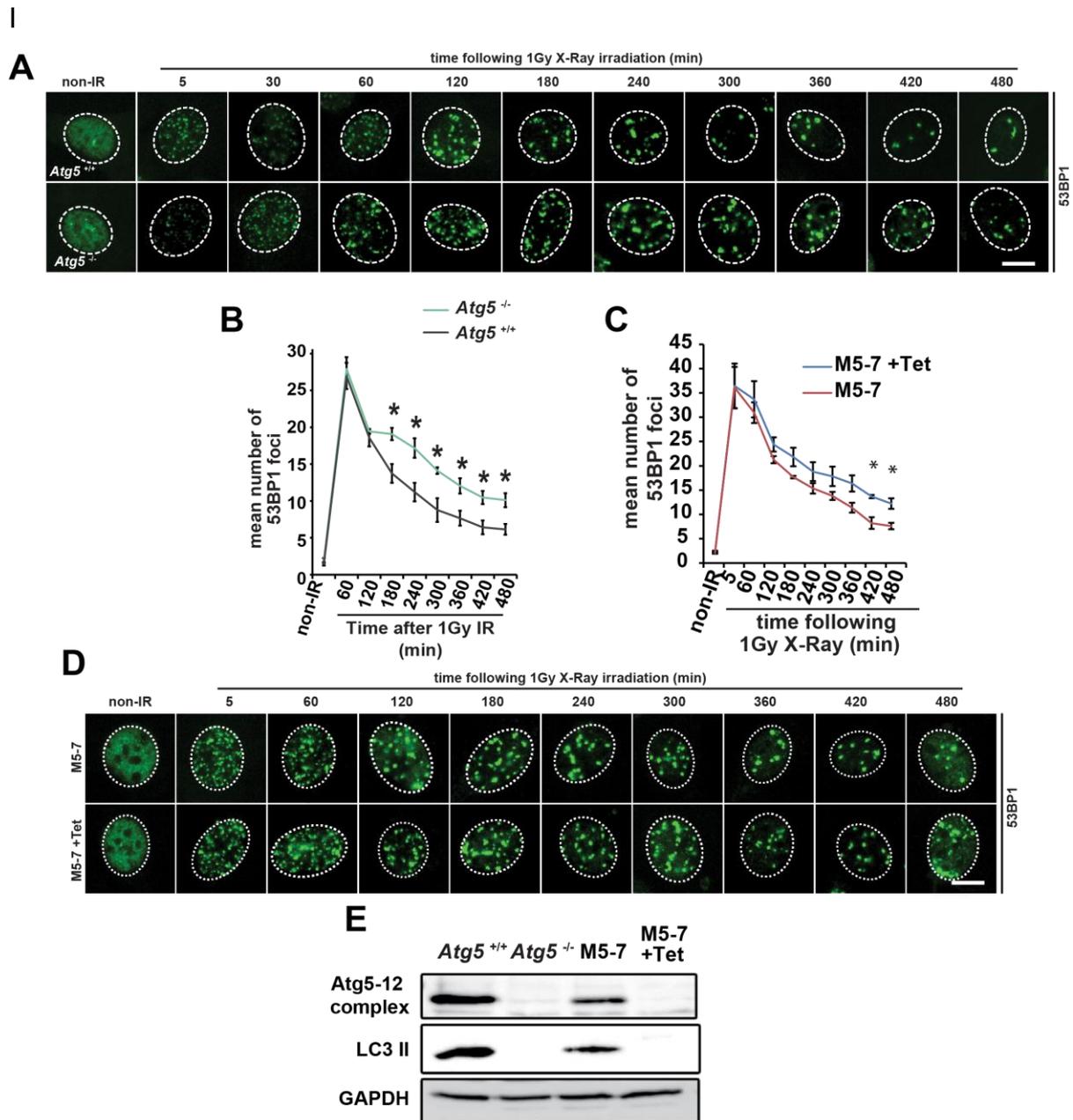
Autophagy is known to play a central role in cellular homeostasis, with impaired autophagy being implicated in many human diseases (Levine and Kroemer, 2008). Autophagy has been shown to be a potent tumor suppressor, with autophagy defects being common in many forms of cancer (Gozuacik and Kimchi, 2004, Kung et al., 2011). However, the exact mechanisms by which autophagy confers protection against transformation are still unclear. Autophagy defects have been shown to lead to genomic instability (Karantza-Wadsworth et al., 2007) and inhibition of autophagy has previously been suggested to suppress DNA repair (Liu et al., 2015, Bae and Guan, 2011, Robert et al., 2011). In this chapter I further explore the link between autophagy and the ability of cells to repair DNA.

### 3.1 Cells Lacking Autophagy Have a Decreased DNA Repair Capacity

To investigate the effect of autophagy on DNA repair, DNA damage was induced in *Atg5<sup>-/-</sup>* and *Atg5<sup>+/+</sup>* MEFs using 1Gy of X-Ray irradiation. Atg5 is part of the Atg5-12/Atg16L1 complex that is essential for the formation of autophagosomes. *Atg5<sup>-/-</sup>* MEFs are unable to form autophagosomes, rendering these cells autophagy deficient (Figure 3-1). Similarly, M5-7 MEFs are a cell line where the knockdown of *Atg5* is under the control of a tetracycline promoter (Tet). The advantage of an inducible knock-down cell system is that it reduces clonal differences between cell lines and the period where cells are able to adapt to the lack of autophagy. DNA damage was assessed using immunostaining for 53BP1 across a 480 minute time course. It was found that *Atg5<sup>-/-</sup>* MEFs show a reduced rate of repair, judged by an increased number of 53BP1 foci at later time points, compared to *Atg5<sup>+/+</sup>* (autophagy competent) MEFs (Figure 3-2A-B). Similarly, M5-7 MEFs treated with Tet to abolish expression of *Atg5*, showed a reduced repair capacity compared to non-treated controls (Figure 3-2C-D). The differences in M5-7 MEFs shown in (Figure 3-2C) are less pronounced than those seen in the *Atg5* MEFs shown in (Figure 3-2B). The reason for this difference is not clear, however M5-7 –Tet cells showed reduced basal levels of both LC3 and the Atg5-Atg12 complex compared to *Atg5<sup>+/+</sup>* (Figure 3-2 E). This suggests a slight suppression of autophagy even in the absence of Tetracycline treatment and could explain the larger differences seen in the *Atg5* cell lines compared to the M5-7 cells. This could be a result of antibiotics present in the FCS used in the culture media. Overall, these results indicate that MEFs lacking autophagy show a slower recovery from X-ray induced DNA damage, suggesting a possible role for autophagy in the DDR.



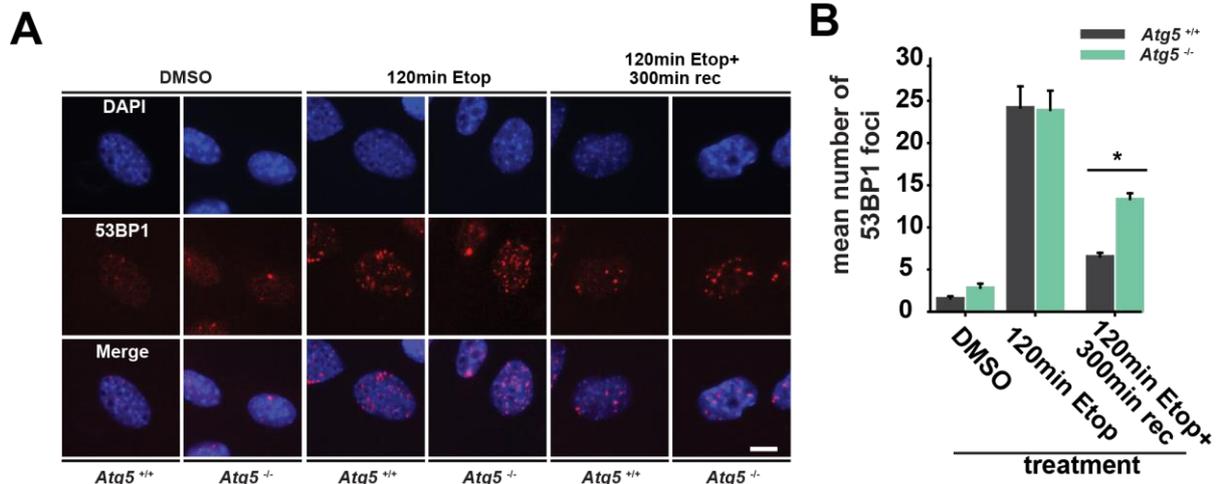
**Figure 3-1: Deletion of *Atg5* leads to a loss of autophagy.** (A) Schematic representation of the initiation of autophagy, showing that loss of *Atg5* results in a loss of functioning autophagy. (B) Representative western blot showing Atg5/12 and LC3 II levels in *Atg5*<sup>+/+</sup> and *Atg5*<sup>-/-</sup> MEFs treated with and without 400nM Bafilomycin A1 for 440 min prior to collection.



**Figure 3-2: Cells lacking autophagy have a reduced repair capacity in response to X-Ray irradiation.** Representative images (A) and quantification (B) of the mean number of 53BP1 foci in *Atg5*<sup>+/+</sup> and *Atg5*<sup>-/-</sup> MEFs 0-480 minutes after 1Gy X-Ray irradiation. (C-D) M5-7 MEFs were treated with tetracycline (Tet) to induce knock-out of *Atg5*. Mean number of 53BP1 foci was quantified (C). Representative images of 53BP1 foci 0-480 minutes after 1Gy X-Ray irradiation are shown in (D). Representative western blot showing Atg5/12 and LC3 II levels in *Atg5*<sup>+/+</sup>, *Atg5*<sup>-/-</sup> and M5-7 MEFs with and without Tetracycline (Tet). Scale bar 10  $\mu$ m; n=3; Error bars represent S.E.M.

In addition to DNA damage, X-Ray irradiation induces damage to other cellular components such as proteins and lipids (Carugo and Djinovic Carugo, 2005). Having shown that cells lacking autophagy show an increased sensitivity to X-Ray induced DNA damage it was important to confirm that these effects were DNA damage specific and not due the accumulation of a different type of cellular damage. This is especially important in light of the role of autophagy in the recycling of many damaged proteins and macromolecules. In order to investigate this, Etoposide was used to induce DNA damage. Etoposide induces double strand breaks via the inhibition of topoisomerases (Pommier et al., 2010), these are enzymes that cleave DNA to allow the rearrangements required for sufficient access of the transcription machinery. Their inhibition results in the enzyme being held at the site of cleavage resulting in improper resolution and generation of double strand breaks (DSB).

Here, *Atg5<sup>-/-</sup>* and *Atg5<sup>+/+</sup>* MEFs were treated with 1 $\mu$ M Etoposide for 120 minutes. Cells were either fixed immediately after treatment or washed and allowed to recover for 300 min, then were fixed and stained with antibody against 53BP1 ( Figure 3-3A). Results shown in Figure 3-3B indicate that treatment with Etoposide induced similar levels of damage in *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs. However, following 5 hours recovery *Atg5<sup>-/-</sup>* MEFs showed significantly more DNA damage. These results confirm that cells lacking autophagy are indeed more sensitive to DNA damage.

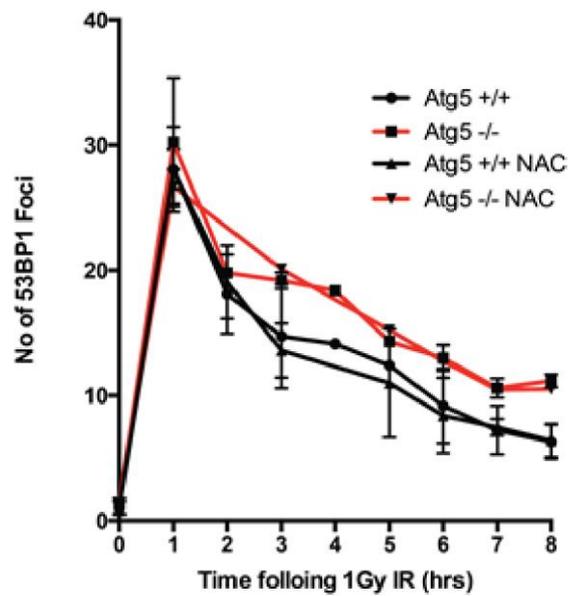


**Figure 3-3: Cells lacking autophagy have a reduced repair capacity in response Etoposide.** DNA damage was induced in *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs by 120 minute incubation with Etoposide and, where indicated, followed by a 300 minute recovery period. Representative images are shown in (A) and the mean number of 53BP1 foci was quantified (B). Scale bar 10  $\mu$ m; n=3; Error bars represent S.E.M; NS: not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

### 3.2 The effect of autophagy on the DDR is independent of ROS

Cells lacking autophagy have been shown to have higher levels of ROS. These are a group of reactive molecules including: superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl radical ( $OH^{\cdot}$ ) and hydrogen peroxide ( $H_2O_2$ ) that are generated as part of normal oxygen metabolism, primarily in mitochondria. Autophagy is involved in the degradation of mitochondria (mitophagy), and inhibition of autophagy has been shown to contribute to an increase in both dysfunctional mitochondria and ROS (Pua et al., 2009). ROS play an important role as signalling molecules in the cell. However, if levels of ROS are elevated they can lead to oxidative stress, causing damage to proteins, lipids and DNA. For this reason, it was important to assess the potential contribution of ROS in the differences in 53BP1 foci kinetics seen between *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs.

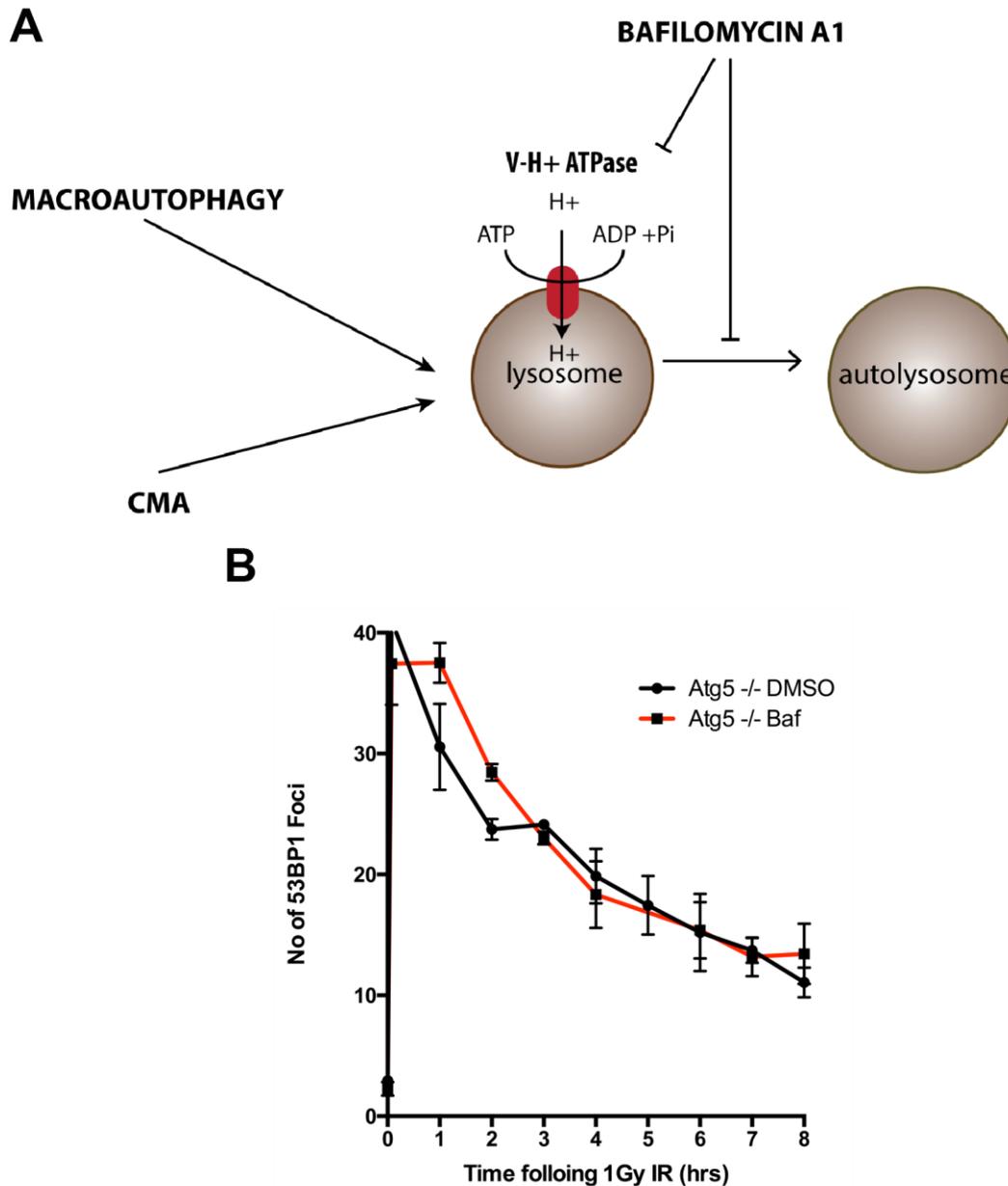
To test whether the increase in DNA damage seen in cells lacking autophagy was ROS dependent, *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs were exposed to 1Gy of X-ray irradiation, treated with and without antioxidant N-acetyl-cysteine (NAC) and fixed every hour for 8h following IR. Cells were then stained with antibody against 53BP1 and DDF quantified by microscopy. Results shown in Figure 3-4 indicate that treatment with NAC had no effect on the number of DNA damage foci in *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs following 1Gy IR. The concentration of 5mM NAC has however, been shown to reduce ROS in response to the higher dose of 10Gy in these cells (Correia-Melo, 2014). These data suggest that the differences seen in *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs are not ROS dependent, however it still remains for us to measure ROS in this system.



**Figure 3-4: Treatment with NAC does not rescue DNA damage phenotype.** Quantification of the mean number of 53BP1 foci in *Atg5*<sup>+/+</sup> and *Atg5*<sup>-/-</sup> MEFs with and without 5mM NAC 0-480 min after 1Gy X-Ray irradiation. n=3; Error bars represent S.E.M.

### 3.3 The effect of Atg5 knock-out on DDR is independent of other lysosomal degradation pathways

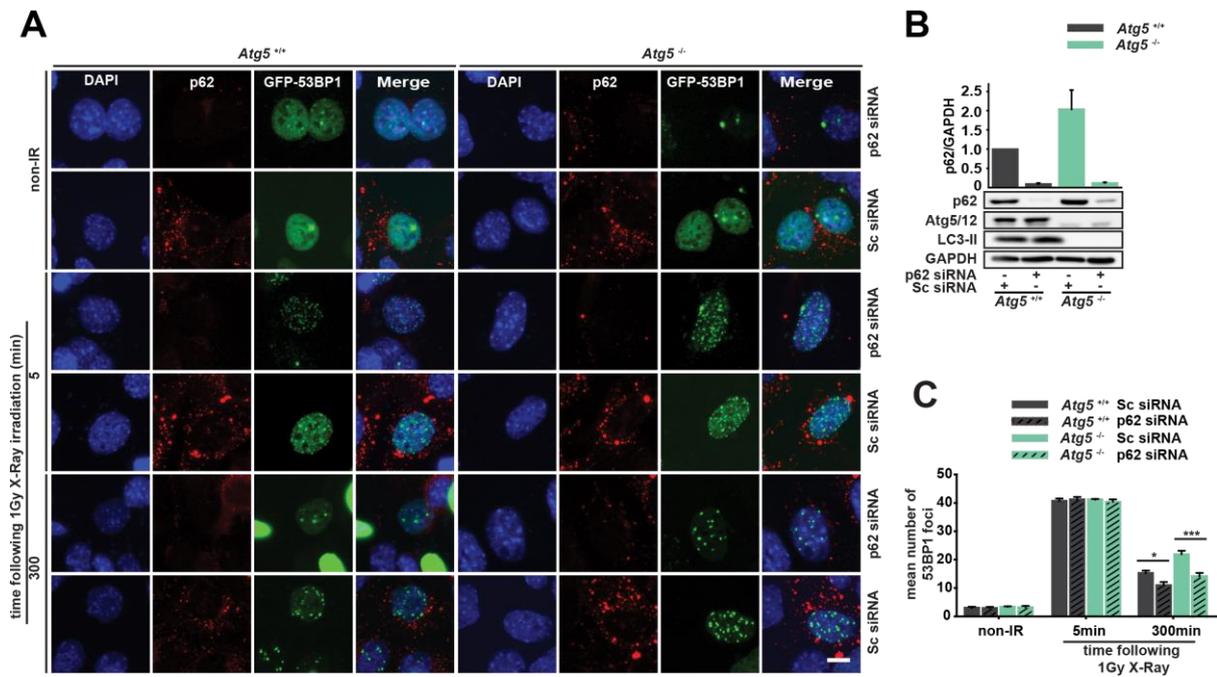
It has been recently suggested the CMA may be involved in the regulation of DNA damage repair through the selective degradation of Chk1 (Park et al., 2015). It has also been suggested that autophagy is able to occur in the absence of Atg5 (Nishida et al., 2009). It was therefore important for us to assess if there was any additive effect of other lysosomal degradation pathways in DNA damage repair in *Atg5*<sup>-/-</sup> MEFs. Here, lysosomal degradation was inhibited (Figure 3-5A) with treatment of *Atg5*<sup>-/-</sup> MEFs with 400nM Bafilomycin A1 (Baf) (Yamamoto et al., 1998). *Atg5*<sup>-/-</sup> MEFs were exposed to 1Gy of X-ray irradiation, treated with and without Baf and fixed every hour for 8h following IR. Cells were then stained with an antibody against 53BP1 and DDF quantified by microscopy. Quantification shown in (Figure 3-5B) indicates that block of lysosomal degradation with Baf had no further effect on DNA damage repair in *Atg5*<sup>-/-</sup> MEFs. These data suggest that CMA as well as Atg5/7 independent autophagy have no effect on DNA damage repair following low dose IR.



**Figure 3-5: Block of lysosomal degradation with Baf has no further effect on DNA damage than genetic knockout of autophagy in *Atg5*<sup>-/-</sup> MEFs.** (A) Schematic representation of the action of Bafilomycin A1 which inhibits V-H ATPases present in the lysosomal membrane leading to deacidification and an inhibition of the fusion of autophagosomes. (B) Quantification of the mean number of 53BP1 foci *Atg5*<sup>-/-</sup> MEFs with and without 400nM Baf 0-480 min after 1Gy X-Ray irradiation. n=3; Error bars represent S.E.M.

### 3.4 Knockdown of p62 rescues Decrease in DNA Repair Capacity in Autophagy Deficient Cells

Assuming that ROS is not responsible for increased levels of DNA damage in autophagy null cells, we decided to look at autophagy adaptor proteins as a potential link between these two spatially separate cellular processes. The autophagy adaptor protein p62 has previously been suggested to play a role in DNA damage repair (Bae and Guan, 2011). In order to further understand the extent of the contribution of p62 to the differences seen in DNA damage between autophagy deficient MEFs, p62 was knocked down using siRNA in *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs expressing a 53BP1-GFP reporter. Cells were then exposed to 1Gy of X-Ray irradiation and fixed at 5 and 300 minutes following IR as well as non-IR control. Cells were stained with antibody against p62 and 53BP1 foci were analysed by microscopy (Figure 3-6A). The knockdown efficiency was tested by immunoblot for p62 as shown in Figure 3-6B. Data in (Figure 3-6C) show that knockdown of p62 rescues the increased DDF seen in *Atg5<sup>-/-</sup>* MEFs as well as reduces the number of DDF at 5h following IR in *Atg5<sup>+/+</sup>* MEFs. These results suggest that differences in DDF in autophagy deficient cells are dependent on p62.

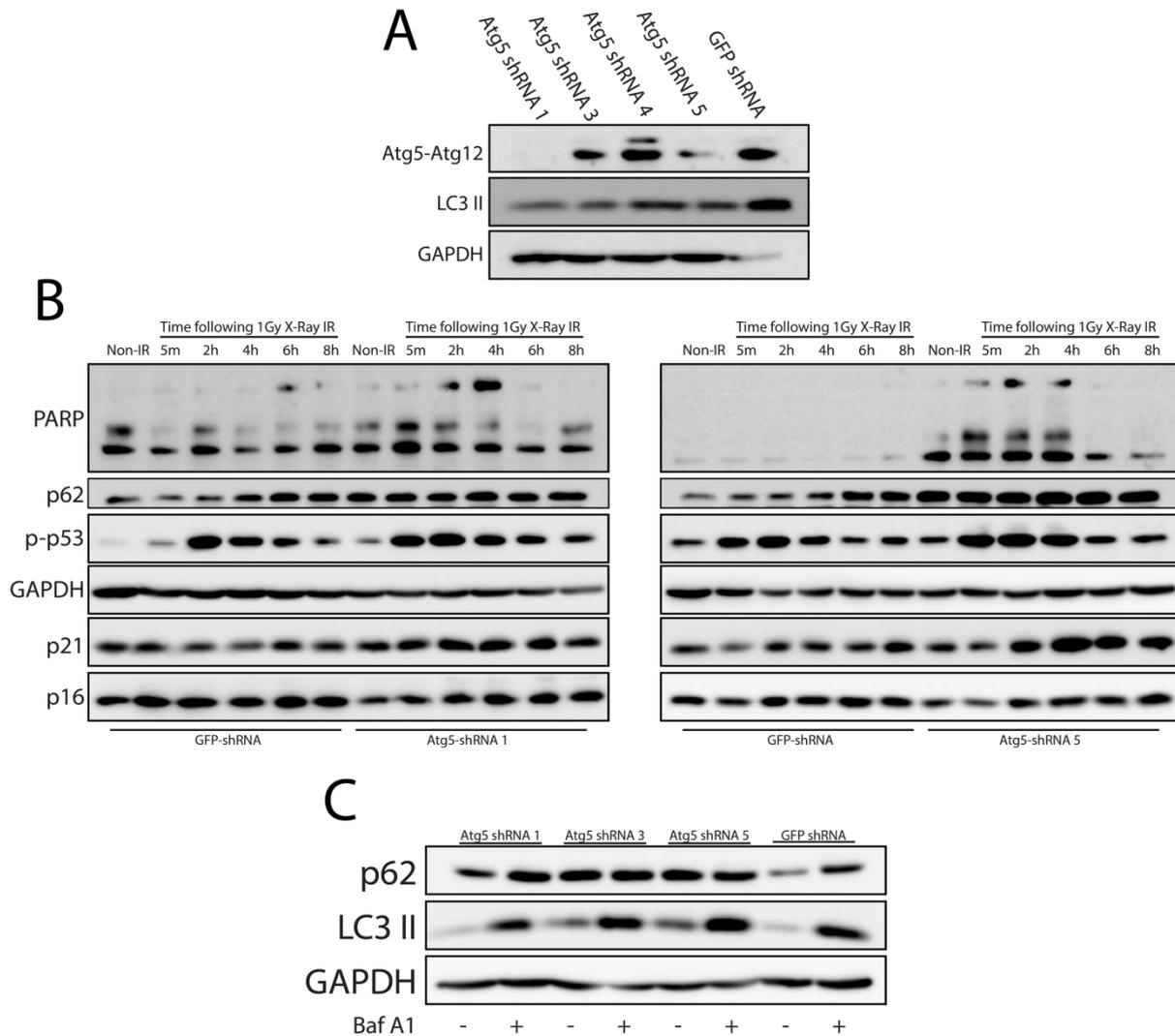


**Figure 3-6: Knockdown of *p62* in autophagy deficient cells rescues the delay in DNA damage repair.** *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs treated with control or *p62* siRNA 5 and 300 min after 1Gy X-Ray irradiation. (A-C) Representative images of GFP-53BP1 foci are shown in (A). Blot showing scrambled control (Sc) and *p62* siRNA in *Atg5<sup>+/+</sup>* and *Atg5<sup>-/-</sup>* MEFs (B). Quantification of the mean number of GFP-53BP1 (C). Scale bar 10  $\mu$ m; n=3; Error bars represent S.E.M; NS: not significant, \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ .

### 3.6 The effect of knockdown of *Atg5* on DDR Downstream Signalling

Having established that cells with compromised autophagy have a slower DNA damage repair kinetic compared to those without, as measured by 53BP1 foci (Figure 3-2 & Figure 3-3), I investigated whether downstream DNA damage signalling was being affected as a result of autophagy inhibition. For this, it was necessary to knock down *Atg5* in primary cells, as downstream signalling is possibly disrupted in *Atg5*<sup>-/-</sup> MEFs as a result of the SV40 immortalisation process (Boichuk et al., 2010). Here, MRC5 human fibroblasts were transduced with shRNA against *Atg5* as a means of inhibiting autophagy. The *Atg5* knockdown efficiency of cell lines derived from chosen virus titre is shown in (Figure 3-7A) following 2 cell passages (to allow cells to recover from transduction), indicating successful *Atg5* knockdown by shRNA#1 and 5. These results indicate a modest effect on LC3II levels; this is in agreement with the observation made in (Hosokawa et al., 2007) that only very low levels of *Atg5* are required for functional autophagy. Next, we wanted to investigate the effect of *Atg5* knockdown on signalling downstream of a DDR. Here, MRC5 human fibroblasts that are stably expressing shRNA against *Atg5* were exposed to 1Gy of X-Ray irradiation and collected at time points 5min-8h following IR. Samples were then run on SDS-PAGE electrophoresis and immunoblot analysis was carried out for PARP, p62, p-p53, p21, p16 and loading control GAPDH. Results shown in Figure 3-7B indicate an increase in p62 in cells expressing shRNA against *Atg5*. Similarly, there was also a trend for an increase in p-p53 and p21 expression in *Atg5* shRNA expressing cells. These results are consistent with data shown previously indicating that impaired autophagy impacts on DDF repair kinetics (Figure 3-2& Figure 3-3). As it was planned to assess senescence in these *Atg5* shRNA-expressing cells, they were not subject to Puromycin selection so that an accurate record of population doubling could be kept. Comparison of the levels of LC3II with and without Bafilomycin A1 treatment allows gauging of the basal rate of autophagic flux. Results shown in Figure 3-7C indicate that *Atg5* shRNA is in fact having little effect of autophagic flux following successive cell passages, suggesting that expression of shRNA had been lost. Another attempt was made to generate these cell lines using higher virus titres with the hope of achieving a greater knockdown of autophagy. Interestingly, cells expressing *Atg5* shRNA in this instance ceased to

proliferate following transduction which precluded follow up experiments. It is possible that inhibition of autophagy has led to a premature induction of senescence in these cells as previously reported in (Kang et al., 2011).



**Figure 3-7: Downstream DDR in MRC5 fibroblasts transduced with shRNA against *Atg5*.** Western blots (A) showing Atg5-12, LC3 II and loading control GAPDH in chosen virus volume (2ml Virus media in a total of 4ml). Western blots (B) showing PARP, p62, p-p53, p21, p16 and loading control GAPDH in MRC5 fibroblasts transduced with *GFP*-shRNA (control) of *Atg5* shRNA as indicated following 1Gy X-ray irradiation at time points 5min-8h. Western blots (C) showing p62, LC3 II and loading control GAPDH in chosen virus volume (2ml Virus media in a total of 4ml) with and without 4h 400nM Bafilomycin A1 treatment.

### 3.6 Discussion

Despite several recent studies that explore the role of autophagy in the DDR few have directly tested the role of autophagy on DNA damage repair (Liu et al., 2015, Bae and Guan, 2011, Robert et al., 2011). Liu *et al* conclude that there is no difference in the rate of DNA repair between autophagy compromised and wt cells (Liu et al., 2015). Here, autophagy is abolished through inducible knock out of Atg7. DNA damage is measured by immunofluorescence analysis of  $\gamma$ H2A.X before, 1 h and 8 h after 10Gy ionizing radiation. In this work, the authors report the complete resolution of  $\gamma$ H2A.X foci 8h following 10Gy irradiation. Our group and others have observed an average of 30 DDF in MEFs at 1 day following 10 Gy X-ray irradiation (Hewitt et al., 2012, Rodier et al., 2011), as well as foci that persist up to 10 days following IR. The striking difference between the numbers observed in the study from Liu *et al* and ours suggests a relatively low sensitivity in the assay used by others to detect DNA damage foci.  $\gamma$ H2A.X is also measured by western blot from whole cells lysed in RIPA buffer. Data from our group (not shown) indicates that samples from MRC5 human fibroblasts lysed in RIPA buffer before or 1h, 6h, and 12h following exposure to 20Gy X-Ray irradiation are positive for  $\gamma$ H2A.X only at 1h and 6h but not 12h. When compared to analysis of DNA damage foci at the same time points (Hewitt et al., 2012, Passos et al., 2010) it can be seen that measurements of DDR using western blotting is extremely insensitive. DNA damage is also measured by Comet assay and data is presented as % of cells with Comet tail. Again, the authors report no significant differences between the levels of DNA damage between ATG7 null and wt MEFs following, in this instance, treatment with Etoposide. Quantification of Comet assay as % of cells with Comet tail is a very crude analysis and results show low levels of damage. This, again, suggests inadequate sensitivity in this analysis. Authors then go on to report increased proteasome activity in ATG7 null MEFs, which results in increased degradation of Chk1, resulting in an inhibition of homologous recombination. They also measure NHEJ using reporter plasmids described in (Seluanov et al., 2004) and report no difference between ATG7 null and wt MEFs. Taken together, it is surprising that an inhibition of HR and no change in NHEJ does not lead to a difference in levels of double strand break repair in cells lacking autophagy and further point to a lack of sensitivity in the methods used to

assess damage. Authors do report an increased level of DNA damage in cells lacking autophagy when NHEJ is inhibited at 4h and 8h following treatment with Etoposide, as measured by COMET assay (Liu et al., 2015).

Contrary to the study from Liu *et al* 2015, Bae and colleagues describe that inhibition of autophagy by FIP200 deletion impairs DNA damage repair (Liu et al., 2015). In this study, similarly to Liu *et al*, high doses of DNA damaging drugs or 10Gy of irradiation is used to induce damage. Damage is assessed by COMET assay, immunostaining and western blot for  $\gamma$ H2A.X (Bae and Guan, 2011). The difference between both studies could be due to a more sensitive analysis of DNA damage carried out by Bae *et al*. In their study the authors detected  $\gamma$ H2A.X positive cells using higher magnification at 24h following 10Gy IR in FIP200 deleted cells. They are also able to detect a much more robust induction of  $\gamma$ H2A.X, as measured by western blot. This could be a result of different sample preparation. Here, cells were lysed directly in boiling sample buffer, giving a more complete lysis, which is especially important when trying to assess hard-to-extract histones and preserves phosphorylation. It should be noted that 10Gy IR is sufficient to cause cell death as concluded by this study (Bae and Guan, 2011) and others (Jo et al., 2015). Similarly Liu *et al* used 10Gy IR to induce DNA damage, however they report a much reduced induction of cell death (Liu et al., 2015). Induction of apoptosis can lead to false positive results when using Comet assay to assess DNA damage induction as shown by (Choucroun et al., 2001). There are a number of mechanisms by which apoptotic cells would appear to have increased DNA damage, as measured by % of tail vs head. It is known that DNA intercalators that are commonly used to visualise Comets such as ethidium bromide and syber gold also recognise RNA (Tuma et al., 1999). As apoptosis is an active process leading to changes in protein synthesis (Clemens et al., 2000), it is possible that changes in levels of mRNA in these cells could lead to incorrect scoring of Comet tails. Moreover, the chromatin degradation occurring at the beginning of apoptosis (Walker et al., 1995) could lead to further migration of stretched DNA into the Comet tail. Comet tails may result due to nuclear blebbing and the formation of micronuclei that are also common to apoptotic cells. Interestingly, work done by our group and others has suggested a role for autophagy in the degradation of micronuclei (Ivanov et al., 2013, Rello-Varona et al., 2012). We have also seen that 10Gy of irradiation is sufficient to lead to the generation of

micronuclei in *Atg5*<sup>-/-</sup> MEFs, presenting another potential confound when using Comet assay to assess autophagy dependent differences in DNA repair using high doses of IR. Contrary to a potential overestimation of DNA damage seen using Comet assay, cell death can lead to an underestimation of DNA damage when using markers such as  $\gamma$ H2A.X measured by microscopy. This is because cells that contain higher level of DNA damage are eliminated from your analysis, as they detach during the fixation and staining process.

Autophagy status has been heavily implicated in cell survival and execution of cell death pathways (Marino et al., 2014), therefore the use of DNA damaging agents at doses able of inducing cell death is inappropriate to elucidate the effect of autophagy on the kinetics of DNA damage repair itself.

As well as inducing cell death, 10Gy X-ray irradiation is also able to induce cellular senescence in MEFs (Hewitt et al., 2012). This induction of senescence is associated with the presence of long lived DNA damage foci (Hewitt et al., 2012, Rodier et al., 2011, Fumagalli et al., 2012), as well as the constant generation and repair of transient DNA that arise due to mitochondrial ROS generation (Passos et al., 2010). The role of autophagy in mitochondrial turnover (Lee et al., 2012a) could indeed influence DNA damage in this context, independent of any role in DNA damage repair. Consistently, knockout of *Atg7* in the hematopoietic system of mice resulted in an accumulation of mitochondria, ROS generation and DNA damage (Mortensen et al., 2011). Similarly, hepatocytes from *Atg5* mosaic knockout mice show increased numbers of swollen mitochondria as well as DNA damage (Takamura et al., 2011). This makes the study of direct effects of autophagy on DNA damage repair extremely challenging in using these models. Interestingly, autophagy status may influence the execution of the senescent phenotype (Gewirtz, 2013) presenting the problem that differences seen in DNA damage may result from the effect of autophagy on the senescence process rather than DNA damage repair itself.

It is possible that the effect of autophagy in processes such as cell death and senescence are mediated, in part, by changes in DNA damage repair. However in this thesis to avoid the possible confounding influences of these processes, DNA damage repair was studied in the context of low level damage induction.

Results presented in this chapter show that cells lacking autophagy due to knockout of *ATG5* show an impaired repair kinetics of 53BP1 foci compared to wt cells when exposed to 1Gy of irradiation. As discussed, M5-7 MEFs were used to help confirm that this result was due to the absence of autophagy and not due to clonal differences between MEFs derived from separate animals. It should be noted that these cells must be cultured for 4 days in the presence of Tet to completely abolish autophagy. This is because only very low levels of Atg5 are required for basal autophagy (Hosokawa et al., 2007). It would be interesting to study the effect of an acute inhibition of autophagy in the context of DNA damage as both *Atg5*<sup>-/-</sup> and M5-7 MEFs cells have impair autophagy prior to induction of DNA damage. This is something that will be discussed further in the coming chapters.

Whole cell X-ray irradiation leads to the damage of many cellular components such as lipids, proteins and DNA. As we are hoping to assess the effect of autophagy, a protein degradation pathway, on DNA repair capacity. It is important to account for the possibility that differences in foci repair kinetics between *Atg5*<sup>+/+</sup> and *Atg5*<sup>-/-</sup> MEFs may arise due to differences in the clearance of other cellular damage other than DNA damage, which may itself influence DNA repair processes. Therefore, damage induced by the topoisomerase inhibitor Etoposide was also assessed. These results indicated that, similar to X-ray IR, cells lacking autophagy had a reduced repair capacity compared to wild type cells. These data suggest that the differences seen in DDF following induction of DNA damage result from differences between *Atg5*<sup>+/+</sup> or *Atg5*<sup>-/-</sup> MEFs in DNA repair pathways and not as a result of damage to other cellular macromolecules.

Data presented above suggest that the differences in DNA damage foci repair kinetics following 1Gy X-ray irradiation are not dependent on ROS as treatment with 5mM NAC had no significant effect on repair kinetics in *Atg5*<sup>+/+</sup> or *Atg5*<sup>-/-</sup> MEFs. It should be noted, however, that levels of intracellular ROS were not assessed in this experiment. Nevertheless, treatment of fibroblasts with 2.5mM NAC has previously been shown to be sufficient to reduce levels of ROS following 20Gy X-Ray IR as well as in *Atg5*<sup>+/+</sup> or *Atg5*<sup>-/-</sup> MEFs following 10Gy X-Ray IR (Correia-Melo, 2014). Therefore, it could be expected that this treatment would be sufficient to suppress ROS in response to 1Gy IR, however further studies would be required to confirm these assumptions.

Recently, it was suggested that CMA may play a role on DNA damage repair (Park et al., 2015). In this paper, similar to those published by 2 other groups (Liu et al., 2015b, Bae and Guan, 2011a) high levels of DNA damaging agents were used and reduced cell viability was observed, again leading to possible confounding effects when assessing DNA damage. The authors propose that CMA is responsible for the regulated degradation of Chk1, concluding that inhibition of CMA leads to an accumulation of Chk1 in the nucleus resulting in a prolonged cell cycle arrest and DNA damage response (Park et al., 2015). For that reason, it was important to assess the potential role of CMA on DNA repair in our system. My results indicate that inhibition of lysosomal degradation, inclusive of CMA, by treatment with Bafilomycin A1 lead to no further change in DNA damage foci kinetics. These data suggest that CMA is not having an effect on DNA damage repair in response to low level radiation. It is possible that CMA is inhibited in *Atg5*<sup>-/-</sup> MEFs and so treatment with Bafilomycin A1 had no further effect, however inhibition of macroautophagy by *Atg5* knockdown has in fact been shown to lead to an increase in the activity of CMA (Wang et al., 2008). Although unlikely, further investigation would be required to rule out the effects of CMA in this process.

There have also been studies that show an *Atg5/7*-independent alternative macroautophagy pathway (Nishida et al., 2009). Here, authors describe the formation of autophagosomes that are devoid of the classical autophagosome marker LC3 II that are generated in a Rab9-dependent manner in both *Atg5*<sup>-/-</sup> and *Atg7*<sup>-/-</sup> MEFs (Nishida et al., 2009). It is possible that this pathway could be upregulated in *Atg5*<sup>-/-</sup> MEFs and influencing DNA damage repair. This possibility was ruled out along with the contribution of CMA as treatments with Bafilomycin A1 in *Atg5*<sup>-/-</sup> MEFs had no effect on the rate of DNA damage repair, suggesting that other lysosomal degradation pathways such as *Atg5/7*-independent autophagy were not important in this context.

It should also be noted that *Atg5* has been shown to be involved in a number of processes independently of its function in autophagy such as: immunity to intracellular pathogens in macrophages via its role in recruiting IFN- $\gamma$ -inducible p47 GTPase IIGP1 (*Irga6*) to the vacuole membrane (Zhao et al., 2008) and late endosome and lysosome biogenesis (Peng et al., 2014). *Atg5* has also recently been shown to play an important role in the induction of mitotic catastrophe through

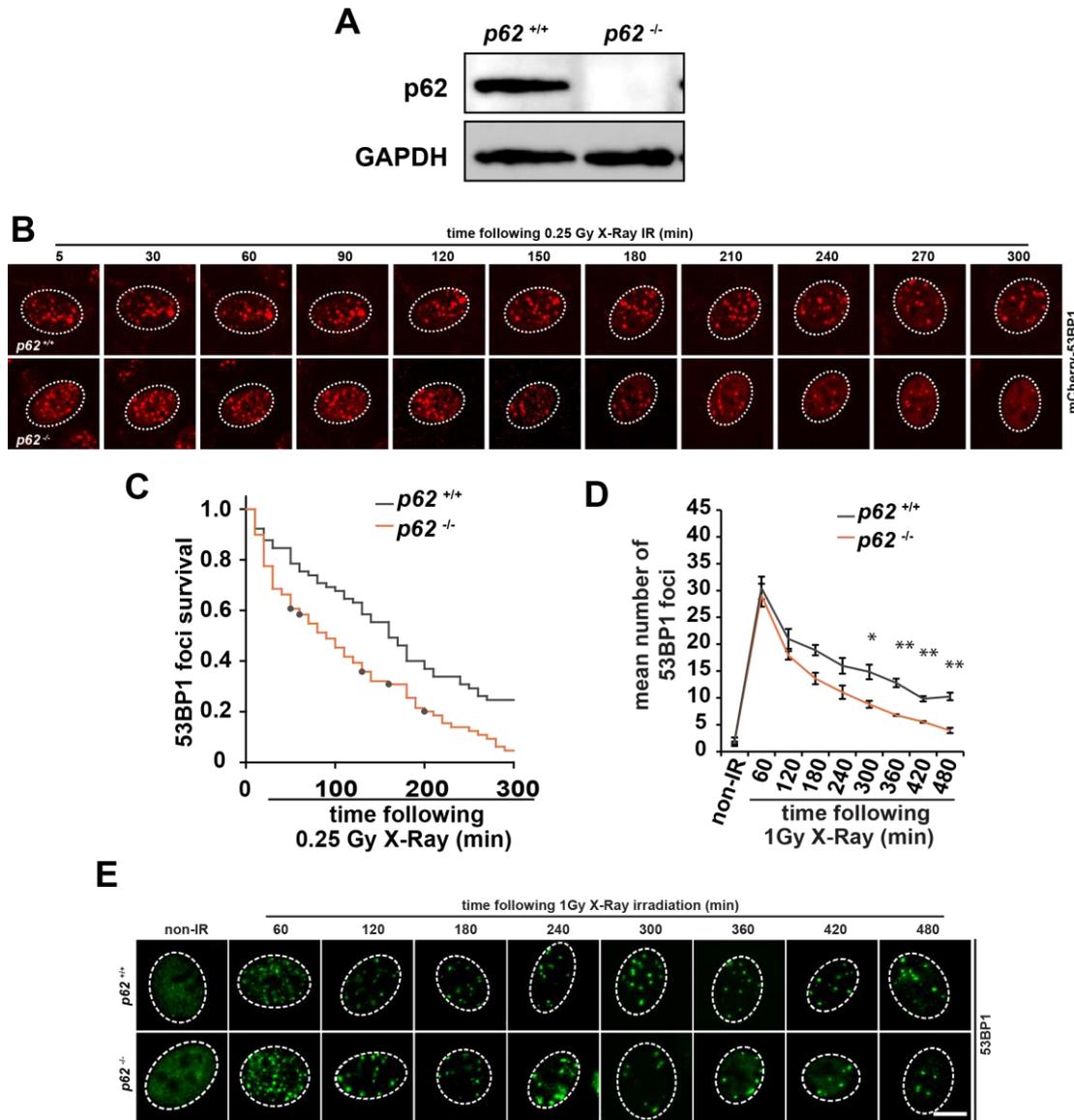
its interaction with survivin, thereby inhibiting the correct formation of the chromosome passenger complex (Simon and Friis, 2014) (Maskey et al., 2013). This effect of Atg5 occurs in the nucleus and is independent of autophagy. This mitotic catastrophe was induced in response to sub-lethal treatment with anti-cancer drugs such as: Etoposide and cystplatin for 24-48 hrs. It is possible that this role for Atg5 could lead to confounding results when studying the role of autophagy in DDR using *Atg5* KO as a means of autophagy ablation. However, Simon and Friis suggest that the effect of Atg5 on mitotic catastrophe occurs independently of DNA damage (Simon and Friis, 2014). The induction of mitotic catastrophe is less likely to be relevant when using more acute treatments (2h to induce DNA damage). Moreover, it would be expected that increased mitotic catastrophe would lead to an increase in DNA damage in *Atg5*<sup>+/+</sup> MEFs. Although my data suggests a link between inhibition of autophagy to a decreased repair of DNA damage foci, further experiments should be conducted using another genetic approach to inhibit autophagy such as knockout of *Atg7* when assessing DNA damage repair in response to 1Gy X-ray IR. Indeed, another reason for the discrepancy with Liu et al is that they have used an *Atg7* KO while in our experiments we used an *Atg5* KO.

## **Chapter 4. Phenotypic characterisation of the role of p62 in the DDR**

In the previous chapter, we demonstrated that cells lacking autophagy have a reduced clearance of 53BP1 foci following induction of damage by both X-ray irradiation and Etoposide treatment (Figure 3-2& Figure 3-3). These data are in agreement with (Bae and Guan, 2011). It has previously been observed by our group (Korolchuk group, unpublished data) and others that cells lacking autophagy have higher levels of reactive oxygen species, especially when cells are exposed to extrinsic stressors (Sun et al., 2013, Kurihara et al., 2012). Reactive oxygen species have long been known to induce DNA damage (Kurihara et al., 2012). However our data indicate that treatment with NAC, a potent antioxidant, had little effect on 53BP1 foci kinetics in our experimental system, suggesting that ROS were not responsible for autophagy-dependent differences in DDF kinetics. Previous studies have suggested a link between the accumulation of p62 in autophagy compromised cells and an increase in DNA damage (Bae and Guan, 2011). Results shown in the previous chapter show that siRNA knockdown of p62 in both autophagy compromised and wt cells does indeed lead to a reduction in 53BP1 foci. Similarly, inhibition of autophagy in primary fibroblasts lead to increased expression of p62 and increased levels of p21 and p-p53 following X-ray irradiation. These data suggest a possible link between p62 and DNA damage repair. The aim of this next chapter is to further investigate the role of p62 in DNA damage repair.

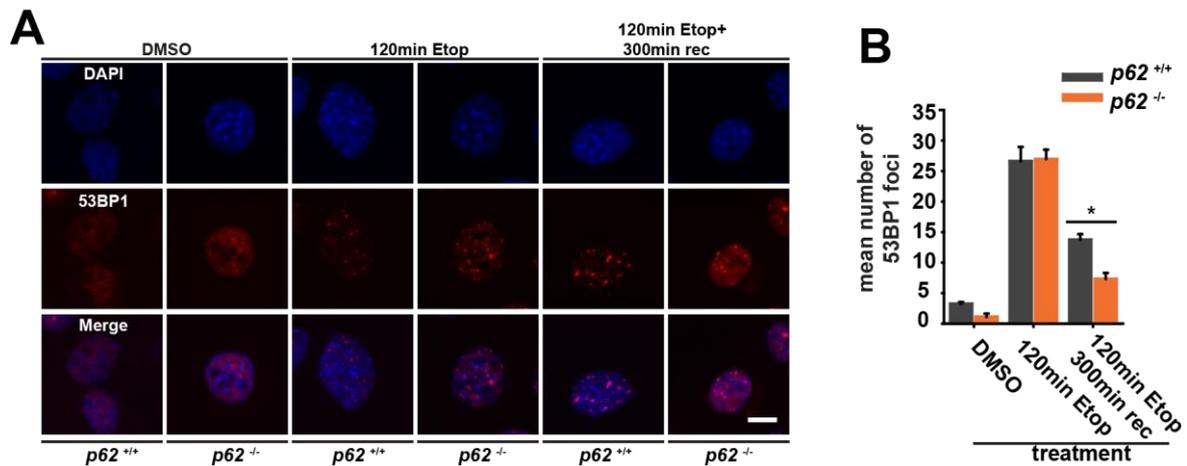
#### 4.1 $p62^{-/-}$ MEFs show a decreased number of DDF following Induction of DNA damage

To assess the role of p62 in the DDR  $p62^{+/+}$  and  $p62^{-/-}$  MEFs were irradiated with 1Gy of X-ray irradiation and fixed every hour for 8h following IR. Cells were then stained with antibody against 53BP1 and DDF quantified by microscopy. p62 levels were assessed by immunoblotting (Figure 4-1A) and loss of p62 in  $p62^{-/-}$  MEFs confirmed. Results shown in Figure 4-1B-C indicate that  $p62^{-/-}$  MEFs have a reduced number of DDF compared to  $p62^{+/+}$  MEFs. These results suggest a possible negative role for p62 in the repair of DNA damage. In order to further understand DNA damage foci dynamics live-cell imaging was performed in  $p62^{+/+}$  and  $p62^{-/-}$  MEFs stably expressing mCherry-53BP1 following 0.25Gy X-Ray irradiation, allowing the tracking of individual foci lifespan. A lower dose of 0.25Gy was used instead of 1Gy like previous experiments to facilitate the accurate tracking of individual foci. Results shown in Figure 4-1D-E indicate that  $p62^{-/-}$  MEFs have an increased rate of foci resolution compared to  $p62^{+/+}$  MEFs. These data support those seen in fixed cells and also help distinguish between foci that are generated by the initial dose of irradiation from those that occur at later time points due to the formation of new DSBs.



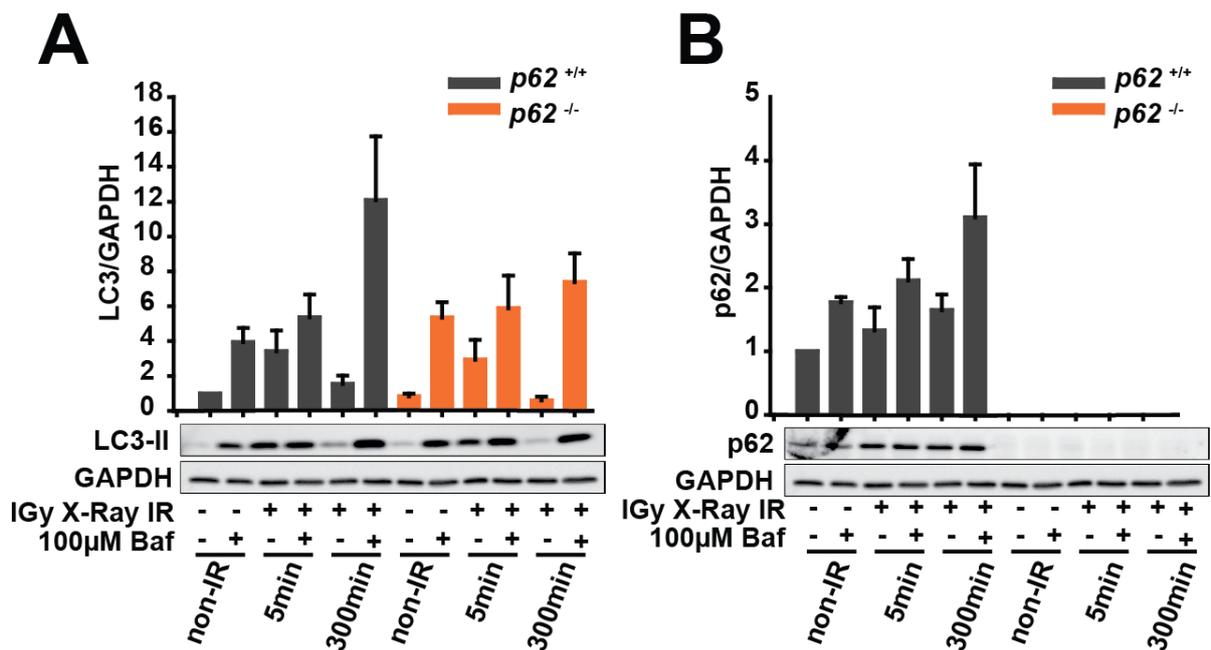
**Figure 4-1: Cells lacking p62 have a faster DNA damage repair kinetic.** (A) Representative immunoblot showing p62 and GAPDH in *p62*<sup>-/-</sup> and *p62*<sup>+/+</sup> MEFs. (B-C) *p62*<sup>-/-</sup> and *p62*<sup>+/+</sup> MEFs stably expressing mCherry-53BP1 were exposed to 0.25Gy X-ray irradiation and 53BP1 foci kinetics were monitored by live cell imaging for 300 min. Representative images are shown in (B), the nucleus is marked by dotted white border. (C) Kaplan-Meier plot showing the survival of individual 53BP1 foci in *p62*<sup>-/-</sup> and *p62*<sup>+/+</sup> MEFs following irradiation. Note that 0.25Gy was used to induce low frequency of DDF and facilitate accurate tracking of foci P=0.003. Representative images (D) and quantification (E) of the mean number of 53BP1 foci in *p62*<sup>-/-</sup> and *p62*<sup>+/+</sup> MEFs 0-480 min after 1Gy X-Ray irradiation. Scale bar 10 µm; n=3; Error bars represent S.E.M; NS: not significant, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

Having shown that knockout of *p62* results in an increased rate of DNA repair in live and fixed cells following X-Ray induced DNA damage, I wanted next to confirm this result using an alternative, more specific means of inducing DNA damage. Here, *p62*<sup>+/+</sup> and *p62*<sup>-/-</sup> MEFs were treated with 1µM Etoposide for 120 min. Cells were either fixed immediately after treatment or washed and allowed to recover for 300 min, then were fixed and stained with antibody against 53BP1 (Figure 4-2A). Results shown in (Figure 4-2B) indicate that treatment with Etoposide induced similar levels of damage in *p62*<sup>+/+</sup> and *p62*<sup>-/-</sup> MEFs. However, following 300 min recovery, *p62*<sup>+/+</sup> MEFs showed significantly more DNA damage. These results confirm that cells lacking p62 repair DNA damage faster.



**Figure 4-2: Cells lacking p62 have an increased repair capacity in response Etoposide.** DNA damage was induced in *p62<sup>-/-</sup>* and *p62<sup>+/+</sup>* MEFs by 120 min incubation with Etoposide and, where indicated, followed by a 300 min recovery period. Representative images are shown in (A) and the mean number of 53BP1 foci was quantified (B). Scale bar 10  $\mu$ m; n=3; Error bars represent S.E.M; NS: not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

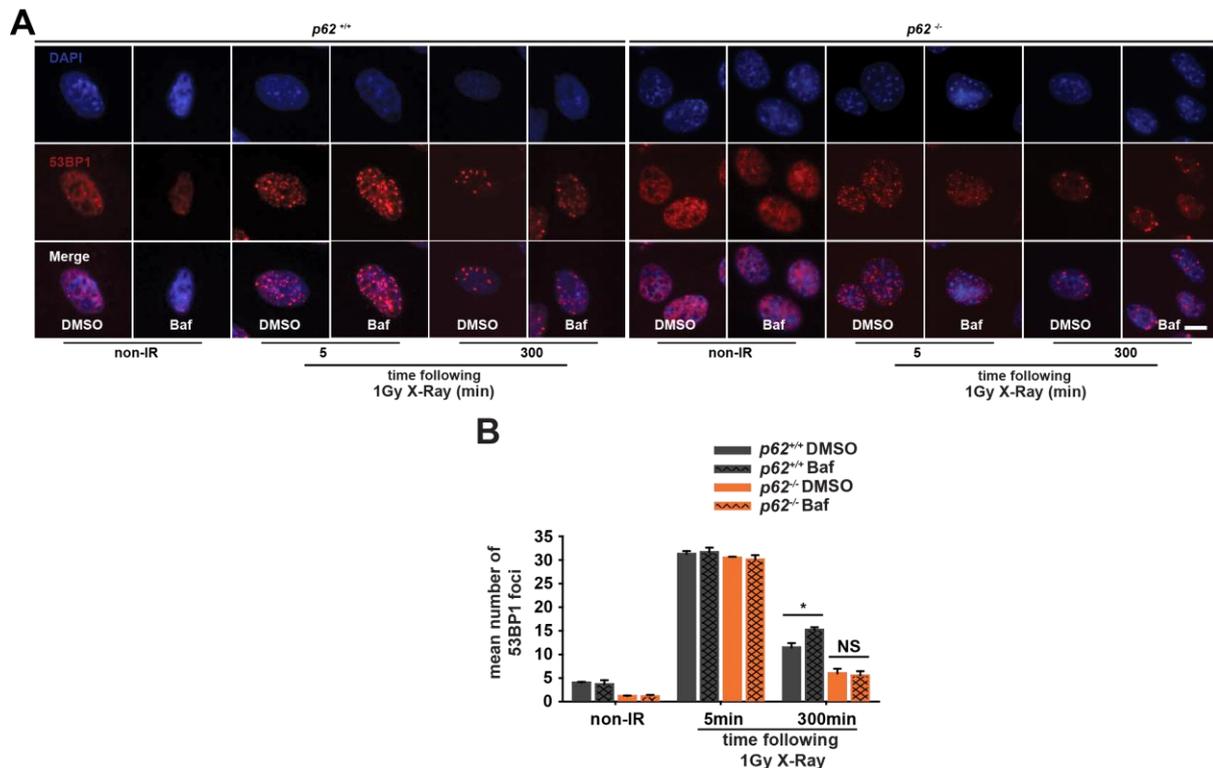
Next, in order to answer whether the differences in DNA damage seen in  $p62^{+/+}$  and  $p62^{-/-}$  MEFs were dependent on autophagy,  $p62^{+/+}$  and  $p62^{-/-}$  MEFs were treated with Baf rendering cells autophagy deficient.  $p62^{+/+}$  and  $p62^{-/-}$  MEFs were pre-treated with 100 $\mu$ M Baf for 180 min in order to block autophagy and allow sufficient accumulation of p62. Cells were then exposed to 1Gy of X-Ray irradiation and cell lysates were collected at 5 min and 300 min following IR as well as non-IR control. The block of autophagy was confirmed by immunoblotting for LC3 (Figure 4-3A) as well as p62 (Figure 4-3B). These results confirm an accumulation of both LC3II and p62 following treatment with Baf in  $p62^{+/+}$  MEFs and LC3II in  $p62^{-/-}$  MEFs indicating a block in autophagy.



**Figure 4-3: The effect of Bafilomycin A1 treatment on autophagy.** Immunoblot analyses showing the levels of LC3 (A) and p62 (B) in  $p62^{+/+}$  and  $p62^{-/-}$  MEFs following treatment with 100µM Bafilomycin A1 following 1Gy X-Ray irradiation as shown. n=3; Error bars represent S.E.M.

Following confirmation of autophagy inhibition (Figure 4-3) as above,  $p62^{+/+}$  and  $p62^{-/-}$  MEFs were pre-treated with 100 $\mu$ M Baf for 180 min. Cells were then exposed to 1Gy of X-Ray irradiation and fixed at 5 min and 300 min following IR as well as non-IR control. Cells were stained with antibody against 53BP1 and DDF were analysed by microscopy (Figure 4-4A-B).

Here, inhibition of autophagy with Baf causes an increase in 53BP1 foci 300 minutes following 1Gy X-Ray irradiation in  $p62^{+/+}$  but not  $p62^{-/-}$  MEFs (Figure 4-4B), indicating that decreased repair capacity seen in autophagy-compromised cells is dependent on p62.

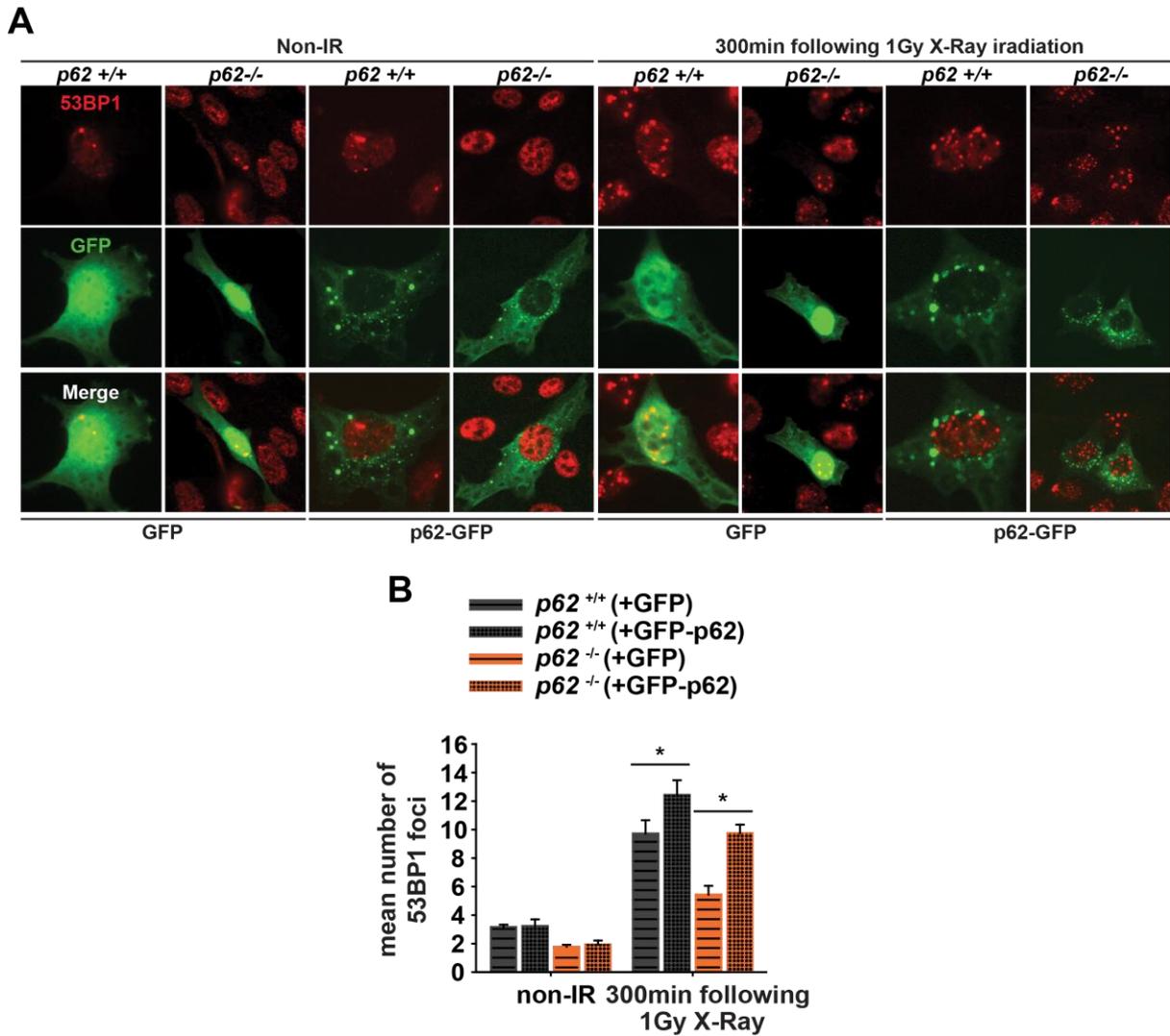


**Figure 4-4: Inhibition of autophagy with Bafilomycin A1 treatment affects DNA damage repair in a p62-dependent manner.** Representative images (A) and quantification (B) of the mean number of 53BP1 foci in  $p62^{-/-}$  and  $p62^{+/+}$  MEFs 5 min and 300 min following 1Gy X-Ray irradiation and treatment with 100 $\mu$ M Bafilomycin A1. Scale bar 10  $\mu$ m; n=3; Error bars represent S.E.M; NS: not significant, \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ .

## 4.2 Reintroduction of p62 returns frequency of DDF to wild type level

Having established that  $p62^{-/-}$  MEFs have a reduced number of DDF following IR compared to their wt counterparts (Figure 4-1A-E) and (Figure 4-2A-B), I determined the effect of reintroducing p62 on DDR as differences between  $p62^{+/+}$  and  $p62^{-/-}$  MEFs could be partly due to clonal differences independent of p62.

Here  $p62^{+/+}$  and  $p62^{-/-}$  MEFs were transfected with p62-GFP or GFP empty vector (control). Cells were then exposed to 1Gy of X-Ray irradiation and then fixed at 5h following IR as well as a non-IR control. Cells were stained with antibody against 53BP1 and DDF analysed by microscopy (Figure 4-5A-B). Results shown in Figure 4-5B demonstrate that re-introduction of GFP-p62 in  $p62^{-/-}$  MEFs increases the number of 53BP1 foci back to the levels seen in  $p62^{+/+}$  transfected with GFP-control. Overexpression of GFP-p62 in  $p62^{+/+}$  MEFs also significantly increased the number of 53BP1 foci 300 min following irradiation (Figure 4-5B). These results show that differences in DNA damage foci seen between  $p62^{+/+}$  and  $p62^{-/-}$  MEFs are indeed dependent on p62.



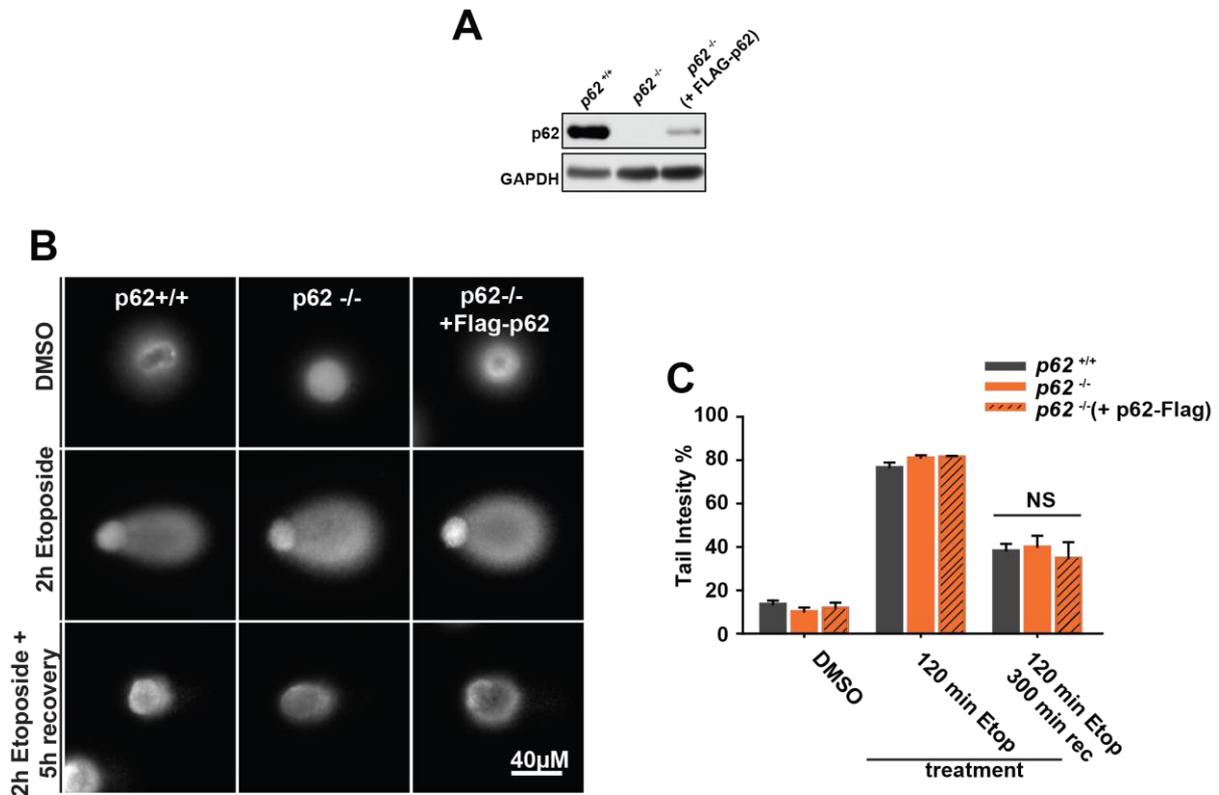
**Figure 4-5: The effect of overexpression or reintroduction of GFP-p62 into *p62*<sup>+/+</sup> and *p62*<sup>-/-</sup> MEFs.** Representative images (A) and quantification (B) of 53BP1 foci 300 min after irradiation of *p62*<sup>-/-</sup> and *p62*<sup>+/+</sup> MEFs transfected overnight with GFP-control or GFP-p62. Scale bar 10  $\mu$ m. n=3; Error bars represent S.E.M; NS: not significant, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

### **4.3 *p62*<sup>-/-</sup> MEFs show a decreased number of DSBs but not SSBs following induction of DNA damage**

Data shown in (Figure 4-1A-E) and (Figure 4-2A-B) indicate that cells lacking p62 have a quicker resolution of 53BP1 foci following induction of DNA damage than wild-types with both X-Ray irradiation and Etoposide treatment, respectively. Although 53BP1 has been shown to aggregate at DSBs and quantification of DNA damage foci is thought to serve as a good approximation for the number of DSBs (Panier and Boulton, 2014) the measurement of DNA damage foci is not a direct measure of DNA damage lesions. This can be done using the Comet assay. The general principle is that neutral Comet can be used to detect DSBs in cells while alkaline Comet may be used to detect both DSBs and SSBs. This will be discussed further later in this chapter.

In order to assess the role of p62 on DNA single stranded lesions, an alkaline Comet assay was performed on  $p62^{+/+}$ ,  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 cell lines following Etoposide treatment for 120 min. Cells were either collected immediately after treatment or washed and allowed to recover for 300 min. The  $p62^{-/-}$ +FLAG-p62 cell line was generated as it allows to separate the effect of p62 knock out from any clonal differences that may be present between  $p62^{+/+}$  and  $p62^{-/-}$  MEFs.

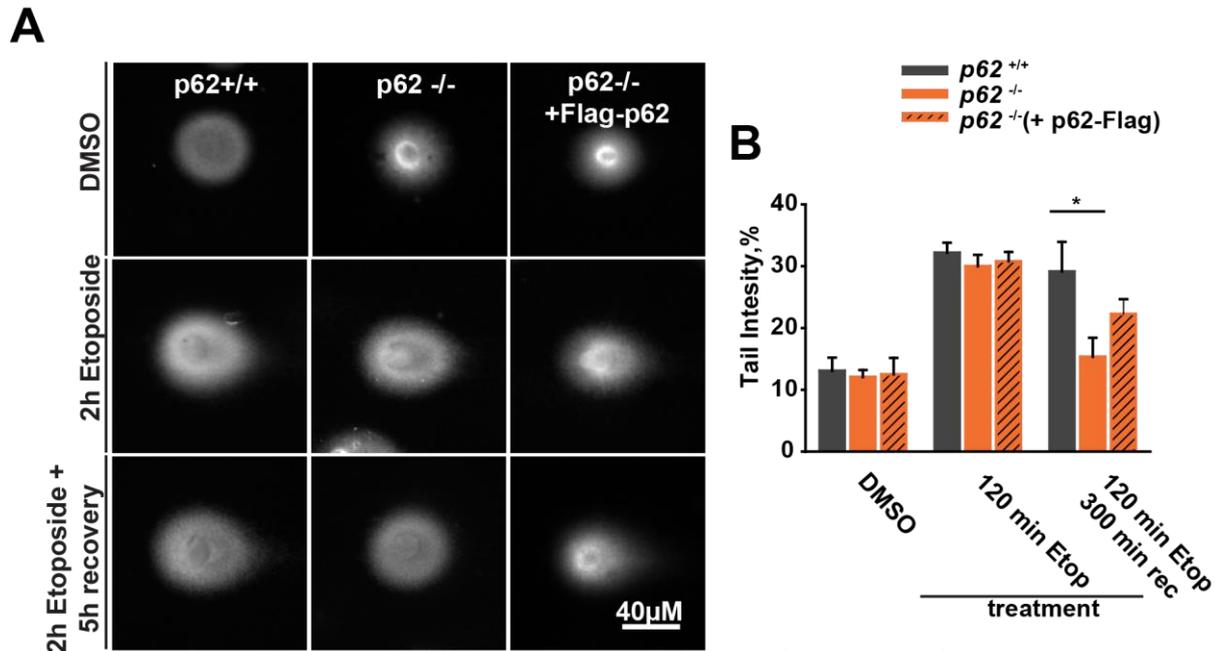
The Immunoblot shown in Figure 4-6A shows the successful reintroduction of p62 in the  $p62^{-/-}$ +FLAG-p62 cell line, however it must be noted that the expression levels of p62 are markedly lower than in  $p62^{+/+}$  MEFs. Results presented in Figure 4-6B-C show a significant induction of DNA damage following Etoposide of around 80% tail intensity in all cell lines. These data indicate that there is no difference in damage induction in  $p62^{+/+}$ ,  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 cell lines in response to Etoposide treatment. Results shown in (Figure 4-6B-C) show equivalent levels of DNA damage around 40% tail intensity in all cell lines at 300 min following Etoposide treatment suggesting that there is no difference in resolution of DNA damage in  $p62^{+/+}$ ,  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 cell lines in alkaline conditions.



**Figure 4-6: Measurement of SSBs in *p62*<sup>+/+</sup>, *p62*<sup>-/-</sup> and *p62*<sup>-/-</sup>+FLAG-p62 MEFs using alkaline Comet assay.** (A) Immunoblot analyses showing the levels of p62 in *p62*<sup>+/+</sup>, *p62*<sup>-/-</sup> and *p62*<sup>-/-</sup>+FLAG-p62 cell lines. Note that transgenic FLAG-p62 is expressed at lower levels than endogenous protein. (B-C) *p62*<sup>+/+</sup>, *p62*<sup>-/-</sup> and *p62*<sup>-/-</sup>+FLAG-p62 following the induction of DNA damage with Etoposide for 120 min either followed with or without a 300 min recovery period (in the absence of Etoposide). Representative images of alkaline Comet analysis shown in (B) whilst % tail intensity is quantified in (C). Scale bar 40  $\mu$ m. n=3; Error bars represent S.E.M; NS: not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

In order to assess DSBs more specifically, neutral Comet assay was performed on  $p62^{+/+}$ ,  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 cell lines following Etoposide treatment for 120 min. Cells were either collected immediately after treatment or washed and allowed to recover for 300 min.

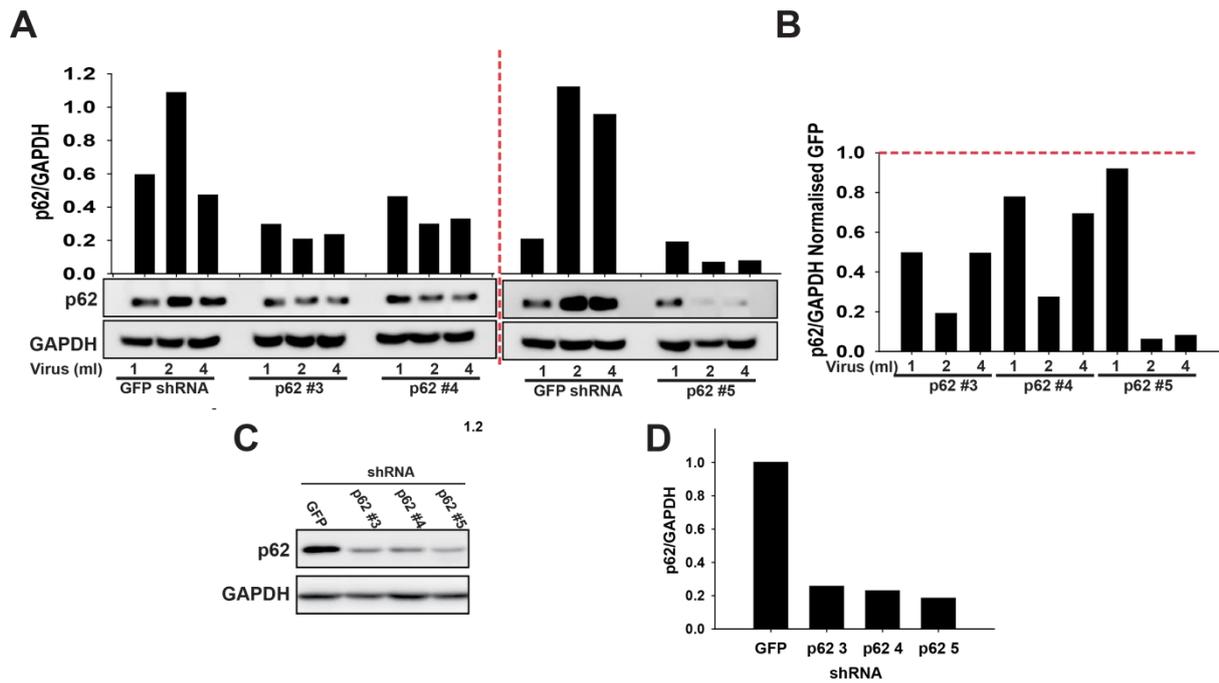
Results shown in Figure 4-7A-B (like in Figure 4-6B-C) show a significant induction of DNA damage following Etoposide of around 35% tail intensity in all cell lines that there is no difference in induction of double stranded DNA damage in  $p62^{+/+}$  and  $p62^{-/-}$  MEFs in response to Etoposide treatment. Results shown in Figure 4-7B indicate a significantly reduced tail intensity of around 17% in  $p62^{-/-}$  MEFs compared to 30% in  $p62^{+/+}$  MEFs, at 300 min following Etoposide treatment, indicating like 53BP1 foci shown in Figure 4-1A-E and Figure 4-2A-B that there is increased resolution of DSBs in  $p62^{-/-}$  compared to  $p62^{+/+}$  MEFs. Interestingly, there is also increased tail intensity in  $p62^{-/-}$ +FLAG-p62 MEFs when compared to  $p62^{-/-}$  MEFs, however the difference is not statistically significant. This is likely due to the relatively low levels of p62 expression seen in  $p62^{-/-}$ +FLAG-p62 MEFs when compared to  $p62^{+/+}$  MEFs (Figure 4-6A).



**Figure 4-7: Measurement of DSBs in *p62*<sup>+/+</sup>, *p62*<sup>-/-</sup> and *p62*<sup>-/-</sup>+FLAG-p62 MEFs using neutral Comet assay. (A-B) *p62*<sup>+/+</sup>, *p62*<sup>-/-</sup> and *p62*<sup>-/-</sup>+FLAG-p62 following the induction of DNA damage with Etoposide for 120 min either followed with or without a 300 min recovery period (in the absence of Etoposide). Representative images of neutral Comet analysis shown in (A) and % tail intensity is quantified in (B). Scale bar 40 µm. n=3; Error bars represent S.E.M; NS: not significant, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.**

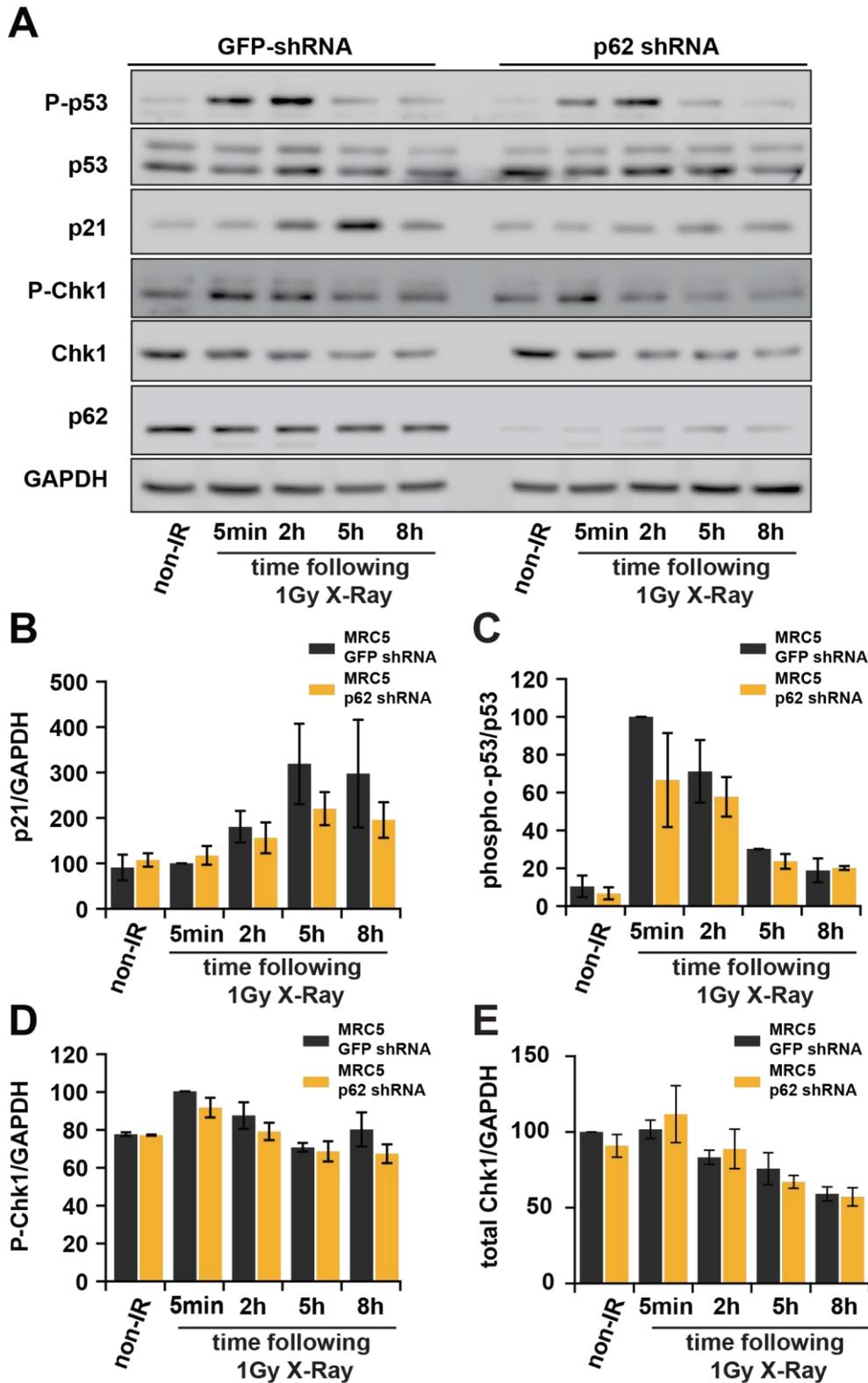
#### 4.4 The Effect of p62 Knockdown on DDR Downstream Signalling

Having established that cells lacking p62 have an increased DNA damage repair kinetic compared to wild type cells, as measured by 53BP1 Foci and neutral Comet (Figure 4-1&Figure 4-5), I intended to investigate the impact of p62 on downstream DNA damage signalling. For this it was necessary to knock down p62 in primary cells as downstream signalling is possibly disrupted in p62<sup>+/+</sup> and p62<sup>-/-</sup> MEFs as a result of the SV40 immortalisation process (Boichuk et al., 2010). Here, MRC5 human fibroblasts were transduced with shRNA for p62. Optimisation of shRNA transduction is shown in Figure 4-8. Lentivirus containing shRNA #s 3,4 and 5 was produced in HEK293FT cells and used at virus titres of 1,2,and 4ml of viral media in a total of 4ml. Knockdown of p62 is shown in Figure 4-8A-B and normalised to GFP shRNA (control) (Figure 4-8B). It can be seen from Figure 4-8A-B that transduction with p62 shRNA lead to a 70-80% knockdown of p62 at the optimal virus titre of 2ml of viral media in a total of 4ml, as measured by immunoblot. The p62 knockdown efficiency of cell lines derived from chosen virus titre is shown in Figure 4-8C and quantified in Figure 4-8D following 2 cell passages (to allow cells to recover from transduction) indicating successful selection and maintenance for p62 shRNA expressing cells.



**Figure 4-8: Optimisation of p62 shRNA transduction in MRC5 fibroblasts.** (A) Western blots and quantification showing p62 and loading control GAPDH for different Virus titres 1, 2 and 4ml of viral media in a total of 4ml. (B) Quantification from (A) normalised to GFP control shRNA. Western blots (C) and quantification (D) showing p62 and loading control GAPDH in p62 shRNA stable cell lines.

Following the successful generation of p62 shRNA-expressing cell lines, next we wanted to investigate the effect of p62 knockdown on the downstream DDR. Here, MRC5 human fibroblasts that are stably expressing shRNA against p62 were exposed to 1Gy of X-Ray irradiation and collected at time points 5min-8h following IR. Samples were then run on SDS page electrophoresis and immunoblot analysis was carried out for p53, p62, p-p53, p21, p-Chk1, Chk1 and loading control GAPDH. Western blots shown in Figure 4-9A indicate successful knockdown of p62, and also indicate that levels of p62 are unchanged across the 8h time course following IR. Quantification shown in Figure 4-9B show a trend for p62 shRNA-expressing cells to have reduced levels of p21 2h and 5h following 1 Gy IR, however these differences are not statistically significant. Similarly, induction of p-p53 is reduced in p62 shRNA-expressing cells but, again, results are not statistically significant. There were no differences between cells with and without p62 shRNA when comparing pChk1 and total Chk1 levels following IR (Figure 4-9D-E). It would be interesting to repeat these experiments using a higher dose of IR in order to ascertain if there are any p62-dependent differences in downstream DDR.



**Figure 4-9: Downstream DDR in MRC5 fibroblasts transduced with shRNA against p62.** Representative western blots (A) showing p62, p53, p-p53, p21, Chk1, p-Chk1 and loading control GAPDH in MRC5 fibroblasts transduced with GFP-shRNA (control) or p62 shRNA as indicated following 1Gy X-ray irradiation at time points 5min-8h. Quantification of p21 is shown in (B), p-p53/p53 in (C), p-Chk1 in (D)

and total Chk1 in (E). n=3; Error bars represent S.E.M.. Done in collaboration with Dr Bernadette Carol.

#### 4.7 Discussion

While previous studies, as well as the previous chapter, have suggested that p62 may mediate autophagy dependent differences in DNA damage repair (Bae and Guan, 2011), the role of p62 in the DNA damage response has not been well characterized (Yu et al., 2011). Here, for the first time, we show a p62-dependent effect on DNA damage repair in cells with active autophagy. p62 has previously been shown to mediate increased tumorigenesis in response to loss of autophagy. In a study from the White group they describe that induction of metabolic stress in cells lacking autophagy leads to increased expression of endoplasmic reticulum (ER), accumulation of damaged mitochondria, p62, ROS and genomic instability. They attribute this effect of increased tumorigenesis in the absence of autophagy to increased levels of p62, as overexpression of p62 in the same cell model leads to increased tumor volume following mouse xenograft experiments. It is possible that in the absence of autophagy increased levels of ROS could, in turn, lead to the induction p62 expression. This p62 overexpression could then contribute to additional ROS generation, leading to genome instability (Mathew et al., 2009). It should be noted that the lack of p62 has also been associated with an increase in ROS, this time through inhibition of NF- $\kappa$ B (Duran et al., 2008). This inhibition of NF- $\kappa$ B is in contrast to that reported in (Sanz et al., 2000a), where small p62 puncta are proposed to serve as signalling hubs leading to NF- $\kappa$ B activation. It is possible that large aggregates actually lead to the sequestration of key proteins involved in signalling, leading to an inhibition of the pathway. Taken together, these data suggest that intracellular levels of p62 play an important role in the modulation of ROS. However, the exact influence appears to be cell type and context specific.

Nonetheless, variances in ROS could well be influencing the differences seen in DNA damage foci repair kinetics in fixed cells. DDF kinetics was followed in live cells. For this, cell lines were generated where  $p62^{-/-}$  and  $p62^{+/+}$  MEFs stably express mCherry-53BP1. This has the added advantage of allowing the analysis of the lifespan of individual foci. It has previously been reported that DNA damage foci that

arise due to ROS are transient in nature (Passos et al., 2010). Here we were able to visualise secondary DNA damage foci that appear independently of those generated by the initial induction of DNA damage using 0.25Gy X-Ray irradiation. There was no difference between  $p62^{-/-}$  and  $p62^{+/+}$  MEFs in the appearance of these secondary DNA damage foci, suggesting that ROS generated DNA damage foci were not the reason for the differences seen in DDF repair kinetics.

Although it has been suggested that p62 may mediate autophagy-dependent differences in DNA damage repair (Bae and Guan, 2011), the effect of p62 on DNA damage has not previously been described independently of autophagy. Results in this chapter indicate that autophagy inhibition is having no effect on DNA damage repair in the absence of p62. These data also indicate that increased levels of p62 do not require active autophagy to affect DDF repair. The lack of effect on DNA damage foci at 5h following 1Gy IR in  $p62^{-/-}$  MEFs with Bafilomycin A1 treatment is in agreement with data from the previous chapter suggesting that other lysosomal degradation pathways are not influencing DNA damage repair kinetics following low dose IR. These data suggest that the CMA mediated degradation of Chk1 that was observed by the Cuervo lab (Park et al., 2015) may only influence the repair of DNA damage induced by higher doses of IR.

Re-introduction of p62 in  $p62^{-/-}$  MEFs and overexpression in  $p62^{+/+}$  MEFs lead to an increase in 53BP1 foci 300 minutes following 1Gy X-Ray IR. These data show that differences seen between  $p62^{+/+}$  and  $p62^{-/-}$  MEFs were indeed dependent on p62 and not due to clonal differences in MEFs derived from different animals. Overexpression of p62 at super physiological levels leads to the formation of large cytoplasmic aggregates; these aggregates are able to sequester a number of proteins leading to a loss in their function (Donaldson et al., 2003). In an attempt to prevent the formation of these aggregates and try to achieve ubiquitous low level expression of GFP-p62 the construct was co-transfected with pcDNA (empty control plasmid) in order to dilute its expression. Cells were also chosen that did not have massive cytoplasmic aggregates when performing analysis of 53BP1 foci. It can be seen that despite efforts to reduce expression levels, as well as select low expressing cells p62 aggregates are still present as a result of overexpression. This is a major limitation when trying to study p62 using mammalian expression vectors. This problem could be avoided by generating cell lines that stably express p62 using

lentiviral transduction. Using this approach, one is able to transduce cells using different virus titres, then select and expand clones with the desired expression level. Newly developed knock-in technology such as CRISPR/Cas now allows the addition of tagged and mutated proteins in place of the wt version, allowing a far more physiological control of transcription and expression. The most recent advances in this technology allow for the creation of cell lines without the need for the selection of successfully altered cells with antibiotics (Zhu et al., 2015). This need for selection and subsequent loss of a percentage of the cell population has been a major limiting factor when using GM modification in primary cells, especially when studying processes such as senescence where accurately tracking the number of population doublings is essential. In order to achieve more physiological levels of expression cell lines were produced using lentiviral transduction of FLAG-p62 and control plasmid in  $p62^{-/-}$  MEFs. It should be noted that when compared to  $p62^{+/+}$  MEFs the level of p62 expression in  $p62^{-/-}$ +FLAG-p62 was lower. The cell line would have to be generated again using a higher virus titer to have comparable levels of p62 to wt MEFs.

Data presented so far have shown comparison of DDF repair kinetics following DNA damage induction using fixed and live-cell microscopy. The measurement of DNA damage foci has been shown to correlate well with the number of DNA lesions; however it is not a direct measure. This is of particular importance as both autophagy and p62 are heavily involved in protein turnover. p62 has also been shown to serve as a scaffold protein (Ciuffa et al., 2015, Johansen and Lamark, 2011). Therefore, differences in DNA damage foci may arise due to differences in protein degradation and foci disassembly potentially uncoupling the link between numbers of DNA damage foci as an accurate measure of DNA lesions. It was therefore important to assess the effect of p62 on DNA lesions directly. This was done using both alkaline and neutral Comet assay. Results show that p62 has no effect on DNA lesions when assessed by alkaline Comet assay. This is a method that is believed to assess both single and double stranded DNA lesions as the alkaline conditions not only unwind DNA but also separate DNA strands (Singh et al., 1988), allowing both DSBs and SSBs to migrate with electrophoresis. Interestingly, when using neutral Comet, cells lacking p62 had a faster resolution of damage as measured by % DNA in the tail. Neutral Comet has been suggested to measure

predominantly double strand breaks as in neutral conditions it is only DNA containing DSBs that are able to migrate with SSBs being held in the head on the Comet by intact complementary strands (Olive et al., 1991). The specificity of this technique for DSBs can be verified by treating cells with hydrogen peroxide which has been shown to create 1000-fold or more SSBs compared to DSBs (Olive and Johnston, 1997). However, the specificity of neutral Comet as a measure of just DSBs is lacking when assessing low levels of damage, as both SSBs and DSBs have been shown to relax the super coiled structure of DNA held within nucleoids, the protein and membrane-depleted nuclear bodies that result following lysis (Ostling and Johanson, 1984). This relaxation has been proposed to mediate the migration of DNA into a Comet tail within the electric field applied even in neutral conditions. The contribution of supercoil relaxation is outweighed by DSB migration at higher levels of damage and this assay has been shown to measure DSBs over a range of 50-10000 breaks per cell (Olive and Banath, 2006). Therefore, in order to achieve higher sensitivity when assessing DSBs a higher concentration of Etoposide was used in these experiments, 10 $\mu$ M instead of 1  $\mu$ M. Assuming that following 10 $\mu$ M Etoposide treatment neutral Comet is an accurate measure of DSBs, these results indicate that p62 is affecting DSB repair directly. These results reinforce the hypothesis that differences in DDF seen in previous experiments arise due to different levels of DSBs and not just as a result in aberrant or impaired foci turnover. The lack of difference seen in the alkaline Comet could result as the number of SSBs present is sufficient to mask any difference in the number of DSBs that can be visualized using this technique. This would be consistent with the finding that Etoposide treatment induces predominantly SSBs (Muslimovic et al., 2009).

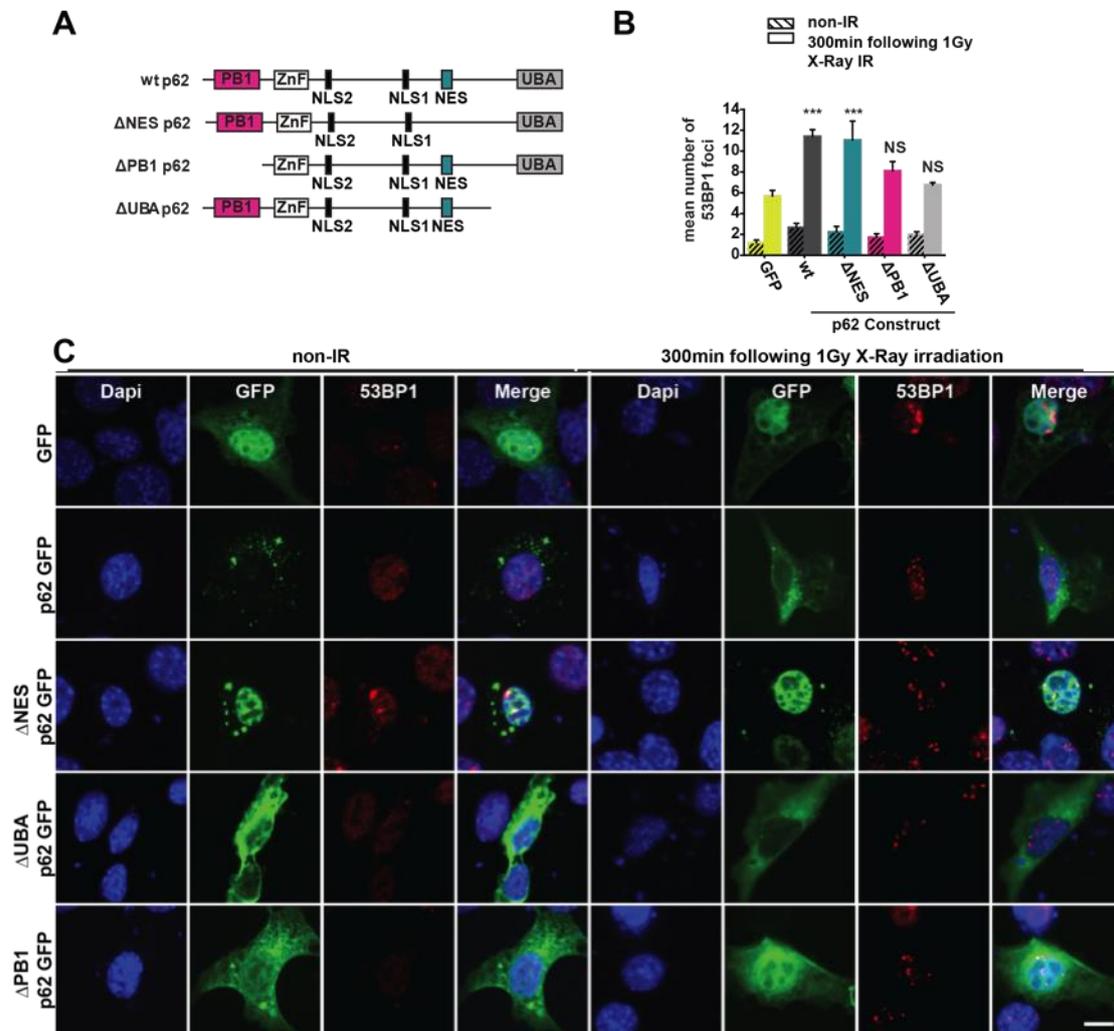
## **Chapter 5. Mechanistic Analysis of the role of p62 in the DDR**

In the previous chapter we established that cells lacking p62 have increased DNA repair capacity as measured by kinetics of 53BP1 foci and Comet assay following X-ray irradiation. It is not yet known how p62 is able to influence the DDR. p62 is primarily a cytoplasmic protein but, as discussed previously, it contains both NLS and NES domains which mediate its transport in and out of the nucleus (Pankiv et al., 2010). Moreover, it has been shown to interact with PML bodies which contain DDR proteins such as BLM/WRN DNA helicases, MRN and DNA topoisomerase II binding protein (TopBP1) (Lallemand-Breitenbach and de The, 2010). On the other hand p62 may interact with proteins in the cytoplasm to elicit its effect on the DDR. It is possible that p62 may have an effect on DNA damage resolution via its role as a scaffold protein, signal transduction or protein degradation. In this following chapter we aim to establish how p62 is able to influence DNA damage repair mechanistically.

## 5.1 Both PB1 and UB1 Protein Domains are required for p62 to impact on DNA Damage Repair

p62 has multiple functional domains that are responsible for its cellular functions. Having established a role for p62 in DNA damage repair I aimed to identify the functional domains involved in the process. Here, *p62*<sup>-/-</sup> MEFs were transfected with GFP empty vector (control), wt p62-GFP or p62 mutants lacking either the PB1 domain responsible for protein oligomerisation ( $\Delta$ PB1), the UBA domain involved in binding to ubiquitylated substrates ( $\Delta$ UBA), or the nuclear export signal ( $\Delta$ NES). The domain structure for these constructs is shown in Figure 5-1A. Cells were then exposed to 1Gy of X-Ray irradiation and then fixed at 300 min following IR as well as non-IR control. Cells were stained with an antibody against 53BP1 and DDF analysed by microscopy (Figure 5-1B-C). Representative images shown in Figure 5-1B indicate the distinctive expression pattern for each of these constructs.  $\Delta$ NES p62 is expressed almost exclusively in the nucleus as it is no longer transported out of the nucleus via nuclear transporter exportin 1.  $\Delta$ UBA p62 lacks the cytoplasmic puncta of the wt protein due to its inability to bind to ubiquitylated substrates.  $\Delta$ PB1 p62, similar to  $\Delta$ UBA p62, has a diffuse cytoplasmic appearance, this time due to its inability to form oligomers. Results shown in Figure 5-1B demonstrate that re-introduction of wt p62 into *p62*<sup>-/-</sup> MEFs increases the number of 53BP1 foci similar to results shown in Figure 4-5. Data shown in Figure 5-1B indicate that both  $\Delta$ UBA and  $\Delta$ PB1 mutants fail to increase levels of 53BP1 foci to the same extent as wt p62, suggesting that both of these domains are important for the effect p62 has on DNA damage repair. Interestingly, *p62*<sup>-/-</sup> MEFs expressing  $\Delta$ NES-p62 show similar levels of 53BP1 foci 300 minutes following IR as those expressing wt p62, meaning that the NES domain is dispensable. These results suggest that p62 does not need to be shuttled from the nucleus into the cytoplasm to have an effect on this process (Figure 5-1B).

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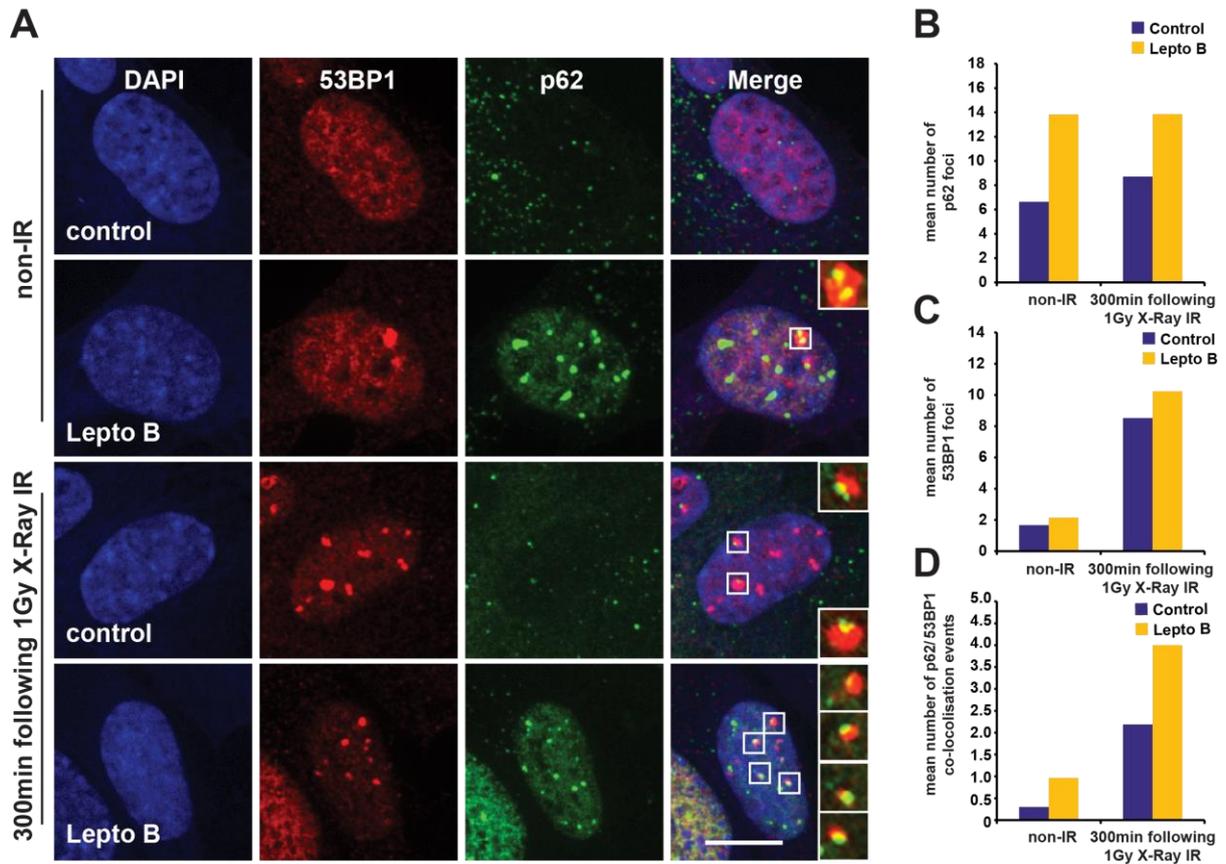


**Figure 5-1: PB1 and UBA domains of p62 are required for a p62 mediated effect on DDR** (A) Schematic representation of the domain structure of p62 constructs. Key structural domains are marked: UBA: ubiquitin-associated domain; PB1: Phox and Bem1p domain; ZnF: ZZ type zinc finger domain; NES: Nuclear export signal; NLS1/2: Nuclear localisation signal. Quantification (B) and representative images (C) of 53BP1 foci in *p62*<sup>-/-</sup> MEFs overexpressing the indicated GFP-tagged p62 mutants 300 minutes after irradiation. Scale bar 10  $\mu$ m. n=3; Error bars represent S.E.M; NS: not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

## 5.2 p62 co-localises with DNA Damage foci *in vitro*

Although p62 is known to shuttle in and out of the nucleus (Pankiv et al., 2010), little is known about its nuclear role. It was reported in Pankiv *et al* that upon inhibition of nuclear export via either treatment with Leptomycin B or deletion of the NES sequence, p62 forms distinctive nuclear puncta (Pankiv et al., 2010). Previous results (Figure 5-1B-C) indicate that both the PB1 and UBA domains of p62 are important for its role in 53BP1 foci repair, both of which are involved in protein-protein interactions. In order to further understand the role of p62 on 53BP1 foci dynamics we decided to investigate interaction between p62 and DNA damage foci.

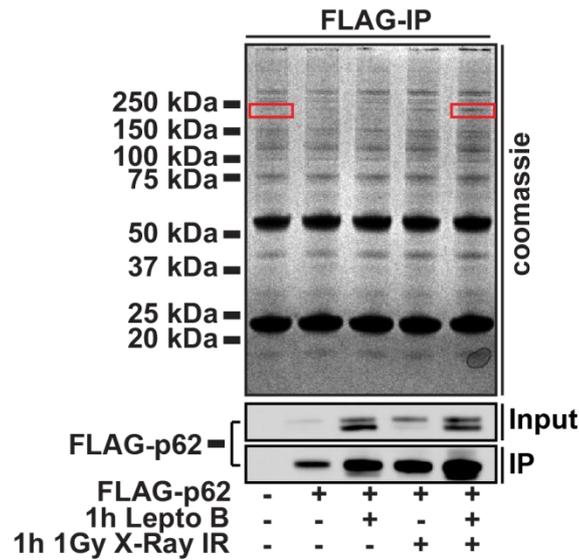
Here, MRC5 human fibroblasts exposed to 1Gy of X-Ray irradiation, as well as non-irradiated controls, were treated with and without Leptomycin B for 1h prior to fixation in order to block nuclear export of p62. Cells were then stained with antibodies against 53BP1 and p62, both DDF and p62 puncta were analysed by microscopy. Representative images shown in Figure 5-2A indicate, consistent with previously published work (Pankiv et al., 2010), that block of nuclear export using Leptomycin B results in the formation of nuclear p62 puncta. Results shown in Figure 5-2B show an elevated number of p62 puncta in cells without Leptomycin B, 300 minutes following IR, indicating an induction of foci formation in response to DNA damage. Cells treated with Leptomycin B show now significant increase in the number of p62 foci following IR (Figure 5-2B). This is likely due to p62 being at a maximal level in the nucleus with Leptomycin B treatment alone. Results shown in Figure 5-2C demonstrate that there is an induction of 53BP1 foci in cells treated with and without Leptomycin B 300 minutes following IR, and there is a slight increase in those treated with Leptomycin B versus control. Interestingly, data shown in Figure 5-2D demonstrate that p62 co-localises with 53BP1 foci. Unsurprisingly, treatment of Leptomycin B increases the number of p62 foci and therefore co-localisation is increased. Taken together these results suggest that induction of irradiation can induce the formation of p62 foci within the nucleus and these foci are able to interact with proteins within DDF.



**Figure 5-2: p62 interacts with DNA damage foci.** (A-D) The colocalisation of p62 and 53BP1 was analysed in human fibroblasts (MRC5) exposed to irradiation (IR) for 0 and 5 hours in the absence or presence of Leptomycin B (Lepto B) as indicated. Representative images are shown in (A) and the mean number of p62 (B), 53BP1 (C) and p62-53BP1 co-localisation (D) foci were quantified. Scale bars 10  $\mu$ m; Data are mean for  $\geq 30$  cells.

### **5.3 p62 Interacts with members of the DDR CHD4 and filamin A**

Having observed that p62 and DDF co-localise following induction of DNA damage by X-Ray irradiation, I then aimed to understand which proteins p62 was interacting with. Here, Hela cells were transfected with FLAG-p62, exposed to 1Gy X-ray irradiation and treated with Leptomycin B as indicated (Figure 5-3). Nuclear fractionation was carried out and the resulting nuclear fractions were subjected to immunoprecipitation using M2-FLAG beads. Samples were then separated by gel electrophoresis and stained using Coomassie blue (Figure 5-3). A band of approximately 250kDa, marked by a red box in Figure 5-3, was evident specifically in the nuclei from IR/Leptomycin B treated cells. This band was excised and sent for mass spectrometry analysis.

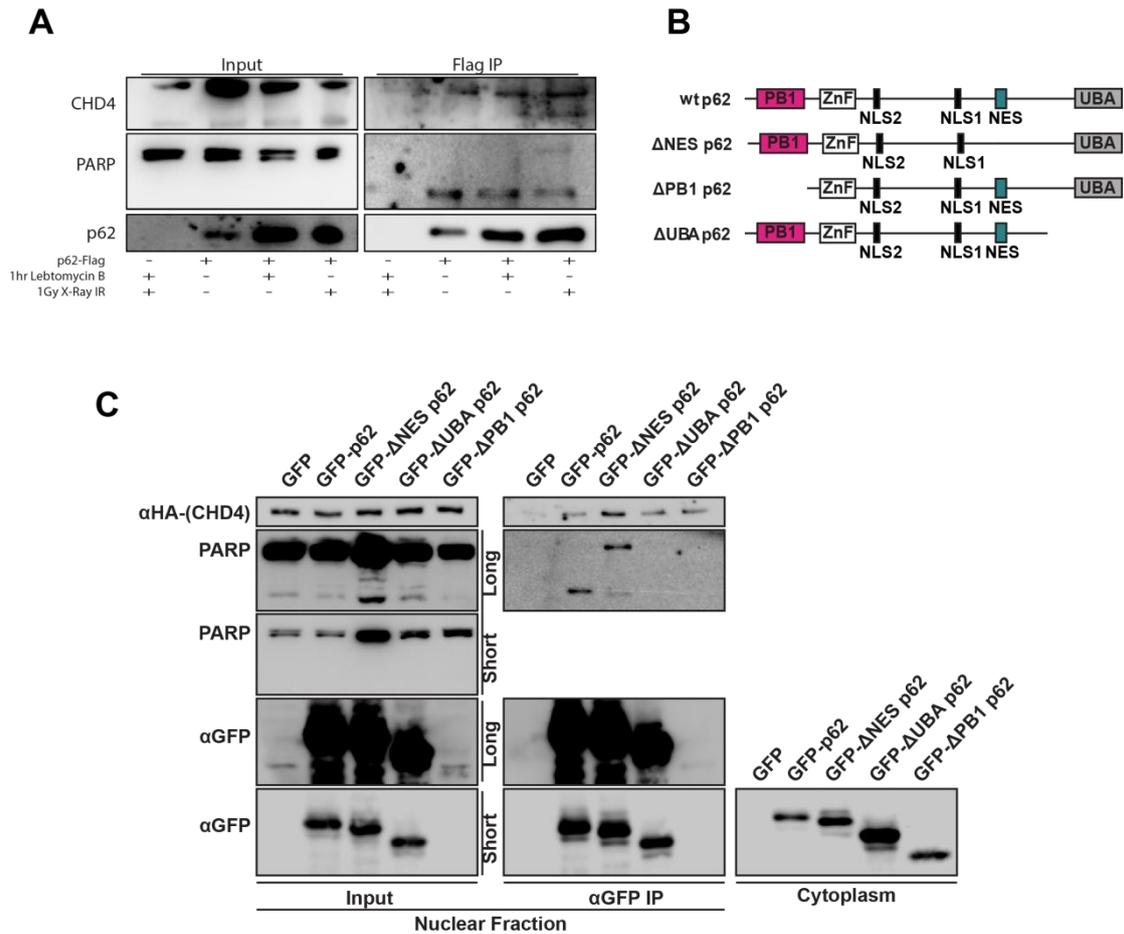


**Figure 5-3: Identification of nuclear p62 interactors.** HeLa cells were transfected with FLAG-p62 overnight and treated as indicated (either non-irradiated or irradiated, in the presence or absence of Leptomycin B). Cells were subjected to immunoprecipitation with anti-FLAG antibody. The band indicated by a red box was identified as being present in association with p62 following irradiation.

#### 5.4 Interaction of p62 with CHD4 does not mediate its effect on DDR

Results shown in Figure 5-3 indicate that p62 interacts with a number of proteins in response DNA damage. One of these proteins, CHD4, has been shown to be involved in the multiple stages of the DNA damage response. CHD4 is recruited to the site of damage and its depletion has been linked to reduced DNA damage-induced histone ubiquitylation, reduced recruitment of BRCA1 and RNF168 (Larsen et al., 2010, Smeenk et al., 2010). CHD4 has also been shown to affect the repair of DSBs (Polo et al., 2010, Pan et al., 2012). Interestingly, CHD4 has been shown to be recruited to the site of damage by PARP (Chou et al., 2010). PARP is a known interactor of p62 (Korolchuk Laboratory, unpublished data) and it is also closely involved in the DNA damage response. CHD4 and PARP are therefore attractive candidates to study when trying to understand a p62- mediated effect on DNA repair. In order to confirm the interaction between p62 and CHD4 immunoprecipitation of p62 was performed in the nuclear fraction  $p62^{-/-}$ +FLAG-p62 MEFs that have been exposed to 1Gy of X-ray irradiation and treated with Leptomycin B as indicated (Figure 5-4A). Immunoblotting was performed for both CDH4 and PARP (Figure 5-4A). It can be seen that p62 interacts with the cleaved form of PARP independently of IR-induced DNA damage. Interestingly, upon inhibition of nuclear export with Leptomycin B, p62 is able to interact with the full-length protein, and there appears to be a reduction in the binding of cleaved PARP to p62. Results in Figure 5-4A show that p62 also interacts with CHD4, confirming the results seen in the mass spectrometry analysis in Figure 5-3. Next, I wanted to investigate which functional domains were responsible for this interaction. Having already found that both the PB1 and UBA domain were needed for p62 to have an effect on DNA damage repair, I hypothesized that these domains could be responsible for the interaction of p62 with CHD4. To investigate this,  $p62^{-/-}$  MEFs were transfected with HA-CDH4 and either GFP empty vector (control), wt p62-GFP or p62 mutants lacking either the PB1 domain ( $\Delta$ PB1), the UBA domain ( $\Delta$ UBA) or the nuclear export signal ( $\Delta$ NES). The domain structure for these constructs is shown in Figure 5-4B. Immunoprecipitation against GFP was performed followed by immunoblot analysis for both HA tag and PARP. Results shown in (Figure 5-4C)

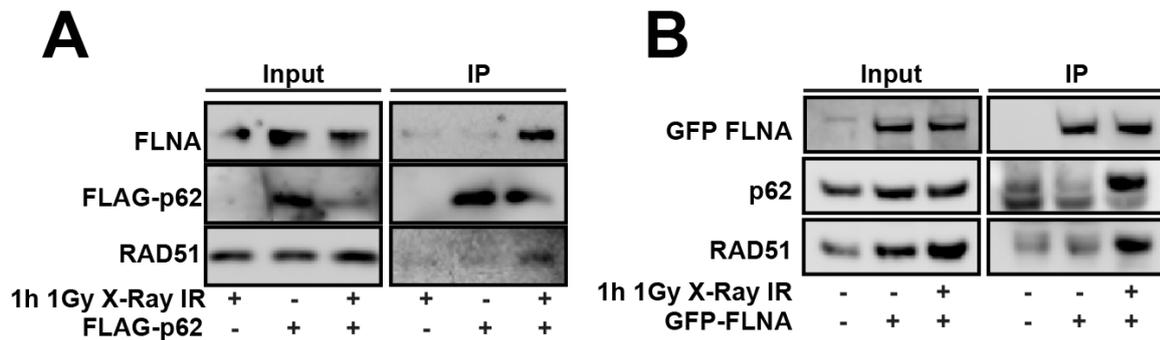
indicate that CHD4 is able to interact with all p62 constructs. When coupled with the data showing that both the PB1 domain and UBA domain are required for the effect of p62 on DNA damage (Figure 5-1B) these data suggest that the interaction between p62 and CHD4 is not what is mediating this effect of p62 on DNA damage repair. Interestingly, both wt p62 and  $\Delta$ NES p62 interact with PARP while PB1 and UBA domain mutants do not. Similar to the results shown with Leptomycin B treatment in Figure 5-4A, when p62 expression is restricted to the nucleus by the deletion of the export signal, it binds preferentially to full-length PARP instead of the cleaved form of the protein.



**Figure 5-4 : Interaction of p62 with CHD4** (A)  $p62^{-/-}$  MEFs stably expressing FLAG-p62 ( $p62^{-/-}$ +FLAG-p62) were irradiated where indicated and 60 min later nuclear fractions were subjected to anti-FLAG IP. The interaction of FLAG-p62 with endogenous CDH4 and PARP was detected by immunoblotting. (B) Schematic representation of the domain structure of p62 constructs. Key structural domains are marked: UBA: ubiquitin-associated domain; PB1: Phox and Bem1p domain; ZnF: ZZ type zinc finger domain; NES: Nuclear export signal; NLS1/2: Nuclear localisation signal. (C)  $p62^{-/-}$  MEFs transfected with GFP-p62 constructs as indicated and HA-CDH4 were irradiated where indicated and 60 min later nuclear fractions were subjected to GFP IP. The interaction of GFP-p62 with HA-CDH4 and endogenous PARP was detected by immunoblotting. GFP-p62 construct expression in the cytoplasmic fraction was detected by immunoblotting as indicated.

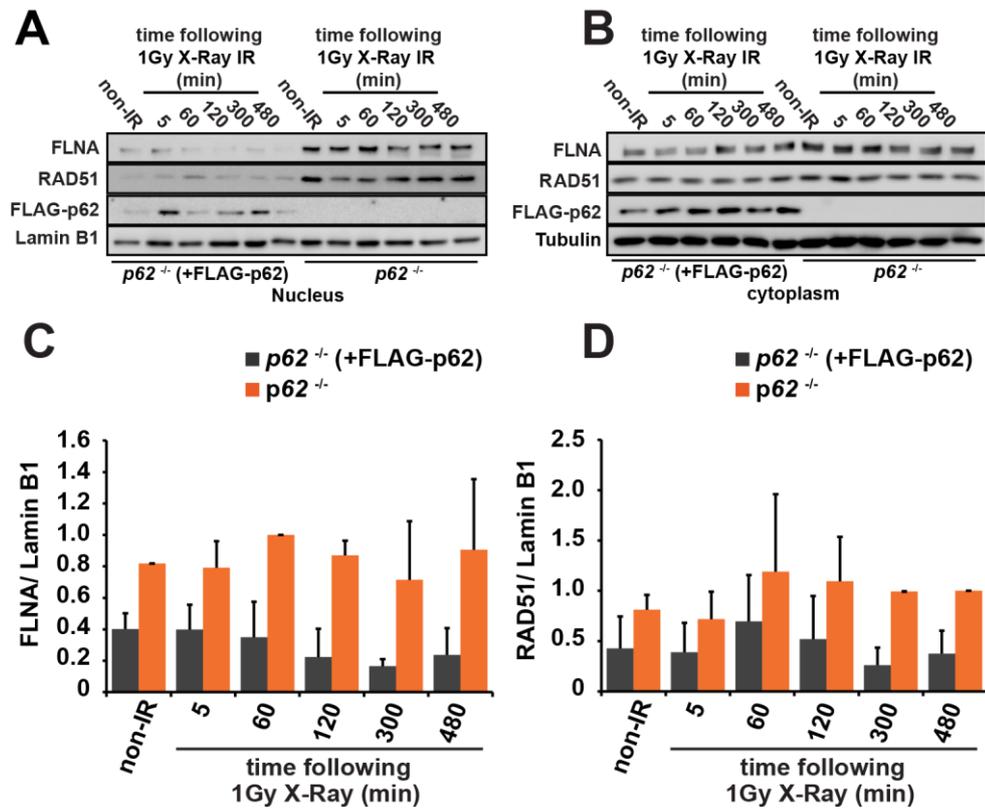
## 5.5 p62-dependent proteasomal degradation of Filamin A (FLNA) and RAD51 regulates DNA repair

Having ruled out the interaction of p62 with CHD4 as a requirement for the differences seen in DNA damage resolution in short time-course experiments following low doses of IR, I decided to investigate Filamin A (FLNA), the second hit from the mass spectrometry analysis. FLNA has previously been shown to be involved in DNA repair, specifically through interaction with BRCA1 and 2 and recruitment of RAD51 in the HR repair pathway (Velkova et al., 2010, Yue et al., 2012, Yue et al., 2009). Firstly, I set out to confirm the interaction between FLNA and p62. Here, *p62*<sup>-/-</sup>+FLAG-p62 MEFs or *p62*<sup>-/-</sup> MEFs were irradiated as indicated (Figure 5-5). Nuclear fractions were then subjected to p62 immunoprecipitation and the resulting IP analysed by immunoblotting for FLNA, RAD51 and Lamin B1 (loading control). Results shown in Figure 5-5 indicate that both FLNA and RAD51 interact with p62 in response to X-Ray irradiation.



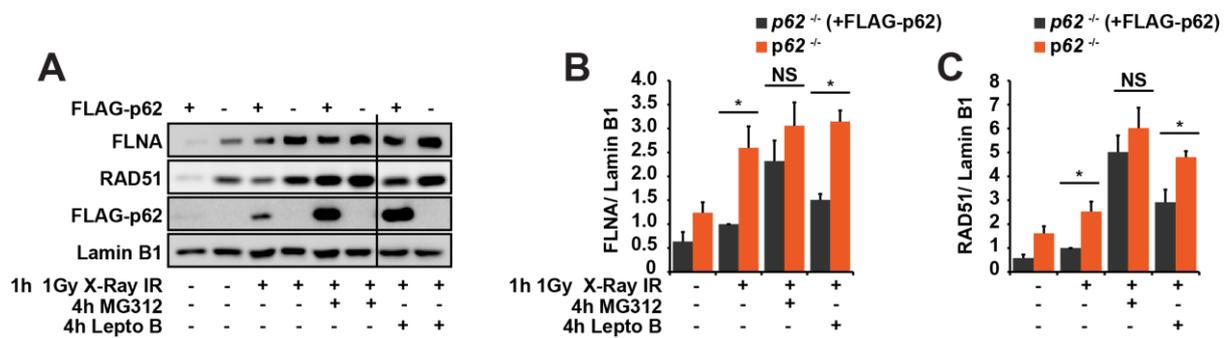
**Figure 5-5: Immunoprecipitation of nuclear p62 and FLNA.** (A)  $p62^{-/-}$  MEFs, stably expressing FLAG-p62 ( $p62^{-/-}$ +FLAG-p62) were irradiated where indicated and 60 min later nuclear fractions were subjected to anti-FLAG IP. The interaction of FLAG-p62 with endogenous FLNA and RAD51 was detected by immunoblotting. (B) HeLa cells transfected with GFP-FLNA were irradiated, where indicated, and 60 min later nuclear fractions were subjected to anti-GFP IP. The interaction of GFP-FLNA with endogenous p62 and RAD51 was detected by immunoblotting.

Having established that p62 and FLNA do indeed interact in response to DNA damage, next I investigated any differences in the levels of FLNA and RAD51 in *p62*<sup>-/-</sup> and *p62*<sup>-/+</sup>FLAG-p62 MEFs. Here, cells were exposed to 1Gy X-Ray irradiation and collected at time points 5-480 min following IR. Nuclear fractionation was performed and the resulting nuclear and cytoplasmic fractions were analyzed by immunoblotting for FLNA, RAD51 and either Lamin B1 or tubulin as a loading control (Figure 5-6A-B). Quantifications shown in Figure 5-6C-D show that cells lacking p62 have higher levels of both FLNA and RAD51 in the nuclear fraction. Interestingly, no difference in the cytoplasmic levels of FLNA and RAD51 was found in *p62*<sup>-/-</sup> and *p62*<sup>-/+</sup>FLAG-p62 MEFs (Figure 5-6B).



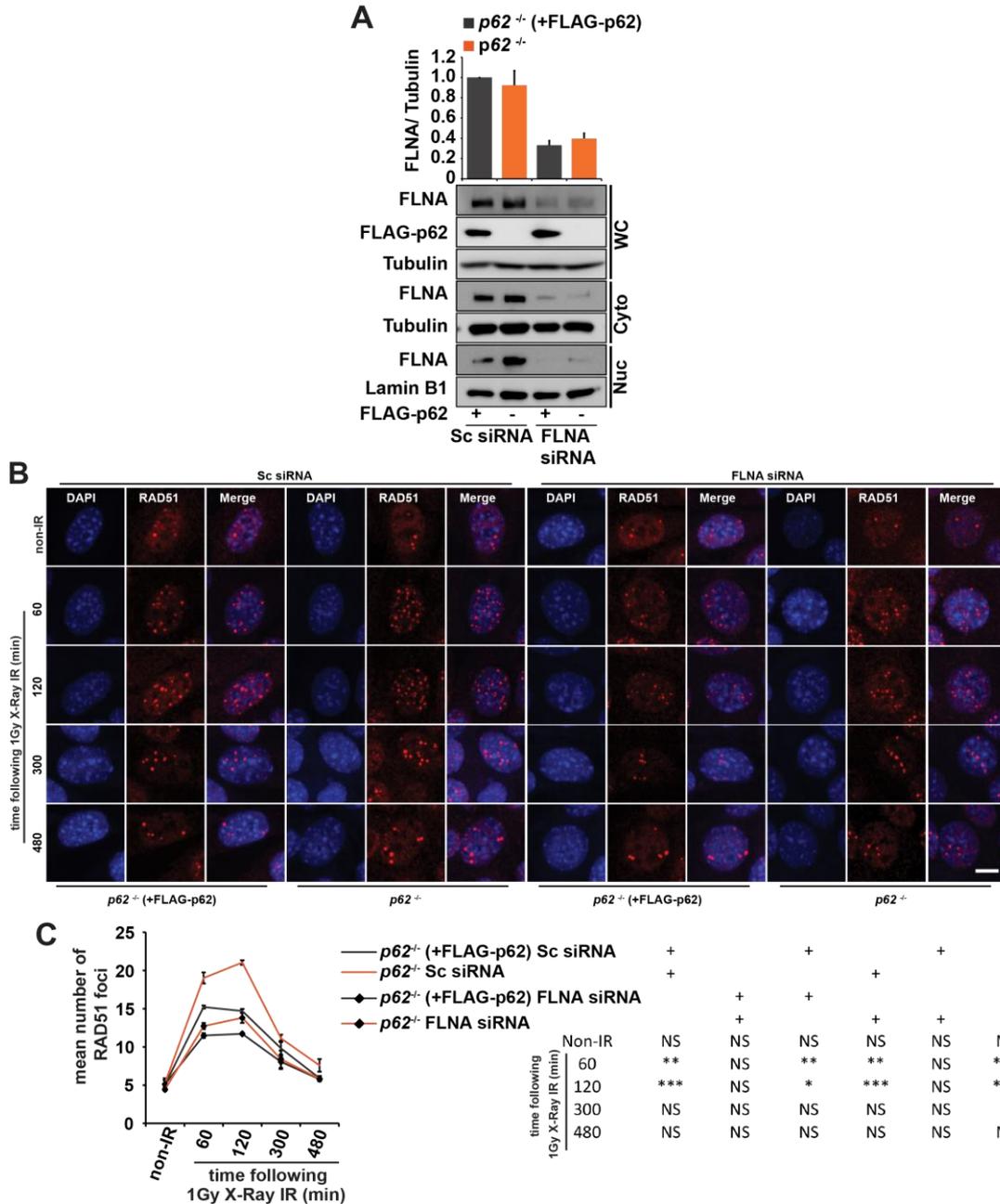
**Figure 5-6: Nuclear and cytoplasmic levels of FLNA and RAD51.** *p62*<sup>-/-</sup> and *p62*<sup>-/-</sup> +FLAG-p62 MEFs were irradiated with 1Gy and subjected to cellular fractionation at the time points indicated. Nuclear (A) and cytoplasmic (B) fractions were analysed for FLNA, RAD51 and Lamin B1 as a loading control. Quantification is shown in (C-D). n=2; Error bars represent S.D.

Having established that p62 was causing a reduction in nuclear levels of both FLNA and RAD51, I next investigated the mechanisms involved. As previously mentioned, p62 is involved in targeting proteins for degradation by both the proteasome and autophagy. Having established that the effect of p62 on DNA damage repair was independent of its role in autophagic degradation (Figure 4-4A-B), I investigated whether this p62-dependent difference in nuclear levels of FLNA and RAD51 was dependent on proteasomal degradation. Although previous results shown in Figure 5-1 suggest that p62 is able to affect DNA damage repair independently of its role in the cytoplasm, it is still possible that p62 is responsible for transporting FLNA and RAD51 to the proteasome within the nucleus. To answer these questions, *p62*<sup>-/-</sup> and *p62*<sup>-/-</sup>+FLAG-p62 MEFs were pre-treated with MG132, a potent proteasomal inhibitor, or Leptomycin B to inhibit nuclear export, for 3h and were then exposed to 1Gy of X-Ray irradiation. Cells were treated for a further 1h with MG132 or Leptomycin B and collected. Following nuclear fractionation the nuclear fraction was analyzed by immunoblotting for FLNA, RAD51 and Lamin B1 as a loading control. Results shown in (Figure 5-7A-C) indicate that treatment with MG132 ablates p62-dependent differences in both FLNA and RAD51 indicating that these differences are dependent on p62-mediated degradation via the proteasome. In keeping with previous results (Figure 5-1) treatment with Leptomycin B did not affect p62-dependent differences in FLNA and RAD51. Taken together, these data suggest that p62 is able to mediate the degradation of FLNA via the proteasome and this is occurring in the nucleus.



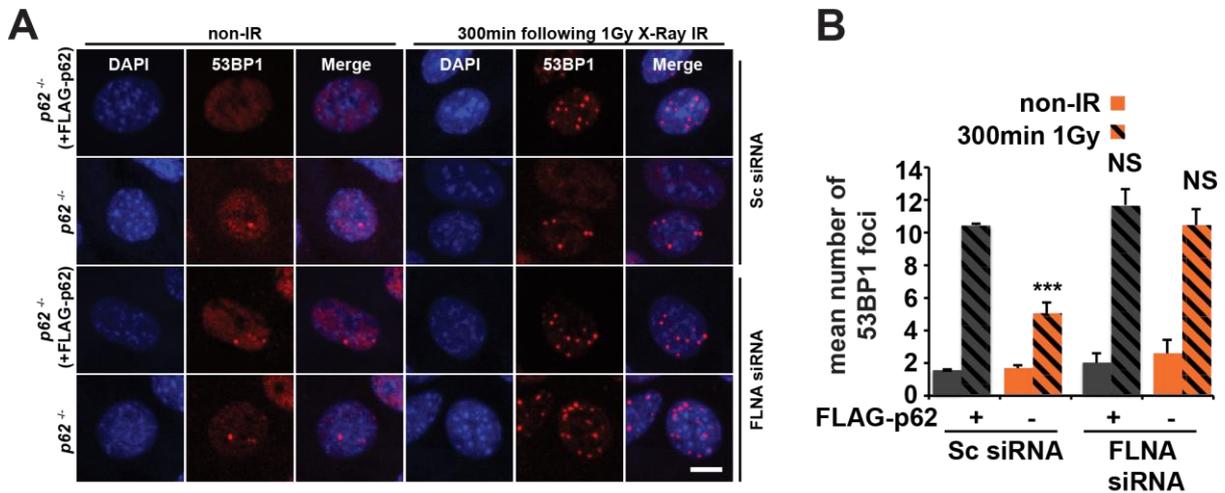
**Figure 5-7: p62-mediated proteasomal degradation of FLNA and RAD51.**  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 MEFs were pre-incubated with MG132 or Leptomycin B where indicated for 3h. Cells were irradiated and incubated in the presence of MG132 or Leptomycin B for further 60 min. Nuclear fractions were analysed for FLNA and RAD51 levels (A) and quantified relative to Lamin B1 (B,C). n=3; Error bars represent S.E.M; NS: not significant, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

Next, having established that p62 was responsible for the nuclear degradation of FLNA, I aimed to investigate the role of FLNA in DNA damage repair. It has been previously reported that cells with reduced levels of FLNA show a reduced formation of RAD51 foci, which is responsible for the reduced ability of these cells to perform HR (Yue et al., 2009). In order to investigate if similar mechanisms were responsible for the differences in DNA damage repair seen between  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 MEFs, I knocked down *FLNA* using siRNA and exposed MEFs to 1Gy of X-Ray irradiation. Cells were then fixed at time points 60-480 min following IR and stained with an antibody against RAD51. The number of RAD51 foci per cell was then analysed by microscopy. Western blot analysis shown in Figure 5-8A indicate successful knockdown of FLNA in whole cells, cytoplasmic and nuclear fractions. Data shown in Figure 5-8B-C indicate that cells lacking p62 show a greater induction of RAD51 foci following X-Ray irradiation compared to wt cells and knockdown of FLNA reduces the number of RAD51 foci in both  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 MEFs. These data are in agreement with those shown by others (Yue et al., 2009), where knockdown of *FLNA* has been shown to result in a reduction of RAD51 foci following DNA damage. The authors also relate this knockdown of FLNA to a reduced ability of cells to perform HR.



**Figure 5-8: The effect of *FLNA* knockdown on RAD51 foci kinetics in  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 MEFs.**  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 MEFs treated with control or *FLNA* siRNA and subjected to 1Gy X-Ray irradiation and collected at time points 60-480 min following IR as indicated (A-C) Blot showing scrambled control (Sc) and *FLNA* siRNA in  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 MEFs in nuclear (Nuc), cytoplasmic (CYTO) and whole cell fractions (WC) (A). Representative images of RAD51 foci are shown in (B). Quantification of the mean number of RAD51 foci with statistical comparison is shown in (C). n=3; Error bars represent S.E.M; NS: not significant, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

Having established that levels of FLNA impacted on RAD51 foci induction following X-Ray irradiation, a process previously shown to be indicative of HR (Li and Heyer, 2008), I next aimed to assess the effect of *FLNA* knockdown on 53BP1 foci repair. Here, *p62*<sup>-/-</sup> and *p62*<sup>-/+</sup>FLAG-p62 MEFs cells were transfected with *FLNA* siRNA and exposed to 1Gy of X-Ray irradiation. Cells were then fixed 300 minutes following IR and stained with an antibody against 53BP1. The number of RAD51 foci per cell was then analysed by microscopy. Data shown in Figure 5-9A-B indicate that knockdown of *FLNA* results in a reduced ability of *p62*<sup>-/-</sup> MEFs to repair 53BP1 foci compared to Scrambled siRNA control similar to that seen in *p62*<sup>-/+</sup>FLAG-p62 MEFs. These data support the idea that increased levels of FLNA in *p62*<sup>-/-</sup> MEFs result in an increase in HR which is able to repair DSBs more readily.



**Figure 5-9: The effect of *FLNA* knockdown on 53BP1 foci kinetics in  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 MEFs.**  $p62^{-/-}$  and  $p62^{-/-}$ +FLAG-p62 MEFs treated with control or *FLNA* siRNA and subjected to 1Gy X-Ray irradiation and collected at 300 min following IR as indicated (A-B). Representative images of 53BP1 foci are shown in (A). Quantification of the mean number of 53BP1 foci is shown in (B). n=3; Error bars represent S.E.M; NS: not significant, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

## 5.5 Discussion

The previous chapter characterized the role of p62 in DNA damage repair; this is to our knowledge the first work reporting a role for p62 in DNA damage repair independent of its role in autophagy. p62 is a multifunctional protein comprised of many functional domains. Understanding which of these domains are required for p62 to illicit an effect on DNA damage repair can help shed light on how this is functionally occurring. Data presented above show cells expressing p62 with a deletion of the PB1 or UBA domain have a reduced number of 53BP1 foci 300 min following 1Gy X-ray IR, implying that both of these domains are required for p62 to influence DNA damage repair.

The PB1 domain is essential for interaction of p62 with other PB1 containing proteins as well as self-oligomerization (Lamark et al., 2003). This has been shown to be central to its role in mediating the selective degradation of proteins via autophagy (Itakura and Mizushima, 2011, Pankiv et al., 2007, Wurzer et al., 2015, Ciuffa et al., 2015). The PB1 domain has been shown to interact with Rpt1, a subunit of the 26S proteasome allowing p62 to serve as a shuttling factor, targeting polyubiquitylated proteins bound by the UBA domain for proteasome degradation (Seibenhener et al., 2004). Interaction of p62 via its PB1 domain has also been shown to mediate its involvement in many signalling cascades (Joung et al., 1996, Lamark et al., 2003). The involvement of the PB1 domain in this process suggests that p62 could be involved in DNA damage repair via scaffolding, signalling or degradation processes. The UBA domain is required for the interaction between p62 and ubiquitylated targets, mediating degradation via both autophagy and the proteasome (Seibenhener et al., 2004, Vadlamudi et al., 1996, Geisler et al., 2010). Taken together the involvement of both of these domains suggests that p62 may be influencing DNA damage repair through its role in protein degradation, signalling or scaffold formation. Deletion of each of these domains only had a partial effect therefore it would be interesting to assess if deletion of both of these domains simultaneously was able to cancel the effect of p62 on DNA damage repair.

Interestingly, p62 lacking the NES domain had the same effect on the number of 53BP1 foci as wild type p62. This additional piece to the puzzle suggests that the action of p62 in this process is occurring in the nucleus, indicating that the

cytoplasmic process of autophagy degradation is not involved, beyond modulating levels of p62 itself. Having eliminated the role of autophagy, the involvement of both the PB1 and UBA domains of p62 suggest that p62 may be eliciting an effect on DNA damage repair via scaffold function, signalling or proteasomal degradation in the nucleus.

Previous work by Pankiv *et al* has described a role for p62 in coordinating the recruitment of proteasomes to nuclear aggregates. They also note that p62 mediates the accumulation of polyubiquitylated proteins at PML bodies when nuclear export is inhibited (Pankiv et al., 2010). PML bodies are known to co-localize with DDF as well as play a role in their processing (Xu et al., 2003, Varadaraj et al., 2007, Munch et al., 2014, Boichuk et al., 2011). This p62-dependent recruitment of proteasomes to PML bodies or DDF may well mediate the differences in DDF repair kinetics seen in *p62*<sup>-/-</sup> and *p62*<sup>-/-</sup> MEFs. Interestingly, we found that p62 co-localizes with a fraction of DDF following 1Gy X-ray IR and the percentage of co-localization was increased following inhibition of nuclear export with Leptomycin B. Further investigation is required to understand if these p62 nuclear puncta are recruited to PML as well as subunits of the 20S proteasome, as was observed by the Johansen lab (Pankiv et al., 2010). Together, these data suggest that p62 is interacting with proteins within DDF. It would be interesting to analyse the importance of both the PB1 domain and UBA domain in mediating this localization of p62 to DDF. It would help to elucidate if this localisation to DDF is indeed important for the function of p62 in DNA damage repair.

Interestingly, the recruitment of different proteasomal subunits has been shown to play an important role in DNA damage foci kinetics (Tsolou et al., 2012), as well as the choice of DNA repair pathway (Gudmundsdottir et al., 2007). Proteasomal subunit Rpn7 has been shown to co-localize with long lived DNA damage foci and is suggested to stabilize DNA damage foci, preventing premature resolution. Knock down of Rpn7 was shown to increase the resolution of DDF (Tsolou et al., 2012). Moreover, Rpn7 has been shown to interact with BRCA2, a key component of HR and inhibition of the proteasome has been shown to inhibit HR in favor of more error prone repair pathways (Gudmundsdottir et al., 2007, Krogan et al., 2004, Murakawa et al., 2007). p62 has been shown to interact with several proteasomal subunits such as Rpt1 (Babu et al., 2005) and S5a (Seibenhener et al., 2004). It is an interesting possibility that p62 may be influencing DNA damage repair

via recruitment or both 19S and 20S proteasomal subunits to DNA damage foci. p62 mediated recruitment of the 20S proteasome could facilitate turnover of DNA damage proteins, while recruitment of 19S subunits could confer foci stability such as was shown with Rpn7 (Tsolou et al., 2012).

Following the observation of p62 localizing to DDF, mass spectrometry analysis identified CHD4 and FLNA as interacting partners of p62 within the nucleus in response to DNA damage. The chromodomain-helicase-DNA-binding 3/4 (CHD3/4) proteins were originally identified as autoantigens in the connective tissue disease dermatomyositis, which is associated with an increased risk of malignancy (Ge et al., 1995, Seelig et al., 1995, Brehm et al., 2000). CHD proteins belong to the SNF2 superfamily of ATPases, which, through ATP hydrolysis, remodel nucleosome structure (Eisen et al., 1995, Seelig et al., 1996). In particular, the ATPase/helicase domain of CHD4 has been shown to facilitate the mobility of nucleosomes along DNA (Wang and Zhang, 2001).

CHD4 is a key component of the nucleosome re-modelling and deacetylation (NuRD) complex. The NuRD complex couples chromatin remodeling activity (CHD4) and deacetylation activity (histone deacetylase 1/2 (HDAC1/2) (Wade et al., 1998, Zhang et al., 1998, Tong et al., 1998). The primary function of NuRD is the remodeling of chromatin to inhibit transcription (Lai and Wade, 2011). It has been shown that NuRD plays an important role in DNA damage repair as well as cell cycle progression. It has also been shown that both CHD4 and HDAC1 interact with ATR (Schmidt and Schreiber, 1999). More recently, it was demonstrated that CHD4 is in fact phosphorylated by ATR and ATM (Matsuoka et al., 2007, Mu et al., 2007) and its expression increases upon exposure to UV irradiation (Burd et al., 2008). These data are suggestive of a role for NuRD and CHD4 in the DDR. A number of studies have now identified that depletion of CHD4 in mammalian cells leads to an increase in markers of replication stress, spontaneous DNA damage as well as hypersensitivity to ionizing radiation (Luijsterburg et al., 2012, Larsen et al., 2010, Sims and Wade, 2011).

The mechanisms by which CHD4 is influencing DDR and DNA repair are beginning to be elucidated. Recently, it has been shown that CHD4 is recruited to sites of DNA damage by two distinct mechanisms (Luijsterburg et al., 2012, Polo et

al., 2010). First, it was suggested that CHD4 was recruited to DNA damage via interaction with poly(ADP-ribosyl)ated proteins, such as PARP1 (Polo et al., 2010). This interaction, in turn, leads to the recruitment of NuRD components HDAC1 and MTA2. Treatment with PARP inhibitors prevented the accumulation of CHD4/NuRD at sites of DNA damage. Interestingly, while this study showed that CHD4 is phosphorylated by ATM, as previously observed, it does not seem to influence its function in the DDR (Polo et al., 2010). The second proposed mechanism for recruitment of CHD4 to the site of DNA damage is via interaction with RNF8 (Luijsterburg et al., 2012). RNF8 is recruited to DNA damage via interaction with MDC1, which itself is recruited to  $\gamma$ H2A.X during the initial activation of a DDR. Following recruitment to RNF8, CHD4 is proposed to mediate chromatin relaxation, stimulating the ubiquitin ligase activity of RNF8 and RNF168 resulting in the formation of ubiquitin conjugates (Luijsterburg et al., 2012). The ubiquitylation activity of RNF8/RNF168 serves as an amplification step in the DDR promoting the recruitment of downstream DNA-damage repair proteins (Smeenk et al., 2010). Knockdown of CHD4 was shown to reduce ubiquitylation as well as RNF168 and BRCA1 accumulation at DSBs highlighting the importance of CHD4 in amplification of the DDR downstream of RNF8. Interestingly, the tethering of RNF8 to chromatin was shown to bypass CHD4 recruitment suggesting that CHD4-mediated chromatin remodelling facilitates access of RNF8 to DNA damage sites allowing the assembly of downstream checkpoint and repair proteins (Luijsterburg et al., 2012). Notably, expression of a CHD4 mutant lacking helicase activity was unable to rescue the effect of depletion on DDR, highlighting the importance of the chromatin remodelling activity of CHD4 in its role in the DDR (Smeenk et al., 2010). Interestingly, reduced levels of some NuRD components were reported in Hutchinson–Gilford progeria syndrome, a disease associated with premature ageing. Similarly, NuRD expression was reduced as a consequence of normal ageing. Higher levels of DNA damage marker  $\gamma$ H2A.X were observed in aged cells as well as following knockdown of NuRD components such as metastasis-associated protein 3 (MTA3), HDAC1 and CHD4 (Pegoraro et al., 2009).

These data indicate a role of CHD4 and the NuRD complex in the DDR as well as organismal ageing. Therefore, the interaction between p62 and CHD4 could be a potential mechanism by which p62 is influencing DNA damage repair. As

PARP1 is a known interactor of p62 (Korolchuk Laboratory, unpublished data), playing a role in the recruitment of CHD4 to the sites of DNA damage (Polo et al., 2010), we hypothesized that it could take part in the interaction between p62 and CHD4 in response to DNA damage induction.

PARP1 is activated in response to DNA damage and catalyses poly(ADP-ribosylation) of a number of proteins (Satoh and Lindahl, 1992). Activation of PARP has been shown to mediate the increased access of DNA repair enzymes and transcription factors to chromatin (Satoh and Lindahl, 1992). PARP1 has been shown to play a crucial role in DNA damage repair as well as cell death. It has been reasoned that in response to high levels of DNA damage, high levels of PARP activity lead to the depletion of cellular levels of NAD<sup>+</sup> and ATP. This can lead to energy collapse and cell death. Recently it has been suggested that PARP1-dependent energy collapse was not dependent on NAD<sup>+</sup> depletion, instead it occurs due to inhibition of glycolysis through inhibition of hexokinase (Andrabi et al., 2014). It has been shown that in response to high levels of PARP activation it is cleaved by caspase 3 and 7 (Cohen, 1997) resulting in separation of the DNA binding domain, contained in a 24KDa fragment, from the catalytic domain within a 89KDa fragment (Cohen, 1997). The remaining 24KDa fragment is still able to bind damaged DNA, however lacks catalytic activity. Therefore, this inactive 24KDa fragment competes for binding with remaining full length PARP setting up a negative feedback reducing PARP activity and NAD<sup>+</sup> and ATP consumption (Yung and Satoh, 2001). The cleavage of PARP has been suggested to facilitate cellular disassembly during apoptosis and its cleavage serves as a marker of active apoptosis (Oliver et al., 1998).

In this chapter we show that p62 and CHD4 interact, however their interaction is not increased upon induction of DNA damage. Moreover, CHD4 was shown to interact with  $\Delta$ PB1 and  $\Delta$ UBA p62 mutants. Both of these constructs were shown to be required for p62 to illicit an effect on DNA damage repair. Interestingly, PARP1 also co-immunoprecipitated with p62 and, like CHD4, this interaction showed no dependence on the induction of DNA damage. These data suggest that the interaction between CHD4 and p62 is not responsible for the effect of p62 on DNA damage repair. Further work is required in order to understand the cellular processes in which this interaction is important. Full-length p62 was shown to interact

predominantly with the cleaved form of PARP whilst, strikingly,  $\Delta$ NES p62 is shown to interact mainly with full length PARP. Similarly, blocking nuclear export on p62 with Leptomycin B treatment resulted in an increased interaction with full length PARP. These data suggest that cytoplasmic p62 may play an active role in PARP cleavage. Interestingly, it has been suggested that autophagy may be involved in PARP cleavage; specifically through an interaction with LC3 and p62 as inhibition of autophagy results in a reduction of PARP cleavage and double silencing of LC3 and p62 resulted in a complete suppression of PARP cleavage (Zhang et al., 2011). Notably, PARP1 has been shown to activate autophagy in response to DNA damage (Munoz-Gamez et al., 2009), as well as nutrient deprivation (Rodriguez-Vargas et al., 2012), and oxidative stress (Huang et al., 2008). Interaction with p62 and subsequent degradation of PARP1 may serve as a negative feedback loop regulating PARP-dependent autophagy.

The cytoskeletal protein Filamin A (FLNA) was first suggested to be involved in DNA damage repair following identification of its interaction with BRCA2 (Yuan and Shen, 2001). Furthermore, it was noted that the melanoma cell line M2 was more sensitive to several genotoxic agents such as: gamma irradiation, bleomycin, and ultraviolet-C light due to the lack of FLNA (Yuan and Shen, 2001). Later, the same group found that FLNA deficiency leads to an 8h delay in recovery from G2 arrest in response to ionizing radiation (Meng et al., 2004). Following these observations, Yue and colleagues reported that FLNA plays a role in HR through its interaction with BRCA2 and recruitment of RAD51 (Yue et al., 2009). FLNA has also been shown to interact with BRCA1, with cells lacking FLNA showing reduced formation of BRCA1 and RAD51 foci in response to IR. Interestingly, FLNA was also reported to interact with other components of the HR pathways DNA-PKcs and Ku86 in a BRCA1-independent manner (Velkova et al., 2010). Recently FLNA was also found to interact with ssDNA-binding proteins RPA1-3 (Marechal et al., 2014). Taken together, these data suggest a role for FLNA in the early stages of HR. Interestingly, levels of FLNA have been shown to negatively correlate with drug sensitivity in melanoma cells in response to both Bleomycin and Cisplatin (Yue et al., 2012).

Work presented in this chapter shows that both FLNA and RAD51 co-immunoprecipitate with p62 in response to X-ray irradiation-induced DNA damage. Similarly, both p62 and RAD51 co-immunoprecipitate with FLNA in the reverse

experiment. Next, data is presented showing a significant reduction of both FLNA and RAD51 in the nuclear fraction of p62<sup>-/-</sup>+FLAG-p62 compared to p62<sup>-/-</sup> MEFs. These data suggest that interaction with p62 is involved in modulating nuclear levels of FLNA and RAD51.

As discussed previously, increased levels of both RAD51 and FLNA have been shown to enhance DNA repair through the HR pathway. It has, however, been shown that elevated levels of RAD51 in some circumstances can lead to hyper-recombination and genome instability (Richardson et al., 2004). Importantly, these data indicate that expression levels of these proteins are equivalent, however p62<sup>-/-</sup> MEFs show an increased nuclear distribution. The nuclear redistribution of RAD51 is a key component of the cellular response to DNA damage. Interestingly, both BRCA2-dependent (Davies et al., 2001, Yuan et al., 1999a) and -independent (Yu et al., 2003, Tarsounas et al., 2000, Lee et al., 2009) mechanisms of RAD51 nuclear redistribution have been observed. Depletion of ATM, ATR and CHK1 has been shown to reduce the nuclear re-localization of RAD51 (Jeyasekharan et al., 2013).

There are a number of possible mechanisms by which p62 could influence the nuclear levels of these proteins. p62 has been shown to promote the turnover of proteins through both the proteasomal and autophagy degradation pathways (Pankiv et al., 2010). Degradation, via either pathway, would result in a p62-dependent reduction in protein levels. p62 has also been shown to shuttle rapidly between the nucleus and cytoplasm (Pankiv et al., 2010). It is possible that through this process p62 could mediate the nuclear export of its binding partners. Additionally, p62 expression is associated with the formation of cytoplasmic aggregates. Sequestration of proteins within these aggregates has been shown to influence a number of cellular processes (Donaldson et al., 2003, Korolchuk et al., 2009). Therefore, p62 could be reducing the nuclear levels of RAD51 and FLNA through their cytoplasmic sequestration.

Since there is no marked increase in cytoplasmic levels of both FLNA and RAD51 in p62<sup>-/-</sup>+FLAG-p62 compared with p62<sup>-/-</sup> MEFs, it is unlikely that p62 is inhibiting the nuclear transport of these proteins through cytoplasmic sequestration. It should be noted, however, that the method of nuclear fractionation employed in this study results in around 5 times enrichment of the nuclear fraction compared to

cytoplasmic (Suzuki et al., 2010). Therefore, comparison between these two cellular fractions should not be used to directly assess cellular distribution as a percentage of total protein. The method does, however, allow the relative comparison of each fraction between different cell types.

Next, data is presented showing that inhibition of proteasomal degradation using MG132 cancelled differences in the nuclear levels of RAD51 and FLNA suggesting that p62 is involved in orchestrating the proteasomal degradation of these proteins. Interestingly, inhibition of nuclear export using Leptomycin B resulted in increased nuclear levels of RAD51 and FLNA in  $p62^{-/-}$ +FLAG-p62 and  $p62^{-/-}$  MEFs but did not cancel the differences between them. This suggests that the nuclear export of proteins via exportin 1 can alter nuclear levels of both RAD51 and FLNA, however, this is not required to mediate p62-dependent differences. These data suggest that p62 is influencing levels of RAD51 and FLNA exclusively through nuclear degradation via the proteasome. These data are in agreement with the previous observation that neither nuclear export of p62 or autophagy are required for its influence on DDF repair kinetics.

While increased nuclear levels of RAD51 in cells lacking p62 is suggestive of increased ability to perform HR, it is not clear if this excess of RAD51 was indeed functioning in HR. Immunofluorescence analysis confirmed that increased nuclear levels of RAD51 seen in  $p62^{-/-}$  MEFs corresponded with an increased number of RAD51 foci following IR. These data suggest that the increased levels of RAD51 do indeed correlate with increased HR activity. While RAD51 foci have been shown to correlate with HR activity (Raderschall et al., 1999), it is not a direct measure. Further analysis is needed to confirm that increased levels of nuclear RAD51 in  $p62^{-/-}$  MEFs facilitate an increase in HR-mediated DNA repair. This could be done using GFP reporter plasmids like those described in (Mao et al., 2008). It would also be of interest to assess genomic stability in  $p62^{-/-}$  MEFs as it is still not clear if increased HR in the context of p62 knock-out would be cytoprotective or if it would, in fact, lead to increased genome instability as has been observed with RAD51 overexpression (Richardson et al., 2004).

We have shown that p62 interacts with both FLNA and RAD51 which appears to influence the nuclear levels of both via proteasomal degradation. It is not clear if

p62 is facilitating the degradation of one or both of these proteins. It has previously been shown that nuclear levels of FLNA are able to influence levels of RAD51 (Yue et al., 2009). Data presented in this chapter show that knockdown of FLNA using siRNA resulted in a reduction in RAD51 foci formation in response to X-Ray IR in p62<sup>-/-</sup>+FLAG-p62 and p62<sup>-/-</sup> MEFs, consistent with previous reports (Yue et al., 2009). It is therefore possible that nuclear levels of RAD51 are reduced in p62<sup>-/-</sup>+FLAG-p62 MEFs as a result of reduced levels of FLNA. Interestingly, FLNA knockdown caused a reduction in RAD51 foci in p62<sup>-/-</sup> MEFs to those below observed in p62<sup>-/-</sup>+FLAG-p62 cells. This reduction in RAD51 foci corresponded with an increase in 53BP1 foci to similar level seen in p62<sup>-/-</sup>+FLAG-p62. These data suggest that the effect of p62 on DNA damage repair could well be mediated by the proteasomal degradation of FLNA within the nucleus.

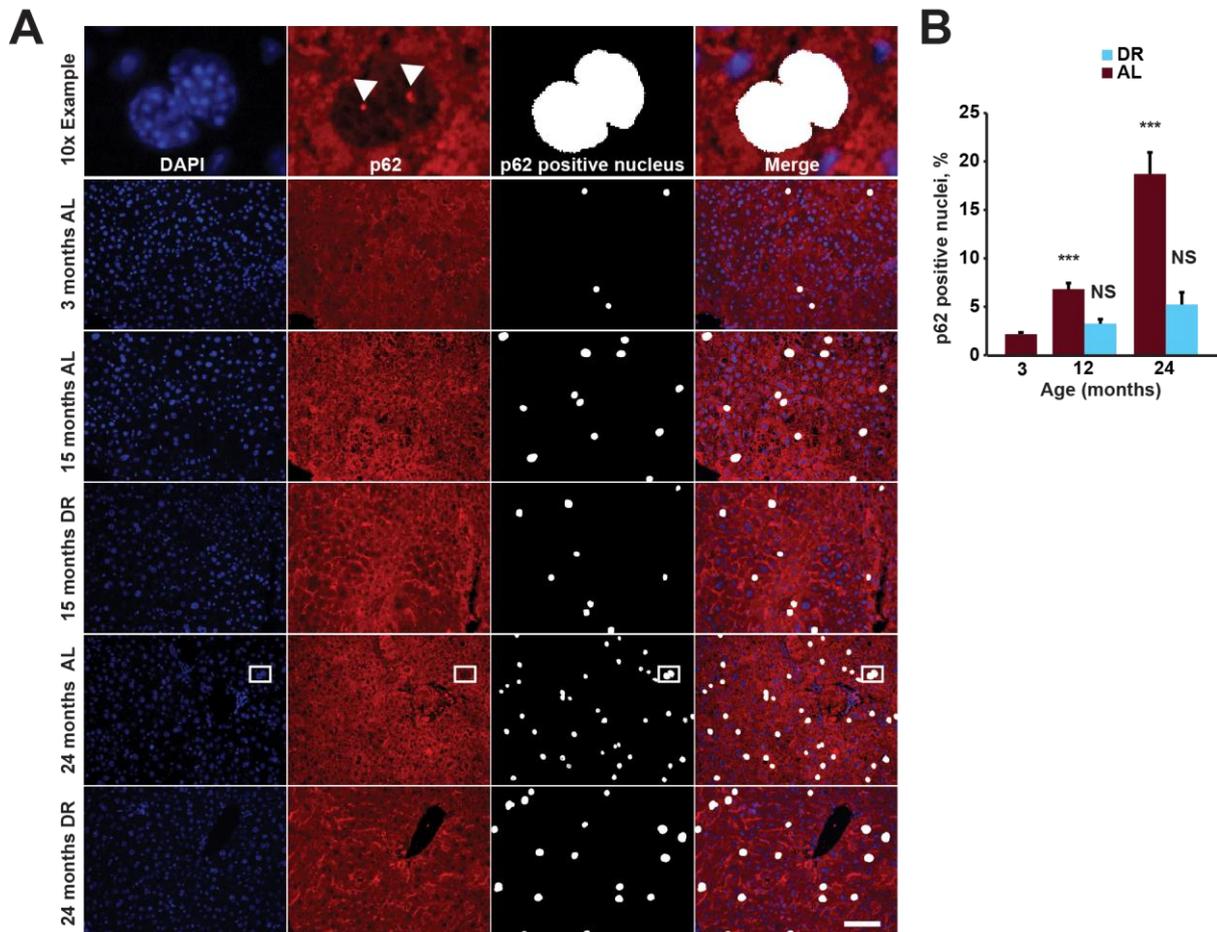
Further work is required to understand the role of autophagy on nuclear levels of RAD51 and FLNA. It is tempting to hypothesize that autophagy, and subsequent increase in p62 levels, would lead to an enhancement of the proteasomal degradation of RAD51 and FLNA within the nucleus. However, it is important to consider cross-talk between these two pathways as it has previously been shown that inhibition of autophagy can also inhibit proteasomal degradation in a p62-dependent manner (Korolchuk et al., 2009). Nuclear levels of RAD51 and FLNA, as well as the contribution of proteasomal degradation, would need to be assessed in the context of autophagy inhibition in order to link the p62-dependent decrease in DDF repair kinetic observed in chapter 3 with the mechanism proposed here.

## Chapter 6. Interaction between p62 and the DDR during Organismal Ageing

In the previous chapters, we have identified that autophagy inhibition was able to reduce DNA repair capacity in a p62-dependent manner *in vitro*. In this chapter, we hypothesise that the p62-dependent effect on DNA damage repair observed *in vitro* may also exist *in vivo*, specifically in the context of cellular ageing.

### 6.1 Levels of p62 in the Nucleus Increase with Age and are reduced with DR.

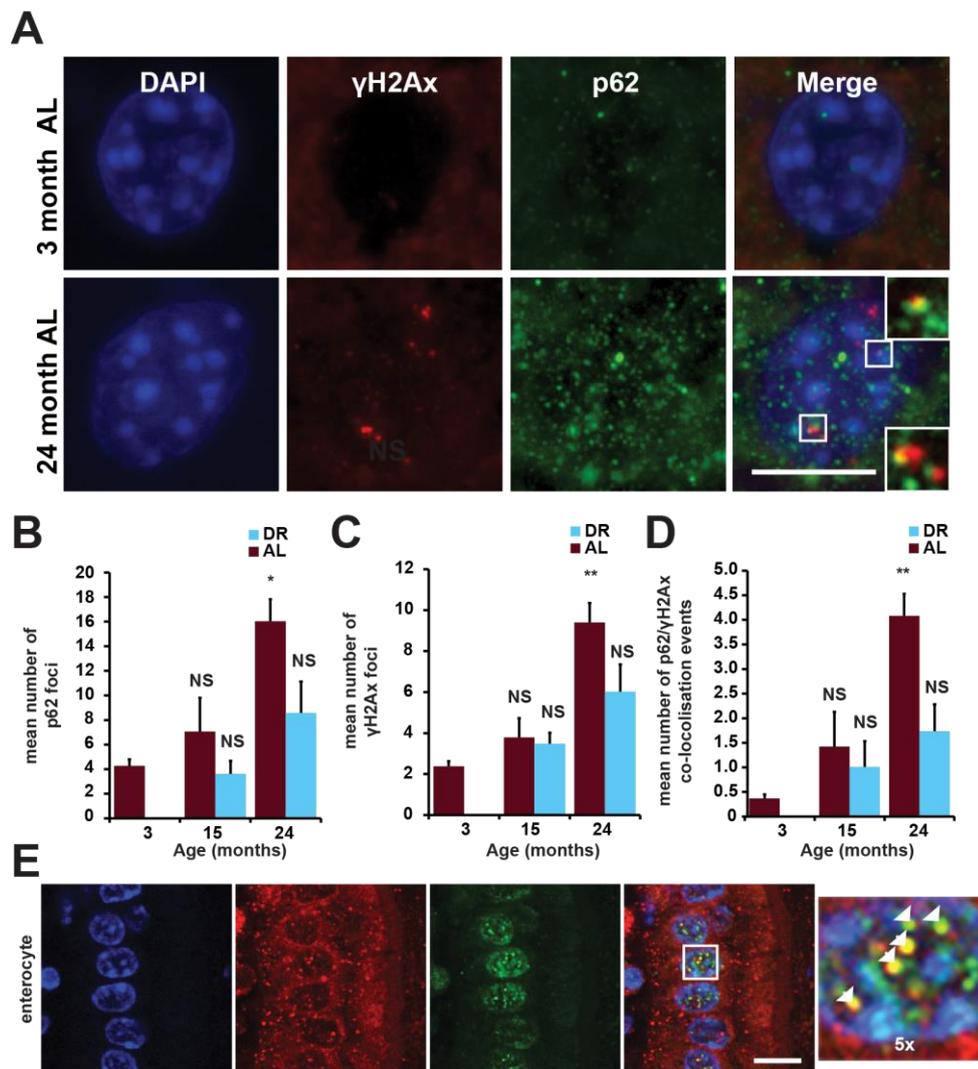
Dietary restriction is an intervention known to increase lifespan in an autophagy-dependent manner in a number of model organisms (Morselli et al., 2010, Rubinsztein et al., 2011, Jia and Levine, 2007). Dietary restriction has also been shown to reduce age-dependent increase in DNA damage (Wang et al., 2010). We hypothesise that increased level of autophagy in DR mice could be affecting DNA damage repair in a p62-dependent manner, similar to *in vitro* data shown in previous chapters. In order to investigate age-dependent changes in p62, liver tissues from C57BL/6 mice of 3, 15 and 24 months age, either fed *ad libitum* (AL) or dietary restricted (DR, 60% of AL), were stained with an antibody against p62. The numbers of cells containing nuclear p62 puncta were quantified by microscopy. Cells that are positive for p62 puncta are marked in white in Figure 6-1, quantification is shown in (Figure 6-1B). Results shown in Figure 6-1A-B demonstrate an age-dependent increase in nuclei positive for p62 puncta. Interestingly, DR mice show no significant increase in p62-positive nuclei compared to AL mice (Figure 6-1B).



**Figure 6-1: Nuclear foci of p62 increase with age in mouse hepatocytes and are decreased by DR.** Representative images of hepatocytes from 3, 15 and 24 month old male C57BL/6 wild type mice maintained on *ad libitum* (AL) or DR. Sections were immunostained with an antibody against p62. p62 positive nuclei are indicated in white (A) and quantified in (B). A higher magnification of a p62 positive nucleus is shown with arrowheads pointing to p62 foci. Scale bars represent 80 $\mu$ m; n=3; Error bars represent S.E.M\* p<0.05, \*\* p<0.01, \*\*\* p<0.001. Done in collaboration with Dr Diana Jurk and Mikolaj Ogradnik.

## 6.2 Co-Localisation of p62 with DDF increases with age and is reduced with DR.

Having established there was an age-dependent increase in nuclear p62 we then set out to establish what role this may be having on the DDR. In previous chapters we have shown that p62 co-localizes with DDF *in vitro*. To test if that is true *in vivo*, liver tissues from 3, 15 and 24 months old C57BL/6 mice on AL or DR diets were stained with antibodies against p62 and DNA damage marker  $\gamma$ H2A.X. p62 and  $\gamma$ H2A.X foci were then analysed using microscopy. Results shown in Figure 6-2A-B indicate an age-dependent increase in the number of nuclear p62 foci that was suppressed by DR, similar to results shown in Figure 6-1B. Further to an age-associated increase in p62 foci, there was a similar increase in the number of  $\gamma$ H2A.X foci (Figure 6-1C). This age-dependent increase in DDF was also suppressed by DR, in agreement with data published earlier (Wang et al., 2010). Interestingly, there was also an age-dependent increase in the number of co-localization events between p62 and  $\gamma$ H2A.X foci (Figure 6-2D). Next, I wanted to ascertain whether recruitment of p62 to DDF was common to different tissue types. Here, intestinal tissues from AhCre; APC f/wt mice were stained with antibodies against p62 and DNA damage marker  $\gamma$ H2A.X. p62 and  $\gamma$ H2A.X foci were then analysed using microscopy. Images shown in Figure 6-1D indicate that p62 is recruited to DDF in enterocytes in a fashion similar to that seen previously in hepatocytes *in vivo* (Figure 6-1C) and fibroblast *in vitro* (Figure 5-2D). These data indicate that p62 is dynamically recruited to DDF. Furthermore, clearance of nuclear p62 foci following DR correlates with the reduction of DDF suggesting a role of p62 in mediating the effect of autophagy on DNA repair *in vivo*.



**Figure 6-2: The interaction of p62 with DDF in mouse hepatocytes increases with age and is decreased by DR.** (A) Representative images of hepatocytes from young (3 months old) and old (24 months old) male C57BL/6 wild-type mice maintained on *ad libitum* (AL) diet. Sections were immunostained with antibodies against p62 and  $\gamma$ H2A.X. The mean number of p62 (B),  $\gamma$ H2A.X (C) and p62- $\gamma$ H2A.X colocalisation (D) foci were quantified in hepatocyte sections from mice maintained on AL or dietary-restricted (DR) diets. (E) Representative images of enterocytes from male C57BL/6 wild type mice. Sections were immunostained with antibodies against p62 and  $\gamma$ H2A.X. Arrowheads in the zoomed merged image indicate points of co-localisation. Scale bar 10  $\mu$ m. Scale bars 10  $\mu$ m; n=3; Error bars represent S.E.M. Done in collaboration with Dr Diana Jurk and Mikloaj Ogrodnik.

### 6.3 Discussion

Data presented in this chapter show an age-dependent increase in the formation of nuclear p62 puncta. The presence of p62 nuclear aggregates has previously been observed in several proteinopathies (Nagaoka et al., 2004, Kuusisto et al., 2003, Pikkarainen et al., 2008). Although these are age-related pathologies, nuclear p62 inclusions have not yet been studied in the context of ageing. This age dependent increase was reduced in mice undergoing dietary restriction. It is tempting to speculate that this age-dependent increase in p62 occurs due to an age-dependent decrease in autophagy as previously observed (Terman, 1995, Vittorini et al., 1999). Dietary restriction is an intervention that is well known to activate autophagy. In fact it has been shown that autophagy activity is required for the ability of DR to extend life span (Jia and Levine, 2007, Rubinsztein et al., 2011). It could also be postulated that the reduction in nuclear p62 foci seen in DR mice is a result of activated autophagy. Similarly, dietary restriction has been shown to result in the upregulation of components of the 20S and 19S proteasome (Lee et al., 1999). As p62 has been shown to be a substrate for proteasomal degradation, an increase in this pathway could also result in reduced levels of p62 (Lee et al., 2012b, Seibenhener et al., 2004). These hypotheses would of course need to be tested experimentally. Alternatively, nuclear levels of p62 could be altered by changes in the rate of nuclear import and export. It was shown by Pankiv *et al.* that phosphorylation of p62 at serine-266, threonine-269 and serine-272 residues regulates the nuclear-cytoplasmic shuttling of p62 (Pankiv et al., 2010). It would be interesting to test the phosphorylation status of p62 in the context of ageing as well as with DR to assess whether this was responsible for the age-dependent changes in nuclear p62 observed here. Our previous data suggest an increase in the number of nuclear p62 foci in response to DNA damage. Data presented in this chapter and in previous studies have shown an age-dependent increase in DNA damage foci (Wang et al., 2010). It is therefore possible that p62 is shuttled to the nucleus in response to increased levels of DNA damage seen with age. Similarly, data shown here indicate that DR leads to a reduced level of DNA damage and so the reduction in p62 foci could be a result of decreased DNA damage (Wang et al., 2010). Further

work is needed to understand the mechanisms driving this age-dependent increase of nuclear p62.

Interestingly, we show that p62 and DNA damage foci co-localise in hepatocytes from aged animals. This co-localisation is reduced in animals under DR. Moreover levels of nuclear p62 and p62-DDF co-localisation correlate with an increase in the number of DDF. These data suggest that p62 may be playing a role in DNA damage *in vivo*. As discussed previously, the co-localisation between DDF and p62 was observed *in vitro*. Further analysis identified an interaction between p62 and RAD51 and FLNA. An attempt was made to assess the co-localisation of p62 and RAD51 *in vivo*, however immunofluorescence staining for RAD51 was unsuccessful in tissue. Further work is required to understand the exact role of the interaction between p62 and DDF in the context of ageing. A tempting hypothesis is that increased levels of nuclear p62 occur with age as a result of declining protein homeostasis. Increased p62 then has an inhibitory effect in DNA damage repair via homologous recombination through its proteasomal deprecation of RAD51 and FLNA. The resulting increase in DNA damage may then contribute to a progression of the ageing phenotype through activation of cellular senescence. This could be one mechanism by which interventions that activate autophagy such as rapamycin treatment and dietary restriction influence DNA damage accumulation and longevity.

## Conclusions

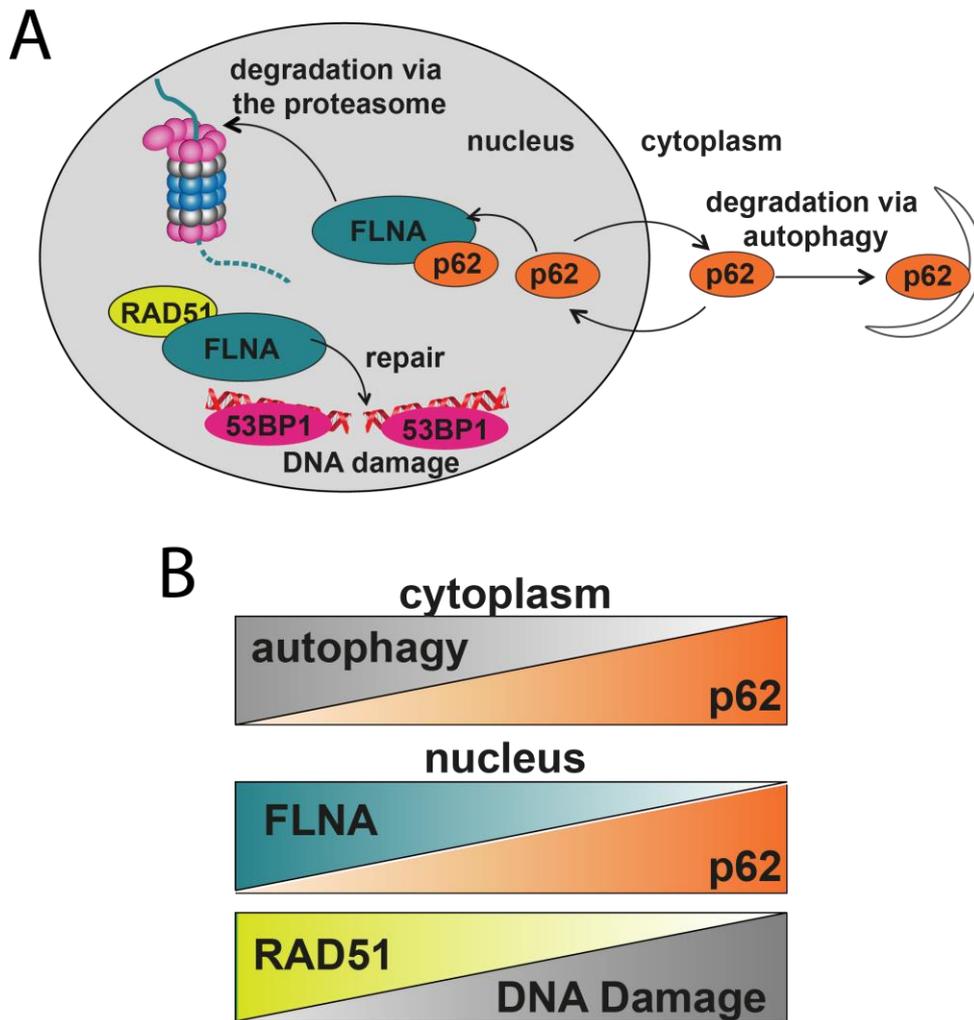
The accumulation of misfolded and damaged proteins is a hallmark of ageing and age-related diseases. This has been attributed to both an increase in the formation of damaged proteins due to ROS and errors in translation, as well as a gradual age-related decline in cellular proteolytic pathways. Autophagy has long been in the spotlight as a modulator of longevity; however the exact mechanisms by which this is mediated are not yet fully understood. Similarly, the accumulation of DNA damage has been suggested as a causal factor in organismal ageing. Again, an increase in the rate of generation of DNA damage as well as a decline in the ability a cell to resolve this damage have been observed in ageing. Data in this thesis identify a novel link between autophagy and DNA repair via HR through its control of intracellular levels of p62. This observation provides a potential link between the gradual decline in autophagy and DNA repair capacity that occurs with age. This interaction could have far reaching influence on a number of age-associated phenotypes and pathologies.

Interestingly, the Gorbunova lab has observed a decline in functional HR in human fibroblast with progressive population doublings and the initiation of cellular senescence. This was accompanied by a decline in proteins from the HR pathway such as RAD51, RAD51L and RAD52. This decline could be halted by the expression of SIRT6 (Mao et al., 2012b). Interestingly expression of SIRT6 has been shown to be a potent activator of autophagy. Moreover, it has been observed that autophagy is required for the anti-senescence effects of SIRT6 overexpression (Takasaka et al., 2014). It is therefore possible that this decline in HR occurs via p62 mediated degradation of RAD51 as observed in this thesis. Thus, activation of autophagy would lead to reduced levels of p62 inhibiting the proteasome-mediated degradation of RAD51 and resulting in reinstated levels of RAD51 and HR activity. It would be interesting to assess the effect of both p62 knockdown and activation of autophagy, via a SIRT6 independent mechanism, on HR in this context to test the relevance of this newly discovered mechanism in this system.

The accumulation of p62-positive nuclear inclusions is common in a number of proteinopathies (Nagaoka et al., 2004, Kuusisto et al., 2003, Pikkarainen et al., 2008). This role for p62 as an inhibitor of HR could provide another mechanism by

which these pathologies lead to cell death and disease progression. Investigation of the relative efficacy of DNA damage repair pathways in the context of proteinopathies could provide further mechanistic insight into these pathologies. Interestingly, mutations in the UBA domain of p62 are a common feature of familial and sporadic ALS (Teyssou et al., 2013) as well as Paget's disease (Laurin et al., 2002, Hocking et al., 2002). Here we identify the UBA domain of p62 as a functional domain required for its effect on DNA damage. It would therefore be interesting to test the relevance of these observations in the context of these diseases.

Lastly, although not the focus of this thesis, both DNA damage and autophagy have been shown to play central roles in cancer development. Indeed, the role of both of these processes in cancer is multifactorial and highly context-dependent. Autophagy has been suggested as a tumour survivor mechanism when tumour cells undergo metabolic and therapeutic stresses (Degenhardt et al., 2006). However many studies have suggested a role for autophagy in tumour suppression (Mathew et al., 2007b, Mathew et al., 2009). Genomic instability is a hallmark of cancer and results from the combined effect of DNA damage, tumour specific DNA repair defects as well as impaired cell cycle checkpoints. Although a central part of cancer progression, genetic instability has also provided many therapeutic opportunities in cancer treatment. The identification of novel mechanisms of crosstalk between autophagy and DNA damage repair will aid our understanding of these complex processes and present further therapeutic opportunities. The ability of autophagy to influence HR-mediated repair, could offer some way to explain its apparent dual role in cancer progression. An increase in HR activity could mediate cell survival in tumour cells with increased autophagy in response to therapeutic stress. Conversely, an activation of this process prior to transformation could infer a reduced accumulation of DNA damage serving as a tumour suppressor mechanism. It is important to note that genome instability has not been assessed in this context. It is possible that a reduction in p62-mediated RAD51 degradation could lead to hyper-recombination and increased genome instability. Nonetheless, both of these possibilities would be relevant in the context of cancer progression.



**Schematic representation of proposed mechanism: (A)** In response to DNA damage p62 interacts with FLNA within the nucleus, targeting it for degradation via the proteasome. Reduced levels of FLNA lead to a reduced recruitment of RAD51 to the site of damage. We propose this results in a reduced activity of the HR repair pathway. **(B)** Cellular levels of p62 are modulated by autophagy; therefore inhibition of autophagy leads to increased levels of p62. We suggest that this mechanisms links autophagy status as a modulator of HR-mediated DNA repair.

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