2009

The Rad9-Rad1-Hus1 complex and Bif-1 regulate multiple mechanisms that affect sensitivity to DNA damage

Cheryl L. Meyerkord
University of South Florida

The Rad9-Rad1-Hus1 Complex and Bif-1 Regulate Multiple Mechanisms that Affect Sensitivity to DNA Damage

by

Cheryl L. Meyerkord

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cancer Biology
College of Graduate School
University of South Florida

Major Professor: Hong-Gang Wang, Ph.D.
Srikumar Chellappan, Ph.D.
W. Douglas Cress, Ph.D.
Lori Hazlehurst, Ph.D.

Date of Approval:
February 9, 2009

Keywords: Programmed cell death, Apoptosis, Autophagy, Endocytosis, BH3-only proteins

Dedication

I would like to dedicate this dissertation to my brave mother and everyone who has battled cancer, whether they won or lost. It is for them that we get up and go to work each day. They are the driving force behind our motivation to some day find a cure.

Acknowledgments

First and foremost, I express my deepest gratitude to my mentor, Dr. Hong-Gang Wang, for all of his patience, help and guidance. I am fortunate to have had him as a mentor. I am very grateful for having an extraordinary committee and would like to thank Dr. W. Douglas Cress, Dr. Srikumar Chellappan and Dr. Lori Hazlehurst for all of their support, direction and constructive criticism. I am deeply honored to have Dr. Robert Weiss of Cornell University as the outside chairperson for my dissertation committee.

I extend many thanks to all of the members of the Wang laboratory, both past and present, who have contributed to my training and provided a work environment that was conducive to my growth as a scientist. I would especially like to thank Dr. Yoshinori Takahashi for always being there for me during the hard times and helping me keep things in perspective. He has not only taught me a lot about science, but also about life.

I deeply appreciate the friends that I have made while at Moffitt, especially Daniele Gilkes, Amy Hazen and Rachel Haviland who have supported me throughout my Ph.D. I would especially like to thank them for treating me like family and always being there for me when I needed someone. I would also like to thank Patricia Massard and Cathy Gaffney for all of their help and support.

Importantly, I would like to thank my family who were always there to support me, no matter what struggle or obstacle they were dealing with in their own lives. Without their love and support, I never would have made it this far.

This work was funded by a predoctoral traineeship from the Department of Defense Breast Cancer Research Program (BC050563).

Note to the Reader

The original of this document contains color that is necessary for understanding the data.

The original dissertation is on file with the USF library in Tampa, Florida.

Table of Contents

List of Figures

List of Abbreviations

9-1-1 complex: the complex composed of Rad9, Rad1 and Hus1
ATG: autophagy-related
ATM: ataxia-telangiectasia mutated
ATR: ATM- and Rad3-related
BAR: Bin-Amphiphysin-Rvs
Bcl-2: B-cell lymphoma 2
BER: base excision repair
BH: Bcl-2 homology domain
Bif-1: Bax-interacting factor-1
Bim: Bcl-2 interacting mediator of cell death
BRCA1: breast cancer gene 1 or breast and ovarian cancer susceptibility gene 1
BSA: bovine serum albumin
Cdc25: cell division cycle 25
cdk: cyclin dependent kinase
Chk: checkpoint kinase
DAPI: 4, 6-diamidino-2-phenylindole
DDR: DNA damage response
DNA: deoxyribonucleic acid
DMEM: Dulbecco's modified Eagle's medium
DMSO: dimethylsulfoxide
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GFP: green fluorescent protein
HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP: horseradish peroxidase
HRR: homologous recombination repair
Hus1: hydroxyurea sensitive 1
HU: hydroxyurea
IR: ionizing radiation
LAMP-1: lysosome-associated membrane protein-1
LC3: microtubule-associated protein light chain 3
MDC1: mediator of DNA damage checkpoint 1
MEF: mouse embryonic fibroblast
MOMP: mitochondrial outer membrane permeablization
MRN: MRE11-Rad50-Nbs1
mRNA: messenger ribonucleic acid

MVB: multivesicular body
Nbs1: Nijmegen breakage syndrome
NGF: nerve growth factor
PARP: poly(ADP-ribose) polymerase
PAS: phagophore assembly sites
PCNA: proliferating cell nuclear antigen
PE: phosphatidylethanolamine
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PI3K: phosphatidylinositol 3-kinase
PI3KC3: phosphatidylinositol 3-kinase class III
Puma: p53 upregulated modulator of apoptosis
Rad: radiation sensitive
RIPA: radioimmunoprecipitation assay buffer
RT-PCR: reverse transcription-polymerase chain reaction
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH3: Src-homology 3
shRNA: short hairpin RNA
siRNA: short interfering RNA
Tor: target of rapamycin
TrkA: tropomyosin-related kinase A
TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UV: ultraviolet radiation
UVRAG: ultraviolet irradiation resistance-associated gene
Vps: vacuolar protein sorting

The Rad9-Rad1-Hus1 Complex and Bif-1 Regulate Multiple Mechanisms that Affect Sensitivity to DNA Damage

Cheryl L. Meyerkord

ABSTRACT

The resistance of cancer cells to traditional chemotherapeutic agents is a major obstacle in the successful treatment of cancer. Cancer cells manipulate a variety of signaling pathways to enhance resistance to anticancer agents; such mechanisms include disrupting the DNA damage response and hyperactivating survival signaling pathways. In an attempt to better understand the molecular mechanisms that underlie resistance to chemotherapeutic agents, we investigated multiple processes regulated by the Rad9-Rad1-Hus1 (9-1-1) complex and Bif-1. The 9-1-1 complex plays an integral role in the response to DNA damage and regulates many downstream signaling pathways. Overexpression of members of this complex has been described in several types of cancer and was shown to correlate with tumorigenicity. In this study, we demonstrate that disruption of the 9-1-1 complex, through loss of *Hus1*, sensitizes cells to DNA damaging agents by upregulating BH3-only protein expression. Moreover, loss of *Hus1* results in release of Rad9 into the cytosol, which enhances the interaction of Rad9 with Bcl-2 to potentiate the apoptotic response. We also provide evidence that disruption of the 9-1-1 complex sensitizes cells to caspase-independent cell death in response to DNA damage. Furthermore, we found that loss of *Hus1* enhances DNA damage-induced autophagy. As

autophagy has been implicated in caspase-independent cell death, these data suggest that the enhanced autophagy observed in *Hus1*-knockout cells may act as an alternate cell death mechanism. However, inhibition of autophagy, through knockdown of Atg7 or Bif-1, did not suppress, but rather promoted DNA damage-induced cell death in *Hus1*-deficient cells, suggesting that in apoptosis-competent cells autophagy may be induced as a cytoprotective mechanism. The aberrant activation of survival signals, such as enhanced EGFR signaling, is another mechanism that provides cancer cells with resistance to DNA damage. We found that knockdown of Bif-1 accelerated the co-localization of EGF with late endosomes/lysosomes thereby promoting EGFR degradation. Our results suggest that Bif-1 may enhance survival not only by inducing autophagy, but also by regulating EGFR degradation. Taken together, the results from our studies indicate that the 9-1-1 complex and Bif-1 may be potential targets for cancer therapy as they both regulate sensitivity to DNA damage.

Chapter One: Introduction

Cancer

Despite major advances in the treatment of cancer, this disease is still the second leading cause of death in the United States (Jemal *et al.*, 2008). It is estimated that in 2008 over 1.4 million people were diagnosed with cancer and over 0.5 million people died from this disease. Cancer is caused by both internal factors, such as inherited mutations, and external factors, such as exposure to DNA damaging agents (American Cancer Society, 2008). Tumorigenesis is a multi-step process that is characterized by the accumulation of genetic mutations that transform normal cells into malignant derivatives (Hanahan and Weinberg, 2000). These mutations can activate oncogenes and inactivate tumor suppressor genes resulting in genomic instability and driving tumor progression. Paradoxically, DNA-damaging agents are some of the most effective drugs used for the treatment cancer. In addition, the efficacy of DNA damage-based chemotherapy may be influenced by the ability of a cell to repair damaged DNA (Helleday *et al.*, 2008). Therefore, deciphering the cellular mechanisms that are activated in response to DNA damage may not only lead to a better understanding of the causes of cancer, but also to better, more effective treatment strategies.

The DNA Damage Response

The genomes of eukaryotic cells are constantly being subjected to endogenous and exogenous genotoxic stresses. Damage resulting from exposure to such stresses threatens cell survival and can lead to cancer, as well as other genetic diseases. In order to preserve genomic integrity and ensure that an accurate copy of the genome is passed on to subsequent generations, cells have evolved a core surveillance machinery that senses damaged or abnormally structured DNA and coordinates cell cycle progression with DNA repair. In cases when damage is excessive or repair is unfavorable, the cell death machinery is activated in order to eliminate damaged cells (Melo and Toczyski, 2002; Niida and Nakanishi, 2006; Zhou and Elledge, 2000).

In response to genotoxic stress, a complex network of interacting checkpoint signaling pathways act in concert to execute an appropriate DNA damage response (DDR) (Harper and Elledge, 2007). In mammalian cells, two related phosphatidylinositol 3-kinase-related serine/threonine kinases play a central role in the regulation of the DDR (Abraham, 2001; Matsuoka *et al.*, 2007). The ATM (ataxia-telangiectasia mutated)-mediated pathway is activated in response to DNA damaging agents that induce double-strand breaks (Lavin, 2008), whereas the ATR (ATM- and Rad3-related)-dependent pathway responds to a broad spectrum of genotoxic stresses including those that inhibit replication and induce single-strand DNA breaks or bulky DNA lesions (Cimprich and Cortez, 2008).

Mutations in ATM lead to the autosomal recessive disorder ataxia-telangiectasia, which is characterized by immunodeficiency, radiosensitivity, neurodegeneration and cancer predisposition (Boder, 1985). Individuals, mice and cells that lack ATM are

viable, which indicates that ATM is not required for differentiation, normal cell cycle progression or other essential cellular functions (Shiloh and Kastan, 2001). Under normal conditions, ATM exists as an inactive homodimer. In response to DNA damage, ATM undergoes a conformational change, which stimulates its kinase activity. ATM is then autophosphorylated at serine 1981 leading to the dissociation of inactive homodimers to form active monomers (Bakkenist and Kastan, 2003). DNA damage also induces the association of ATM with the MRE11-Rad50-Nbs1 (MRN) complex, which acts as an adaptor for the recruitment of downstream signaling proteins and facilitates the full activation and proper localization of ATM (Berkovich *et al.*, 2007; van den Bosch *et al.*, 2003). ATM-mediated phosphorylation of H2AX (γH2AX) recruits MDC1, which acts as a positive feedback loop to facilitate further ATM phosphorylation of H2AX and the recruitment of additional ATM-MRN complexes, thereby propagating the DDR (Stucki and Jackson, 2006).

In contrast to ATM, ATR is required for viability (Brown and Baltimore, 2000); however, hypomorphic mutations of ATR are associated with Seckel syndrome (O'Driscoll *et al.*, 2003). ATR is constitutively bound to ATRIP (ATR-interacting protein) even in the absence of DNA damage or replicative stress (Cortez *et al.*, 2001). ATRIP binds to the single-stranded DNA-coating protein, RPA (replication protein A), which facilitates the recruitment of ATR to DNA and the activation of downstream signaling (Zou and Elledge, 2003). However, recruitment of ATR to the site of DNA damage is not sufficient to activate ATR signaling, several other proteins must be present in order for ATR to execute all of its cellular functions. Rad17, the Rad9-Rad1-Hus1 (9-1-1) complex, TopBP1 (topoisomerase-binding protein-1) and Claspin are all required for

the full activation of ATR-mediated downstream signaling (Chini and Chen, 2003; Delacroix *et al.*, 2007; Kumagai *et al.*, 2006; Weiss *et al.*, 2002; Zou *et al.*, 2002). The loading of the 9-1-1 complex onto the DNA results in the recruitment of TopBP1 through its interaction with Rad9 (Delacroix *et al.*, 2007; Lee *et al.*, 2007). TopBP1 then binds to the ATR-ATRIP complex and enhances the kinase activity of ATR (Kumagai *et al.*, 2006; Mordes *et al.*, 2008).

Upon activation, ATM and ATR are responsible for relaying the DNA damage signal to downstream transducer and effector proteins (Chen *et al.*, 2001; Niida and Nakanishi, 2006; Zou *et al.*, 2002). ATM and ATR may regulate as many as 700 substrates in response to DNA damage (Matsuoka *et al.*, 2007). While ATM and ATR share some substrate specificity, these kinases have also been shown to selectively phosphorylate different substrates (Cimprich and Cortez, 2008; Kim *et al.*, 1999; Zhou and Elledge, 2000). Two proteins that play a key role in conveying the DNA damage response are the checkpoint proteins, Chk2 and Chk1, which are phosphorylated by ATM and ATR, respectively. Along with ATM and ATR, Chk1 and Chk2 are responsible for transducing the DNA damage signal to downstream effector proteins, such as p53, MDM2, BRCA1, E2F1, Cdc25A and Cdc25C (Bartek and Lukas, 2003; Kastan and Bartek, 2004). Through the phosphorylation of these, and many other proteins, ATM and ATR respond to DNA damage in order to regulate cell cycle arrest/progression, facilitate DNA repair, regulate transcriptional events and induce apoptosis (Zhou and Elledge, 2000) (Figure 1).

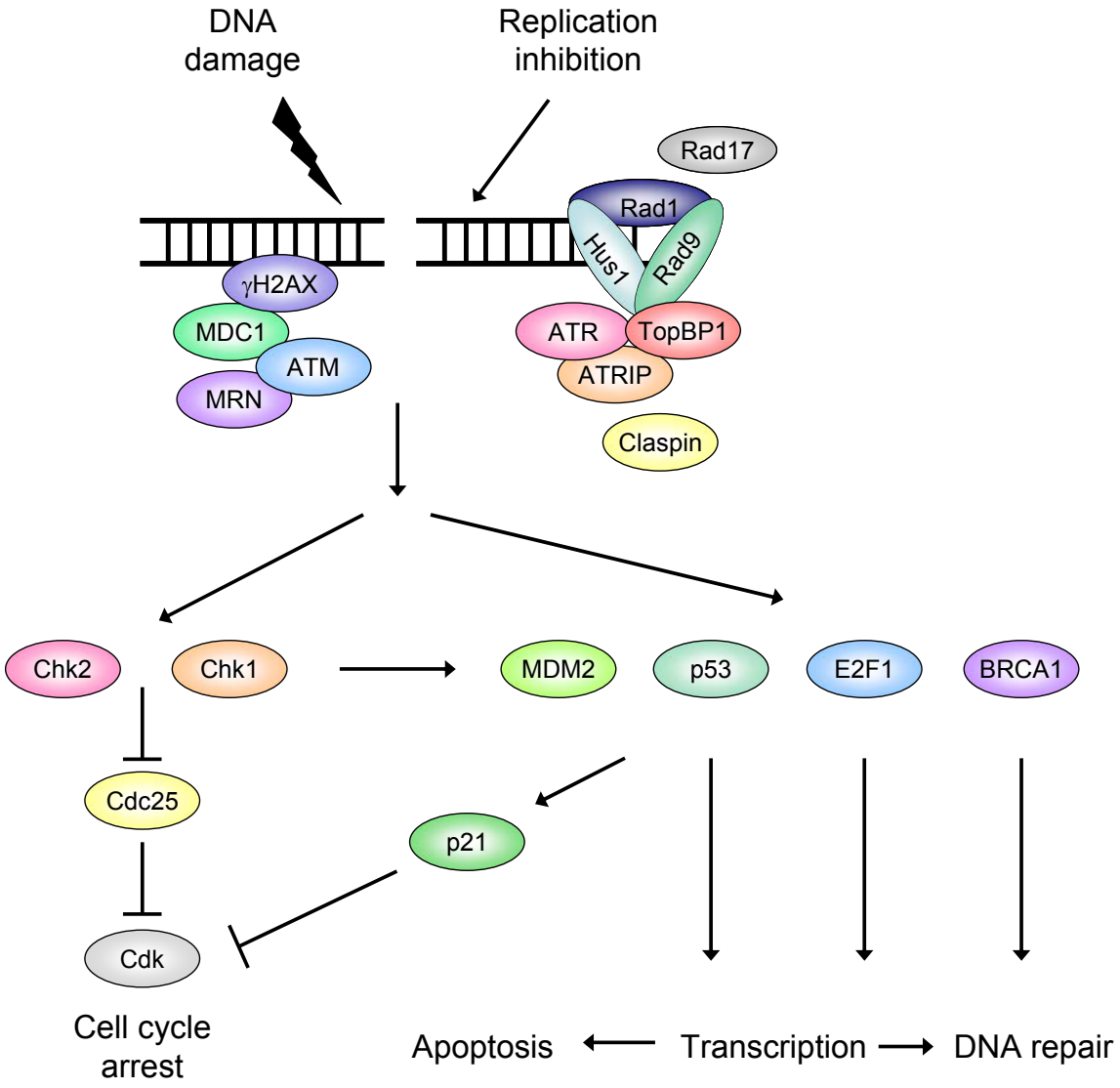

Figure 1. Simplified schematic of the DNA damage response.

The Rad9-Rad1-Hus1 Complex

The members of the Rad (radiation sensitive) family are key regulators in sensing DNA damage and regulating checkpoint activation (Parrilla-Castellar *et al.*, 2004). In fission yeast, certain members of the Rad family including Rad9, Rad1, Hus1, Rad17 and Rad3 (ATR) are essential for both the DNA damage and DNA replication checkpoints (Rhind and Russell, 1998). Evidence suggests that the functions of these proteins are

conserved in mammals, which highlights the critical role for these proteins in the DDR (Parrilla-Castellar *et al.*, 2004).

Structure and Role in the DNA Damage Response

Three members of the Rad family, Rad9, Rad1 and Hus1, form a heterotrimeric clamp that acts as a putative sensor for DNA damage (Burtelow *et al.*, 2001; Hang and Lieberman, 2000; Roos-Mattjus *et al.*, 2002; St. Onge *et al.*, 1999; Volkmer and Karnitz, 1999). Biochemical and molecular modeling data suggest that the 9-1-1 complex bears structural similarity to the homotrimeric PCNA (proliferating cell nuclear antigen) sliding clamp (Venclovas and Thelen, 2000), which is loaded onto DNA during replication, recombination and repair (Tsurimoto, 1999). Another member of the Rad family, Rad17, along with the four small subunits of the replication factor C complex (RFC), is responsible for loading the 9-1-1 complex onto DNA in response to various types of DNA damage (Bermudez *et al.*, 2003; Lindsey-Boltz *et al.*, 2001; Rauen *et al.*, 2000) (Figure 2). Together with Rad17, the 9-1-1 complex is responsible for facilitating ATR-mediated signaling pathways that are required for an appropriate response to DNA damage. While the 9-1-1 complex is required for full activation of the ATR-mediated DDR, it appears to be dispensable for activation of the ATM-mediated pathway (Weiss *et al.*, 2002). However, it has been shown that ATM can phosphorylate Rad9 on serine 272. This event is required for checkpoint activation in response to IR (Chen *et al.*, 2001), suggesting that 9-1-1- and ATM-mediated responses may not be completely exclusive. Thus, the 9-1-1 complex collaborates with ATR and ATM to activate downstream signaling pathways and cell cycle checkpoints.

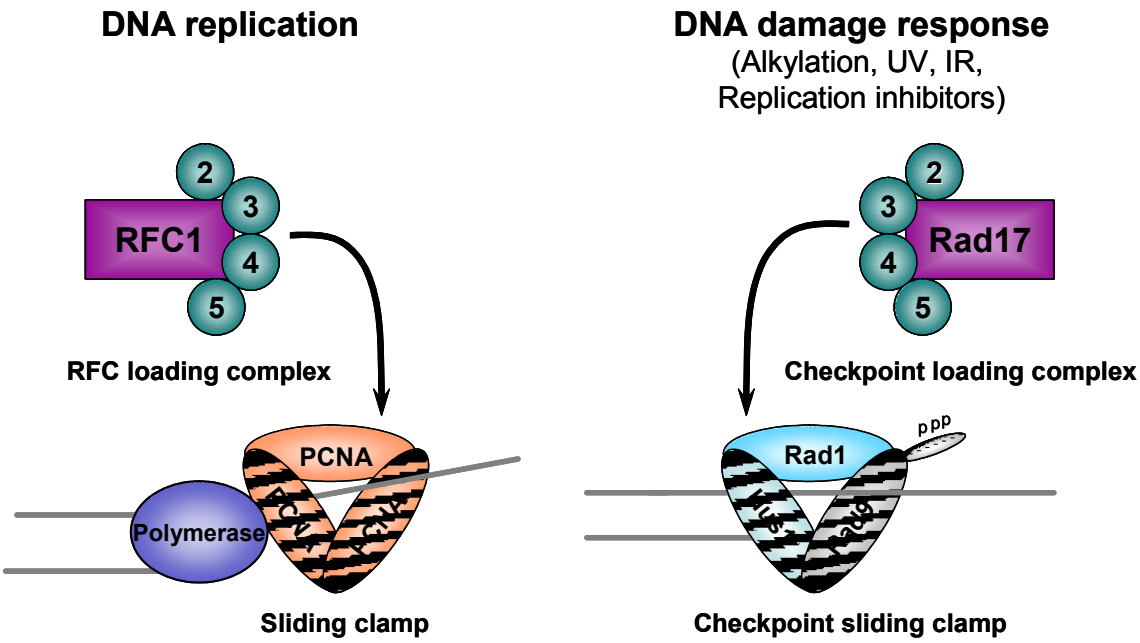

Figure 2. Comparison of the Rad9-Rad1-Hus1 complex and PCNA.

Unlike the other members of the 9-1-1 complex, Rad9 possesses a carboxy-terminal region that is constitutively phosphorylated and inducibly hyperphosphorylated in response to DNA damage (Chen *et al.*, 2001; Roos-Mattjus *et al.*, 2003; St Onge *et al.*, 2001; St Onge *et al.*, 2003). While this region is not required for interaction with Rad17, Rad1 or Hus1, it is required for Chk1 phosphorylation and downstream signaling (Roos-Mattjus *et al.*, 2003). It has been suggested that the C-terminus of Rad9, in which the "phospho-tail" is located, is responsible not only for translocation of the 9-1-1 complex to the nucleus (through a nuclear localization signal) (Hirai and Wang, 2002), but also for the recruitment of signaling proteins to DNA lesions, thereby facilitating the activation of downstream signaling pathways (Roos-Mattjus *et al.*, 2003).

Regulation of DNA Repair

In addition to playing a central role in DDR signaling pathways, the 9-1-1 complex has also been shown to play a direct role in several DNA repair mechanisms. A role for the 9-1-1 complex in base excision repair (BER) (especially long-patch BER) has been well described (Helt *et al.*, 2005). It has been shown that the 9-1-1 complex regulates the early steps of BER by binding to and enhancing the activity of the DNA glycosyslase MutY homologue (MYH) (Chang and Lu, 2005; Shi *et al.*, 2006) and apurinic/apyrimidinic endonuclease 1 (Gembka *et al.*, 2007), which results in the removal of damaged bases. The 9-1-1 complex also interacts with DNA polymerase β to augment its activity (Toueille *et al.*, 2004). In addition, the 9-1-1 complex can also bind to flap endonuclease 1 (Friedrich-Heineken *et al.*, 2005; Wang *et al.*, 2004a) and DNA ligase I (Smirnova *et al.*, 2005; Wang *et al.*, 2006a), thereby stimulating the cleavage of flaps and the sealing of the final nick, respectively. Thus the 9-1-1 complex plays an integral role in the regulation of all of the steps of BER. In addition to regulating BER, members of the 9-1-1 complex may also regulate homologous recombination repair (HRR), as knockdown of either Rad9 or Hus1 decreases the efficiency of HRR (Pandita *et al.*, 2006; Wang *et al.*, 2006b). Moreover, the 9-1-1 complex may also regulate translesion synthesis in yeast by binding to translesion polymerases (Kai and Wang, 2003; Sabbioneda *et al.*, 2005). Furthermore, Rad9 and Rad1 possess 3′ to 5′ exonuclease activity and therefore may facilitate the processing of double-stranded DNA to single-stranded DNA (Bessho and Sancar, 2000; Parker *et al.*, 1998). These results suggest that the 9-1-1 complex may respond to DNA damage both by activating downstream signaling pathways and by directly mediating various forms of DNA repair.

Regulation of Additional Cellular Processes

In addition to regulating the processes described above, members of the 9-1-1 complex have also been shown to play a role in the maintenance of telomeres (Francia *et al.*, 2006; Nabetani *et al.*, 2004; Pandita *et al.*, 2006). Indeed, this role is evolutionarily conserved as progressive telomere shortening has been observed in *Caenorhabditis elegans* strains that lack *Hus1* or *MRT-2* (the orthologue of Rad1) (Ahmed and Hodgkin, 2000; Hofmann *et al.*, 2002). Interestingly, Rad9 has been shown to function as a transcription factor that can transactivate p53 target genes, including p21, which is a well known regulator of the cell cycle (Yin *et al.*, 2004). Therefore, in addition to playing a role in checkpoint signaling pathways as a member of the 9-1-1 complex, Rad9 may also be able to directly affect cell cycle arrest at the G_1 to S-phase transition through the transcriptional activation of p21. In addition to playing a key role in the regulation of DNA damage checkpoints, Rad9 also has been shown to induce apoptosis through its interaction with the anti-apoptotic proteins, Bcl-2 and Bcl-xL (Ishii *et al.*, 2005; Komatsu *et al.*, 2000a; Komatsu *et al.*, 2000b; Yoshida *et al.*, 2002; Yoshida *et al.*, 2003). Moreover, phosphorylation of Rad9 by either c-Abl (Yoshida *et al.*, 2002) or protein kinase Cδ (Yoshida *et al.*, 2003) promotes the binding of Rad9 to anti-apoptotic proteins thereby enhancing apoptosis. Furthermore, Rad9 can be cleaved by caspase-3, resulting in disruption of the 9-1-1 complex and release of the BH3 domain-containing fragment of Rad9 into the cytosol where it binds to Bcl-xL to potentiate the apoptotic response (Lee *et al.*, 2003).

Defects Resulting from Loss of a Functional 9-1-1 Complex

As described above, the 9-1-1 complex plays an integral role in the regulation of a multitude of cellular processes including the DDR, DNA repair and apoptosis. Therefore, disruption of the 9-1-1 complex affects many downstream signaling processes. Impaired function of the 9-1-1 complex results in defects in cell cycle arrest at both the S and G_2/M checkpoints, an increase in chromosomal abnormalities and increased sensitivity to genotoxic stresses including topoisomerase poisons, ultraviolet radiation (UV), hydroxyurea (HU) and ionizing radiation (IR) (Bao *et al.*, 2004; Dang *et al.*, 2005; Hopkins *et al.*, 2004; Kinzel *et al.*, 2002; Loegering *et al.*, 2004; Pandita *et al.*, 2006; Roos-Mattjus *et al.*, 2003; Wang *et al.*, 2004b; Wang *et al.*, 2006b; Wang *et al.*, 2003; Weiss *et al.*, 2000; Weiss *et al.*, 2003). Moreover, loss of *Rad9* or *Hus1* results in embryonic lethality, which is, at least in part, attributable to widespread apoptosis during embryogenesis (Hopkins *et al.*, 2004; Weiss *et al.*, 2000). Although $Hus1^{-/-}$ mouse embryonic fibroblast (MEF) cells have a cellular proliferative defect, crossing of $Hus1^{+/-}$ mice to a $p21^{-/-}$ background results in $Hus1^{-/-}p21^{-/-}$ MEFs that are viable and can be grown in culture (Weiss *et al.*, 2000). The defects described above emphasize the importance of the role of the 9-1-1 complex in the regulation of the DDR and cell cycle checkpoints, maintenance of genomic integrity, proper embryonic development and continued viability in culture.

While loss of *Hus1* has been shown to increase sensitivity to DNA damage-induced cell death, the molecular mechanisms by which this occurs have yet to be elucidated. In this study, we demonstrate for the first time that loss of *Hus1* sensitizes cells to etoposide treatment through the upregulation of the BH3-only proteins, Bim and

Puma. Furthermore, loss of *Hus1* results in a defect in the binding of Rad9 to chromatin and release of Rad9 into the cytosol, which in turn enhances the interaction of Rad9 with Bcl-2 to amplify the apoptotic response.

Programmed Cell Death

The term programmed cell death can be defined as "a genetically controlled cell-death process that is turned on in response to external or internal signals" (Maiuri *et al.*, 2007b). Programmed cell death has been shown to play an important role in development by regulating the formation and deletion of structures, controlling cell numbers and eliminating abnormal and damaged cells (Baehrecke, 2002). For years, the term apoptosis has been used interchangeably with programmed cell death (Chipuk and Green, 2005; Edinger and Thompson, 2004). However, accumulating evidence from more recent studies suggest that several forms of cell death are regulated or "programmed" (Edinger and Thompson, 2004; Kroemer *et al.*, 2009). Programmed cell death now encompasses processes such as autophagic cell death and programmed necrosis, in addition to apoptosis (Degterev and Yuan, 2008; Edinger and Thompson, 2004; Kroemer *et al.*, 2009; Lockshin and Zakeri, 2004; Okada and Mak, 2004).

Apoptotic Cell Death

Apoptosis is an evolutionarily conserved form of programmed cell death that results in the self-destruction of a cell. This physiological "cell suicide" program is essential for development and plays an important role in the regulation tissue homeostasis, as it allows for the elimination of damaged or redundant cells (Zimmermann

et al., 2001). Disruption of the apoptotic pathway can lead to the development of numerous pathological conditions. Increased apoptosis is associated with diseases such as neurodegenerative disorders, myelodysplastic syndromes, acquired immune deficiency syndrome and ischemic injury, while impaired apoptosis can lead to cancer and autoimmune disorders (Thompson, 1995).

Apoptotic cell death is defined by certain morphological and biochemical features that are distinct from other forms of cell death and include membrane blebbing, chromatin condensation, nuclear fragmentation, loss of adhesion and cell shrinkage and the externalization of phosphatidylserine. The resulting apoptotic bodies are removed by phagocytes, thereby avoiding the initiation of an immune response (Kerr *et al.*, 1972; Kroemer *et al.*, 2009). Apoptosis is a tightly regulated process that eventually leads to the activation of cysteinyl aspartate-specific proteases, known as caspases (Nicholson, 1999). The apoptotic caspases can be separated into two functional groups: the initiator caspases (caspase-2, -8, -9 and -10) and the effector caspases (caspase-3, -6 and -7). Cleavage of procaspase zymogens results in their activation allowing them in turn to cleave hundreds of downstream proteins (Luthi and Martin, 2007). The cleavage of these substrates results in the biochemical and morphological changes that are associated with apoptotic cell death (Taylor *et al.*, 2008). Apoptosis is primarily mediated through two pathways: the extrinsic pathway, which is activated by ligation of death receptors, and the intrinsic or stress-induced, mitochondrial pathway (Figure 3).

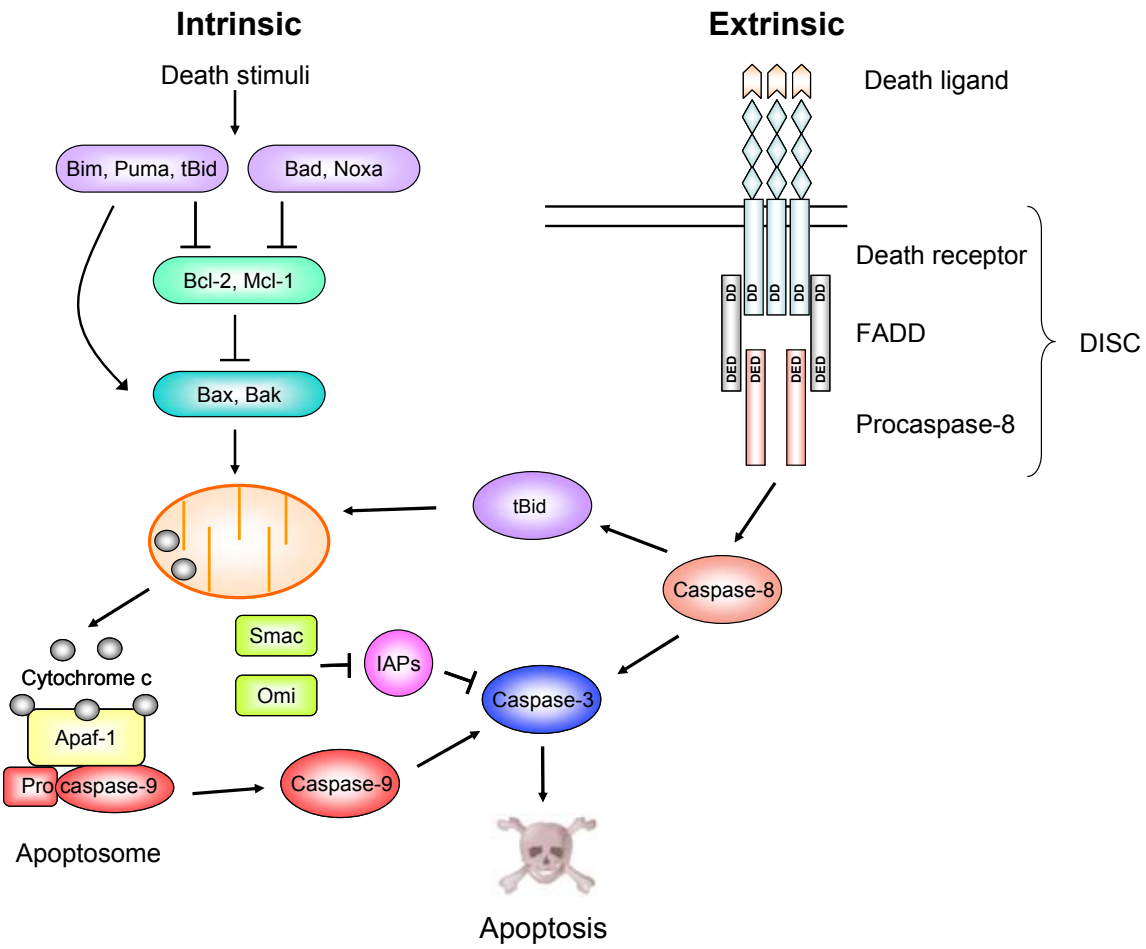

Figure 3. Overview of the extrinsic and intrinsic apoptotic pathways.

The extrinsic pathway

The extrinsic pathway is activated by the binding of a ligand to its cognate death receptor. The tumor necrosis factor receptor (TNFR) family consists of more than 20 proteins including TNFR, Fas (CD95 or Apo-1), the TNF-related apoptosis-inducing ligand (TRAIL), DR3, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), among others (Ashkenazi, 2002). Members of the TNFR family contain cysteine-rich extracellular domains and an intracellular death domain (DD). While the extracellular domain is important for receptor trimerization (which requires the pre-ligand assembly domain) and

provides ligand specificity, the intracellular death domain is critical for transmitting the death signal to downstream signaling pathways (Jin and El-Deiry, 2005). Ligand binding results in receptor activation, which recruits adaptor proteins, such as Fas-associated death domain (FADD) or TNF-associated death domain (TRADD). The DD of the receptor binds to the DD of FADD, which exposes the death effector domain (DED) of FADD. The DED of FADD in turn binds to the DED of procaspase-8 (and procaspase-10) to form the death-inducing signaling complex (DISC). Aggregation of procaspase-8 within the DISC leads to autoproteolysis resulting in the activation of caspase-8 and subsequent cleavage and activation of effector caspases to elicit the apoptotic response (Ashkenazi, 2002; Jin and El-Deiry, 2005; Zimmermann *et al.*, 2001). In addition to cleaving downstream effector caspases, caspase-8 has also been shown to cleave Bid; this truncated form of Bid (tBid) then translocates to the mitochondria to activate the intrinsic pathway and amplify the apoptotic response (Li *et al.*, 1998; Luo *et al.*, 1998).

The intrinsic pathway

The members of the Bcl-2 family play a central role in the regulation of apoptosis induced through the intrinsic pathway (Adams and Cory, 2007). The Bcl-2 family consists of both anti-apoptotic and pro-apoptotic members. Proteins, such as Bcl-2, Bcl-xL, Bcl-w and Mcl-1, are anti-apoptotic and thus prevent activation of apoptosis through the mitochondrial pathway. Most of these proteins share structural similarity within all four of the conserved Bcl-2 homology (BH) domains. The pro-apoptotic Bcl-2 family members can be subdivided into the multi-domain proteins, including Bax, Bak and Bok, and the BH3-only proteins, which include Bim, Puma, Bid, Bad, BNIP3 and Noxa,

among others. The multi-domain proteins are structurally similar to the anti-apoptotic proteins, but lack the BH4 domain, and are essential for inducing apoptosis through the mitochondrial pathway. The members of the BH3-only family lack structural similarity to other Bcl-2 family members, except within their BH3 domain. The BH3-only proteins act as sensors for damage signals and induce apoptosis by neutralizing the anti-apoptotic proteins or by directly activating the multi-domain, pro-apoptotic proteins of the Bcl-2 family to release apoptogenic factors from the mitochondria (Galonek and Hardwick, 2006; Strasser, 2005). Therefore, the members of the Bcl-2 family ultimately control the decision of whether a cell is to live or die, based on the relative ratio of anti- to pro-apoptotic proteins (Cory *et al.*, 2003; Oltvai and Korsmeyer, 1994).

As mentioned above, the BH3-only proteins act as sensors for various stress stimuli, including cytokine deprivation, hypoxia, oncogene activation and DNA damage, and are potent inducers of mitochondrial apoptosis (Willis and Adams, 2005). Therefore, these proteins must be regulated in order to prevent inappropriate activation of apoptosis and also to ensure that the apoptotic response is fully activated when necessary. It has been shown that BH3-only proteins can be regulated by a variety of mechanisms, including transcriptional upregulation, post-translational modification, sequestration to cytoskeletal components and proteasomal degradation (Puthalakath and Strasser, 2002). The regulation of the BH3-only proteins is tightly orchestrated and ensures that activation of Bax/Bak, and thus the intrinsic pathway, occurs only when appropriate.

Activation of the multi-domain proteins is required for mitochondrial outer membrane permeablization (MOMP), which results in the release of apoptogenic factors from the mitochondria (Green and Evan, 2002; Reed, 2003). MOMP results in the release

of cytochrome c into the cytosol where it binds to Apaf-1. Apaf-1 can then oligomerize and recruit procaspase-9 to form the apoptosome. The clustering of procaspase-9 in the apoptosome results in the cleavage of procaspase-9 to its active form and subsequent cleavage and activation of effector caspases. In addition to release of cytochrome c, MOMP also results in the release of other toxic proteins (Saelens *et al.*, 2004), such as Smac/Diablo and Omi/Htr2A, which antagonize the inhibitor of apoptosis (IAP) proteins. The binding of Smac/Diablo and Omi/HtrA2 to IAPs, such as XIAP and cIAP, abrogates their inhibitory effects on caspases (such as caspase-3 and -9), thus augmenting caspase activation and the apoptotic response. AIF and endonuclease G are also released from the mitochondria and aid in DNA fragmentation and chromatin condensation, respectively. Together, these mitochondrial proteins act in concert to ensure that the apoptotic response is effectively executed to completion.

DNA Damage-Induced Programmed Cell Death

The intrinsic apoptotic pathway can be triggered by various intracellular and extracellular stresses including those caused by exposure to DNA-damaging agents, such as the chemotherapeutic drugs, camptothecin and etoposide (Cory *et al.*, 2003; Reed, 2003). In addition to activating apoptosis, camptothecin and etoposide have also been shown to activate other forms of programmed cell death, such as autophagy (Abedin *et al.*, 2007; Shimizu *et al.*, 2004) (see below). Camptothecin and etoposide are two commonly used chemotherapeutic agents that target topoisomerases to induce DNA damage. Topoisomerases are a family of enzymes that regulate the topology of DNA by inducing transient single-strand (topoisomerase I enzymes) or double-strand

(topoisomerase II enzymes) breaks in DNA to resolve torsional strains (Wang, 2002). Campothecin and etoposide target topoisomerase I and II, respectively. The cytotoxicity of these drugs is a result of their ability to stabilize the covalent interaction between a topoisomerase and DNA, known as the cleavage complex (Montecucco and Biamonti, 2007; Pommier, 2006). Camptothecin and etoposide can both induce single- and double-strand breaks. When the replication machinery encounters the camptothecin-topoisomerase I-DNA complex, topoisomerase I releases the cleaved strand resulting in a single- and double-strand break in the DNA (Kaufmann, 1998; Pommier, 2006). The molar ratio between topoisomerase II and etoposide determines whether a single-strand or double-strand break will be induced (Montecucco and Biamonti, 2007). Thus, both of these agents are able to elicit a DNA damage response that activates both ATM- and ATR-mediated signaling to induce apoptosis via the intrinsic apoptotic pathway; however, the mechanisms by which these topoisomerase poisons induce autophagy are not well understood.

Autophagic Cell Death

As mentioned above, mechanisms of programmed cell death, such as autophagy, play an essential role during development. Autophagy is an evolutionarily conserved process for the bulk degradation of subcellular constituents (Levine and Klionsky, 2004; Yoshimori, 2004). Evidence is accumulating that suggest that autophagy is involved in a wide variety of physiological processes and conditions, including aging, neurodegenerative diseases, infectious diseases and cancer (Kundu and Thompson, 2008; Levine and Kroemer, 2008; Mizushima *et al.*, 2008) (for a detailed description of

autophagy see below). Although autophagy is generally thought to play a cytoprotective role, excess induction of autophagy could result in the digestion of essential proteins and organelles, thereby promoting the collapse of cellular functions and leading to cell death (Levine and Yuan, 2005; Tsujimoto and Shimizu, 2005). In addition, it has been suggested that while cells may preferentially die by activating the apoptotic machinery, cell death will be induced by any available route, including autophagy, if cells are exposed to harsh enough conditions (Lockshin and Zakeri, 2004).

Autophagic cell death is morphologically and biochemically different from apoptotic cell death. Characteristics of autophagic cell death include the absence of chromatin condensation, massive vacuolization of the cytoplasm, accumulation of double-membraned vacuoles and little or no uptake by phagocytic cells (Kroemer *et al.*, 2009). While the expression "autophagic cell death" implies that death is actually executed by autophagy, it is generally accepted that the term simply describes cell death with autophagy (Kroemer *et al.*, 2009; Levine and Yuan, 2005). Currently, the direct causative role of autophagy in cell death remains a key and controversial issue.

Crosstalk between Apoptotic and Autophagic Cell Death

Recent evidence suggests the functional relationship between the apoptotic and autophagic cell death pathways is quite complex (Maiuri *et al.*, 2007b). Depending on the stimulus or cellular context, the interplay between autophagy and apoptosis could occur through several mechanisms: autophagy could induce apoptosis or act in the later stages of apoptosis to aid in the elimination of apoptotic bodies, autophagy could delay or prevent apoptosis through the clean-up of damaged mitochondria or the two processes

may be mutually exclusive, acting as a back-up in case the other pathway fails (Scarlatti *et al.*, 2009). In addition, common cellular stresses can activate various signaling pathways that elicit both the induction of autophagy and apoptosis (Maiuri *et al.*, 2007b). As such, several proteins have been identified that regulate both the autophagic and apoptotic pathways. Beclin 1 was originally identified through a yeast two-hybrid screen aimed at identifying novel Bcl-2 and Bcl-xL binding partners (Liang *et al.*, 1999). Recently, the BH3 domain of Beclin 1, which is required for its interaction with anti-apoptotic Bcl-2 family members, has been described, thus Beclin 1 can be classified as a BH3-only protein (Maiuri *et al.*, 2007a). Furthermore, other BH3-only proteins, specifically Bad and BNIP3, as well as the pharmacological BH3 mimetic, ABT-737, were shown to disrupt the interaction between Beclin 1 and Bcl-2/Bcl-xL thereby stimulating autophagy (Maiuri *et al.*, 2007a). Atg5, a key component of the ubiquitin-like conjugation system in autophagy (see below), can be cleaved by calpain resulting in the N-terminal fragment of Atg5 translocating to the mitochondria where it triggers MOMP (Yousefi *et al.*, 2006). p53, a transcription factor that is known to regulate DNA damage-induced apoptosis, was recently shown to induce DRAM (damage-regulated autophagy modulator). Knockdown of DRAM not only abrogated the induction of autophagy, but also inhibited the initiation of apoptosis (Crighton *et al.*, 2006). Other proteins, such as DAPk (death-associated protein kinase), act as regulators of cell death and can mediate processes that are involved in both the apoptotic and autophagic pathways including membrane blebbing (a characteristic of apoptosis) and cytoplasmic vesicle formation (a characteristic of autophagy) (Inbal *et al.*, 2002). In addition, activation of the DNA damage-responsive transcription factor, E2F1 results in the upregulation of the

expression of autophagic proteins, including LC3, Atg1 and Atg5 (Polager *et al.*, 2008). Thus, numerous proteins play a role in both the apoptotic and autophagic pathways, which highlights the intricate crosstalk between these two pathways.

In this report, we demonstrate that inhibition of apoptosis, through treatment with Z-VAD-FMK, results in the induction of caspase-independent cell death in response to camptothecin treatment. Furthermore, disruption of the 9-1-1 complex, through loss of *Hus1*, enhanced DNA damage-induced autophagy. These results suggest that in response to genotoxic stresses, autophagy may be induced as a cell death mechanism. Surprisingly, inhibition of autophagy, through knockdown of Atg7 or Bif-1, enhanced cell death in response to camptothecin treatment, suggesting that the induction of autophagy observed in *Hus1*-deficient cells is actually a cytoprotective mechanism. It is of interest to determine whether the inhibition of caspase activity in these autophagy-deficient cells would suppress cell death in response to DNA damage.

Autophagy

Autophagy is a tightly regulated process for the bulk degradation of cytoplasmic constituents (Yoshimori, 2004). This evolutionarily conserved process plays a role in the maintenance of cellular homeostasis by recycling nutrients and removing damaged organelles, misfolded proteins and invasive microorgansisms. In addition, recent studies have shown that autophagy is involved in a variety of physiological processes, including development, differentiation, tissue remodeling and cell survival, whereas the deregulation of autophagy has been implicated in the pathogenesis of certain diseases, such as cancer, cardiomyopathy, muscular diseases and neurodegenerative disorders

(Kundu and Thompson, 2008; Levine and Klionsky, 2004; Levine and Kroemer, 2008). At least three distinct types of autophagy have been described; chaperone-mediated autophagy, microautophagy and macroautophagy (Cuervo, 2004; Klionsky *et al.*, 2007). The studies included in this report focus on marcroautophagy, hereafter referred to as autophagy.

Upon the initiation of autophagy, a portion of the cytoplasmic components is sequestered into cup-shaped membrane structures known as isolation membranes or phagophores (Levine and Klionsky, 2004). The isolation membrane elongates and the edges eventually fuse to form a double-membraned vesicle known as an autophagosome. The autophagosome matures when it fuses with endosomes and lysosomes to become an autolysosome, within which the enclosed components are degraded by lysosomal hydrolases. Autophagy occurs at basal levels in virtually all cells, but can be upregulated in response to environmental changes, such as starvation and exposure to DNA damaging agents (Crighton *et al.*, 2006; Levine and Kroemer, 2008; Polager *et al.*, 2008; Shimizu *et al.*, 2004).

Mechanisms that Regulate Autophagy

As mentioned above, the process of autophagy involves multiple steps, including initiation, cargo selection and packaging, vesicle nucleation, vesicle expansion and completion, retrieval, docking and fusion and lysosomal degradation of vesicles and their contents (Levine and Klionsky, 2004). The initiation of autophagy is mainly regulated by downstream signaling through the target of rapamycin (TOR) and the autophagy-related (Atg) proteins, including the phosphatidylinositol 3-kinase class III (PI3KC3)-Atg6

(yeast homologue of mammalian Beclin 1) complex (Levine and Klionsky, 2004; Xie and Klionsky, 2007) (Figure 4) .

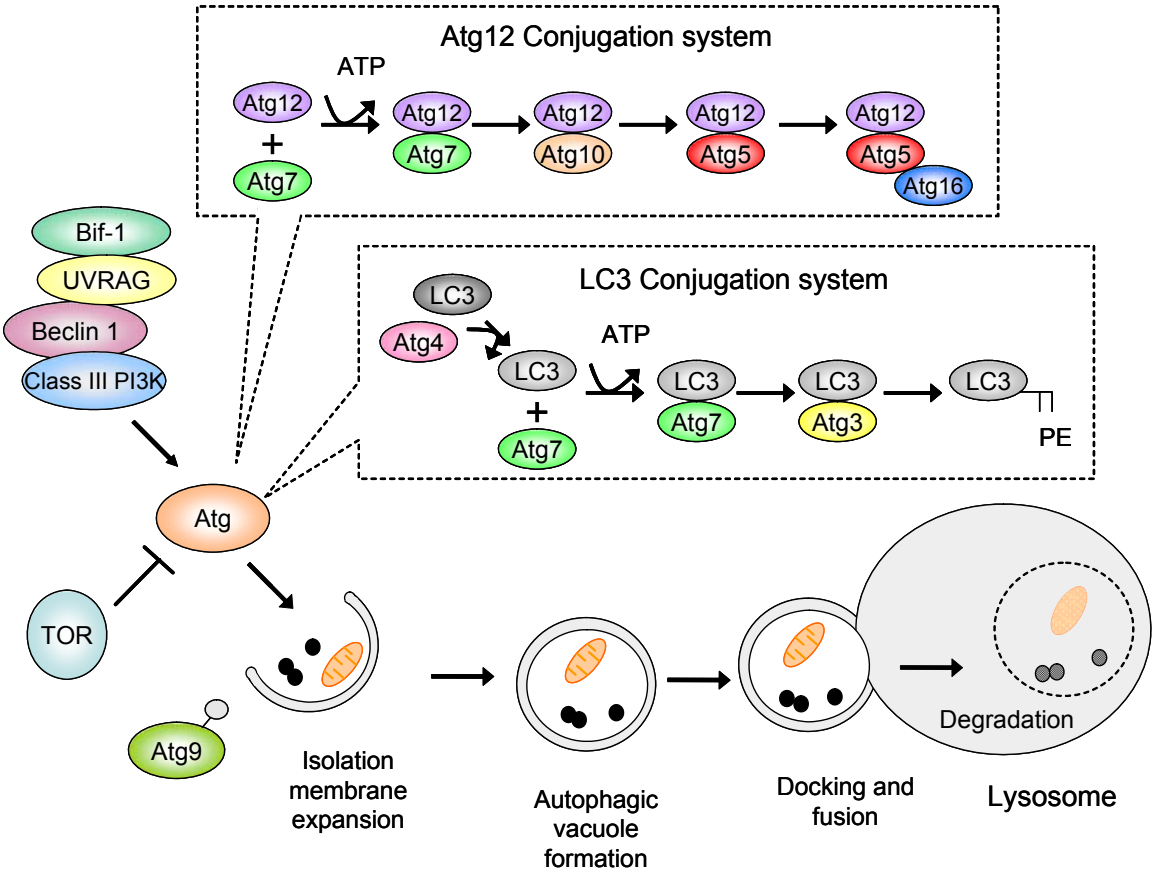

Figure 4. Model of the mechanisms that regulate autophagy.

ATG genes

Genetic screening in yeast has lead to the discovery of at least 30 autophagy-related genes, many of which have known orthologues in higher eukaryotes (Klionsky *et al.*, 2003; Xie and Klionsky, 2007). The corresponding gene products of a subset of the ATG genes form what has been described as the core autophagy machinery. This machinery can be subdivided into three main functional groups: (1) the Atg9 cycling

system, (2) the PI3KC3-Beclin 1 complex and (3) the ubiquitin-like (Ubl) protein system (Xie and Klionsky, 2007).

Atg9 is one of the most well characterized molecules for the investigation of the biogenesis of autophagosomes and is the only known transmembrane Atg protein (Noda *et al.*, 2000). In yeast, Atg9 has been shown to shuttle between perivacuolar sites, known as phagophore assembly sites (PAS), and peripheral sites which include mitochondria (Reggiori *et al.*, 2005). In contrast, the mammalian orthologue of Atg9 localizes to the *trans*-Golgi network and late endosomes, but not to mitochondria (Yamada *et al.*, 2005; Young *et al.*, 2006). These results suggest that Atg9-containing vesicles could be a source of membranes for the biogenesis or expansion of autophagosomes by delivering donor membranes to the PAS. While the efficient transport of Atg9 to the PAS requires the Atg9 transport proteins Atg23 and Atg27, the retrieval of Atg9 from the PAS depends on the Atg1-Atg2-Atg18 complex (Xie and Klionsky, 2007).

Vesicle nucleation is an early step in autophagosome formation and results in the formation of double-membraned structures. The class III phosphatidylinositol 3-kinase (PI3KC3), Vps34 (vacuolar protein sorting 34), interacts with Beclin 1 to form a complex that is required for vesicle nucleation (Volinia *et al.*, 1995). This complex interacts with p150 (the mammalian homologue of yeast Vps15), a protein kinase that is thought to activate Vps34 and mediate the binding of the complex to membranes. The Vps34 complex most likely functions at the PAS by recruiting PtdIns(3)P-binding proteins. The process of autophagosome formation can be blocked by treatment with PI3K inhibitors, such as 3-MA and wortmannin (Mizushima *et al.*, 2001), suggesting that Vps34 is an essential regulator of autophagosome formation.

In addition to the Vsp34-Beclin 1 complex and Atg9, two ubiquitin-like conjugation systems, Atg12-Atg5 and Atg8-phosphatidylethanolamine (PE), also play a role in the regulation of autophagic vesicle formation (Ohsumi, 2001). The first system consists of Atg12 (the ubiquitin-like protein), Atg7 (similar to an E1 ubiquitin-activating enzyme) and Atg10 (similar to an E2 ubiquitin-conjugating enzyme), which are responsible for transferring Atg12 to Atg5 (Mizushima *et al.*, 1998; Shintani *et al.*, 1999; Tanida *et al.*, 1999). This complex then recruits Atg16, which can homooligomerize to mediate the formation of large protein complexes containing Atg12, Atg5 and Atg16 (Mizushima *et al.*, 2003; Mizushima *et al.*, 1999). The second system is composed of Atg8 (the ubiquitin-like protein), Atg4 (which cleaves Atg8 exposing a glycine residue that is then accessible for activation by Atg7), Atg7 (the E1-like protein) and Atg3 (the E2-like enzyme), which are required for transferring Atg8 to PE (Ichimura *et al.*, 2000; Kirisako *et al.*, 2000). Unlike the Atg12-Atg5 system, Atg8 conjugation to PE is reversible through Atg4-mediated cleavage of Atg8 (Kirisako *et al.*, 2000). It has been shown that the Atg12-Atg5 conjugate is required for the stability and proper localization of Atg8 (Mizushima *et al.*, 2001; Suzuki *et al.*, 2001). While the Atg12-Atg5 complex was found to localize to forming autophagosomes and dissociate before vesicle completion, the Atg8-PE conjugate is located on autophagosomes during their formation and after completion and is eventually degraded within the autolysosome (Kabeya *et al.*, 2000; Kirisako *et al.*, 1999; Mizushima *et al.*, 2003; Mizushima *et al.*, 2001). Accordingly, Atg8 is one of the best markers of autophagosomes and has been used extensively as an indicator for the initiation of the autophagic pathway and for autophagosome formation. The mammalian homologue of Atg8, the microtubule-

associated protein light chain 3 (LC3), is modified in a similar mechanism to that of Atg8 (Ichimura *et al.*, 2000; Kabeya *et al.*, 2000). Upon the induction of autophagy LC3 is cleaved by Atg4 to produce a cytosolic form known as LC3-I. LC3-I is then conjugated to PE to form LC3-II, which is recruited to the autophagosomal membrane. Thus examining the modification and localization of LC3 are well described methods to monitor the induction of autophagy (Mizushima and Yoshimori, 2007; Tasdemir *et al.*, 2008).

Bif-1

Bif-1, also known as Endophilin B1 or SH3GLB1, was originally discovered as a Bax-interacting protein (Cuddeback *et al.*, 2001; Pierrat *et al.*, 2001). In addition to regulating apoptosis through its interaction with Bax, Bif-1 has also been found to play an integral role in the regulation of autophagy. Bif-1 interacts with Beclin 1 through UVRAG (ultraviolet radiation resistance-associated gene) to promote the activation of PI3KC3/Vps34 and the formation of autophagosomes (Takahashi *et al.*, 2007). Furthermore, Bif-1 has an intrinsic ability to induce membrane curvature (Farsad *et al.*, 2001), suggesting that Bif-1 may collaborate with the Beclin 1-UVRAG-PI3KC3 complex to provide the driving force for the curvature of the isolation membrane. In response to nutrient starvation, Bif-1 accumulates in foci in the cytosol where it co-localizes with LC3 and Atg5 (Takahashi *et al.*, 2007). As mentioned above, LC3 is a well-known marker for autophagosomes and Atg5 has been shown to locate to phagophores throughout the elongation step of autophagosome formation, but is removed before the completion/sealing of the autophagosome (Kabeya *et al.*, 2000; Klionsky *et*

al., 2008; Mizushima *et al.*, 2001). Taken together, these results implicate Bif-1 in the regulation of the early stages of autophagosome formation and suggest that Bif-1 may play a role in the biogenesis or expansion of phagophores. Indeed, Bif-1 was found to localize to Atg9-positive vesicles (Takahashi *et al.*, 2008). As the formation and trafficking of Atg9-positive vesicles is essential for the biogenesis and expansion of autophagosomal membranes during the induction of autophagy (Noda *et al.*, 2000; Young *et al.*, 2006), these results provide further evidence that Bif-1 is involved in these processes. Further studies are required to determine the precise molecular mechanisms by which Bif-1 regulates autophagosome formation.

Deregulation of Autophagy in Cancer

Accumulating evidence suggests that autophagy may both enhance and inhibit tumor development and progression (Mizushima, 2005). In the early stages of tumor development, tumors are limited in growth by a lack of blood vessels, which provide necessary oxygen and nutrients. In this setting, induction of autophagy would provide cells with nutrients in a starvation setting and thus be cytoprotective (Degenhardt *et al.*, 2006). Alternatively, autophagy could protect cells from undergoing apoptotic cell death induced by various chemotherapeutic agents (Abedin *et al.*, 2007; Amaravadi *et al.*, 2007; Carew *et al.*, 2007; Paglin *et al.*, 2001). Conversely, autophagy may hinder tumor progression by removing damaged or malfunctioning organelles, such as mitochondria, thereby limiting exposure to genotoxic substances, such as reactive oxygen species, which would result in enhanced genetic mutations and favor tumorigenesis (Edinger and Thompson, 2003). Indeed, the concept that autophagy may be beneficial during the early

stages of tumorigenesis and detrimental during later stages is consistent with the findings that the rate of autophagy is decreased in malignant pancreatic adenocarcinoma cells as compared to premalignant cells (Toth *et al.*, 2002).

Recent studies have shown that genetic deregulation of autophagy regulatory proteins contributes to tumorigenesis. While monoallelic deletions of Beclin 1 are frequently detected in breast, ovarian and prostate cancers (Aita *et al.*, 1999), monoallelic mutations in UVRAG occur at a high frequency in colon cancer cells (Ionov *et al.*, 2004). Furthermore, homozygous deletion of Bif-1 has been confirmed in mantle cell lymphomas (Balakrishnan *et al.*, 2006) and decreased Bif-1 expression has been described in gastric carcinomas, colorectal adenocarcinomas, urinary bladder and gallbladder cancers (Coppola *et al.*, 2008a; Kim *et al.*, 2008; Lee *et al.*, 2006). A recent study demonstrated that Bif-1 expression is decreased in a significant portion of prostate cancers, although the majority of prostate cancer samples examined had high levels of Bif-1 (Coppola *et al.*, 2008b). In addition, *Beclin* $1^{+/-}$ and *Bif-*$1^{-/-}$ mice both have a significantly enhanced occurrence of spontaneous tumor development (Qu *et al.*, 2003; Takahashi *et al.*, 2007; Yue *et al.*, 2003) and ectopic expression of UVRAG suppresses tumorigenesis in nude mice (Liang *et al.*, 2006). Moreover, it was shown that Atg4C and Atg5 also possess tumor suppressive capabilities (Marino *et al.*, 2007; Yousefi *et al.*, 2006). In addition to the involvement of autophagy genes in the regulation of tumorigenesis, it has also been shown that tumor suppressor proteins, such as PTEN, p53 and DAPk, activate autophagy while oncogenes, such as Akt and Bcl-2, suppress autophagy (Botti *et al.*, 2006). These studies highlight the strong correlation that exists between proteins that regulate the induction of autophagy and tumor suppression and

those that inhibit autophagy and oncogenesis (Levine and Kroemer, 2008). While great strides have been made to determine the connection between the deregulation of autophagy and cancer, further studies are needed to determine the precise mechanisms by which autophagy functions in tumorigenesis and tumor suppression. Once the association between autophagy and cancer has been determined, it will allow for the manipulation of autophagy to enhance therapeutic treatments for cancer.

Crosstalk between the Autophagic and Endocytic Pathways

The autophagic and endocytic pathways represent branches of the lysosomal degradation system; these pathways are responsible for the degradation of cytoplasmic constituents and exogenous substances/macromolecules, respectively. It has been shown in yeast that two distinct complexes form to regulate these processes; complex I (Vps15, Vps34, Atg14 and Atg6) and complex II (Vps15, Vps34, Vps38 and Atg6) that are involved in autophagy and vacuolar protein sorting/endocytosis, respectively (Kihara *et al.*, 2001). Complex I and II share three common members that are evolutionarily conserved: the adaptor protein, Vps15 (p150 in mammals), a class III PI3K, Vps34, and Atg6 (the yeast orthologue of mammalian Beclin 1). Certain members of these complexes have been shown to play a role in both the autophagic and endocytic pathways, while others have been found to only be involved in the regulation of one process, but not the other.

Mammalian Vps34 has been implicated not only in the regulation of autophagy, but also in endocytic trafficking and sorting of cell-surface receptors and the formation of internal vesicles in multivesicular endosomes (Backer, 2008). While Vps34 is required

for the production of PtdIns(3)P for membrane trafficking, particularly at late endosomes/multivesicular bodies, it is dispensable for the internalization of cell surface receptors and the uptake of fluid phase markers (Johnson *et al.*, 2006). Another member of the mammalian Vps34 complex, UVRAG, has been shown to interact with the class C Vps complex, a key component of the endosomal fusion machinery (Peterson and Emr, 2001), to promote autophagosome maturation by enhancing fusion with late endosomes/lysosomes (Liang *et al.*, 2008). Similar to Vps34, UVRAG is not involved in the internalization of endocytic cargo, however it does accelerate intracellular trafficking and degradation (Liang *et al.*, 2008). In addition, the role of UVRAG in the class C Vps complex, which regulates endocytic vesicle trafficking, was shown to be distinct from its role in the Vps34-Beclin 1-Bif-1 complex, which induces autophagy by regulating autophagosome formation (Liang *et al.*, 2008). Whereas Vps34 and UVRAG both play a role in the autophagic and endocytic pathways, as mentioned above, Beclin 1 has only been shown to be required for autophagy and is expendable for endocytosis and vesicle trafficking (Zeng *et al.*, 2006).

In addition to various proteins being involved in the regulation of both the autophagic and endocytic pathways, these pathways have been shown to converge at the prelysosomal and lysosomal level for degradation (Gordon *et al.*, 1992; Gordon and Seglen, 1988) (Figure 5). Indeed, it has been shown that autophagic vacuoles can directly fuse with vesicular and multivesicular bodies (MVB) (before fusing with lysosomes) to form what are known as amphisomes (Fader and Colombo, 2008; Liou *et al.*, 1997). While much has been discovered about the interplay between the autophagic and

endocytic pathways, the molecular mechanisms that regulate vesicular trafficking and the convergence between these two pathways are not fully understood.

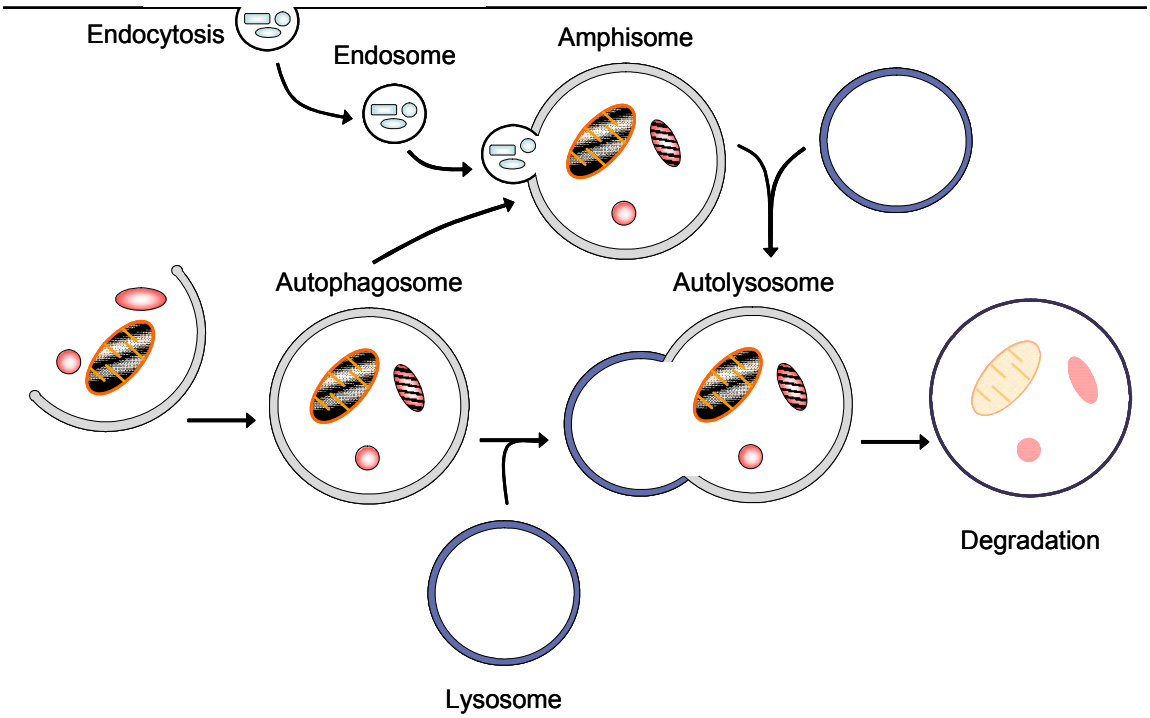

Figure 5. Convergence of the autophagic and endocytic pathways for lysosomal degradation.

Endocytosis and Vesicle Trafficking

The endocytic pathway functions in cellular homeostasis through the regulation of internalization, transport, sorting and degradation of macromolecules (Fader and Colombo, 2008). In addition to a role for endocytosis in vesicle trafficking and degradation, evidence is accumulating that suggest that endocytosed receptors may be able to activate specialized signaling complexes that are not assembled at the cell surface

(Sorkin and Von Zastrow, 2002). Indeed, adaptor and effector proteins have been found to localize to endosomes containing epidermal growth factor receptors (EGFR) (Di Guglielmo *et al.*, 1994; Wiley, 2003) and EGFR can maintain signaling even after internalization and fusion with endosomes (Miaczynska *et al.*, 2004). Therefore, it is possible that endocytic trafficking could regulate signaling pathways that are distinct from those initiated at the cell surface (Vieira *et al.*, 1996). Although evidence suggests that signaling through endocytosis may activate specialized signaling pathways, receptor endocytosis is generally considered to downregulate growth factor signaling through lysosomal degradation (Citri and Yarden, 2006).

EGFR as a Model for Endocytic Trafficking and Degradation

The EGFR was one of the first growth factor receptors that was observed to be internalized following ligand binding (Gorden *et al.*, 1978; Haigler *et al.*, 1979). Based on extensive studies, which have investigated the mechanisms behind the internalization, sorting and degradation of this receptor, the EGFR is now a prototype for endocytic vesicle trafficking (Citri and Yarden, 2006; Sorkin and Von Zastrow, 2002). Internalization and sorting of the EGFR leads to the removal of activated receptors from the cell surface. Receptors can then be recycled back to the membrane; alternatively, the receptors can be degraded, thereby downregulating EGFR-mediated proliferation and survival signaling (Katzmann *et al.*, 2002) (Figure 6).

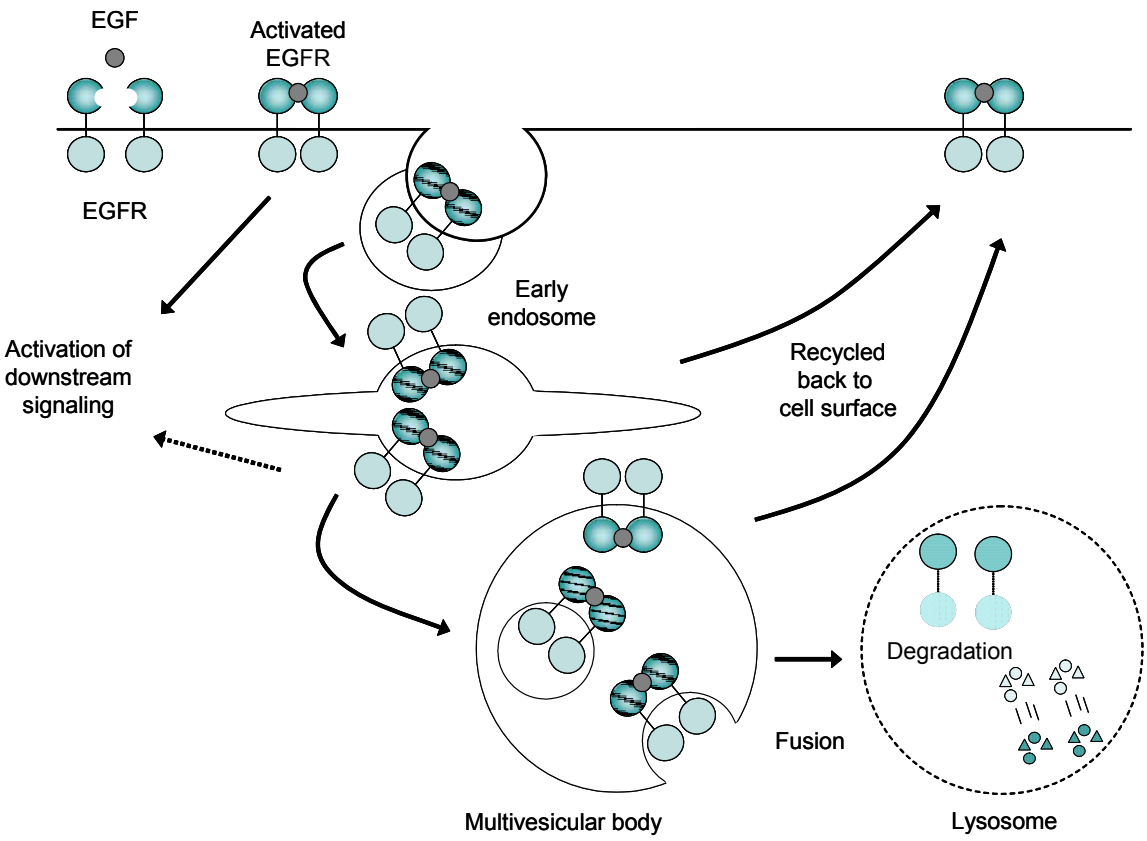

Figure 6. Endcoytic trafficking of the EGFR.

Ligand binding results in EGFR dimerization and phosphorylation, which provides docking sites that are required for the recruitment of adaptor and effector proteins that are involved in the regulation of the endocytic pathway (Grandal and Madshus, 2008). The E3 ubiquitin ligase, Cbl, is recruited to phosphorylated tyrosine 1045 of EGFR (Levkowitz *et al.*, 1999). Cbl-mediated EGFR ubiquitination results in further recruitment of other signaling molecules and ubiquitin-binding proteins, including Eps15 and CIN85 (Kirisits *et al.*, 2007), which act as a scaffold for endophilins. Together these proteins are responsible for inducing the curvature of the plasma membrane and thus, regulate the formation of clathrin coated pits (Soubeyran *et al.*, 2002). Dynamin mediates vesicle fission from the plasma membrane, which releases the vesicle into the

cytoplasm (McNiven *et al.*, 2000). The clathrin then dissociates from the vesicle, which then fuses with a tubular-vesicular network located at the periphery of the cell. This fusion results in the delivery of the ligand-bound receptors to early endosomes (Katzmann *et al.*, 2002; Sorkin and Von Zastrow, 2002). The EGFR is then sorted through various intracellular trafficking events, which depend on Cbl-mediated ubiquitin signals (Levkowitz *et al.*, 1998). PI3KC3 is responsible for membrane invagination and thus the formation of internal vesicles to form MVBs (Futter *et al.*, 2001). The ligand-bound receptors then accumulate in the limiting (outer) and lumenal membranes of MVBs. The limiting membrane then fuses with lysosomal membranes resulting in the delivery of lumenal contents to the hydrolytic interior of the lysosome where the contents are then degraded (Futter *et al.*, 1996). In contrast, proteins that remain on the limiting membrane of MVBs avoid degradation and are subsequently recycled back to the plasma membrane or transported to other sites within the cell (Katzmann *et al.*, 2002). Thus, the integrity of the endocytic pathway can be monitored by tracking the fate of activated EGFR.

In this study, we have found that knockdown of Bif-1 does not affect the uptake of a fluid phase marker, horse radish peroxidase, or the internalization of epidermal growth factor (EGF). Interestingly, knockdown of Bif-1 accelerated the co-localization of internalized EGF with late endosomes/lysosomes. Furthermore, EGFR degradation was enhanced by loss of Bif-1. These results indicate a novel role for Bif-1 in vesicle trafficking within the endocytic pathway. Further studies are needed to determine the molecular mechanisms by which loss of Bif-1 accelerates vesicle trafficking and receptor degradation.

Deregulation of Endocytosis in Cancer

Deregulation of EGFR signaling occurs in nearly 50% of all human tumors (Rodemann *et al.*, 2007). In addition to overexpression and gain-of-function mutations, the evasion of downregulation by endocytic/lysosomal degradation is another mechanism that enhances EGFR signaling and drives tumorigenesis (Grandal and Madshus, 2008; Kirisits *et al.*, 2007; Yarden and Sliwkowski, 2001). Defective downregulation of EGFR can prolong signaling and thus positively regulate survival and proliferation. Indeed, it has been shown that preventing the downregulation of EGFR facilitates cell transformation (Levkowitz *et al.*, 1998). Furthermore, EGFR mutations that impair ubiquitination and thereby stabilize the EGFR have been described in cancer patients. These mutations have been shown to protect cells from apoptosis and promote growth (Grandal and Madshus, 2008). Therefore, elucidating the mechanisms that regulate the endocytic trafficking and degradation of EGFR could lead to more lucrative therapeutic approaches for the treatment of cancer.

Summary

As I mentioned above, a functional 9-1-1 complex is required for the maintenance of genomic stability and thus prevents the accumulation of mutations that could lead to cancer. Additionally, cells with a disrupted 9-1-1 complex are hypersensitive to genotoxic stress-induced apoptosis. However, the molecular mechanism by which loss of a functional 9-1-1 complex activates the apoptotic pathway had yet to be determined. Our data indicate that loss of *Hus1* sensitizes cells to etoposide-induced apoptosis through the upregulation of Bim and Puma. Furthermore, loss of *Hus1* enhances the interaction of

Rad9 with Bcl-2 to potentiate the apoptotic response. Interestingly, our data suggest that disruption the 9-1-1 complex not only sensitizes cells to caspase-dependent cell death, but also to caspase-independent cell death in response to DNA damage. Moreover, the results presented in this study indicate that loss of *Hus1* enhances DNA damage-induced autophagy. Since excessive induction of autophagy could result in cell death, autophagy may be the mechanism underlying caspase-independent cell death in *Hus1*-deficient cells. However, inhibition of autophagy, by knockdown of Atg7 or Bif-1, enhanced the cytotoxicity of camptothecin, suggesting that autophagy is being induced as a cytoprotective mechanism rather than a pro-death mechanism in response to DNA damage. Finally, our results describe a novel role for Bif-1 in endocytic vesicle trafficking and receptor degradation. It is therefore likely that Bif-1 promotes survival not only through its regulation of autophagy, but also by affecting EGFR signaling through its regulation of the endocytic pathway. Importantly, the results described here better define the mechanisms that are regulated by the 9-1-1 complex and Bif-1 that affect sensitivity to DNA damage.

Chapter Two: Loss of *Hus1* Sensitizes Cells to Etoposide-Induced Apoptosis by Regulating BH3-Only Proteins[1]

Abstract

The Rad9-Rad1-Hus1 (9-1-1) cell cycle checkpoint complex plays an integral role in the DNA damage response. Cells with a defective 9-1-1 complex have been shown to be sensitive to apoptosis induced by certain types of genotoxic stress. However, the mechanism linking the loss of a functional 9-1-1 complex to the cell death machinery has yet to be determined. Here, we report that etoposide treatment dramatically upregulates the expression of the BH3-only proteins, Bim and Puma, in *Hus1*-deficient cells. Inhibition of either Bim or Puma expression in *Hus1*-knockout cells confers significant resistance to etoposide-induced apoptosis, while knockdown of both proteins results in further resistance, suggesting that Bim and Puma cooperate in sensitizing *Hus1*-deficient cells to etoposide treatment. Moreover, we found that Rad9 collaborates with Bim and Puma to sensitize *Hus1*-deficient cells to etoposide-induced apoptosis. In response to DNA damage, Rad9 localizes to chromatin in *Hus1*-wild-type cells, whereas in *Hus1*-deficient cells Rad9 is predominantly located in the cytoplasm where it binds to Bcl-2. Taken together, these results suggest that loss of *Hus1* sensitizes cells to etoposide-induced apoptosis, not only by inducing Bim and Puma expression, but also by releasing Rad9 into the cytosol to augment mitochondrial apoptosis.

[1] Meyerkord CL, Takahashi Y, Araya R, Takada N, Weiss RS, Wang HG (2008). Loss of Hus1 sensitizes cells to etoposide-induced apoptosis by regulating BH3-only proteins. *Oncogene* **27:** 7248-59.

Results

Loss of Hus1 Sensitizes Cells to Etoposide-Induced Apoptosis

Knockout of *Hus1* results in cell cycle checkpoint defects and enhanced cell death in response to DNA damage induced by hydroxyurea (HU) and ultraviolet (UV) radiation (Weiss *et al.*, 2000; Weiss *et al.*, 2003; Weiss *et al.*, 2002). In this study, we examine the sensitivity of *Hus1*-deficient cells to etoposide, one of the most potent drugs used for cancer therapy (Montecucco and Biamonti, 2007). In order to determine whether loss of *Hus1* would sensitize cells to etoposide-induced cell death, $Hus1^{+/+}p21^{-/-}$ and $Hus1^{-/-}p21^{-/-}$ MEFs were treated with increasing doses of etoposide for 24 h. Measurement of cell death by trypan blue exclusion assay revealed that knockout of *Hus1* greatly enhanced the dose-dependent susceptibility of MEFs to etoposide (Figure 7a). Consistently, the hypersensitivity of *Hus1*-deficient cells to etoposide-induced cell death also occurred in a time-dependent manner (Figure 7b).

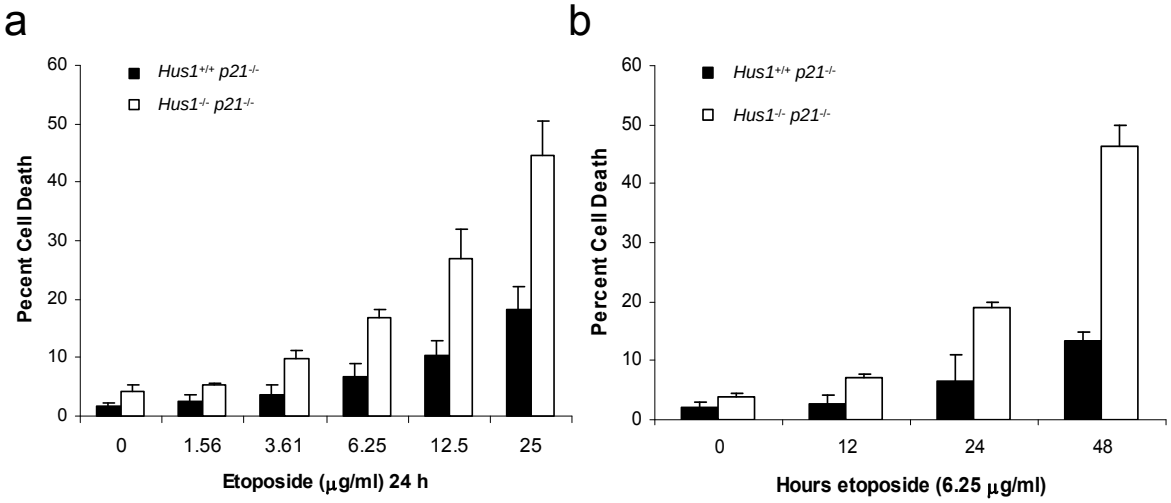

Figure 7. Loss of *Hus1* sensitizes cells to etoposide-induced cell death. (**a**) $Hus1^{+/+}$ $p21^{-/-}$ and $Hus1^{-/-}p21^{-/-}$ MEFs were treated with increasing doses of etoposide for 24 h. Viability was determined by trypan blue exclusion assay (mean ± s.d.; n=3). (**b**) $Hus1^{+/+}p21^{-/-}$ and $Hus1^{-/-}p21^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for varying time points and subjected to trypan blue exclusion assay (mean ± s.d.; n=3).

To determine whether the increase in cell death observed in *Hus1*-deficient cells is due to the enhanced induction of apoptosis, activation of caspase-3, as well as cleavage of its downstream substrate, poly(ADP-ribose) polymerase (PARP), were examined by immunoblot analysis. *Hus1*$^{-/-}$*p21*$^{-/-}$ cells exhibited a robust induction of caspase-3 processing, which correlated with PARP cleavage, upon 24 h treatment with 12.5 μg/ml etoposide that was further enhanced at a higher dose (Figure 8). In contrast, *Hus1*$^{+/+}$*p21*$^{-/-}$ cells showed only slight activation of caspase-3 and minimal PARP cleavage even upon treatment with the highest dose of etoposide (Figure 8). Induction of apoptosis in response to etoposide treatment was further analyzed by examination of nuclear morphology for chromatin condensation and nuclear fragmentation. As shown in Figures 9a and b, *Hus1*-deficient cells were almost three times more sensitive to etoposide-induced apoptosis. Taken together, these results suggest that MEFs that lack *Hus1* are not only sensitive to hydroxyurea and UV radiation, as previously described, but that these cells are also sensitive to DNA damage induced by topoisomerase II poisons, such as the chemotherapeutic drug, etoposide.

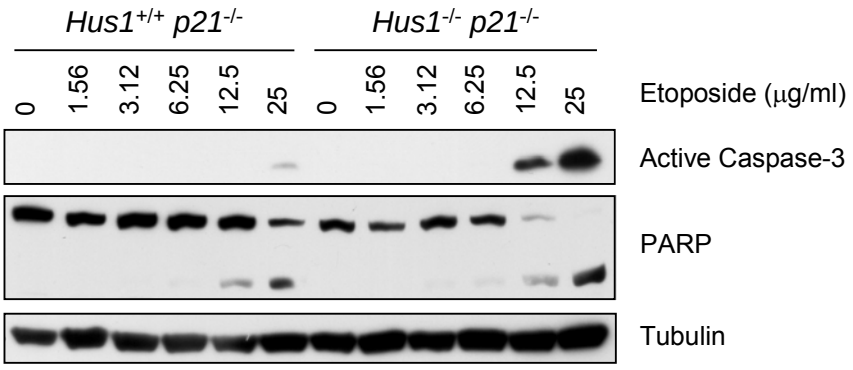

Figure 8. Loss of *Hus1* enhances the cleavage of caspase-3 and PARP. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEF cells were treated with varying doses of etoposide for 24 h. Total cell lysate was normalized for protein content and subjected to SDS-PAGE/immunoblot analysis.

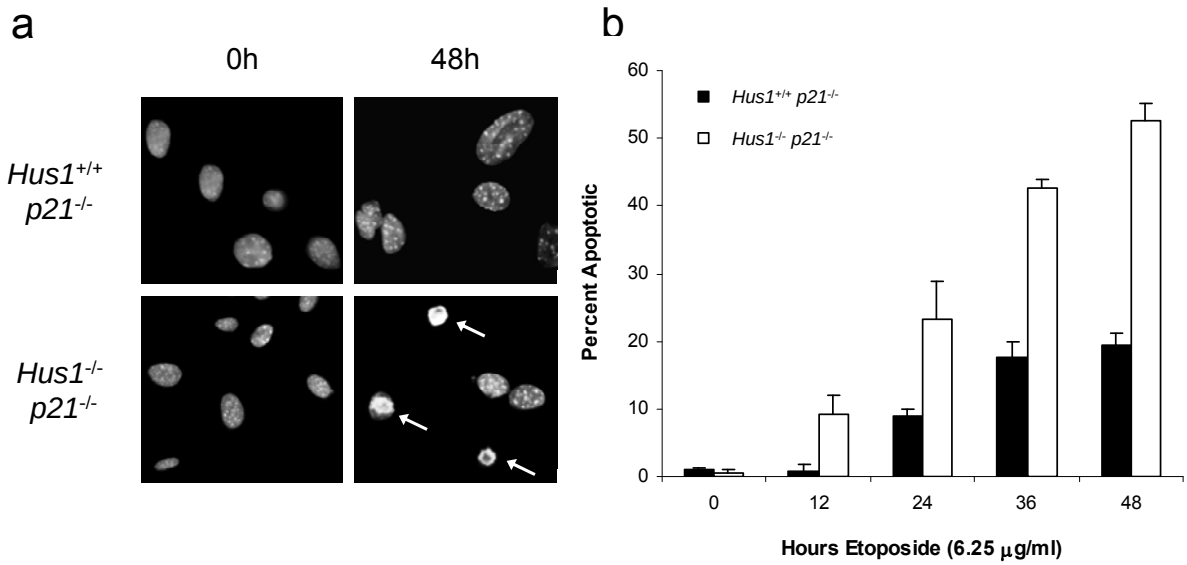

Figure 9. Loss of *Hus1* sensitizes cells to etoposide-induced apoptosis. (a) *Hus1*$^{+/+}$ *p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for 0 or 48 h. Apoptosis was determined by examination of nuclear morphology. Arrows indicate apoptotic nuclei. (b) *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for the times indicated. The percent of apoptotic cells was quantified based on nuclear morphology (mean ± s.d.; n=3).

Loss of Hus1 Enhances Bim and Puma Expression at Both the Protein and mRNA Level in Response to DNA Damage

Since our results indicate that loss of *Hus1* sensitizes cells to etoposide-induced apoptosis, the expression levels of members of the Bcl-2 family were examined (Figure 10). In response to etoposide treatment, the expression levels of anti-apoptotic Bcl-2-like proteins and pro-apoptotic multi-domain proteins remained relatively stable in both *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs. Notably, the basal level of Bcl-xL was higher in *Hus1*$^{-/-}$*p21*$^{-/-}$ cells compared to *Hus1*$^{+/+}$*p21*$^{-/-}$ cells, presumably to neutralize elevated Bax expression in cells lacking *Hus1* (Weiss *et al.*, 2000). Interestingly, the expression of the BH3-only proteins, Bim and Puma, was induced following etoposide treatment. Whereas

the expression of these proteins was only slightly induced and peaked at 24 h in *Hus1*-wild-type cells, the upregulation of all three isoforms of Bim, as well as Puma, was much more dramatic and persisted to later time points in *Hus1*-deficient cells. Similar results were observed after treatment with other DNA damaging agents including camptothecin, an inhibitor of topoisomerase I, and hydroxyurea, an inhibitor of DNA replication (Figures 11 and 12, respectively).

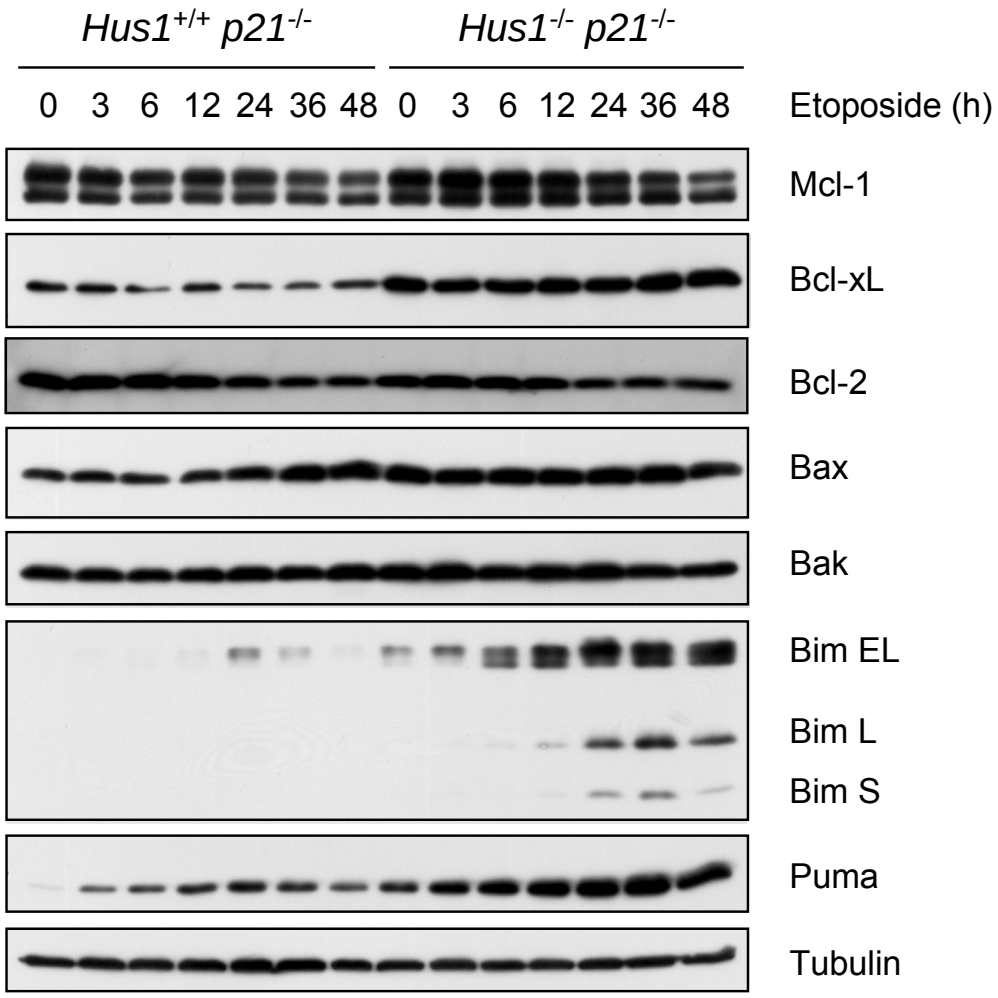

Figure 10. Expression of Bcl-2 family members in response to etoposide treatment. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for the indicated time points. Total cell lysate was prepared and analyzed by SDS-PAGE/immunoblot using the indicated antibodies.

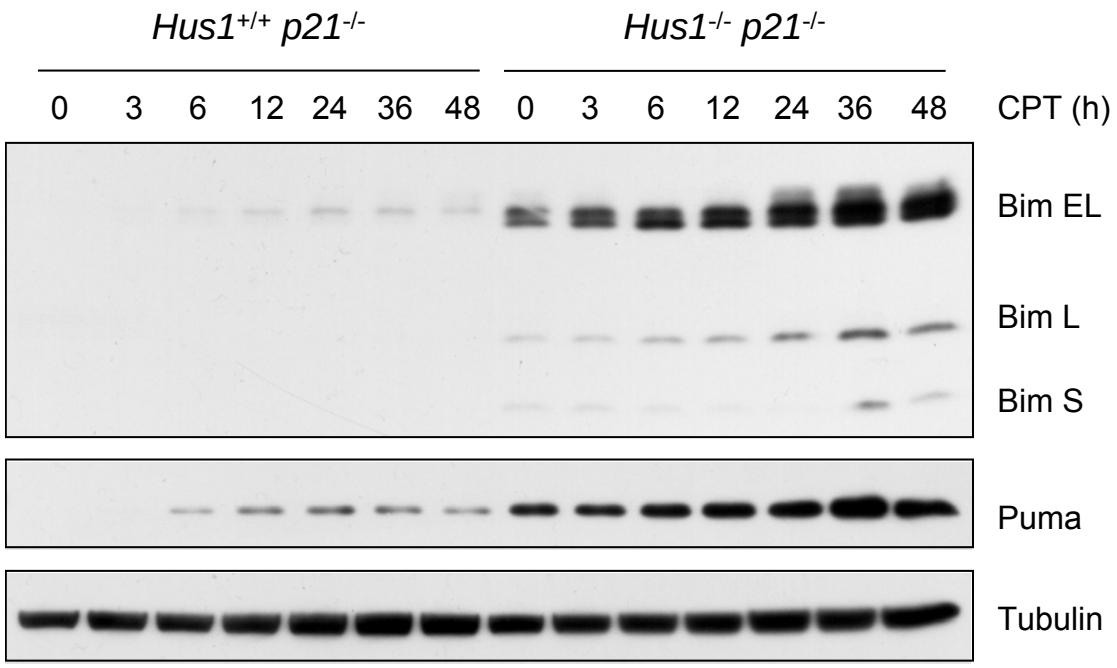

Figure 11. Loss of *Hus1* results in upregulation of Bim and Puma expression in response to camptothecin treatment. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 500 nM camptothecin (CPT) for the times indicated. Total cell lysate was prepared and analyzed by SDS-PAGE/immunoblot using the indicated antibodies.

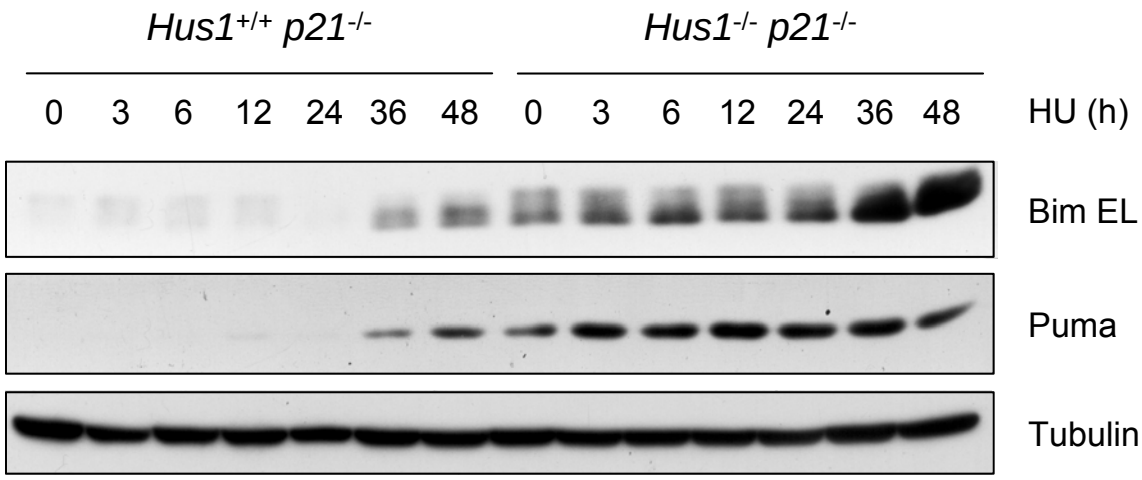

Figure 12. Loss of *Hus1* results in upregulation of Bim and Puma expression in response to hydroxyurea treatment. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 50 μM hydroxyurea (HU) for the times indicated. Total cell lysate was prepared and analyzed by SDS-PAGE/immunoblot using the indicated antibodies.

To confirm that the etoposide-induced upregulation of Bim and Puma expression is a direct result of loss of *Hus1*, we examined whether restoration of Hus1 expression would suppress the induction of these BH3-only proteins in response to etoposide treatment. To this end, *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs that were infected with retrovirus to express either Hus1 (*Hus1*$^{-/-}$*p21*$^{-/-}$ *Hus1*) or control GFP (*Hus1*$^{-/-}$*p21*$^{-/-}$ *GFP*) (Weiss *et al.*, 2002) were treated with etoposide for varying time points and the expression of Bim and Puma was examined. Expression of Hus1, but not control GFP, significantly reduced etoposide-induced expression of Bim and Puma in *Hus1*-deficient MEFs (Figure 13). These results indicate that upregulation of Bim and Puma in response to etoposide-induced DNA damage is indeed a direct result of loss of *Hus1*.

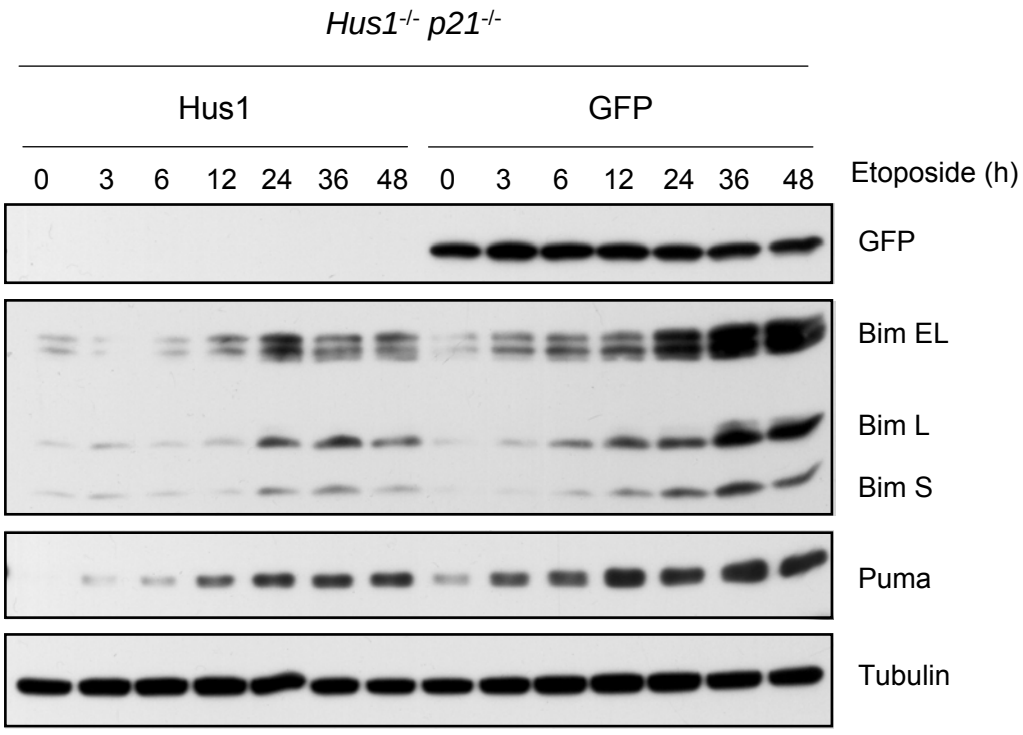

Figure 13. Restoration of *Hus1* suppresses the upregulation of Bim and Puma in response to DNA damage. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing Hus1 or GFP were treated with 6.25 μg/ml etoposide for varying time points. Total cell lysate was prepared and analyzed by SDS-PAGE/immunoblot using the indicated antibodies.

The activity of BH3-only proteins can be regulated by various mechanisms including transcriptional upregulation, post-translational modification, proteasomal degradation and sequestration to cytoskeletal components (Puthalakath and Strasser, 2002; Willis and Adams, 2005). To examine whether the induction of Bim and Puma expression observed in *Hus1*-deficient cells is regulated at the transcriptional level after exposure to etoposide, *Hus1*$^{-/-}$*p21*$^{-/-}$ cells were treated with etoposide in the presence of a transcriptional inhibitor, actinomycin D, a translational inhibitor, cycloheximide, or control DMSO. As shown in Figure 14, treatment with either actinomycin D or cycloheximide abrogated the expression of Bim, as well as Puma, even in the presence of etoposide. In contrast, the expression of these BH3-only proteins was significantly induced in response to etoposide treatment in the control DMSO-treated cells (Figure 14), indicating that upregulation of both Bim and Puma, in response to etoposide-induced DNA damage, occurs at the transcriptional level. Indeed, semi-quantitative RT-PCR analyses revealed that the expression of Bim and Puma mRNAs are increased in response to etoposide treatment (Figures 15-18). The levels of Bim and Puma mRNAs continued to increase until approximately 24 to 36 h and then decreased at later time points, presumably due to induction of cell death (Figures 15 and 16). Notably, a greater upregulation of Bim, especially Bim S, the most potent isoform, and Puma mRNAs was observed in *Hus1*-deficient cells, as compared to *Hus1*-wild-type cells. Consistently, when cells were treated with a higher dose of etoposide for a shorter time course, a clear induction of Bim and Puma mRNAs occurred in a time-dependent manner, with more dramatic increases seen in *Hus1*-deficient cells (Figures 17 and 18). Notably, the increase in Bim and Puma protein levels (Figure 10) in *Hus1*-deficeint cells after etoposide

treatment is greater than the increase in their mRNA levels (Figures 15 and 16), suggesting that etoposide-mediated upregulation of Bim and Puma is regulated through both transcriptional and post-transcriptional mechanisms.

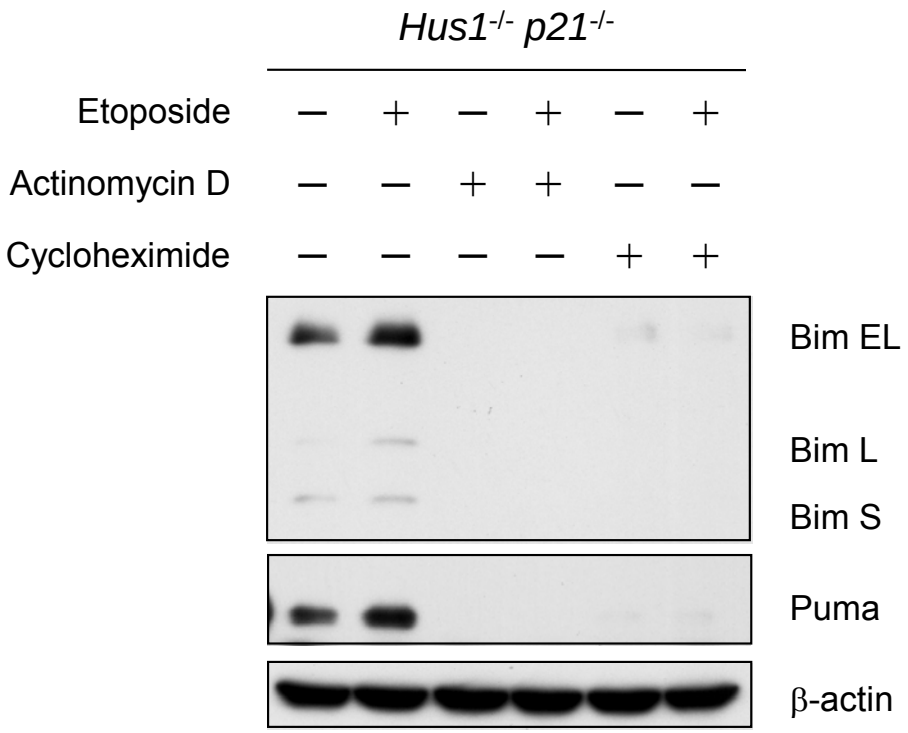

Figure 14. Induction of Bim and Puma expression in response to etoposide treatment is regulated at the transcriptional level. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with control DMSO (-), 1 μg/ml actinomycin D or 5 μg/ml cycloheximide alone or in combination with 3.125 μg/ml etoposide for 24 h. Total cell lysate was prepared and the expression of Bim and Puma was analyzed by SDS-PAGE/immunoblot.

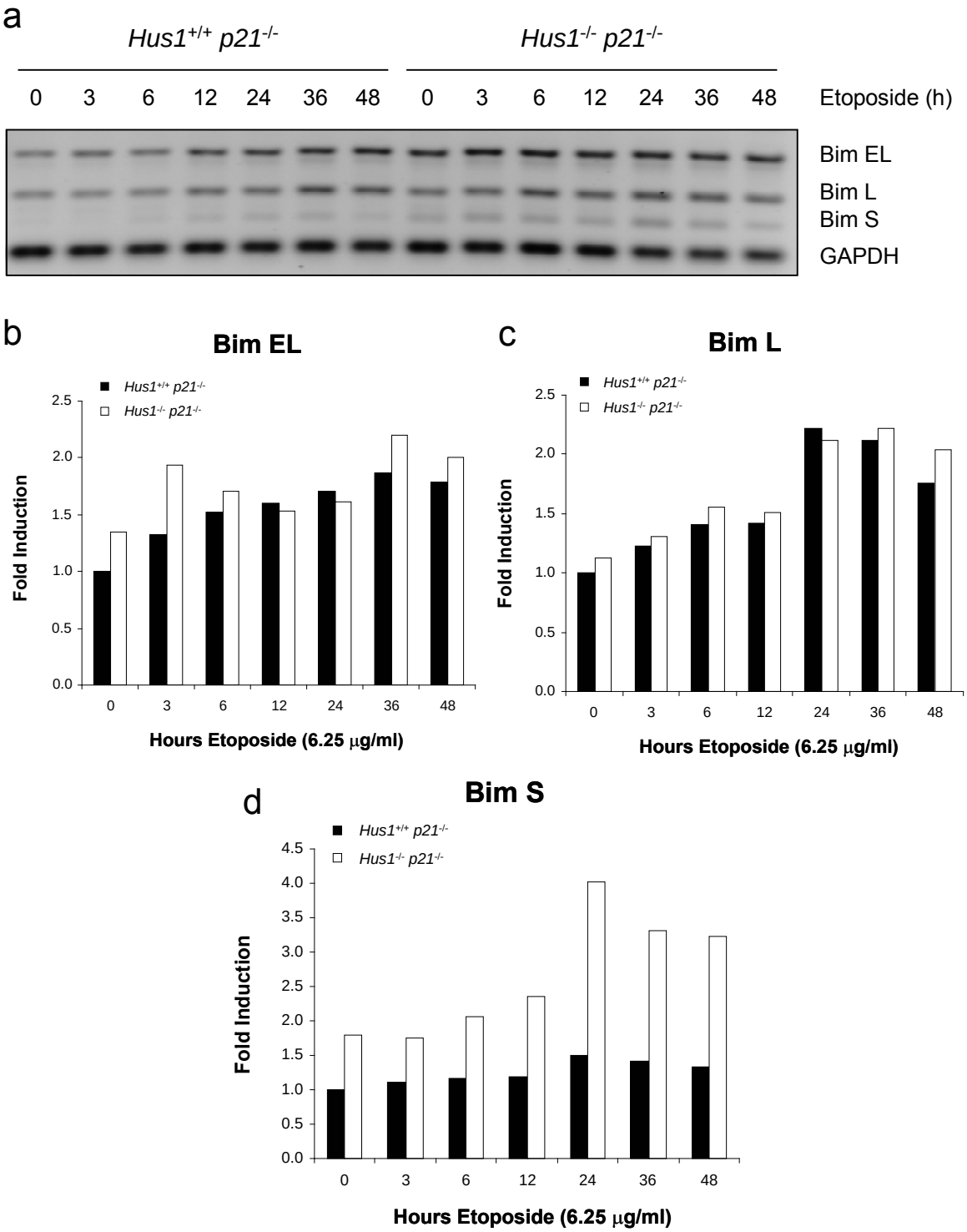

Figure 15. Induction of Bim expression in response to etoposide treatment is regulated at the mRNA level. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for 0, 3, 6, 12, 24, 36 or 48 h. (a) Semi-quantitative RT-PCR was used to examine the mRNA level of Bim. (b, c, d) Quantification of the levels of (b) Bim EL, (c) Bim L and (d) Bim S.

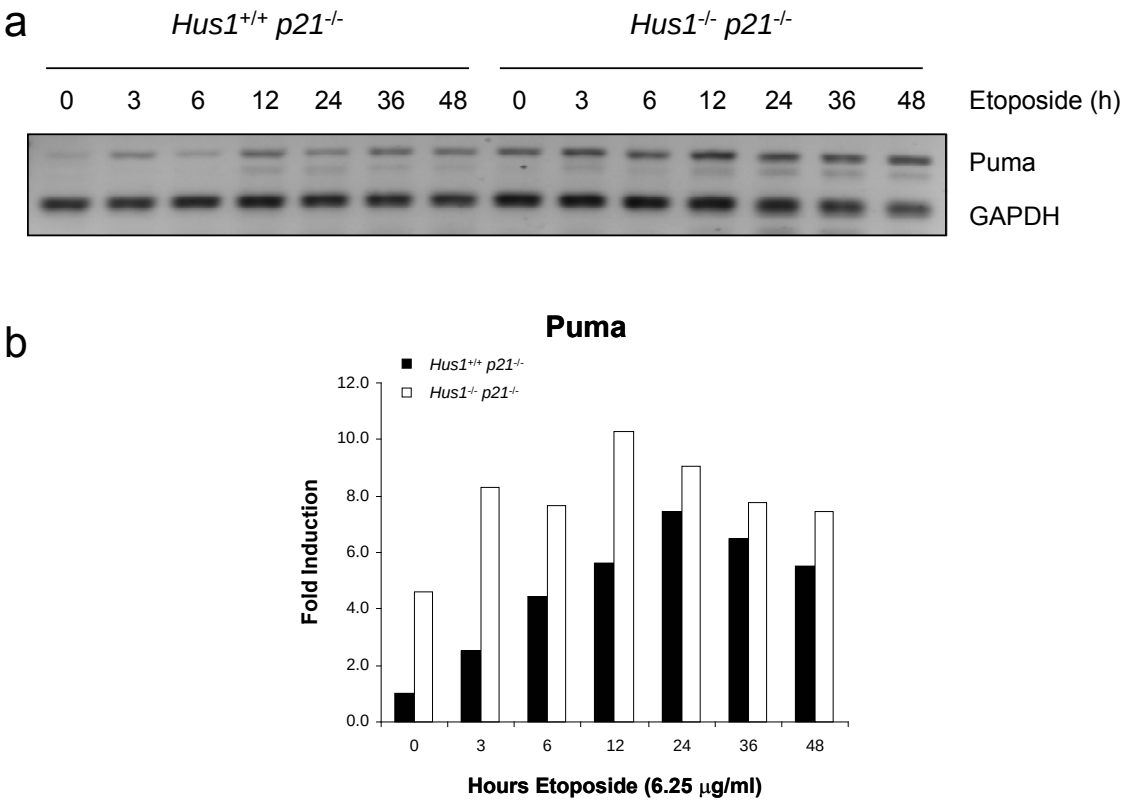

Figure 16. Induction of Puma expression in response to etoposide treatment is regulated at the mRNA level. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for 0, 3, 6, 12, 24, 36 or 48 h. (a) Semi-quantitative RT-PCR was used to examine the mRNA level of Puma. (b) Quantification of Puma expression.

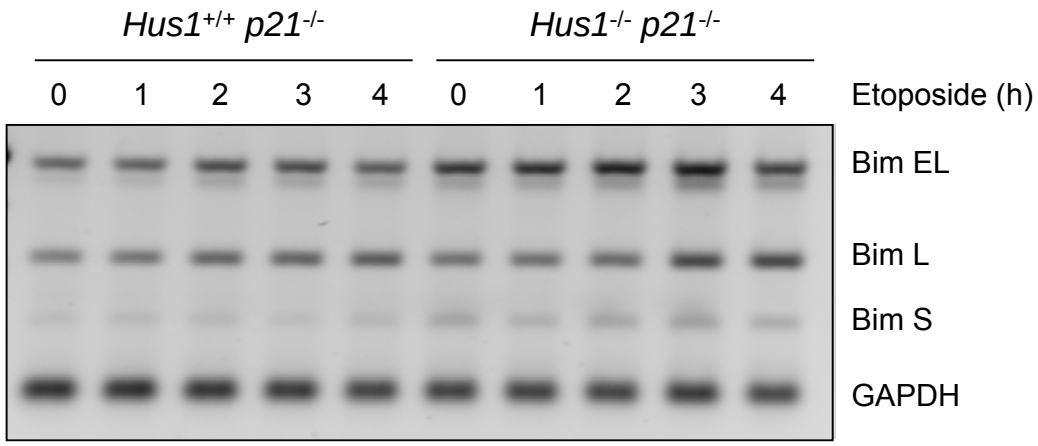

Figure 17. Etoposide-induced upregulation of Bim expression occurs at the transcriptional level. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 25 μg/ml etoposide for 0, 1, 2, 3 or 4 h. Semi-quantitative RT-PCR was used to examine the mRNA level of Bim.

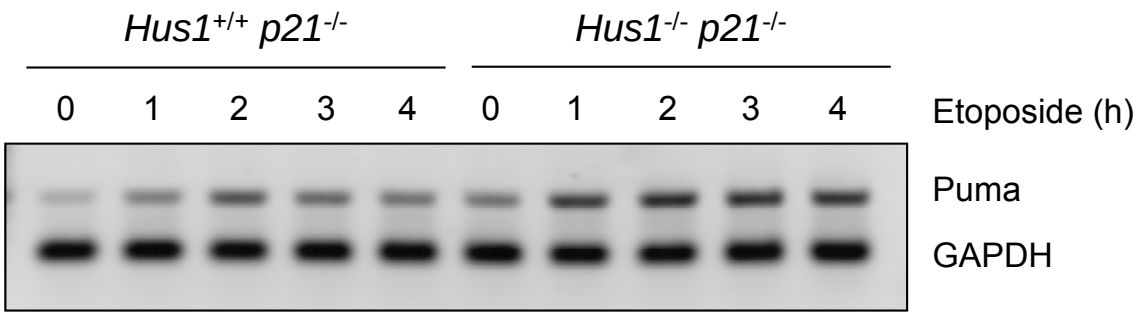

Figure 18. Etoposide-induced upregulation of Puma expression occurs at the transcriptional level. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 25 μg/ml etoposide for 0, 1, 2, 3 or 4 h. Semi-quantitative RT-PCR was used to examine the mRNA level of Puma.

Since we found that Bim and Puma are regulated, at least in part, at the transcriptional level, we investigated which transcription factors are responsible for the upregulation of these BH3-only proteins in response to DNA damage. As it has been shown that p53 can transcactivate both Bim and Puma in response to DNA damage (Burns and El-Deiry, 2003; Nakano and Vousden, 2001), we first examined the possibility that p53 is responsible for the etoposide-induced upregulation of these BH3-only proteins. To this end, we examined the effect of loss of *p53* on DNA damage-induced Bim and Puma expression by treating *Hus1*$^{-/-}$ *p53*$^{-/-}$ and *Hus1*$^{-/-}$ *p21*$^{-/-}$ MEFs with etoposide for varying time points. As shown in Figure 19, loss of *p53* resulted in a slight inhibition of Bim expression and moderate inhibition of Puma expression. However, knockout of *p53* did not affect etoposide-induced cell death or apoptosis, regardless of *Hus1* status (Figures 20 and 21, respectively). Taken together, these results suggest that p53 is involved in inducing Puma expression and to a lesser extent Bim expression, but that other factors are also responsible for the induction of Bim and Puma in response to

etoposide treatment. FoxO3a and E2F1 are also candidates for transcription factors that may regulate Bim and Puma expression, as both have been shown to upregulate the expression of these BH3-only proteins in response to DNA damage (Dijkers *et al.*, 2000; Hershko and Ginsberg, 2004; Sunters *et al.*, 2003; Yang *et al.*, 2006). However, knockdown of either FoxO3a or E2F1 in *Hus1*-deficient cells did not inhibit etoposide-induced upregulation of Bim or Puma expression (Figure 22 and 23, respectively). These results indicate that FoxO3a and E2F1, along with p53, are not essential for the upregulation of Bim and Puma in response to etoposide treatment in *Hus1*-deficient cells. Therefore, further studies are needed to identify the transcription factors that are responsible for etoposide-induced Bim and Puma expression in cells lacking *Hus1*.

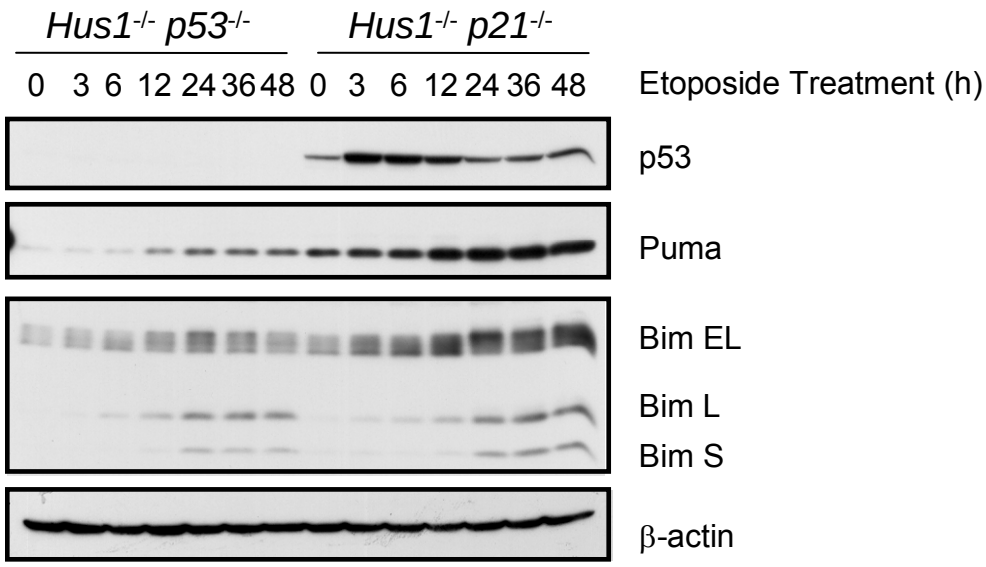

Figure 19. Loss of *p53* suppresses DNA damage-induced Puma expression. *Hus1*$^{-/-}$ *p53*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for the times indicated. Total cell lysate was prepared and the expression of p53, Bim and Puma were examined by SDS-PAGE/immunoblot analysis.

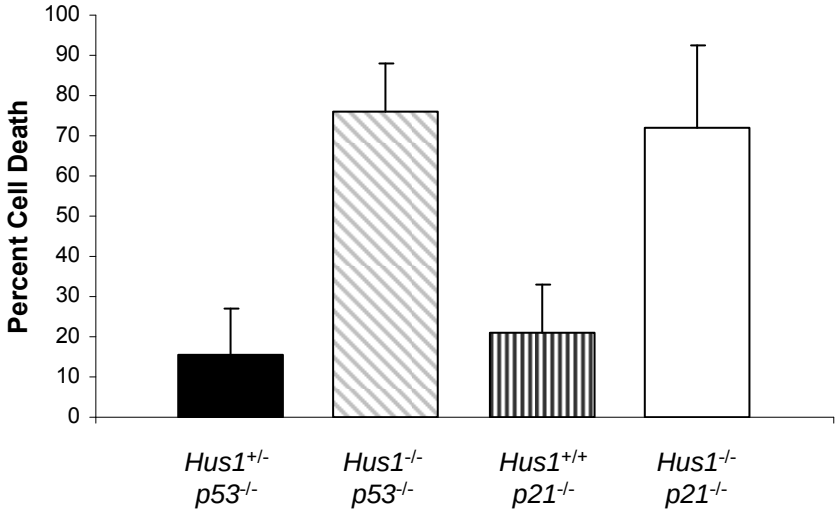

Figure 20. Loss of *p53* does not affect etoposide-induced cell death. *Hus1*$^{+/-}$*p53*$^{-/-}$, *Hus1*$^{-/-}$*p53*$^{-/-}$, *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide or control DMSO for 48 h. Cell death was measured by trypan blue exclusion assay. The data shown represent the percent cell death of etoposide-treated cells minus the percent cell death of control DMSO-treated cells (mean ± s.d.; n=3).

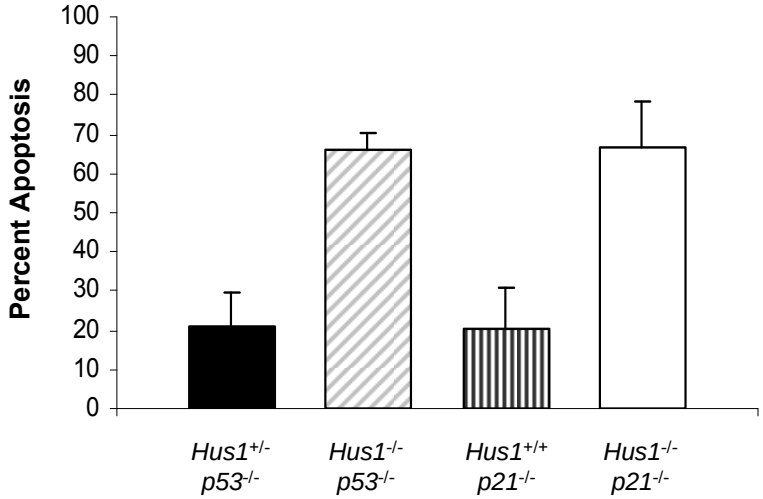

Figure 21. Loss of *p53* does not affect etoposide-induced apoptosis. *Hus1*$^{+/-}$*p53*$^{-/-}$, *Hus1*$^{-/-}$*p53*$^{-/-}$, *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for 48 h. The cells were harvested and prepared for TUNEL staining and analysis by flow cytometry. The data shown represent the percent apoptosis of etoposide-treated cells minus the percent apoptosis of control DMSO-treated cells (mean ± s.d.; n=3).

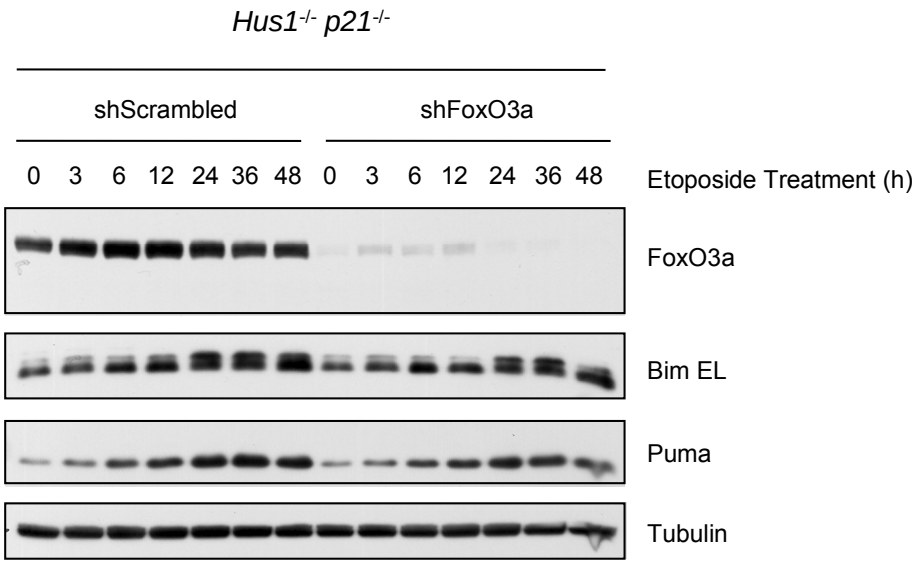

Figure 22. FoxO3a is not responsible for the upregulation of Bim and Puma expression in response to etoposide treatment. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shRNA targeting FoxO3a or a control scrambled shRNA were treated with 6.25 μg/ml etoposide for the times indicated. Total cell lysate was prepared and the expression of FoxO3a, Bim and Puma were examined by SDS-PAGE/immunoblot analysis.

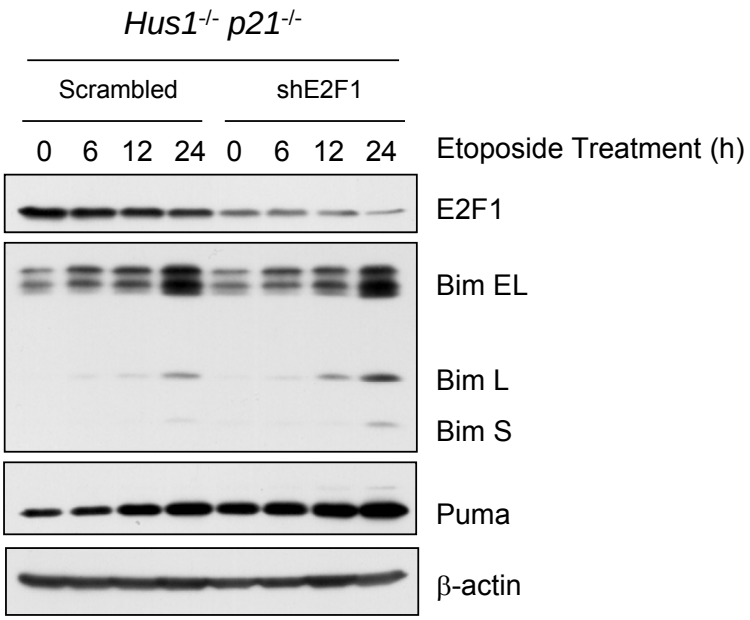

Figure 23. E2F1 is not responsible for the upregulation of Bim and Puma expression in response to etoposide treatment. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shRNA targeting E2F1 or a control scrambled shRNA were treated with 6.25 μg/ml etoposide for the indicated times. Total cell lysate was prepared and the expression of E2F1, Bim and Puma were examined by SDS-PAGE/immunoblot analysis.

Knockdown of Bim and Puma Confers Resistance to Etoposide-Induced Apoptosis in Hus1-Deficient Cells

Our results clearly show that loss of *Hus1* not only results in the upregulation of Bim and Puma expression, but also promotes caspase-3 activation and cell death induced by etoposide treatment. Since BH3-only proteins play a key role in the initiation of apoptosis (Huang and Strasser, 2000; Puthalakath and Strasser, 2002; Willis and Adams, 2005), we examined whether the upregulation of Bim and Puma is responsible for sensitizing *Hus1*-deficient cells to etoposide treatment. As a dramatic induction of all three isoforms of Bim was observed in response to etoposide treatment, we first investigated whether inhibition of Bim expression would suppress DNA damage-induced cell death in $Hus1^{-/-}p21^{-/-}$ MEFs. Transfection of siRNA specific for Bim abrogated its expression, even upon treatment with etoposide (Figure 24). Knockdown of Bim expression resulted in a decrease in PARP cleavage (Figure 24) and partial resistance to DNA damage-induced cell death (Figure 25), as compared to siGFP or mock transfected cells. These results suggest that upregulation of Bim expression contributes to the sensitivity of *Hus1*-deficient cells to etoposide-induced apoptosis. We next examined whether the upregulation of Puma expression is also involved in sensitizing *Hus1*-deficient cells to etoposide treatment. To this end, a lentiviral delivery system was used to transduce $Hus1^{-/-}p21^{-/-}$ MEFs with shRNA targeting Puma or Bim, or a control scrambled shRNA. Whereas etoposide treatment resulted in the induction of Bim and Puma expression in control shScrambled expressing cells, the upregulation of these proteins was suppressed by their respective shRNA (Figure 26). Consistent with the siBim results shown in Figure 24, expression of shBim suppressed etoposide-induced apoptosis

(Figures 26 and 27). Moreover, knockdown of Puma expression significantly suppressed etoposide-induced caspase-3 activation and apoptosis, as compared to control cells (Figures 26 and 27). Since knockdown of either Bim or Puma alone only partially suppressed etoposide-induced cell death, we next examined whether Bim and Puma act redundantly or synergistically to induce apoptosis in response to etoposide treatment. To this end, shBim expressing *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were infected with lentivirus expressing shRNA targeting Puma, which resulted in efficient knockdown of Puma expression (Figure 26). Importantly, knockdown of both Bim and Puma resulted in further inhibition of caspase-3 processing and apoptosis, when compared to *Hus1*$^{-/-}$*p21*$^{-/-}$ cells expressing shBim or shPuma alone (Figures 26 and 27). Taken together, these results indicate that Bim and Puma cooperate in sensitizing *Hus1*-deficient cells to etoposide-induced apoptosis.

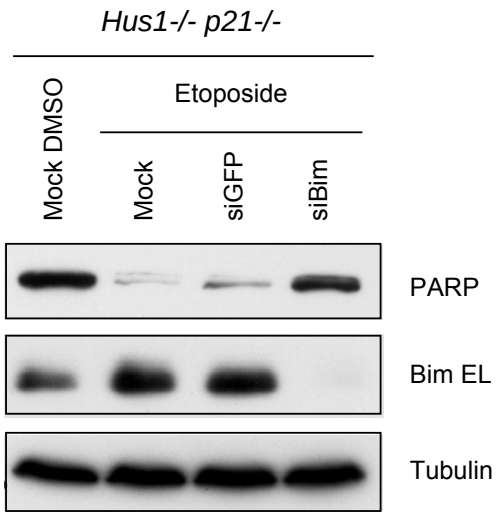

Figure 24. Knockdown of Bim expression suppresses PARP cleavage in *Hus1*-deficient cells. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were mock transfected or transiently transfected with siRNA targeting GFP or Bim. Thirty-six hours after transfection, cells were treated with control DMSO or 6.25 μg/ml etoposide for 30 h. Whole cell lysate was subjected to SDS-PAGE/immunoblot analysis with antibodies to PARP (full length PARP is shown), Bim, Puma and Tubulin. The expression of Bim in DMSO-treated mock transfected cells represents the basal level of Bim expression.

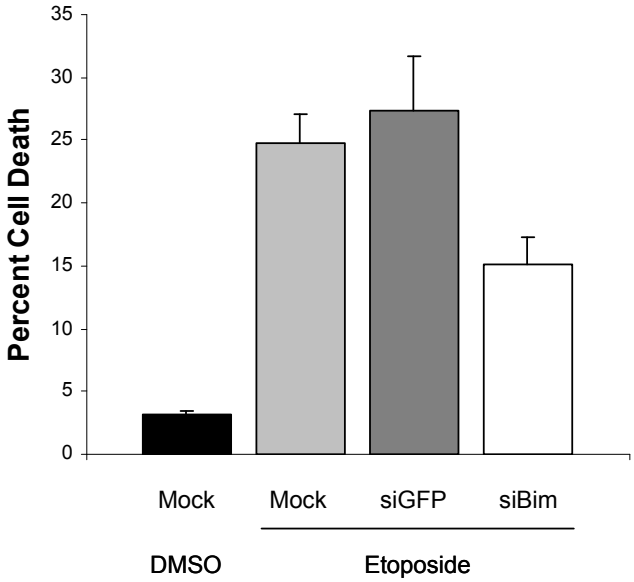

Figure 25. Knockdown of Bim expression confers resistance to etoposide-induced cell death in *Hus1*-deficient cells. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were mock transfected or transiently transfected with siRNA targeting GFP or Bim. Thirty-six hours after transfection, cells were treated with control DMSO or 6.25 μg/ml etoposide for 30 h. Viability was determined by trypan blue exclusion assay (mean ± s.d.; n=2). The cell death of DMSO-treated mock transfected cells represents the basal level of cell death.

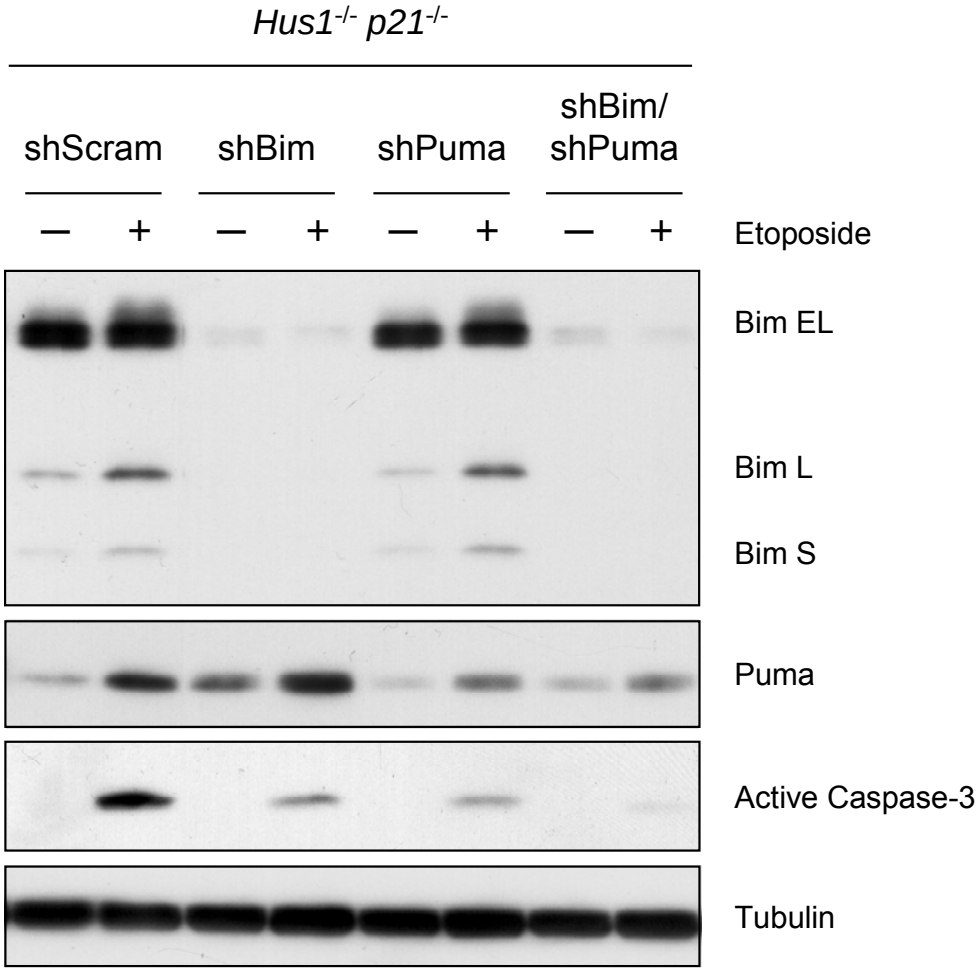

Figure 26. Knockdown of Bim and Puma expression suppresses etoposide-induced caspase-3 cleavage in *Hus1*-deficient cells. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were infected with lentivirus expressing shRNA targeting Bim, Puma, Bim and Puma, or a control scrambled shRNA (shScram). After selection on puromycin, cells were treated with 12.5 μg/ml etoposide or control DMSO for 16 h. Knockdown of Bim and Puma was confirmed by SDS-PAGE/immunoblot analysis.

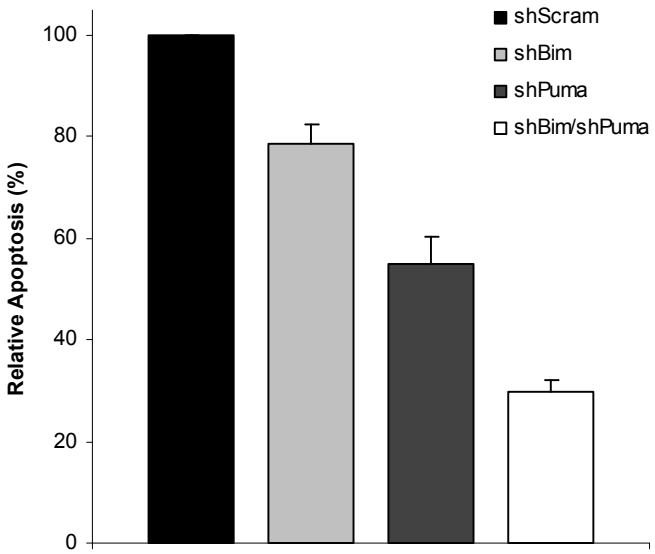

Figure 27. Knockdown of Bim and Puma expression confers resistance to etoposide-induced apoptosis in *Hus1*-deficient cells. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shRNA targeting Bim, Puma, Bim and Puma, or a control scrambled shRNA (shScram) were treated with 12.5 μg/ml etoposide or control DMSO for 16 h. Induction of apoptosis was measured by caspase-3 activity assay. The caspase-3 activity of control DMSO-treated cells was subtracted from the amount of caspase-3 activity observed in the etoposide treated cells. The data are represented as percent relative apoptosis as normalized to the control infected cells (mean ± s.d.; n=3).

Loss of Hus1 Enhances the Binding of Rad9 to Bcl-2 to Potentiate the Apoptotic Response

It has been shown that DNA damage promotes the binding of the 9-1-1 checkpoint complex to chromatin to initiate the DNA damage response and facilitate the activation of downstream proteins (Parrilla-Castellar *et al.*, 2004; Zhou and Elledge, 2000). Consistently, Rad9 binding to chromatin was enhanced in *Hus1*-wild-type cells in a time-dependent manner after etoposide treatment (Figure 28). In contrast, chromatin bound Rad9 was barely detectable in *Hus1*-deficient cells, although a slight increase was noticeable after etoposide treatment. These results are consistent with previous findings

which show that hydroxyurea- and UV-induced binding of Rad9 to the chromatin is decreased in *Hus1*$^{-/-}$ cells (Zou *et al.*, 2002). Taken together, these results indicate that loss of *Hus1* results in a defect in the binding of Rad9 to chromatin in response to DNA damage.

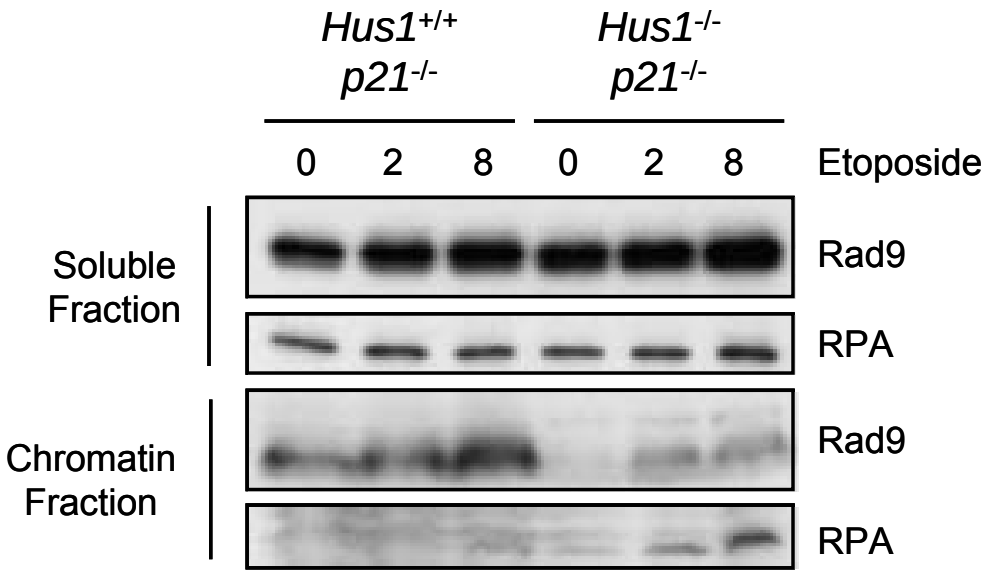

Figure 28. Loss of *Hus1* results in a defect in the binding of Rad9 to chromatin. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 12.5 μg/ml etoposide for 0, 2 or 8 h and subjected to subcellular fractionation. The resulting chromatin bound and soluble fractions were analyzed by SDS-PAGE/immunoblot using antibodies specific for Rad9 and RPA as a control.

Previous evidence from our laboratory and others demonstrate that Rad9 can interact with Bcl-2 or Bcl-xL through a BH3-like domain within its N-terminus to promote apoptosis following DNA damage (Ishii *et al.*, 2005; Komatsu *et al.*, 2000a; Komatsu *et al.*, 2000b; Lee *et al.*, 2003; Yoshida *et al.*, 2002; Yoshida *et al.*, 2003). These results indicate that Rad9 not only has functions in the nucleus as a member of a DNA damage checkpoint complex, but also in the cytosol as an inducer of apoptosis. Therefore, the effect of loss of *Hus1* on the intracellular localization of Rad9 was

examined. Consistent with previous studies (Burtelow *et al.*, 2000), Rad9 was detected in both the nuclear and cytosolic fractions of *Hus1*$^{+/+}$*p21*$^{-/-}$ cells when analyzed by subcellular fractionation (Figure 29). Moreover, etoposide treatment resulted in Rad9 accumulation and hyperphosphorylation in the nucleus of *Hus1*$^{+/+}$*p21*$^{-/-}$ cells (Figure 29). In contrast, Rad9 was primarily detected in the cytosolic fraction of *Hus1*-deficient cells and remained hypophosphorylated even upon DNA damage (Figure 29). These results suggest that Rad9 chromatin binding and hyperphosphorylation are *Hus1*-dependent. Furthermore, immunofluorescent analysis revealed that Rad9 formed punctate nuclear foci in *Hus1*-wild-type cells after etoposide treatment, whereas the Rad9 signal accumulated in perinuclear foci upon treatment with etoposide in *Hus1*-deficient cells (Figure 30).

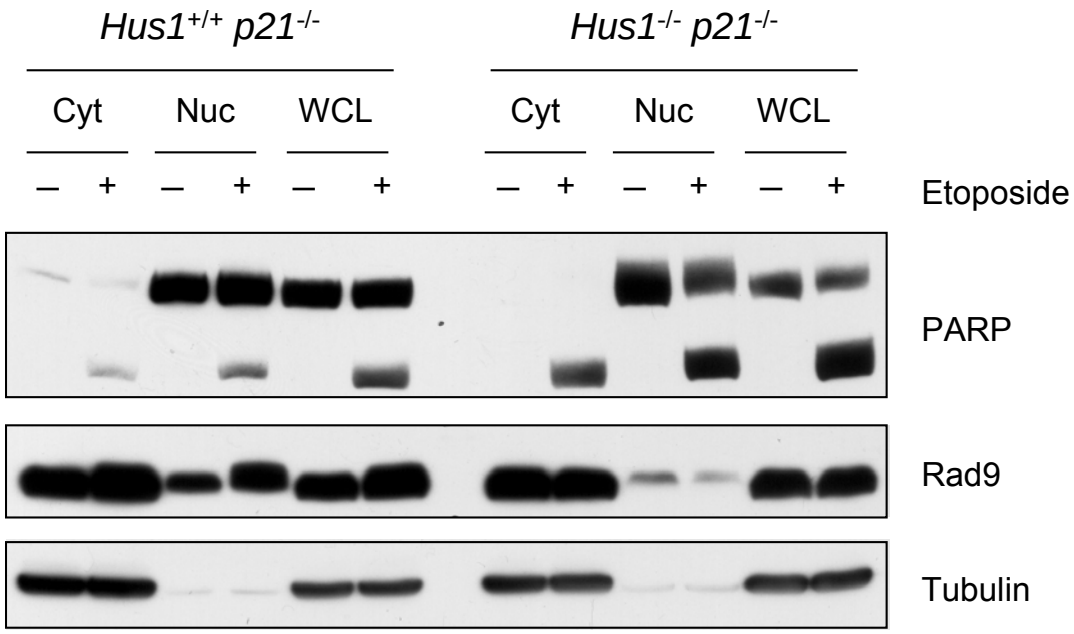

Figure 29. Rad9 is predominantly detected in the cytosolic fraction of *Hus1*-deficient cells. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 12.5 μg/ml etoposide or control DMSO for 12 h and subjected to subcellular fractionation. The resulting cytosolic (Cyt) and nuclear (Nuc) fractions, along with whole cell lysate (WCL), were analyzed by SDS-PAGE/immunoblot.

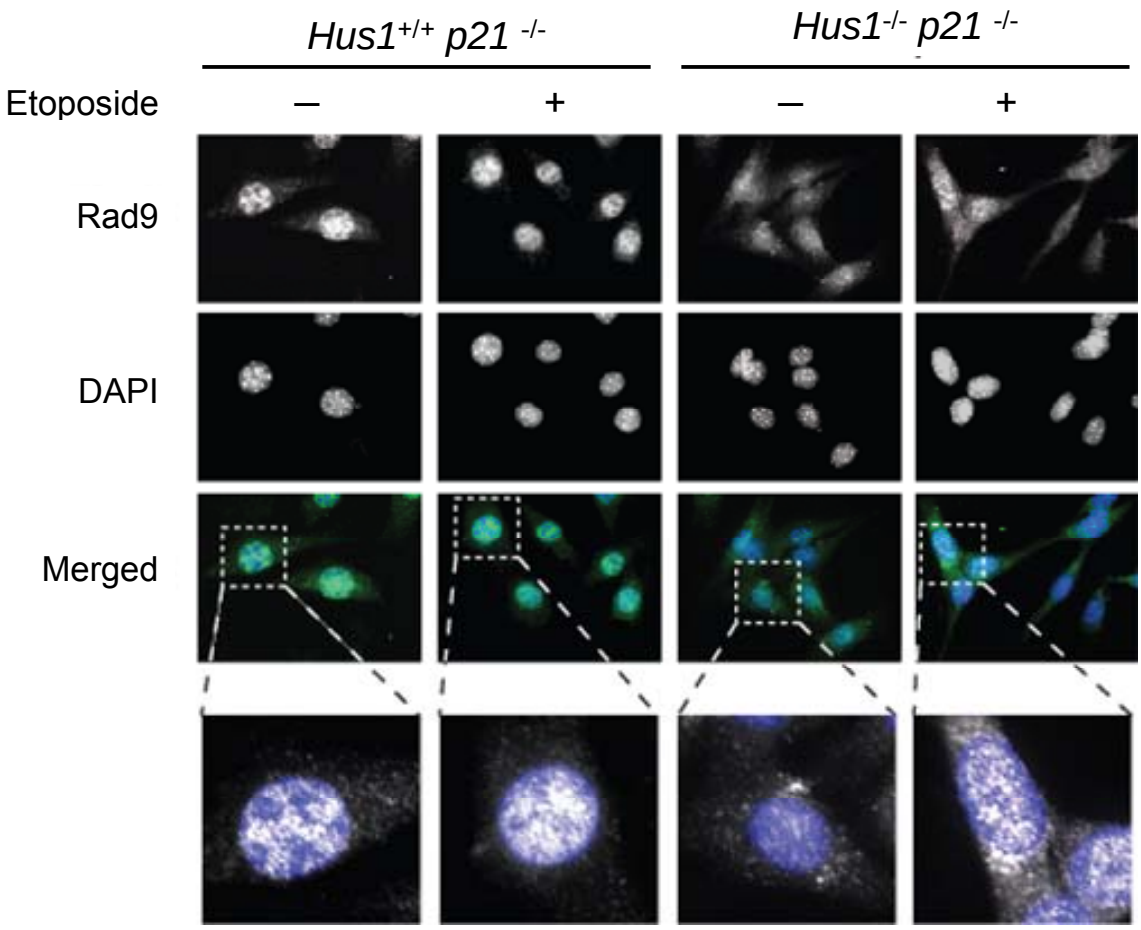

Figure 30. Rad9 is predominantly located in the cytosol of *Hus1*-deficient cells. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 12.5 μg/ml of etoposide or control DMSO for 12 h. Localization of Rad9 was analyzed using fluorescence microscopy.

In order to determine whether Rad9 binds to Bcl-2 family members during apoptosis, the interaction between Rad9 and Bcl-2 was examined in *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs in response to etoposide treatment. Since the majority of Rad9 was detected in the cytosolic fraction of *Hus1*-deficient cells, coimmunoprecipitation of cytosolic Rad9 with Bcl-2 was performed. While etoposide treatment enhanced Rad9 interaction with Bcl-2, a significant amount of cytosolic Rad9 was bound to Bcl-2 even in the absence of DNA damage (Figure 31). Thus, it is possible that subcellular

fractionation using a hypotonic buffer may alter the conformation or localization of Rad9 and Bcl-2, which affects their interaction. Indeed, it has been shown that Rad9 can leak from the nucleus during subcellular fractionation even in the absence of DNA damage (Burtelow *et al.*, 2000). In order to confirm the interaction of Rad9 with Bcl-2, the coimmunoprecipitation was repeated using whole cell lysates. As shown in Figure 32, a minimal amount of Rad9 was bound to Bcl-2 in the absence of DNA damage, regardless of *Hus1* status. Treatment with etoposide resulted in an induction of Rad9 binding to Bcl-2 that was much greater in *Hus1*-deficient cells as compared to *Hus1*-wild-type cells. These results suggest that, in response to DNA damage, Rad9 may also contribute to the enhanced sensitivity of *Hus1*-deficient cells through its interaction with anti-apoptotic Bcl-2 family members.

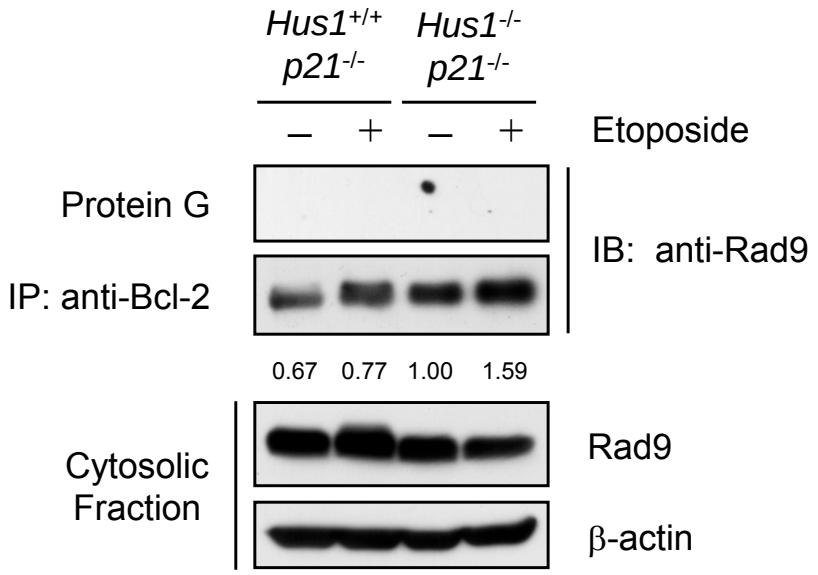

Figure 31. Binding of cytosolic Rad9 to Bcl-2 is increased upon treatment with etoposide and enhanced by loss of *Hus1*. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 12.5 μg/ml etoposide or control DMSO for 12 h. The cytosolic fractions were subjected to immunoprecipitation in the presence or absence of anti-Bcl-2 monoclonal antibody. The resulting immunocomplexes were analyzed by SDS-PAGE/immunoblot. The amount of Rad9 in the immunocomplexes was quantified and normalized to cytosolic Rad9. The levels of Bcl-2 bound Rad9 are listed relative to those of untreated *Hus1*$^{-/-}$*p21*$^{-/-}$ cells, which was set as 1.0.

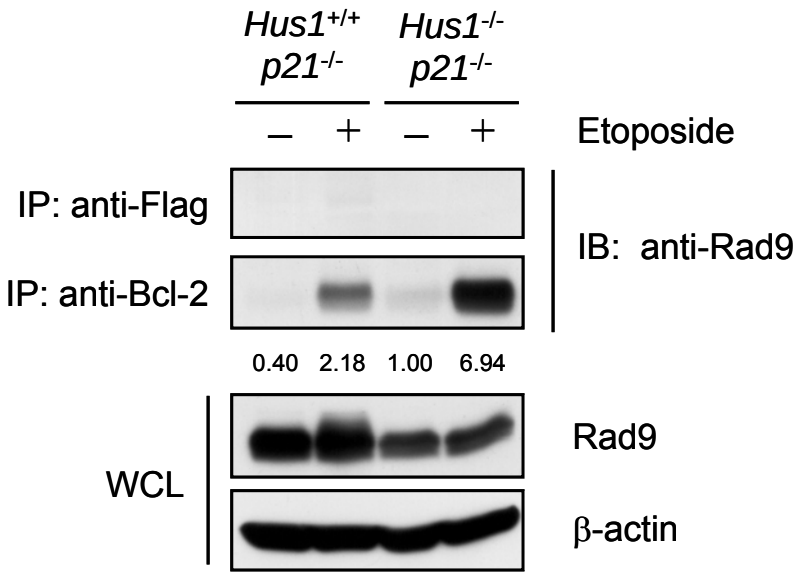

Figure 32. Etoposide-induced binding of Rad9 to Bcl-2 is enhanced by loss of *Hus1*. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 12.5 μg/ml etoposide or control DMSO for 12 h. Whole cell lysate (WCL) was subjected to immunoprecipitation with anti-Bcl-2 or control anti-Flag monoclonal antibodies. The resulting immunocomplexes and WCL were analyzed by SDS-PAGE/immunoblot. The amount of Rad9 in the immunocomplexes was quantified and normalized to total Rad9. The levels of Bcl-2 bound Rad9 are listed relative to those of untreated *Hus1*$^{-/-}$*p21*$^{-/-}$ cells, which was set as 1.0.

We therefore examined whether Rad9 cooperates with Bim and Puma to sensitize *Hus1*-deficient cells to etoposide-induced apoptosis. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shBim and shPuma were infected with lentivirus expressing shRNA targeting Rad9. Indeed, knockdown of Rad9 resulted in further inhibition of caspase-3 activation, as compared to the shBim and shPuma expressing cells (Figures 33 and 34). Consistently, suppression of Rad9 expression in the shBim and shPuma expressing cells conferred further resistance to etoposide-induced cell death (Figure 35). Taken together, these results indicate that Rad9 acts in collaboration with Bim and Puma to sensitize *Hus1*-deficient cells to etoposide-induced apoptosis.

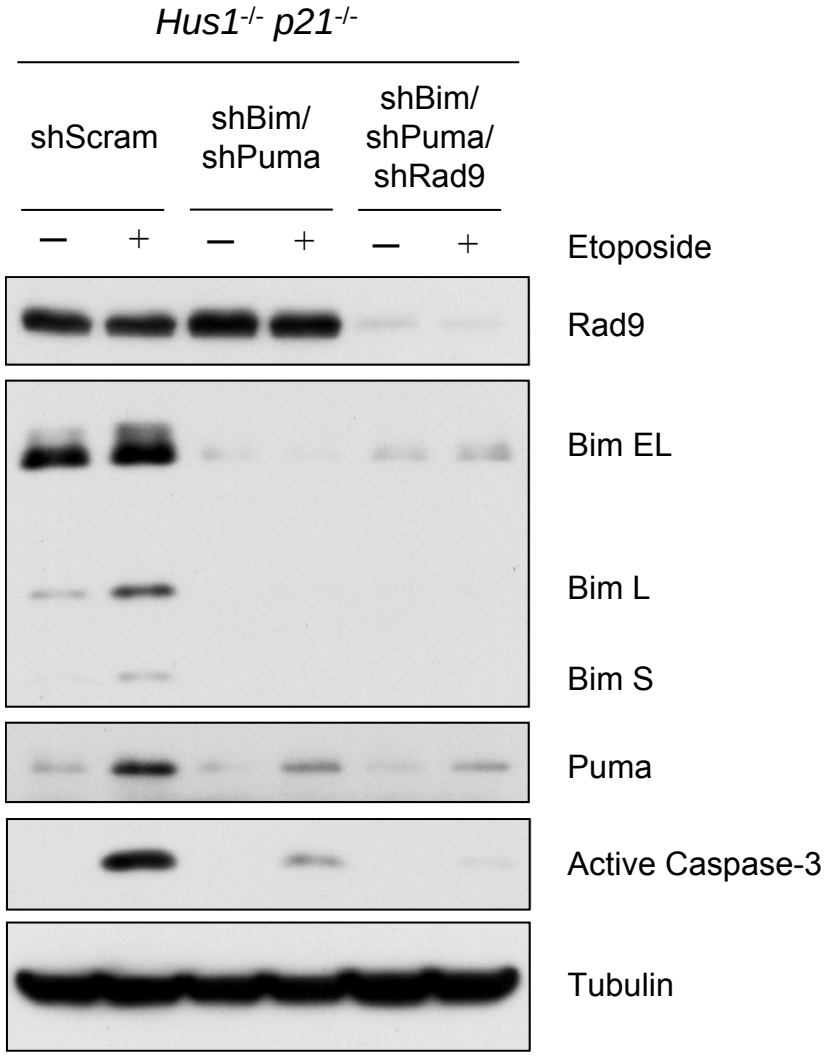

Figure 33. Knockdown of Rad9 further suppresses caspase-3 activation in shBim and shPuma expressing cells. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shBim and shPuma were infected with lentivirus expressing shRNA targeting Rad9. Cells were treated with 12.5 μg/ml etoposide or control DMSO for 16 h. Knockdown of Rad9, as well as Bim and Puma, was confirmed by SDS-PAGE/immunoblot analysis.

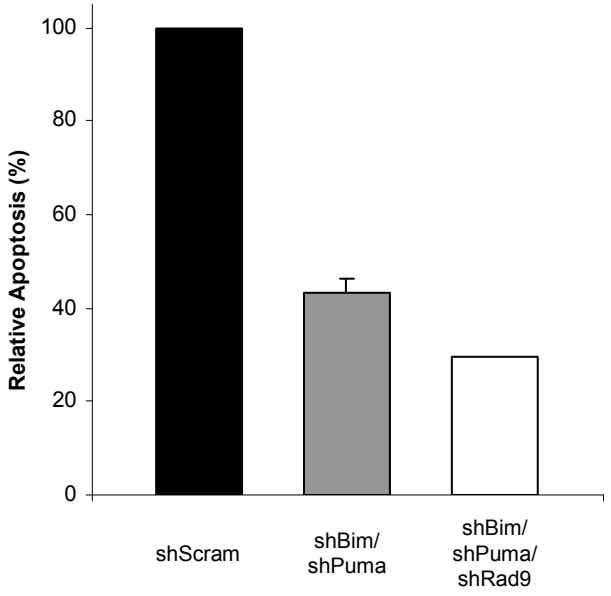

Figure 34. Rad9 collaborates with Bim and Puma to sensitize *Hus1*-deficient cells to etoposide-induced apoptosis. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shBim and shPuma were infected with lentivirus expressing shRNA targeting Rad9. Cells were treated with 12.5 μg/ml etoposide or control DMSO for 16 h. Induction of apoptosis was measured using a caspase-3 activity assay.

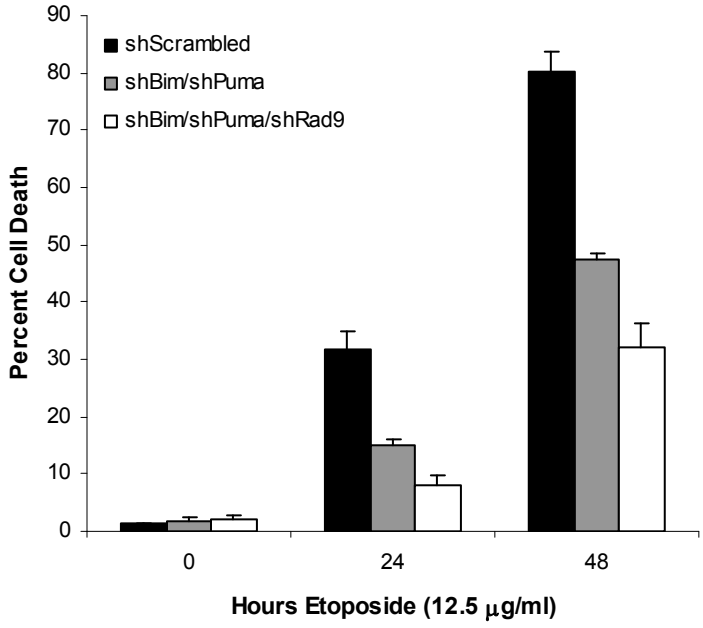

Figure 35. Rad9 collaborates with Bim and Puma to sensitize *Hus1*-deficient cells to etoposide-induced cell death. *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing control shRNA, shBim and shPuma, or shBim and shPuma plus shRad9 were treated with 12.5 μg/ml etoposide for 0, 24 or 48 h. Cell death was determined by trypan blue exclusion assay (mean ± s.d.; n=3).

Discussion

In this study, we have demonstrated that loss of *Hus1* results in the upregulation of the BH3-only proteins, Bim and Puma, which is partially responsible for sensitizing *Hus1*-deficient cells to etoposide-induced apoptosis. In addition, we found that in the absence of *Hus1*, Rad9 functions as a BH3-only protein and cooperates with Bim and Puma to promote apoptosis in response to etoposide treatment. There are currently two models for the activation of apoptosis by the Bcl-2 family members: the direct model and the hierarchy model (Galonek and Hardwick, 2006). The direct model proposes that BH3-only proteins have varying levels of potency due to their ability to bind various Bcl-2-like family members (Certo *et al.*, 2006; Chen *et al.*, 2005; Willis *et al.*, 2007). Thus, Bim, Puma and tBid are the most potent as they can bind all of the anti-apoptotic Bcl-2 family members, whereas Noxa and Bad are less potent as they can only bind to a subset of the Bcl-2-like proteins. In the hierarchy model, on the other hand, Bim and Puma, as well as tBid, are more potent as they act downstream of the other BH3-only proteins and the Bcl-2-like proteins and can bind directly to the multi-domain pro-apoptotic proteins, resulting in their activation and the induction of apoptosis (Kim *et al.*, 2006; Kuwana *et al.*, 2005; Letai *et al.*, 2002). This model suggests that loss of both Bim and Puma would result in complete inhibition of apoptosis mediated through the intrinsic pathway. Our results show that knockdown of both Bim and Puma diminishes the hypersensitivity of *Hus1*-deficient cells to etoposide-induced apoptosis, indicating that both Bim and Puma indeed play a central role in the activation of this programmed cell death pathway. Interestingly, suppression of Rad9 expression in the Bim and Puma double-knockdown cells resulted in further inhibition of DNA damage-induced apoptosis. Therefore, our data

argue in favor of the direct model and suggest that it is the ratio of the BH3-only proteins to the Bcl-2-like proteins that regulates apoptosis.

Our results demonstrate that Bim and Puma mRNA expression is induced by etoposide treatment. Among known transcription factors, p53, FoxO3a and E2F1 were the most likely candidates for regulators of Bim and Puma expression in response to etoposide treatment, as these transcription factors have been shown to upregulate both Bim and Puma in response to DNA damage (Burns and El-Deiry, 2003; Dijkers *et al.*, 2000; Hershko and Ginsberg, 2004; Nakano and Vousden, 2001; Sunters *et al.*, 2003; Yang *et al.*, 2006). However, our results suggest that these transcription factors are not required for the upregulation of Bim and Puma in *Hus1*-deficient cells in response to DNA damage. While we cannot rule out the possibility that loss of p53, FoxO3a or E2F1 results in compensation by other transcription factors, our data indicate that these transcription factors are not essential for the upregulation of Bim and Puma in response to etoposide treatment in *Hus1*-deficient cells. Several other transcription factors have been shown to regulate BH3-only expression (Shibue and Taniguchi, 2006). It has been reported that Myc plays a role in the enhanced expression of Bim (Egle *et al.*, 2004). Additionally, JNK and its downstream pathway, c-Jun/AP-1, have been shown to be involved in the transcriptional upregulation of Bim (Jin *et al.*, 2006; Putcha *et al.*, 2003; Whitfield *et al.*, 2001). Furthermore, JNK was reported to phosphorylate p73 at several residues and this phosphorylation was required for p73-mediated induction of Puma in response to DNA damage (Jones *et al.*, 2007). It is of interest to determine whether these proteins are responsible for etoposide-induced Bim and Puma expression in cells lacking *Hus1*.

Rad9 has been shown to play a role in multiple cellular processes including: regulation of cell cycle checkpoints, transcriptional activation of p53 targets, initiation of DNA repair and when DNA repair is unfavorable, induction of apoptosis (Lieberman, 2006). It has been suggested that the primary function of Rad9 is to act as a sensor in the DNA damage response pathway to promote survival by initiating cell cycle arrest and facilitating DNA repair (Brandt *et al.*, 2006; Loegering *et al.*, 2004). Thus, it is not surprising that loss of Rad9 sensitizes cells to DNA damage as these cells lack the ability to activate appropriate cell cycle checkpoints and DNA repair. On the other hand, Rad9 may function as a pro-apoptotic factor in cells with unrepairable, excessively damaged DNA or a disrupted 9-1-1 complex, such as through loss of *Hus1*. *Hus1*-deficient cells have a defective cell cycle checkpoint, thus making them more sensitive to DNA damage. In the absence of *Hus1*, Rad9 was found to be mostly located in the cytosol, where it formed perinuclear foci and associated with Bcl-2 in response to DNA damage. These results suggest that loss of *Hus1* results in an abrogation of the nuclear functions of Rad9 and an augmentation of its pro-apoptotic functions.

Taken together, the results presented here indicate that the 9-1-1 complex plays a critical role in the suppression of etoposide-induced apoptosis by regulating the induction of the BH3-only proteins, Bim and Puma. Loss of *Hus1* results in enhanced upregulation of these BH3-only proteins that initiate mitochondrial apoptosis in response to DNA damage. Moreover, disruption of the 9-1-1 complex, through loss of *Hus1,* switches Rad9 from functioning as a mediator of cell cycle checkpoints and DNA repair to an inducer of apoptosis. Thus, the 9-1-1 complex may act as a checkpoint sensor to decide whether a cell should survive or undergo apoptosis in response to DNA damage (Figure 36).

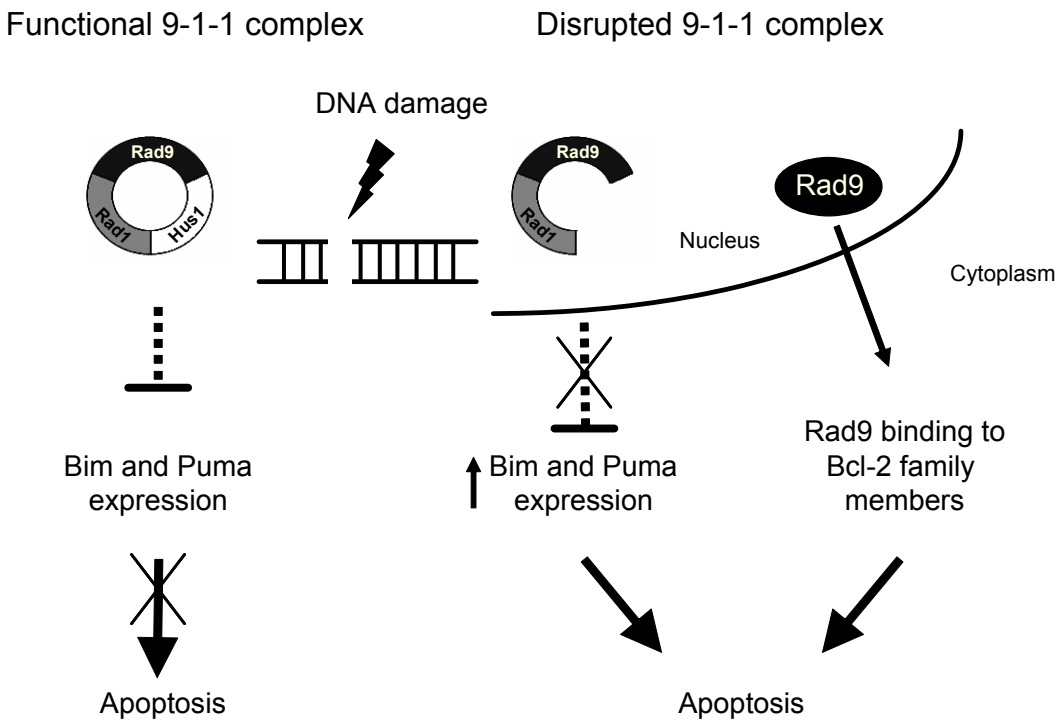

Figure 36. Proposed model for the role of the Rad9-Rad1-Hus1 complex in the regulation of DNA damage-induced apoptosis. Cells with a functional 9-1-1 complex can suppress Bim and Puma expression in response to DNA damage, which results in resistance to apoptosis. In cells with a disrupted 9-1-1 complex, through loss of *Hus1*, exposure to DNA damaging agents results in the upregulation of Bim and Puma expression, which activates the mitochondrial apoptotic pathway. Loss of *Hus1* also results in the cytoplasmic localization of Rad9 and enhances the interaction of Rad9 with Bcl-2 to potentiate the apoptotic response.

Materials and Methods

Reagents

Etoposide, camptothecin, hydroxyurea, protease inhibitor cocktail, phosphatase inhibitor cocktails I and II, cycloheximide and actinomycin D were purchased from Sigma (St. Louis, MO). Puromycin was purchased from Calbiochem (San Diego, CA). Antibodies were purchased from the following commercial sources: anti-tubulin, anti-β-actin, anti-Flag and FITC-conjugated goat anti-rabbit secondary antibody from Sigma, anti-Bim and anti-Puma from Calbiochem, anti-Mcl-1 from Rockland Immunochemicals (Gilbertsville, PA), anti-PARP and anti-cleaved caspase-3 from Cell Signaling (Danvers, MA), anti-Bcl-2 from BD Pharmingen (San Diego, CA), anti-RPA from Oncogene (Cambridge, MA), anti-GFP from Clontech (Mountain View, CA), anti-Bak and anti-FoxO3a from Upstate (Lake Placid, NY), anti-Bax and goat anti-rabbit IgG-horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Rad9 and anti-Bcl-xL polyclonal rabbit antisera have been previously described (Komatsu *et al.*, 2000b). Protein G agarose beads, trypan blue and culture medium were purchased from Invitrogen (Carlsbad, CA). The Nucleofector machine, as well as all Nucleofector solutions, was purchased from Amaxa Biosystems (Gaithersburg, MD).

Cell Culture, Transfection and Infection

Hus1$^{+/+}$*p21* $^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ cells (Weiss *et al.*, 2000), *Hus1*$^{-/-}$*p21*$^{-/-}$ *GFP* and *Hus1*$^{-/-}$ *p21*$^{-/-}$ *Hus1* cells (Weiss *et al.*, 2002), as well as *Hus1*$^{+/-}$*p53*$^{-/-}$ and *Hus1*$^{-/-}$*p53*$^{-/-}$cells (Weiss *et al.*, 2002) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1.0 mM L-glutamine, 0.1 mM MEM non-

essential amino acids, 100 μg/ml streptomycin and 100 U/ml penicillin. The 21-nucleotide siRNA duplexes targeting Bim and GFP were synthesized and purified by Dharmacon (Lafayette, CO). The siRNA sequence targeting mouse Bim mRNA was 5'-AAUCAUGUACAAUCUCUUCAU-3'. The siRNA specific for GFP has previously been described (Hirai and Wang, 2002). *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were transfected with 10 μg of siRNA per 1 x 10^6 cells using the Nucleofector system. After transfection, cells were allowed to recover for 36 h. The medium, containing any cells which may have died due to transfection, was removed and replaced with treatment medium containing control DMSO or etoposide.

The pLKO.1-based lentiviral shRNAs targeting Bim (TRCN0000009692), Puma (TRCN0000009710) and Rad9 (TRCN0000012638) were purchased from Open Biosystems (Huntsville, AL). The pLKO.1-based scrambled control shRNA vector was purchased from Sigma (St. Louis, MO). Recombinant lentivirus was produced by co-transfecting the appropriate shRNA plasmid with the ViraPower Packaging Mix (Invitrogen) into 293FT cells. The resulting supernatant containing shRNA-expressing lentivirus was used to transduce *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs according to the manufacturer's protocol.

Analysis of Cell Death and Apoptosis

Cell death was assessed by trypan blue exclusion assay. Apoptosis was scored by the presence of nuclear chromatin condensation and DNA fragmentation and evaluated by fluorescence microscopy. Briefly, cells were harvested, fixed in 4% paraformaldehyde for 10 min at room temperature and washed with PBS. Cell nuclei were stained with 0.5

μg/ml bis-benzimide trihydrochloride (Hoechst 33258, Molecular Probes, Eugene, OR). At least 200 cells were counted for each sample and percent apoptosis was calculated [(apoptotic nuclei) / (all nuclei) x 100]. The induction of apoptosis was analyzed using a Caspase-3 Assay Kit (Sigma), an *In Situ* Cell Death Detection (TUNEL) kit (Roche Applied Science, Indianapolis, IN) and by examination of caspase-3 processing and PARP cleavage by SDS-PAGE/immunoblot analysis.

Semi-Quantitative Reverse Transcription-PCR

Semi-quantitative reverse transcription-PCR was performed using the Qiagen (Valencia, CA) OneStep RT-PCR system according to the manufacturer's recommendations. The primers for Bim and GAPDH have been previously described (Wong *et al.*, 2005). The primers for Puma are 5'-GTGATCCGGACACGAAGACT-3' and 5'-GACTCTAAGTGCTGCTGGGC-3'. For quantification, Bim and Puma mRNA levels were normalized to GAPDH mRNA.

Chromatin Fractionation

Chromatin fractionation was carried out as described previously (Mendez and Stillman, 2000). Briefly, cells were washed twice with PBS and resuspended in buffer A (10 mM HEPES, pH 7, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT) containing protease inhibitors. Triton X-100 was added to a final concentration of 0.1% and the cells were incubated for 5 min on ice. The nuclei were collected by low-speed centrifugation (4 min, 1,500 x g, 4 ºC). The supernatant was clarified by high-speed centrifugation (15 min, 12,500 x g, 4 ºC) to remove insoluble

aggregates and the resulting lysate was designated the soluble fraction. The nuclei were washed once in buffer A and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT) with protease inhibitors for 10 min on ice. Insoluble chromatin was collected by centrifugation at 2,000 x g for 4 min at 4 ºC and washed once in buffer B. The final pellet was resuspended in 2X Laemmli buffer, boiled for 10 min and used as the chromatin fraction.

Subcellular Fractionation and Coimmunoprecipitation

Subcellular fractionation was performed as previously described (Wang *et al.*, 1996). Briefly, cells were washed with PBS then resuspended in hypotonic lysis buffer (5 mM Tris HCl, pH 7.5, 5 mM NaCl, 1.5 mM $MgCl_2$, 0. 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin A). After incubation on ice for 30 min, cells were homogenized using a Dounce homogenizer. Samples were spun down at 510 x g for 5 min at 4 °C. The supernatant (cytosolic fraction) was cleared by centrifugation at 720 x g for 5 min at 4 °C, while the pellet (nuclear fraction) was washed with hypotonic lysis buffer, centrifuged at 720 x g for 5 min at 4 °C, then lysed in radioimmunoprecipitation assay buffer.

Coimmunoprecipitation of Rad9 with Bcl-2 was performed as previously described with minor modifications (Yoshida *et al.*, 2002; Yoshida *et al.*, 2003). Briefly, whole cell lysate was prepared in 1% NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10μ g/ml pepstatin A). One milligram

of lysate was precleared by incubation with protein G agarose beads for 1 h at 4 °C. The precleared lysate was then incubated with anti-Bcl-2 (BD Pharmingen), or anti-Flag monoclonal antibody as negative control, overnight at 4 °C. The immunocomplexes were then incubated with protein G agarose beads for 1.5 h at 4 °C. After extensive washing in lysis buffer, the resulting immunocomplexes were subjected to SDS-PAGE/immunoblot analysis with anti-Rad9 polyclonal antibody. For coimmunoprecipitation analysis of the cytosolic fraction, subcellular fractionation was performed as described above, then NP-40, SDS and NaCl were added to a final concentration of 1%, 0.1% and 150 mM, respectively. Coimmunoprecipitation was performed as described above.

Immunofluorescence

Hus1$^{+/+}$*p21* $^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEF cells were grown on gelatin-coated glass coverslips. After treatment with etoposide, cells were washed once with PBS and fixed in 4% paraformaldehyde for 20 min at 4 °C. Cells were then washed three times with PBS and permeabilized in 0.5% Triton X-100 plus 1% normal goat serum (NGS) for 30 min at room temperature. After four washes with PBS, the cells were blocked in 5% milk/ 3% BSA/ 1% NGS for several hours at 4 °C. The cells were then incubated in primary antibody in blocking solution overnight at 4 °C. After several washes with PBS, cells were incubated in blocking solution for 30 min at 37 °C, then in FITC-conjugated goat anti-rabbit secondary antibody for one hour at 37 °C. The cells were washed several times with PBS before the addition of mounting media containing DAPI (4', 6'-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA). The fluorescent images were analyzed using an automated Zeiss Axiovert fluorescence microscope.

Chapter Three: Loss of *Hus1* Enhances DNA Damage-Induced Caspase-Independent Cell Death and Autophagy

Abstract

The Rad9-Rad1-Hus1 (9-1-1) complex plays a central role in the decision of whether a cell should survive or undergo cell death in response to DNA damage. Although it has been shown that disruption of the 9-1-1 complex sensitizes cells to certain genotoxic stresses, the precise mechanisms that are responsible for enhanced cell death are not fully understood. We have recently described the mechanism behind the sensitivity of *Hus1*-deficient cells to etoposide-induced apoptosis. Here, we provide evidence that loss of *Hus1* also sensitizes cells to caspase-independent cell death. Treatment with the pan-caspase inhibitor, Z-VAD-FMK, only moderately inhibited camptothecin-induced cell death, suggesting that *Hus1*-knockout cells die through a caspase-independent mechanism in response to camptothecin-induced DNA damage. Moreover, we found that loss of *Hus1* enhances LC3 foci formation and modification, indicating that disruption of the 9-1-1 complex enhances DNA damage-induced autophagy. Interestingly, inhibition of autophagy, by knockdown of Atg7 or Bif-1, does not suppress, but rather promotes, camptothecin-induced cell death. Taken together, these results suggest that the 9-1-1 complex plays a key role in the regulation of both caspase-dependent and caspase-independent cell death in response to DNA damage and that autophagy is induced as a survival mechanism when this complex is disrupted.

Results

Loss of Hus1 Enhances Caspase-Independent Cell Death in Response to DNA Damage

As mentioned above, we have found that disruption of a functional 9-1-1 complex, through loss of *Hus1*, sensitizes cells to DNA damage-induced cell death (Meyerkord *et al.*, 2008). Treatment of *Hus1*-deficient cells with DNA damaging agents promotes the upregulation of the BH3-only proteins, Bim and Puma, and enhances the interaction of Rad9 with Bcl-2 thereby inducing the activation of caspase-3 and apoptosis (Meyerkord *et al.*, 2008). In order to determine if apoptosis is the only mechanism that sensitizes *Hus1*-deficient cells to DNA damage-induced cell death, $Hus1^{+/+}p21^{-/-}$ and $Hus1^{-/-}p21^{-/-}$ MEFs were treated with camptothecin in the presence or absence of the pan-caspase inhibitor, Z-VAD-FMK. Consistent with a previous report (Wang *et al.*, 2004b), knockout of *Hus1* greatly enhanced the susceptibility of MEFs to camptothecin-induced cell death (Figure 37). Moreover, the induction of caspase-3 activity observed in *Hus1*-deficient cells was greater than that in *Hus1*-wild-type cells (Figure 38). Whereas caspase-3 was activated in a time-dependent manner in *Hus1*-wild-type cells, caspase-3 activity increased until the 24 h time point in *Hus1*-deficient cells, after which, it decreased presumably due to cell death. Importantly, treatment with Z-VAD-FMK completely inhibited the induction of caspase-3 activity, regardless of *Hus1* status (Figure 38). Interestingly, the DNA damage-induced cell death observed in *Hus1*-deficient MEFs was only slightly suppressed by addition of Z-VAD-FMK (Figure 37), suggesting that an alternate cell death mechanism is activated when apoptosis is inhibited.

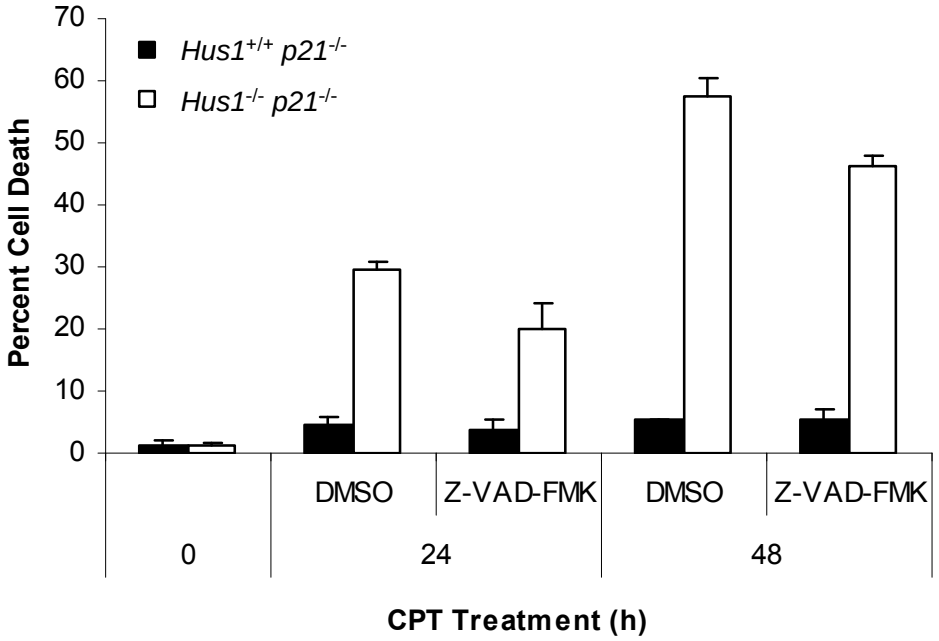

Figure 37. Camptothecin-induced cell death is moderately inhibited by Z-VAD-FMK. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 1 μM camptothecin (CPT) for 24 or 48 h in the presence or absence of 50 μM Z-VAD-FMK. At the indicated times, cells were harvested and cell death was determined by trypan blue exclusion assay (mean ± s.d.; n=3).

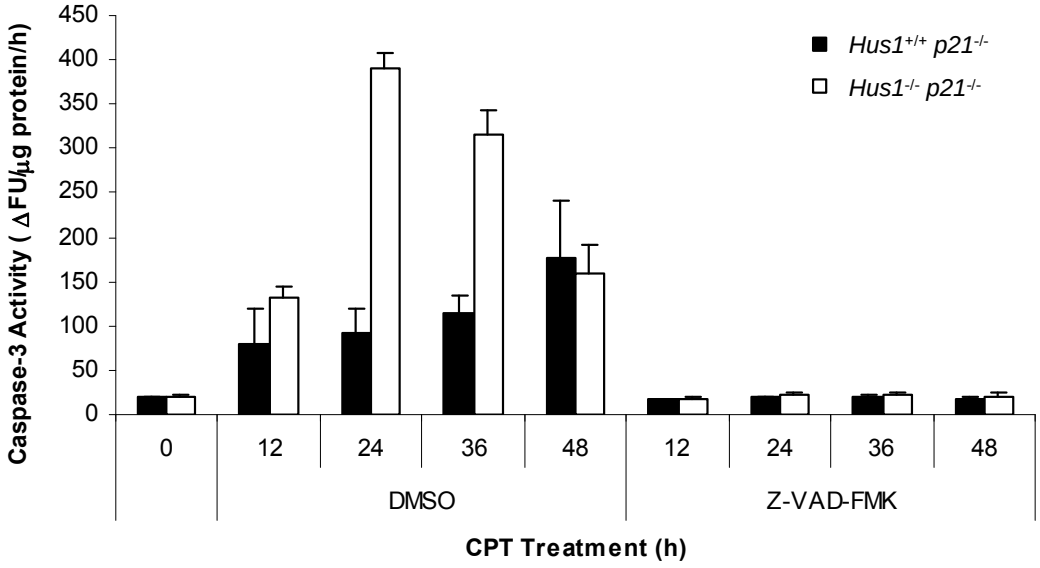

Figure 38. Camptothecin-induced caspase-3 activity is abrogated by Z-VAD-FMK. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 1 μM camptothecin (CPT) for the times indicated in the presence or absence of 50 μM Z-VAD-FMK. At the indicated times, cells were harvested and the induction of apoptosis was measured by caspase-3 activity assay (mean ± s.d.; n=3).

Loss of Hus1 Enhances DNA Damage-Induced Autophagy

It has been shown that cells that are resistant to apoptosis initiated through the mitochondrial pathway, such as those that lack both *Bax* and *Bak* (Wei *et al.*, 2001; Zong *et al.*, 2001), undergo caspase-independent, but autophagy-dependent cell death in response to DNA damage (Shimizu *et al.*, 2004). Autophagy is an evolutionarily conserved intracellular process for the bulk degradation of cytoplasmic components that is initiated in response to environmental changes (Levine and Klionsky, 2004; Yoshimori, 2004). Although autophagy is generally thought to play a cytoprotective role by recycling nutrients under starvation conditions and preventing the accumulation of damaged organelles, excessive autophagy could result in the overconsumption of functional proteins and organelles, thus leading to cell death (Levine and Yuan, 2005; Tsujimoto and Shimizu, 2005). In order to examine whether autophagy is involved in the enhanced caspase-independent cell death observed in *Hus1*-deficient cells, we established $Hus1^{+/+}p21^{-/-}$ and $Hus1^{-/-}p21^{-/-}$ MEFs that stably expressed GFP-LC3, a well characterized marker for autophagy that is used to visualize autophagosomes (Kabeya *et al.*, 2000). In control DMSO-treated cells, GFP-LC3 was mostly located diffusely throughout the cell; although, the basal level of GFP-LC3 foci formation was higher in *Hus1*-deficient cells (Figure 39). Treatment with rapamycin, which inhibits mTOR to induce autophagy (Noda and Ohsumi, 1998), resulted in the formation of GFP-LC3 foci in both *Hus1*-wild-type and *Hus1*-deficient MEFs, indicating that both of these cell lines are capable of inducing autophagy. Interestingly, in response to treatment with either etoposide or camptothecin, GFP-LC3 foci formation was greatly induced in *Hus1*-deficient cells, while relatively few foci were observed in *Hus1*-wild-type cells (Figure 39). These results suggest that

autophagy is induced in response to DNA damage and that the level of DNA damage-induced autophagy is enhanced by loss of *Hus1*. In order to confirm these results, we next examined the effect of *Hus1*-deficiency on LC3 modification. During the induction of autophagy, LC3 is processed from a cytosolic form, LC3-I, to the phosphatidylethanolamine (PE)-conjugated, membrane-bound form, LC3-II (Kabeya *et al.*, 2000; Klionsky *et al.*, 2008). Consistent with the results from the analysis of GFP-LC3 foci formation, an accumulation of LC3-II was observed in response to etoposide treatment (Figure 40), indicating that autophagy is indeed being induced in response to DNA damage. Moreover, the modification of LC3 was much greater in *Hus1*-deficient cells, as compared to *Hus1*-wild-type cells (Figure 40). Taken together, these results indicate that loss of a functional 9-1-1 complex promotes the induction of DNA damage-induced autophagy.

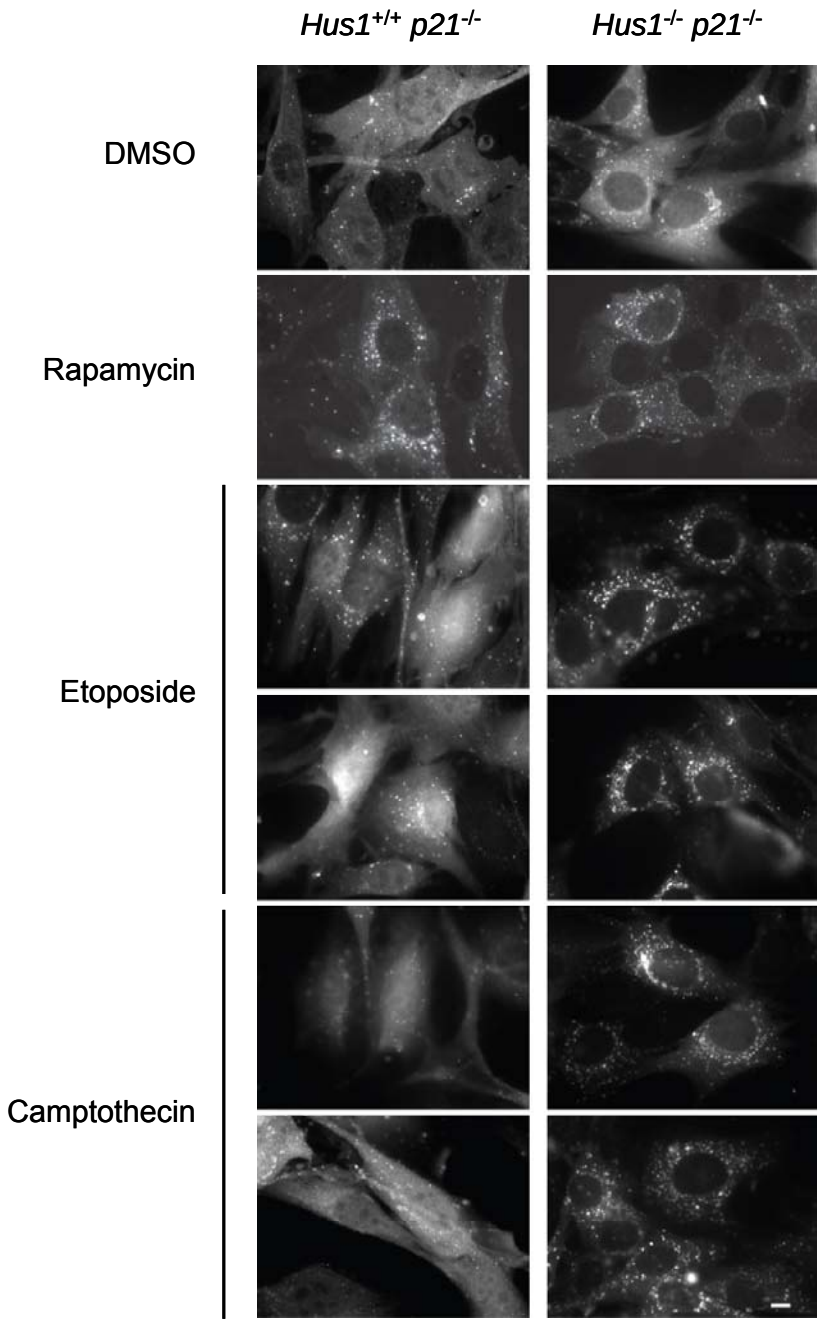

Figure 39. Loss of *Hus1* enhances DNA damage-induced GFP-LC3 foci formation. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing GFP-LC3 were treated with 12.5 μg/ml etoposide or 500 nM camptothecin for 12 h or 500 nM rapamycin for 3 h. The localization of GFP-LC3 was examined by fluorescence microscopy.

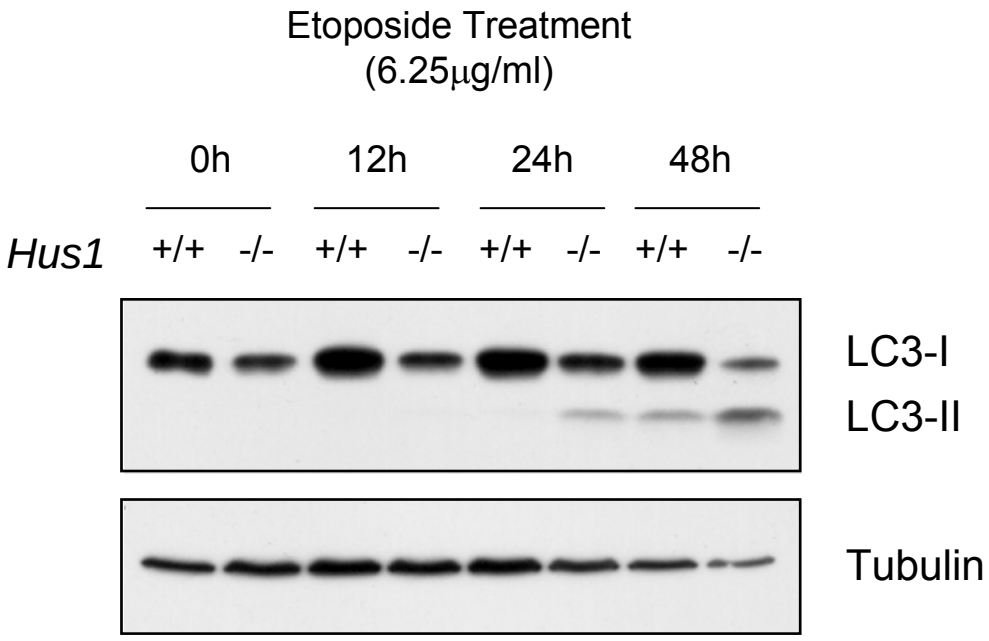

Figure 40. DNA damage-induced LC3 modification is enhanced by loss of *Hus1*. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were treated with 6.25 μg/ml etoposide for the times indicated. Total cell lysate was prepared and the modification of LC3 was examined by SDS-PAGE/immunoblot analysis.

Autophagy Plays a Cytoprotective Role in Response to DNA Damage

As loss of *Hus1* resulted in enhanced autophagy in response to DNA damage, we next investigated whether autophagy is responsible for sensitizing *Hus1*-deficient cells to caspase-independent cell death induced by camptothecin treatment. To this end, *Hus1*$^{+/+}$*p21* $^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs were infected with lentivirus expressing shRNA targeting Atg7, Bif-1 or a control scrambled shRNA. Both Atg7 and Bif-1 are required for autophagosome formation and are thus key regulators of the induction of autophagy. While Atg7 acts as the E1-like enzyme that mediates both of the ubiquitin-like conjugation systems (Tanida *et al.*, 1999), Bif-1 binds to UVRAG to regulate the activation of PI3KC3 (Takahashi *et al.*, 2007). As shown in Figure 41, the expression of Atg7 and Bif-1 was efficiently knocked down in cells stably expressing shAtg7 or shBif-

1, respectively. Consistent with etoposide treatment (Figure 40), LC3 modification was enhanced in shScrambled-expressing cells after camptothecin treatment (Figure 41). Similar to a previous study (Komatsu *et al.*, 2005), loss of Atg7 expression completely abolished the induction of autophagy, as determined by modification of LC3-I to LC3-II (Figure 41). Moreover, knockdown of Bif-1 significantly suppressed LC3 modification (Figure 41), as previously reported (Takahashi *et al.*, 2007). Consistently, in response to camptothecin treatment a greater number of LC3 foci were observed in *Hus1*-deficient cells expressing shScrambled (Figure 42). Notably, LC3 foci formation was suppressed by knockdown of either Atg7 or Bif-1 (Figure 42), suggesting that the initiation of autophagy is indeed being inhibited in these cells. To determine whether the induction of autophagy results in cell death in response to DNA damage, we treated the shBif-1, shAtg7 and control shScrambled-expressing cells with camptothecin and examined cell death as determined by trypan blue exclusion. Surprisingly, inhibition of autophagy, by suppression of either Atg7 or Bif-1, significantly enhanced cell death when compared to shScrambled-expressing control cells (Figure 43). These results suggest that in response to treatment with DNA damaging agents, autophagy is induced to promote cell survival, rather than cell death.

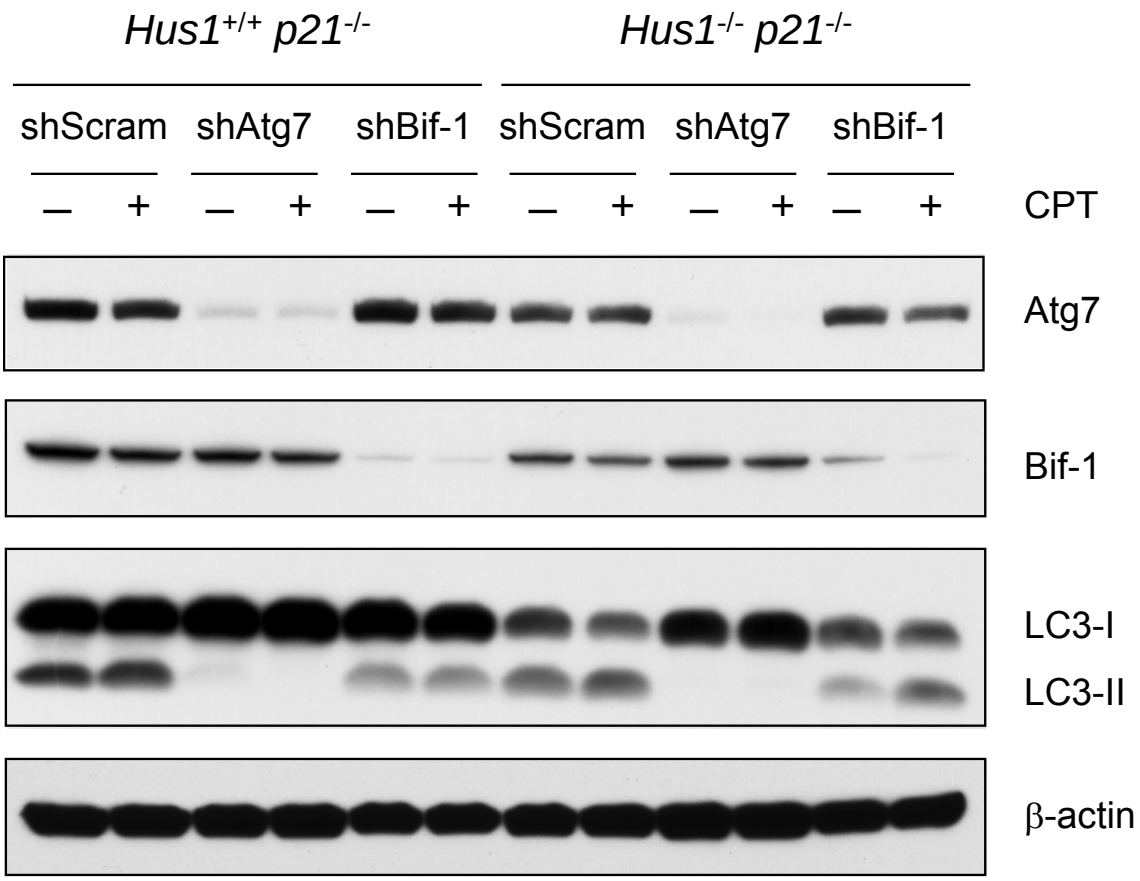

Figure 41. Knockdown of Atg7 or Bif-1 suppresses LC3 modification. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shAtg7, shBif-1 or a control scrambled shRNA (shScram) were treated with treated with 1 μM camptothecin (CPT) for 36 h. Total cell lysate was prepared and LC3 modification was examined by SDS-PAGE/immunoblot analysis.

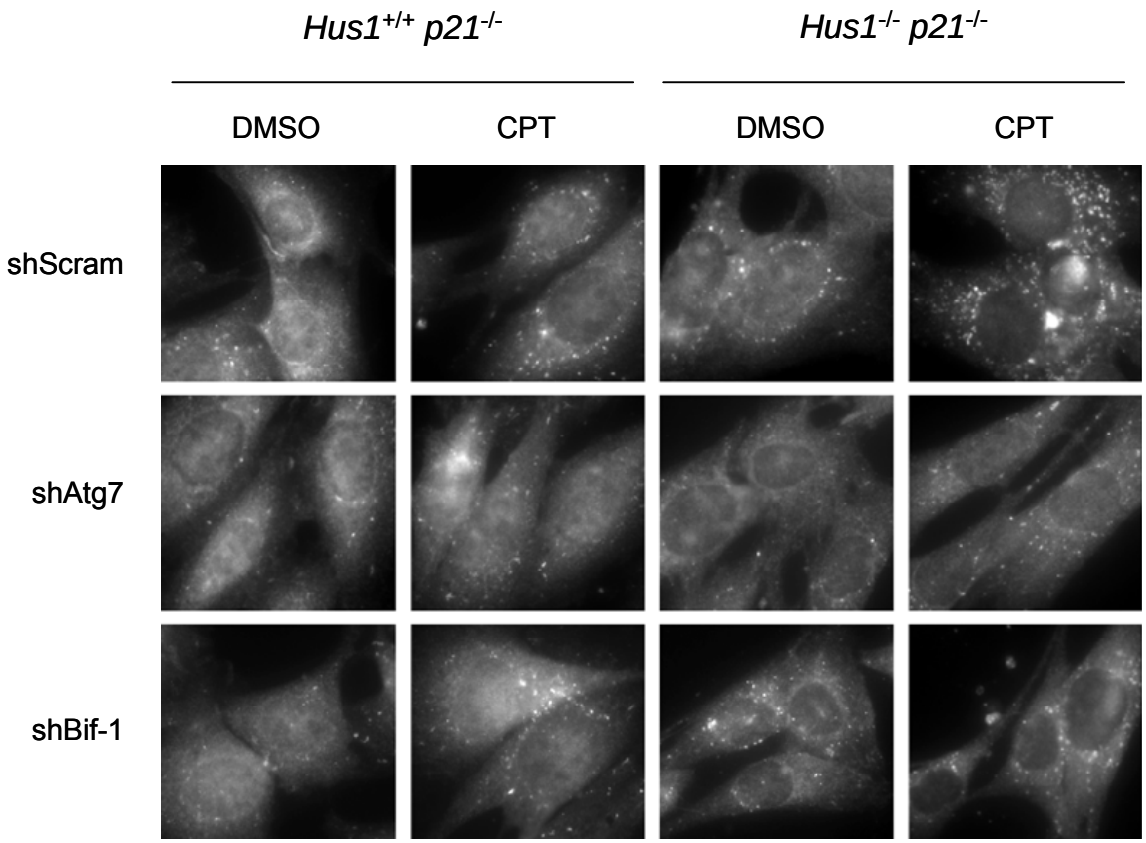

Figure 42. Knockdown of Atg7 or Bif-1 suppresses DNA damage-induced LC3 foci formation. *Hus1*$^{+/+}$ *p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shAtg7, shBif-1 or a control scrambled shRNA (shScram) were treated with 500 nM camptothecin (CPT) for 12 h. Immunocytochemistry was used to examine endogenous LC3 localization.

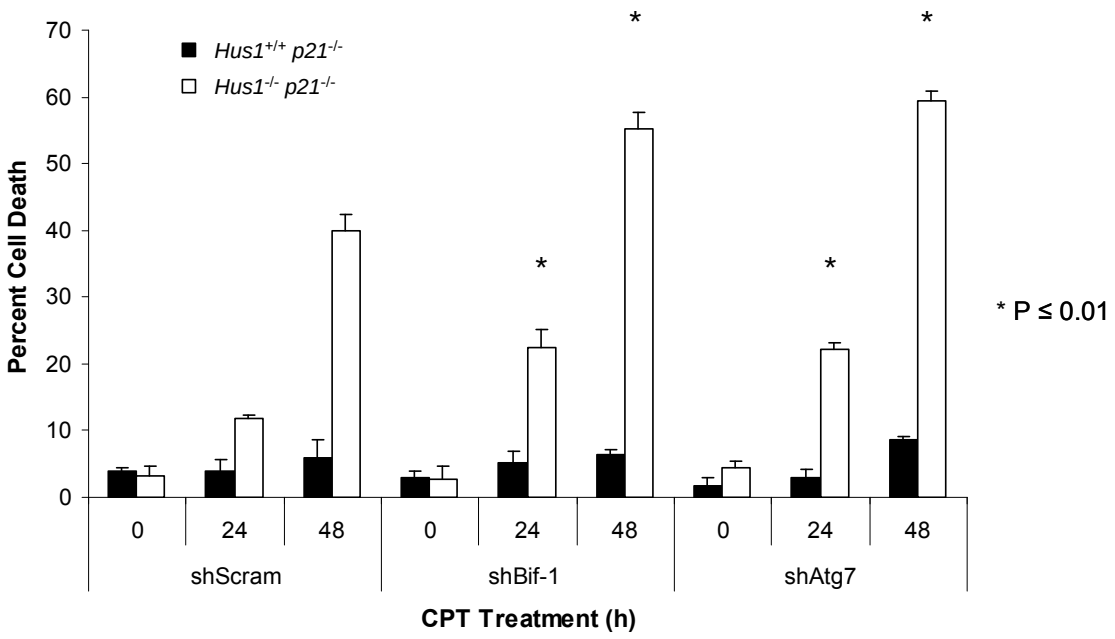

Figure 43. Inhibition of autophagy results in enhanced cell death in response to camptothecin treatment. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing shAtg7, shBif-1 or a control scrambled shRNA (shScram) were treated with 1 μM camptothecin (CPT) for the times indicated. Cell death was determined by trypan blue exclusion assay (mean ± s.d.; n=3).

The BH3 Mimetic, ABT-737, Does Not Significantly Induce Autophagy

As described above, BH3-only proteins can regulate autophagy by binding to Bcl-2 thereby releasing Beclin 1 to induce autophagosome formation and thus the initiation of autophagy (Liang *et al.*, 1999; Maiuri *et al.*, 2007a). We have shown that the expression of the BH3-only proteins, Bim and Puma, are dramatically upregulated in *Hus1*-deficient cells in response to camptothecin treatment (Meyerkord *et al.*, 2008). In order to determine whether the upregulation of these BH3-only proteins is the underlying mechanism behind the enhanced autophagy seen in *Hus1*-deficient cells, we treated *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing GFP-LC3 with the pharmacological BH3-mimetic, ABT-737. ABT-737 binds to Bcl-2 and Bcl-xL and

mimics BH3-only protein binding (Oltersdorf *et al.*, 2005), thus releasing Beclin 1 to induce autophagy (Maiuri *et al.*, 2007a). Therefore, if the excess binding of BH3-only proteins to Bcl-2-like proteins is the underlying mechanism of enhanced autophagy induction in *Hus1*-deficient cells, ABT-737 should promote GFP-LC3 foci formation in camptothecin-treated *Hus1*-wild-type cells, such that the GFP-LC3 foci formation in these cells would be similar to that of *Hus1*-deficient cells treated with camptothecin alone. However, co-treatment of ABT-737 and camptothecin resulted in only a slight induction of autophagy in *Hus1*-wild-type cells, indicating that upregulation of BH3-only proteins is not the main mechanism responsible for DNA damage-induced autophagy in these cells (Figure 44).

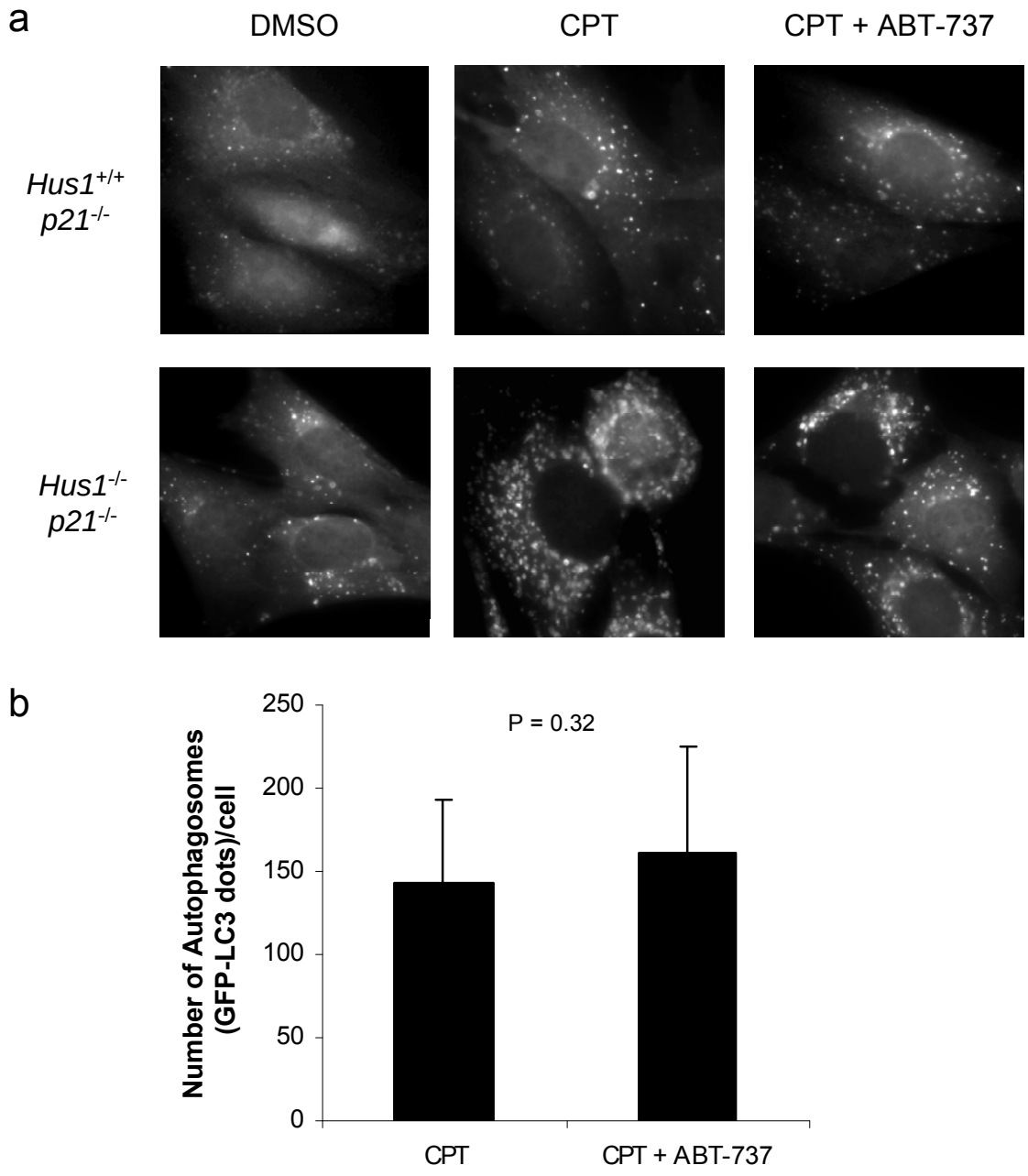

Figure 44. ABT-737 does not significantly enhance GFP-LC3 foci formation. *Hus1*$^{+/+}$*p21*$^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs stably expressing GFP-LC3 were treated with 500 nM camptothecin (CPT) in the presence or absence of 10 μM ABT-737 for 12 h. (a) The localization of GFP-LC3 was examined by fluorescence microscopy. (b) The number of GFP-LC3 dots per *Hus1*$^{+/+}$ *p21*$^{-/-}$ cell was determined by analyzing images from a fluorescent microscope.

Discussion

In the present study, we have demonstrated that the 9-1-1 complex plays an integral role in the regulation of autophagy and cell death induced by treatment with DNA damaging agents. Co-treatment of Z-VAD-FMK with camptothecin resulted in only a moderate inhibition of cell death, regardless of *Hus1* status. These results suggest that DNA damage not only promotes apoptosis, as mentioned previously (Meyerkord *et al.*, 2008), but also induces a caspase-independent form of cell death when caspase activity is inhibited. Notably, a greater induction of cell death was observed in camptothecin-treated *Hus1*-deficient MEFs as compared to *Hus1*-wild-type MEFs, even in the presence of Z-VAD-FMK, indicating that the 9-1-1 complex may play a role in the suppression of both caspase-dependent and caspase-independent cell death induced by DNA damage.

In addition to the role of the 9-1-1 complex in the suppression of DNA damage-induced cell death, we have also found that the 9-1-1 complex is responsible for the suppression of autophagy. DNA damage-induced autophagosome formation was enhanced when the 9-1-1 complex was disrupted through loss of *Hus1*. Notably, loss of *Hus1* enhanced the level of autophagosome formation, even in the absence of DNA damage. Interestingly, it has been shown that *Hus1*-deficient MEFs have an increased frequency of spontaneous chromosomal abnormalities and increased expression of DNA damage-responsive genes (Levitt *et al.*, 2007; Weiss *et al.*, 2000; Zhu and Weiss, 2007). Therefore, one possible explanation for the enhanced autophagy observed in these cells is to dispose of mitochondria that have been damaged through activation of the DDR. Indeed, it has been shown that DNA damage-induced autophagy is involved in the early

removal of damaged mitochondria (Abedin *et al.*, 2007). Consistent with previous reports (Abedin *et al.*, 2007; Kanzawa *et al.*, 2004; Katayama *et al.*, 2007; Paglin *et al.*, 2001), we found that autophagy can be induced after exposure to DNA damaging agents. Furthermore, while both *Hus1*-wild-type and *Hus1*-deficient cells are capable of inducing autophagy, DNA damage-induced autophagy was enhanced in cells lacking *Hus1*. It has also been shown that inhibition of DNA-PK, which plays a major role in the repair of IR-induced double-strand DNA breaks, sensitizes cells to IR through the induction of autophagy (Daido *et al.*, 2005). Taken together, these results suggest that loss of either DNA repair machinery or a functional DDR can sensitize cells to DNA damage-induced autophagy. Alternatively, loss of *Hus1* could affect the induction of autophagy by disrupting signaling upstream of Akt and mTOR. Akt phosphorylation of mTOR results in the suppression of autophagy induction (Kondo *et al.*, 2005). In addition, it has been reported that knockdown of ATR prevents Akt activation in response to genotoxic stress (Caporali *et al.*, 2008). Moreover, Hus1 has been shown to be required for signaling downstream of ATR (Weiss *et al.*, 2002), suggesting that loss of *Hus1* may also disrupt signaling pathways that are required for the suppression of autophagy. Furthermore, inhibition of mTOR, by treatment with rapamycin, induced similar levels of autophagy in both *Hus1*-wild-type and *Hus1*-deficient cells. Taken together, these results indicate that signaling downstream of Akt and mTOR is important for the suppression of autophagy and that disruption of this signaling, by loss of *Hus1*, could promote autophagy. While the precise mechanism linking the 9-1-1 complex to the autophagic machinery has yet to be determined, our results suggest that this may occur independent of the upregulation of

BH3-only proteins in *Hus1*-deficient cells, which conversely was shown to be required for DNA damage-induced apoptosis (Meyerkord *et al.*, 2008).

Evidence from various studies suggests that autophagy plays a dual role in cell survival and cell death (Baehrecke, 2005; Tsujimoto and Shimizu, 2005). Indeed, it has been shown that autophagy is induced in *Bax*/*Bak*$^{-/-}$ cells after treatment with etoposide (Shimizu *et al.*, 2004) and in L929 fibroblast cells treated with Z-VAD-FMK (Yu *et al.*, 2004). Furthermore, the induction of autophagy observed in these cells is required for cell death (Shimizu *et al.*, 2004; Yu *et al.*, 2004). In contrast, we found that inhibition of autophagy, through knockdown of Atg7 or Bif-1, resulted in enhanced cell death in response to treatment with camptothecin, suggesting that autophagy is induced as a cytoprotective mechanism in these cells in response to DNA damage. Therefore, it is possible that autophagy may function as an alternate cell death mechanism only when the apoptotic pathway is inhibited, such as through loss of *Bax*/*Bak* or by treatment with Z-VAD-FMK. However, if the apoptotic machinery is intact, such as in shAtg7- and shBif-1-expressing *Hus1*$^{-/-}$*p21*$^{-/-}$ cells, DNA damage-induced autophagy may play a pro-survival role. Accumulating evidence now suggests that this may indeed be the case, that autophagy may act primarily as a survival mechanism by which the cell rids itself of potentially harmful constituents in order to maintain cellular homeostasis (Kroemer and Levine, 2008; Levine and Kroemer, 2009). Further studies, in which *Hus1*$^{-/-}$*p21*$^{-/-}$ cells expressing shAtg7 or shBif-1 are treated with camptothecin in the presence or absence of Z-VAD-FMK, will be required to determine whether DNA damage-induced autophagy acts as a cell death or survival mechanism in apoptosis-impaired cells.

Materials and Methods

Reagents

Etoposide, camptothecin, digitonin, protease inhibitor cocktail and phosphatase inhibitor cocktails I and II were purchased from Sigma (St. Louis, MO). Z-VAD-FMK and rapamycin were purchased from Alexis Biochemicals (San Diego, CA). Puromycin was purchased from Calbiochem (San Diego, CA). Trypan blue and culture medium were purchased from Invitrogen (Carlsbad, CA). ABT-737 was a kind gift from Abbott Laboratories. Antibodies were purchased from the following commercial sources: anti-tubulin, anti-β-actin and FITC-conjugated goat anti-rabbit secondary antibody from Sigma, anti-LC3 from Novus Biologicals (Littleton, CO) and MBL International (Naka-ku Nagoya, Japan), anti-Bif-1 from GeneTex (San Antonio, TX), bovine anti-goat and bovine anti-mouse IgG-horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA) and goat anti-rabbit IgG-horseradish peroxidase from Amersham Biosciences (Piscataway, NJ). The anti-Atg7 antibody was a generous gift from Dr. Isei Tanida (Tanida *et al.*, 1999).

Cell Culture, Transfection and Infection

Hus1$^{+/+}$*p21* $^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEFs (Weiss *et al.*, 2000) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1.0 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 100 μg/ml streptomycin and 100 U/ml penicillin. To generate the GFP-LC3 expression vector, cDNA encoding a GFP-LC3 fusion protein was subcloned into the *Bgl* II-*Eco*R I site of the pK1-IRES-puro

vector. Amphotrophic 293T cells were transfected with the GFP-LC3 vector. The resulting recombinant retrovirus was used to infect *Hus1*$^{+/+}$*p21* $^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ cells.

The pLKO.1-based lentiviral shRNAs targeting Atg7 (TRCN0000007586) and Bif-1 (TRCN0000093178) were purchased from Open Biosystems (Huntsville, AL). The pLKO.1-based scrambled control shRNA vector was purchased from Sigma. The appropriate shRNA plasmid, along with the ViraPower Packaging Mix (Invitrogen), was co-transfected into 293FT cells to produce recombinant lentivirus. The supernatant containing the shRNA-expressing lentivirus was used to infect *Hus1*$^{+/+}$*p21* $^{-/-}$ and *Hus1*$^{-/-}$ *p21*$^{-/-}$ cells MEFs according to the manufacturer's protocol.

Analysis of Cell Death and Apoptosis

Cell death was assessed by trypan blue exclusion assay. The induction of apoptosis was analyzed using a Caspase-3 Assay Kit (Sigma) according to the manufacturer's protocol.

Analysis of LC3 Localization

For analysis of GFP-LC3 localization, *Hus1*$^{+/+}$*p21* $^{-/-}$ and *Hus1*$^{-/-}$*p21*$^{-/-}$ MEF cells were grown on gelatin-coated chamber slides. After treatment, cells were washed once with PBS, then fixed in 3.7% formaldehyde for 7 min at room temperature. The cells were washed three times with PBS before being mounted with media containing DAPI (4', 6'-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA). The fluorescent images were obtained using an automated Zeiss Axiovert fluorescence microscope.

To examine the localization of endogenous LC3, cells were washed once with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were then washed three times with PBS and permeabilized in 100 μg/ml digitonin for 15 min at room temperature. After three washes with PBS, the cells were blocked in 3% BSA for one hour at room temperature. Cells were then incubated in primary antibody in blocking solution overnight at 4 °C. After three washes with PBS, cells were incubated in 3% BSA blocking solution for 30 min, then in FITC-conjugated goat anti-rabbit secondary antibody for three hours at room temperature. The cells were washed three times with PBS before the addition of mounting media containing DAPI. The fluorescent images were analyzed using an automated Zeiss Axiovert fluorescence microscope.

Analysis of LC3 Modification

Modification of LC3 was analyzed by SDS-PAGE/immunoblot. After treatment, cells were harvested, washed once in ice-cold PBS, then lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris-HCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate and 5 mM EDTA pH 8.0) containing protease and phosphatase inhibitor cocktails. Total cell lysate was subjected to SDS-PAGE/immunoblot analysis with anti-LC3 antibody.

Chapter Four: Knockdown of Bif-1 Accelerates Endocytic Vesicle Trafficking and Enhances EGFR Degradation

Abstract

The Endophiln proteins are well known regulators of intracellular membrane dynamics. While the members of the Endophilin A subfamily regulate the formation of endocytic vesicles at the plasma membrane, members of the Endophilin B subfamily are involved in regulating the membrane dynamics of organelles, such as the Golgi complex, mitochondria and autophagosomes. While the mechanisms by which Bif-1/Endophilin B1 regulates membrane dynamics are well studied, the role of Bif-1 in endocytic trafficking is not well defined. In this study, we report that knockdown of Bif-1 expression does not affect the uptake of a fluid phase marker, horseradish peroxidase, or the internalization of EGF. However, loss of Bif-1 results in the premature localization of EGF to late endosomes/lysosomes. Moreover, knockdown of Bif-1 accelerates the degradation of EGFR in response to EGF stimulation. We found that EGFR degradation is regulated by both the lysosomal and proteasomal pathways in Bif-1-knockdown cells. Taken together, these results identify Bif-1 as a novel regulator of endocytic vesicle trafficking and receptor degradation.

Results

Knockdown of Bif-1Does Not Affect Internalization of Endocytic Cargo, but Accelerates EGF Co-Localization with Late Endosomes/Lysosomes

It has been shown that Bif-1 can interact with Beclin 1 through UVRAG to promote the activation of PI3KC3/Vps34 and the induction of autophagy (Takahashi *et al.*, 2007). The activation of Vps34 plays an essential role not only in the regulation of autophagy, but also in vesicle transport, including endocytic trafficking (Backer, 2008). As loss of Bif-1 significantly reduces the activity of Vps34, we investigated whether Bif-1 also plays a role the regulation of endocytic trafficking. To determine whether loss of Bif-1 would affect the integrity of the early endocytic pathway, we first examined the effect of knockdown of Bif-1 expression on the uptake of a fluid phase marker, horseradish peroxidase (HRP). As shown in Figure 45, knockdown of Bif-1 did not affect the kinetics of HRP uptake, suggesting that Bif-1 is not involved in the regulation of the early endocytic pathway and HRP internalization.

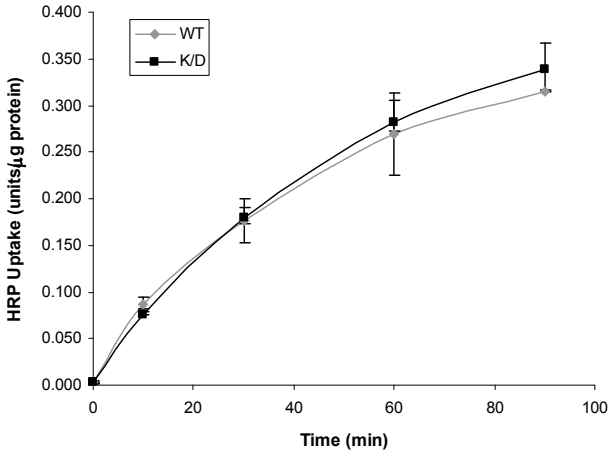

Figure 45. Knockdown of Bif-1 does not affect the internalization of a fluid phase marker. Wild-type (WT) and Bif-1-knockdown (K/D) HeLa cells were incubated in uptake media for the times indicated. HRP activity was measured using a 1-Step Turbo TMB-ELISA kit. Enzyme activity was normalized to protein concentration (mean ± s.d.; *n*=3).

In order to directly investigate the role of Bif-1 in endocytic trafficking, we examined EGF internalization and endocytic transport to lysosomes. In response to serum starvation, the EGFR accumulates on the cell surface. After stimulation with EGF, the EGFR is rapidly activated by phosphorylation of the C-terminal cytoplasmic domain. The EGF-EGFR complex is then internalized, sorted into multivesicular bodies (MVBs), delivered to lysosomes and degraded, which efficiently downregulates EGFR signaling pathways (Katzmann *et al.*, 2002). Therefore, disruptions in the endocytic pathway can be identified by changes in the localization of the EGF-EGFR complex. As shown in Figure 46, similar amounts of fluorescently conjugated EGF were initially bound to the plasma membranes of Bif-1-knockdown cells and control wild-type HeLa cells, indicating that EGF-receptor binding was not affected by loss of Bif-1. Fifteen minutes after stimulation the EGF signal formed small foci that were located throughout wild-type cells. In contrast, the EGF signal accumulated in large aggregates and partially co-localized with LAMP-1, a marker of late endosomes/lysosomes, in Bif-1-knockdown cells after 15 min of EGF stimulation. Notably, the difference in EGF localization between Bif-1-knockdown cells and control wild-type cells was more pronounced at 30 min after stimulation. In wild-type cells the EGF signal formed larger foci and began to co-localize with LAMP-1, whereas the EGF signal was decreased in Bif-1-knockdown cells, suggesting that the degradation of EGF is accelerated by loss of Bif-1. To determine if the observed effect of loss of Bif-1 on the endocytic system was due to the inhibition of autophagy, through knockdown of Bif-1, we established HeLa cells that stably expressed shRNA targeting Atg5 or a control scrambled shRNA. Atg5 plays a role in one of the ubiquitin-like conjugation systems and is thus required for autophagosome

formation and the induction of autophagy (Mizushima *et al.*, 2001). Importantly, knockdown of Atg5 did not result in an appreciable difference in the localization of EGF or co-localization with LAMP-1 at any of the times examined (Figure 47), indicating that inhibition of autophagy does not affect the endocytic trafficking and degradation of EGF. Taken together, these results describe a novel role for Bif-1 in the regulation of EGF trafficking to late endosomes/lysosomes through an autophagy-independent mechanism.

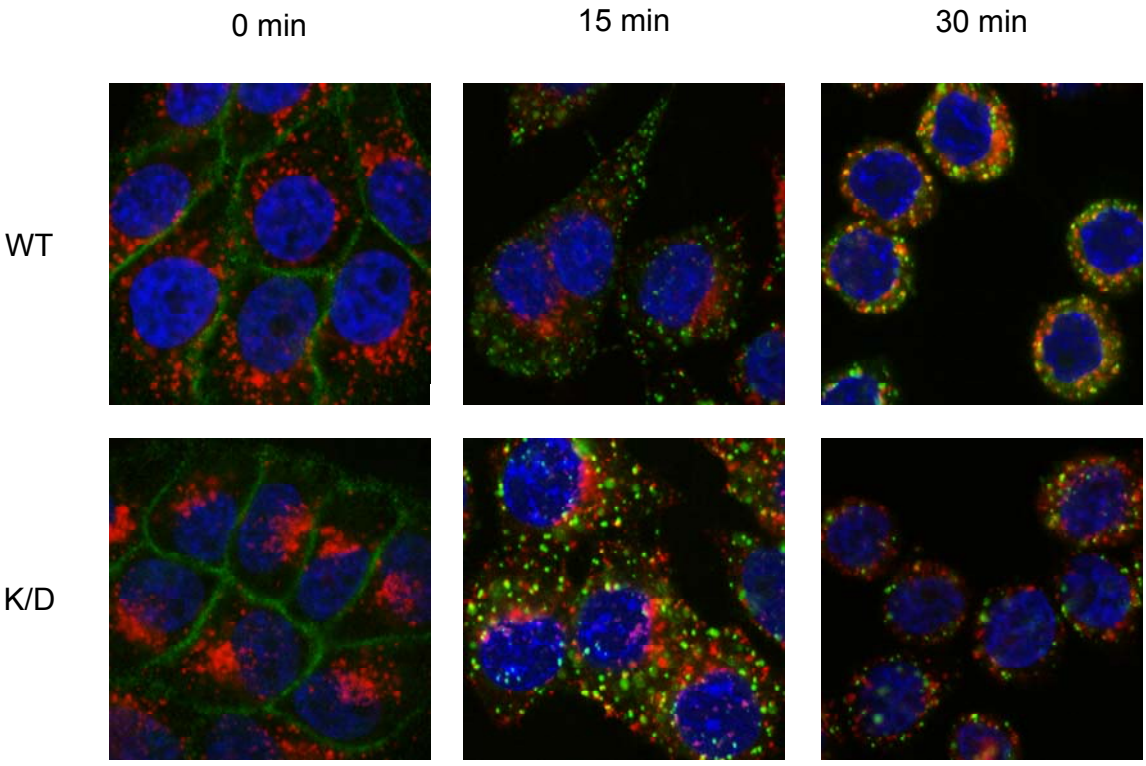

Figure 46. Knockdown of Bif-1 accelerates the co-localization of EGF with LAMP-1-positive vesicles. The localization of Alexa Fluor 488-EGF (green) in wild-type (WT) and Bif-1-knockdown (K/D) HeLa cells was detected by confocal microscopy. At the times indicated, the cells were fixed, permeabilized, stained for LAMP-1 (red) and mounted in media containing DAPI (blue). All images were taken at the same exposure setting for the 488-EGF signal.

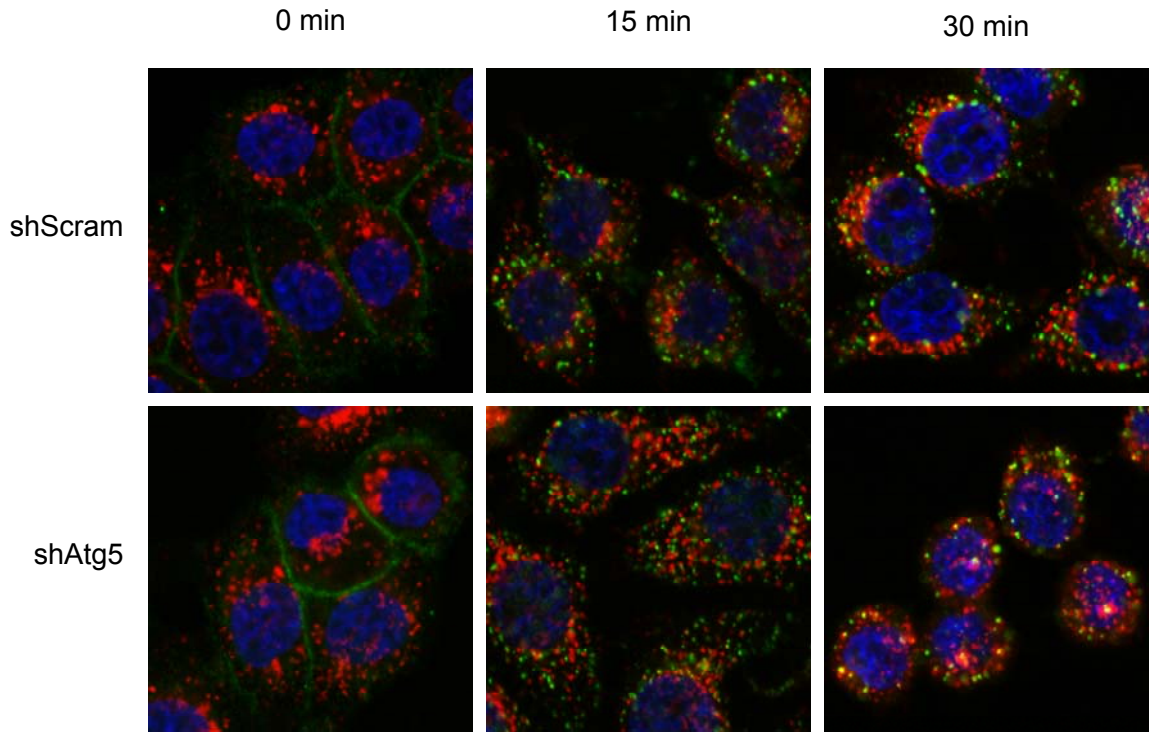

Figure 47. Knockdown of Atg5 does not affect EGF localization. The localization of Alexa Fluor 488-EGF (green) in control shScrambled (shScram) or shAtg5-expressing HeLa cells was monitored by confocal microscopy. At the times indicated, the cells were fixed, permeabilized, stained for LAMP-1 (red) and mounted in media containing DAPI (blue). All images were taken at the same exposure setting for the 488-EGF signal.

Knockdown of Bif-1 Promotes EGFR Degradation

Since loss of Bif-1 accelerated the co-localization of EGF to late endosomes/lysosomes, we next investigated the effect of loss of Bif-1 on the degradation of EGFR. To this end, we examined the expression of total and phosphorylated EGFR in response to stimulation with EGF. Indeed, the degradation of EGFR was enhanced by knockdown of Bif-1 (Figures 48 and 49). Moreover, the rate of degradation of activated EFGR was markedly accelerated in Bif-1-knockdown cells (Figures 48 and 50), suggesting that upon EGF stimulation, EGFR-mediated signaling is downregulated more

rapidly in cells lacking Bif-1. Interestingly, the expression of EGFR before stimulation with EGF was lower in Bif-1-knockdown cells as compared to control wild-type cells, suggesting that loss of Bif-1 also affects the stability or turnover of EGFR. Notably, knockdown of Atg5 did not affect the degradation of total or phosphorylated EGFR (Figure 51), which is consistent with the EGF localization data (Figure 47). In support of our results, a recent study has shown that loss of Bif-1 resulted in the premature targeting of the TrkA receptor to late endosomes and lysosomes, which resulted in the accelerated the degradation of TrkA (Wan *et al.*, 2008). Taken together, these results suggest that Bif-1 not only plays a role in autophagy, as previously described, but that Bif-1 also regulates endocytic vesicle trafficking and the degradation of EGFR.

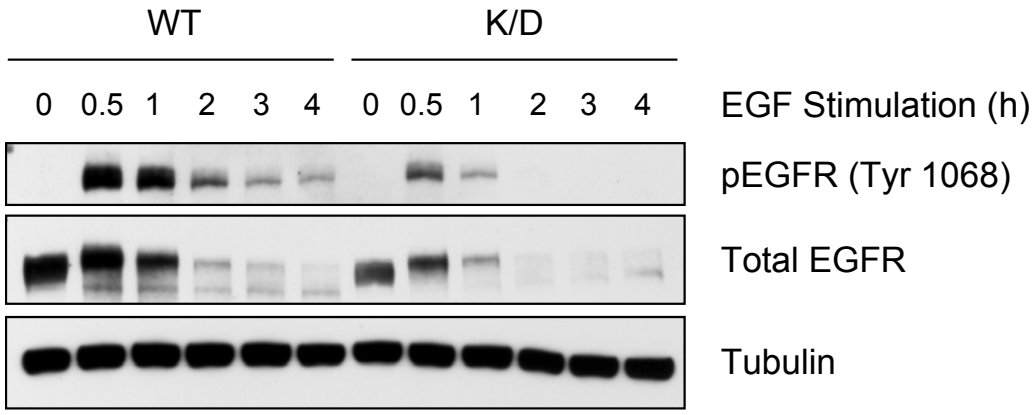

Figure 48. Knockdown of Bif-1 enhances EGFR degradation. Wild-type (WT) and Bif-1-knockdown (K/D) HeLa cells were serum-starved overnight. Cells were incubated with 100 ng/ml EGF for the times indicated, then washed and harvested. Total cell lysates were subjected to SDS-PAGE/immunoblot analysis with antibodies specific for total and phosphorylated EGFR (Tyr 1068).

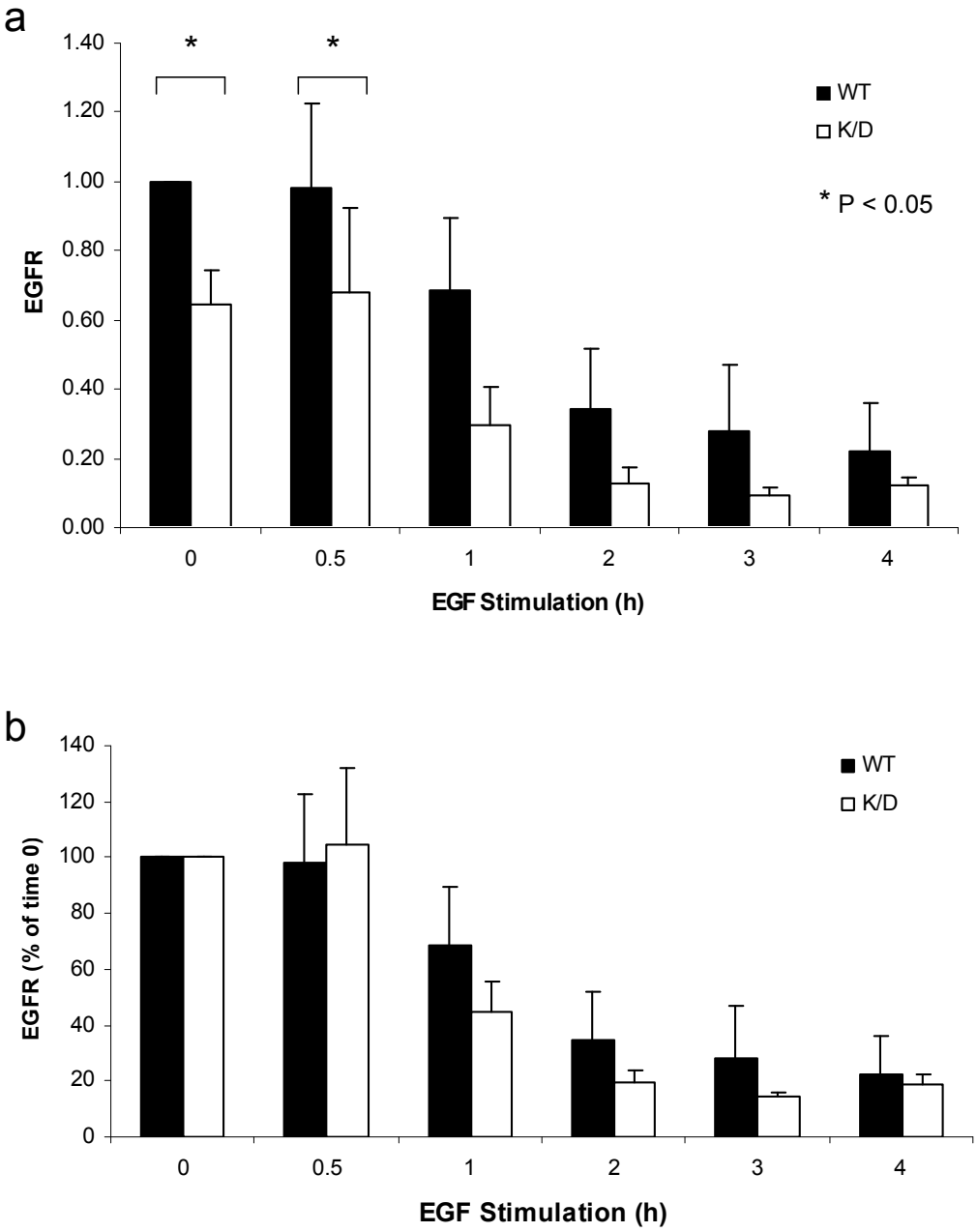

Figure 49. Quantification of total EGFR levels and degradation. Cells were treated and lysate prepared as in Figure 48. Data from three independent experiments were analyzed (mean ± s.d.). (a) Quantification of total EGFR levels. The levels of EGFR are listed relative to that of unstimulated wild-type cells, which was set as 1.
(b) Quantification of the percent EGFR relative to time 0 h.

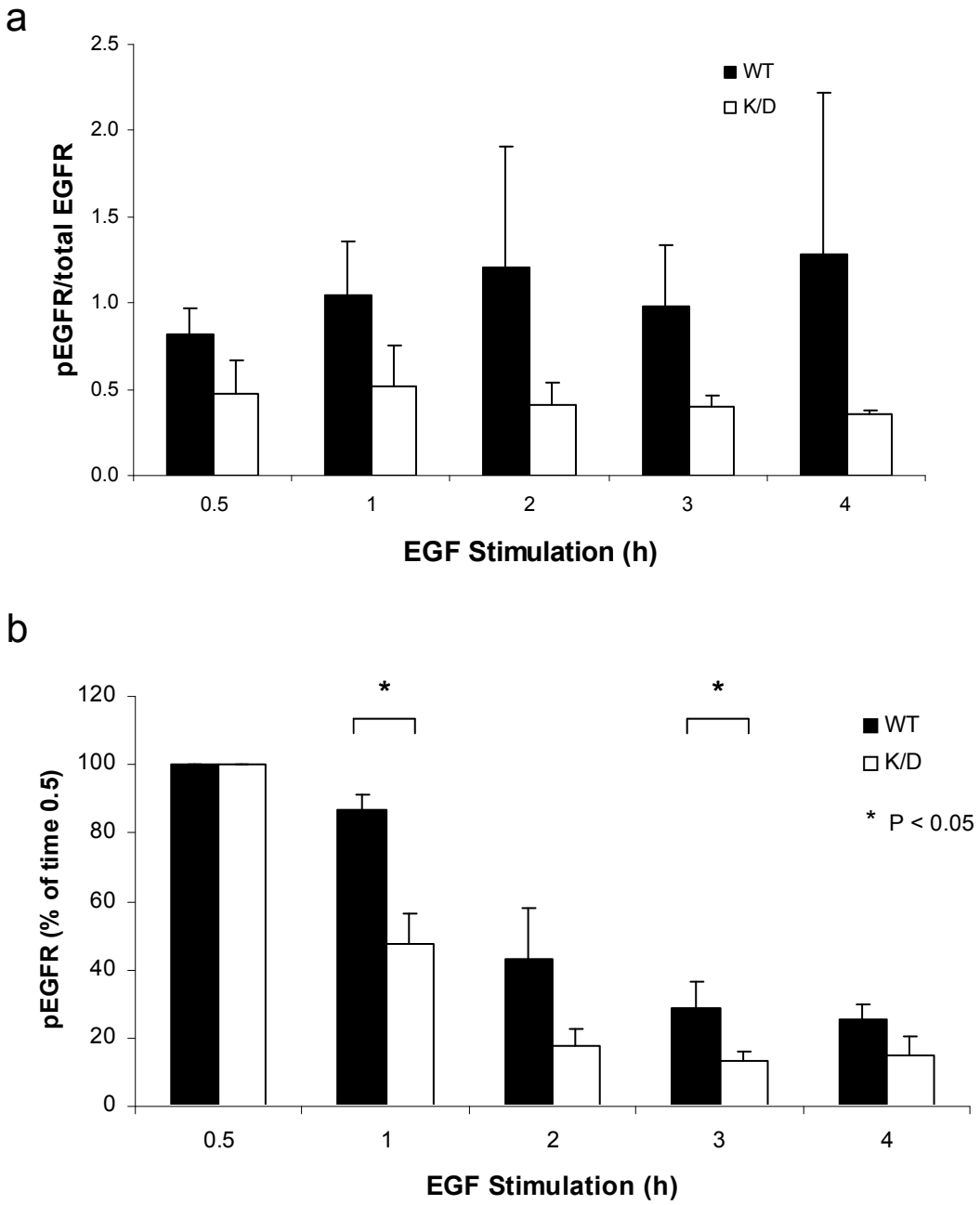

Figure 50. Quantification of phosphorylated EGFR levels and degradation. Cells were treated and lysate prepared as in Figure 48. Data from three independent experiments were analyzed (mean ± s.d.). (a) Quantification of the ratio of phospho-EGFR to total EGFR. (b) Quantification of the percent phospho-EGFR relative to time 0.5 h.

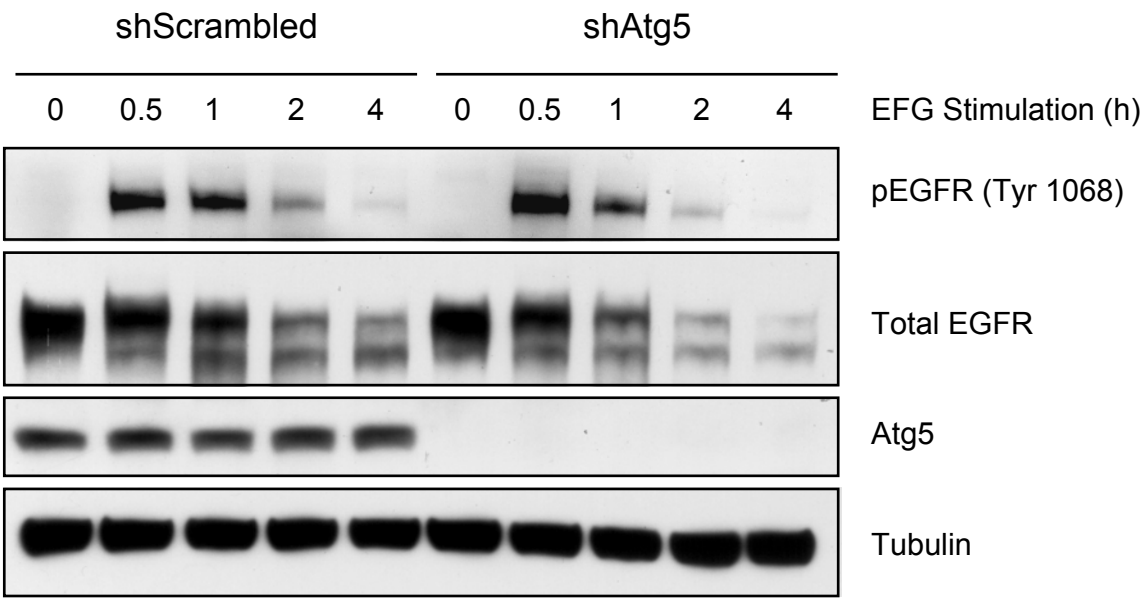

Figure 51. Knockdown of Atg5 does not affect EGFR degradation. HeLa cells stably expressing shAtg5 or a control scrambled shRNA were cultured in serum-free DMEM overnight. Cells were incubated with 100 ng/ml EGF for the times indicated, then washed and harvested. Total cell lysates were subjected to SDS-PAGE/immunoblot analysis with antibodies specific for total and phosphorylated EGFR (Tyr 1068).

EGFR Degradation is Mediated through Both Proteasomal and Lysosomal Mechanisms

The degradation of EGFR has been shown to be regulated by both the lysosomal and proteasomal pathways (Ettenberg *et al.*, 2001; Levkowitz *et al.*, 1999; Levkowitz *et al.*, 1998; Longva *et al.*, 2002). However, the precise mechanisms that regulate EGFR degradation are not fully understood. In order to investigate the mechanism by which the degradation of the EGFR is enhanced by knockdown of Bif-1, we treated Bif-1-knockdown and wild-type cells with bafilomycin A1 or MG132, which are well known inhibitors of the lysosomal and proteasomal pathways, respectively (Lee and Goldberg, 1998; Yoshimori *et al.*, 1991). As shown in Figure 52, treatment with either bafilomycin A1 or MG132 inhibited the degradation of EGFR. In contrast to a previous study (Alwan *et al.*, 2003), our results indicated that treatment with bafilomycin A1 does not

completely inhibit EGFR degradation. Interestingly, the EGFR was still substantially degraded in Bif-1-knockdown cells, despite treatment with bafilomycin A1 or MG132, suggesting that the lysosomal and proteasomal pathways may be functionally compensating for one another. To examine this possibility, Bif-1-wild-type and -knockdown cells were treated with either bafilomycin A1 or MG132 alone or in combination and the degradation of EGFR was examined. Co-treatment of bafilomycin A1 and MG132 further suppressed the degradation of EGFR as compared to treatment with either inhibitor alone (Figure 53). These results suggest that the lysosomal and the proteasomal pathways collaborate to regulate the degradation of EGFR after stimulation with EGF.

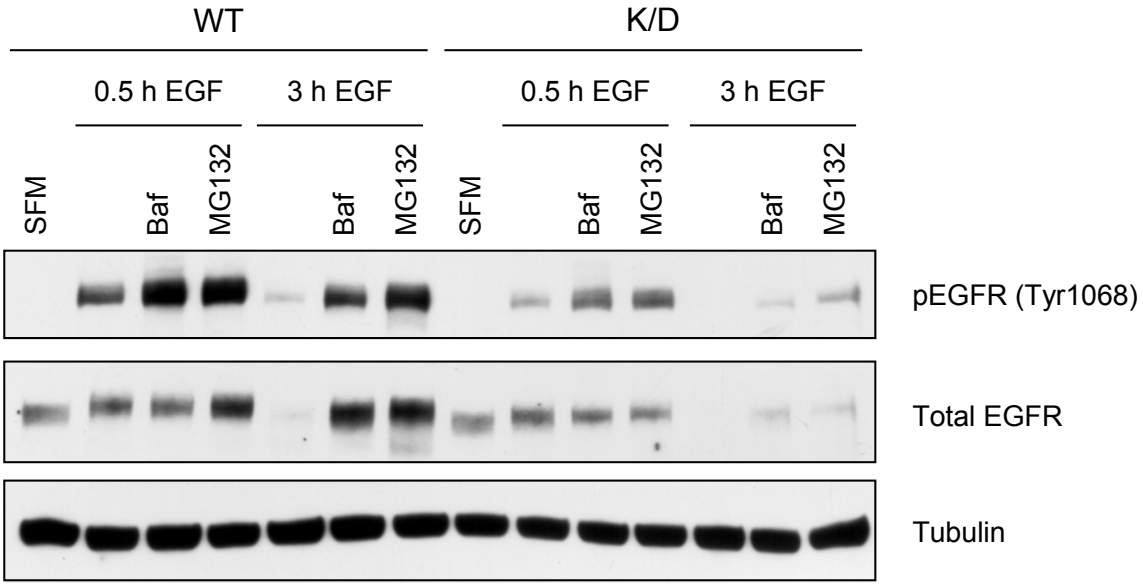

Figure 52. The lysosomal and proteasomal pathways regulate EGFR degradation. Wild-type (WT) and Bif-1-knockdown (K/D) HeLa cells were serum-starved overnight, then treated with 100 ng/ml EGF either alone or in combination with either 250 nM bafilomycin A1 or 10 μM MG132 for 0, 0.5 or 3 h. The degradation and phosphorylation of EGFR was examined by SDS-PAGE/immunoblot analysis with antibodies specific for total and phosphorylated EGFR (Tyr1068).

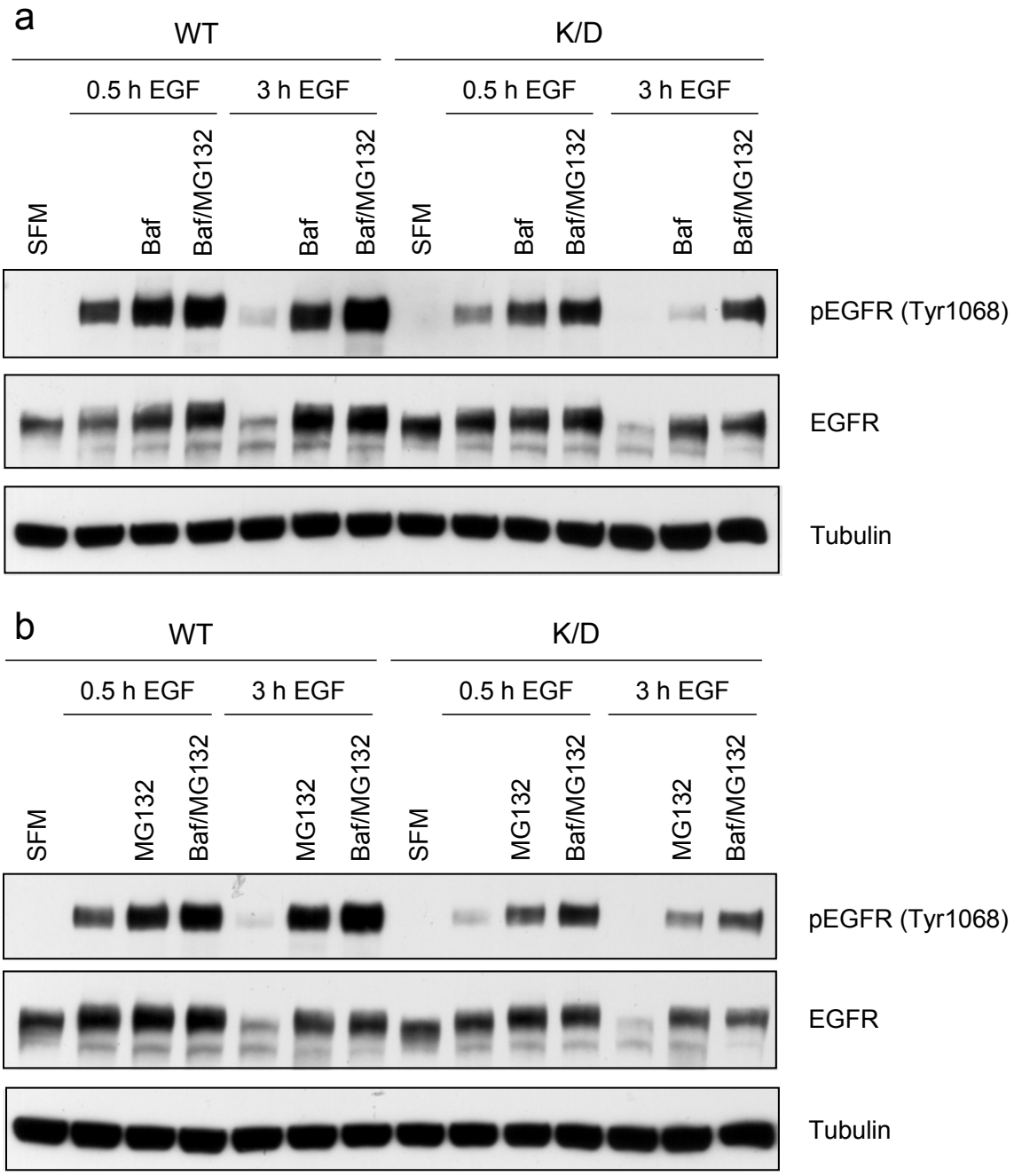

Figure 53. The lysosomal and proteasomal pathways collaborate to regulate EGFR degradation. Wild-type (WT) and Bif-1-knockdown (K/D) HeLa cells were serum-starved overnight then stimulated with 100 ng/ml EGF in the presence of either 250 nM bafilomycin A1 (Baf) or 10 μM MG132 or a combination of bafilomycin A1 and MG132 for 0, 0.5 or 3 h. The levels of phosphorylated and total EGFR were analyzed by SDS-PAGE/immunoblot analysis. (a) Cells were treated with bafilomycin A1 alone or in combination with MG132. (b) Cells were treated with MG132 alone or in combination with bafilomycin A1.

Discussion

In this study, we have described a novel role for Bif-1 in the regulation of endocytic trafficking and receptor degradation. Our observations indicate that Bif-1 plays a critical role in the regulation of the later stages of the endocytic pathway with little affect on internalization. Examination of the levels of both activated and total EGFR, by SDS-PAGE/immunoblot analysis, revealed that knockdown of Bif-1 enhanced the degradation of EGFR, especially the activated form. Furthermore, knockdown of Bif-1 lead to the premature targeting of EGF to LAMP-1-positive foci, suggesting that the accelerated degradation of the EGF-EGFR complex could be the result of precocious localization to late endosomes/lysosomes. Degradation of the EGFR appears to be mediated by both lysosomal and proteasomal mechanisms, as co-treatment with bafilomycin A1 and MG132 resulted in further suppression of EGFR degradation than treatment with either inhibitor alone. Taken together, our results suggest that although loss of Bif-1 accelerates intracellular trafficking and receptor degradation, it does not affect internalization of endocytic cargo. However, further studies are needed to rule out the possibility that Bif-1 may play a role in vesicular trafficking through the early steps of the endocytic pathway.

In support of our findings, a recent report described a role for Bif-1 in the regulation of the endocytic trafficking of nerve growth factor (NGF)-tropomyosin-related kinase A (TrkA) (Wan *et al.*, 2008). Consistent with our results, Ip and colleagues found that knockdown of Bif-1 did not affect the internalization of a fluid phase marker; however, they demonstrated that Bif-1 may regulate the size of early endosomes. In addition, knockdown of Bif-1 resulted in the premature trafficking of internalized NGF-

TrkA to lysosomes. Furthermore, loss of Bif-1 enhanced the degradation of TrkA and diminished signaling downstream of NGF-TrkA. Taken together, these studies indicate that Bif-1 plays a critical role in the regulation of endocytic vesicle trafficking and the degradation of internalized receptors.

Our laboratory has demonstrated that Bif-1 can interact with Beclin 1 through UVRAG to promote the activation of PI3KC3/Vps34 (Takahashi *et al.*, 2007). As mentioned above, the activation of Vps34 plays an critical role not only in the induction of autophagy, but also in the regulation of vesicle transport, including endocytic trafficking (Backer, 2008). It has recently been shown that knockdown of Vps34 reduced the rate of EGFR degradation and disrupted the invagination of late endosomes preventing the formation of MVBs (Johnson *et al.*, 2006). As Bif-1 has been shown to promote the activity of Vps34, it would be assumed that loss of Bif-1 should therefore result in a decrease in endocytic vesicle trafficking and a reduction in EGFR degradation. However, our results suggest that knockdown of Bif-1 does not suppress, but rather accelerates, endocytic trafficking and EGFR degradation. One possible explanation for this discrepancy could be that Bif-1 suppresses these processes by binding to UVRAG and interrupting the formation of the UVRAG-C-Vps complex as described below.

UVRAG is a Beclin 1-binding protein that regulates the activity of PI3KC3/Vsp34 and thus the formation of autophagosomes (Liang *et al.*, 2006; Liang *et al.*, 2007). In addition, a recent report has shown that UVRAG also regulates the maturation of autophagosomes and the trafficking of endocytic vesicles through its interaction with the class C Vps complex (Liang *et al.*, 2008). In stark contrast to our results, which suggest that cells expressing wild-type Bif-1 exhibit delayed EGFR

degradation as compared to knockdown cells, UVRAG expression was shown to enhance EFGR localization to endosomes, resulting in the accelerated degradation of EGFR (Liang *et al.*, 2008). In addition, it was shown that the role of UVRAG in C-Vps-mediated autophagosome/endosome maturation is independent of the role of UVRAG in Beclin 1-mediated autophagosome formation and maturation (Liang *et al.*, 2008). Furthermore, Beclin 1 has been shown to exclusively regulate the autophagic pathway, not endocytic trafficking (Zeng *et al.*, 2006). Taken together, these results suggest that targeting of UVRAG to the Beclin 1-Bif-1 complex could sequester UVRAG from interacting with C-Vps, thereby augmenting its role in the autophagic pathway and abrogating its role in endocytic trafficking. Indeed, Bif-1 has been shown to interact with the proline rich domain of UVRAG, which is located next to the phospholipid-interacting C2 domain (Takahashi *et al.*, 2007). The C2 domain of UVRAG, along with the C-terminus, corresponds to the region that is required for the interaction of UVRAG with C-Vps (Liang *et al.*, 2008). Therefore, it is possible that binding of Bif-1 changes the conformation of UVRAG which could decrease its affinity for C-Vps and would thus decrease the maturation and fusion capabilities of UVRAG. Further studies are necessary to determine whether the expression of a Bif-1 mutant that cannot bind to UVRAG would affect the localization of UVRAG, its interaction partners and its ability to regulate endosomal maturation and fusion.

In addition, Bif-1 could also regulate UVRAG localization by tethering UVRAG to autophagosomes, rather than endosomes. Bif-1 contains an N-BAR domain, which is required for membrane binding, driving membrane curvature and perhaps regulating subcellular localization (Masuda *et al.*, 2006). It is therefore possible that Bif-1 may bind

to UVRAG and regulate its localization thereby targeting UVRAG to autophagosomes, not endosomes. Therefore, in the absence of Bif-1, UVRAG would be released from autophagosomes, allowing it to localize to endosomes and regulate the endocytic pathway. While our results demonstrate that Bif-1 plays an integral role in the later stages of endocytic trafficking, further studies are needed to determine the molecular mechanisms by which loss of Bif-1 accelerates EGF trafficking and enhances the degradation of EGFR.

Methods and Materials

Reagents

Horseradish peroxidase was from purchased from Sigma (St. Louis, MO). The 1 step-Turbo TMB-ELISA kit was purchased from Pierce (Rockford, IL). Bafilomycin A1 was purchased from Wako Chemicals USA (Richmond, VA). MG132 and puromycin were purchased from Calbiochem (San Diego, CA). Epidermal growth factor, Alexa Fluor 488 conjugated EGF, culture medium and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Antibodies were purchased from the following commercial sources: anti-tubulin from Sigma, anti-LAMP-1 from BD Pharmingen (San Diego, CA), total EGFR and phospho-EGFR from Cell Signaling (Danvers, MA), anti-Atg5 from MBL International (Naka-ku Nagoya, Japan), Alexa Fluor 594 chicken anti-mouse IgG from Invitrogen, bovine anti-mouse IgG-horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA) and goat anti-rabbit IgG-horseradish peroxidase from Amersham Biosciences (Piscataway, NJ).

Cell Culture, Transfection and Infection

Wild-type and Bif-1-knockdown HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 1.0 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (Takahashi *et al.*, 2005). The pLKO.1-based lentiviral shRNA targeting shAtg5 (TRCN0000151963) was purchased from Open Biosystems. The pLKO.1-based scrambled control shRNA vector was purchased from Sigma. Recombinant lentivirus was produced by co-transfecting the appropriate shRNA plasmid with the ViraPower Packaging Mix (Invitrogen) into 293FT

cells. The resulting supernatant containing shRNA-expressing lentivirus was used to infect wild-type HeLa cells according to the manufacturer's protocol.

Endocytosis of HRP

Analysis of enodcytosis by HRP uptake was performed as previously described (Johnson *et al.*, 2006; Zeng *et al.*, 2006). Briefly, Bif-1-knockdown and control wild-type HeLa cells were washed once in DMEM, then incubated with 2 mg/ml HRP in DMEM containing 1% BSA for the indicated times. Cells were washed three times in ice-cold PBS containing 1% BSA and once with PBS. Cells were then scraped into PBS and collected by centrifugation at 400 x g for 4 min at 4ºC. The pellet was washed once with PBS, then lysed in PBS containing 0.5% Triton X-100 and protease inhibitors. HRP activity was measured using the 1-Step Turbo TMB-ELISA kit according to the manufacturer's protocol. Enzyme activity was normalized to protein concentration.

EGFR Endocytosis and Degradation

To monitor endocytosis, the internalization of EGF coupled to Alexa Flour 488 was examined as previously described (Liang *et al.*, 2008). Briefly, Bif-1-knockdown and control wild-type HeLa cells were seeded on 2-well chamber slides. The following day, the cells were washed in DMEM, then cultured in serum-free DMEM overnight in order to allow the EGFR to accumulate at the cell surface. Cells were washed once in ice-cold PBS and then incubated in uptake medium (DMEM, 2% BSA and 20 mM HEPES, pH 7.5) containing 5 μg/ml Alexa Fluor 488-EGF for one hour on ice. Unbound ligand was removed by washing the cells three times in ice-cold PBS. At the indicated times, the

cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were then washed three times with PBS and permeabilized in 100 μg/ml digitonin for 15 min at room temperature. After three washes with PBS, the cells were blocked for one hour at room temperature in 3% BSA. Cells were then incubated in primary antibody in blocking solution overnight at 4 °C. After three washes with PBS, cells were incubated in 3% BSA blocking solution for 30 min, then in FITC-conjugated goat anti-rabbit secondary antibody for three hours at room temperature. The cells were washed three times with PBS before being mounted with media containing DAPI (4', 6'-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA). The fluorescent images were obtained using a Leica confocal microscope.

To monitor the degradation of the EGFR, Bif-1-knockdown and control wild-type HeLa cells were serum-starved overnight. Receptor internalization and degradation were stimulated by incubating the cells in 100 ng/ml EGF (Invitrogen) in DMEM containing 20 mM HEPES and 0.2% bovine serum albumin at 37 ºC. At the indicated times, cells were washed once with ice-cold PBS, then collected in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 mM *p*-nitrophenyl phosphate, 1% Triton X-100) as previously described (Ren *et al.*, 2004). Total cell lysates were subjected to SDS-PAGE/immunoblot analysis with antibodies specific for total and phosphorylated EGFR (Tyr 1068). The levels of total and phosphorylated EGFR, as well as degradation, from three independent experiments were quantified by densitometry.

Scientific Significance

Genomic DNA is constantly being subjected to genotoxic stresses. DNA damage triggers the activation of complex, highly coordinated DNA damage response (DDR) pathways, which can initiate cell cycle arrest and promote DNA repair or if DNA repair is unfavorable, activate the apoptotic machinery to induce cell death (Zhou and Elledge, 2000). Thus the DDR acts as a safeguard to protect genomic integrity and to prevent the accumulation of mutations which could lead to cancer, as well as other genetic diseases. A vast amount of data has accumulated that provides insight into the regulation of the DDR. However, research is still being conducted to elucidate the precise mechanisms by which DNA damage is sensed and translated into a signal that results in the decision of whether to arrest the cell cycle or undergo cell death. Further investigation into the mechanisms that regulate the DDR are imperative, as a better understanding of these pathways will allow more educated approaches for novel treatment strategies that will hopefully contribute to the cure of cancer and possibly other diseases.

At the forefront of the DDR is the Rad9-Rad1-Hus1 complex (9-1-1). This complex is a key mediator of the DDR and regulates many downstream signaling pathways that are not only involved in cell cycle arrest and DNA repair, but also in the induction of cell death. It is well accepted that the 9-1-1 complex confers resistance to a variety of genotoxic stresses, including several traditional chemotherapeutic agents. Accordingly, disruption of the 9-1-1 complex has been shown to sensitize cells to DNA

damaging agents (Hopkins *et al.*, 2004; Kinzel *et al.*, 2002; Loegering *et al.*, 2004; Wang *et al.*, 2004b; Wang *et al.*, 2006b; Weiss *et al.*, 2000). Importantly, the data presented in this report describe for the first time the mechanism by which loss of a functional 9-1-1 complex sensitizes cells to etoposide-induced apoptosis. We have shown that etoposide treatment dramatically upregulates the expression of the pro-apoptotic, BH3-only proteins, Bim and Puma, in *Hus1*-deficient cells. The upregulation of these proteins is responsible for sensitizing *Hus1*-knockout cells to etoposide-induced apoptosis, as knockdown of either Bim or Puma confers resistance to etoposide treatment. Interestingly, knockdown of both Bim and Puma results in further resistance, indicating that these BH3-only proteins collaborate in sensitizing *Hus1*-deficient cells to apoptosis induced by genotoxic stress. Furthermore, loss of *Hus1* results in a defect in the binding of Rad9 to chromatin and release of Rad9 into the cytosol, which enhances the interaction of Rad9 with Bcl-2 to potentiate the apoptotic response. However, future studies are necessary to determine the precise mechanisms linking the loss of a functional 9-1-1 complex to the upregulation of Bim and Puma. Taken together, our results clearly demonstrate a role for the 9-1-1 cell cycle checkpoint complex in the suppression of genotoxic stress-induced apoptosis and suggest that this complex may play a pivotal role in determining whether a cell should survive or undergo apoptosis.

Importantly, the results from our studies also reveal a role for the 9-1-1 complex in the regulation of caspase-independent cell death. Our data indicate that loss of a functional 9-1-1 complex sensitizes cells to camptothecin treatment, which is only moderately suppressed by co-treatment with the pan-caspase inhibitor, Z-VAD-FMK. These results indicate that loss of a functional 9-1-1 complex not only sensitizes cells to

apoptosis, as described above, but also to caspase-independent cell death when the apoptotic machinery is inhibited. In addition, we found that loss of *Hus1* results in enhanced autophagy in response to treatment with the DNA damaging agents, camptothecin and etoposide, suggesting that a functional 9-1-1 complex is required for suppression of DNA damage-induced autophagy. However, the mechanism by which loss of a functional 9-1-1 complex activates the autophagic machinery has yet to be discovered. It has previously been shown that BH3-only proteins, as well as the pharmacological BH3 mimic, ABT-737, can disrupt the interaction between Bcl-2 and Beclin 1, which releases Beclin 1 to induce apoptosis. Therefore, it is possible that the upregulation of BH3-only proteins observed in *Hus1*-deficienct cells in response to genotoxic stress could be responsible for enhancing autophagy in these cells. However, this is unlikely as we found that co-treatment of *Hus1*-wild-type cells with ABT-737 and camptothecin did not significantly enhance DNA damage-induce autophagy. These results suggest that the mechanism by which loss of *Hus1* induces autophagy occurs independent of the upregulation of BH3-only protein expression.

As loss of *Hus1* both enhanced caspase-independent cell death and the initiation of autophagy in response to DNA damage, we predicted that the induction of autophagy was responsible for the caspase-independent cell death observed in *Hus1*-knockout cells. Interestingly, inhibition of autophagy, through knockdown of Atg7 or Bif-1, did not suppress, but rather enhanced DNA damage-induced cell death in *Hus1*-deficient cells. These results indicate that the induction of autophagy observed in *Hus1*-knockout cells may play a cytoprotective role. Interestingly, damaged mitochondria, which could release apopotogenic factors and activate the caspase cascade, have been shown to be removed

by autophagy (Abedin *et al.*, 2007; Mijaljica *et al.*, 2007). Therefore, it is not difficult to imagine that inhibition of the clearance of damaged mitochondria by suppressing autophagy could amplify the apoptotic response. It is of interest to determine whether prevention of caspase activation (e.g. by treatment with Z-VAD-FMK) could suppress cell death when the induction of autophagy is inhibited. Taken together, our results highlight a role for the 9-1-1 complex in the regulation of various cell death mechanisms and suggest that targeting the 9-1-1 complex may be an effective treatment strategy for cancer, even in cells with impaired apoptosis (see below).

While an immense amount of research has focused on investigating the role of the 9-1-1 complex in the DDR, much less is known about the role of the 9-1-1 complex in cancer progression. Interestingly, overexpression of members of the 9-1-1 complex has been observed in various types of cancer, including breast cancer, non-small cell lung carcinoma and prostate cancer (Cheng *et al.*, 2005; de la Torre *et al.*, 2008; Maniwa *et al.*, 2005; Zhu *et al.*, 2008). Moreover, high Hus1 expression in ovarian tumors was found to correlate with poor prognosis and advanced stage (de la Torre *et al.*, 2008). In addition, aberrantly high levels of Rad9 mRNA in breast cancer cells were associated with tumor size and local recurrence, suggesting that Rad9 overexpression may play a role in tumor proliferation and local invasion (Cheng *et al.*, 2005). Consistently, Rad9 protein abundance has been shown to strongly correlate with advanced stage prostate cancer and knockdown of Rad9 led to decreased tumorigenicity in nude mice (Zhu and Lieberman 2008). The concept that overexpression of Rad9 enhances tumorigenesis may seem contradictory given the well-described role of Rad9 in apoptosis (Ishii *et al.*, 2005; Komatsu *et al.*, 2000a; Komatsu *et al.*, 2000b; Yoshida *et al.*, 2002; Yoshida *et al.*, 2003).

However, as Rad9 was found to accumulate in the nucleus of non-small cell lung carcinoma cells (Maniwa *et al.*, 2005), it is not unreasonable to assume that overexpression of members of the 9-1-1 complex could result in enhanced complex formation and retention of the complex in the nucleus. Enforced nuclear localization would augment the nuclear functions of Rad9, such as activation of cell cycle checkpoints and initiation of DNA repair, and abrogate the cytosolic functions of Rad9 in activating the apoptotic machinery. It is reasonable to assume that rapidly dividing cancer cells may benefit from, or even require, additional DNA repair mechanisms (e.g. elevated expression of DDR proteins, such as Rad9 and Hus1) in order to offset the high level of DNA replication and the corresponding DNA damage that can occur during this process. Alternatively, the increase in DNA repair mechanisms could provide resistance by rapidly and efficiently repairing DNA damage incurred by exposure to chemotherapeutic agents. Thus, overexpression of members of the 9-1-1 complex could provide cancer cells with a survival advantage and resistance to chemotherapy thereby enhancing tumorigenicity.

As described above, evidence is accumulating that suggests that overexpression of members of the 9-1-1 complex promotes tumorigenesis. Conversely, disruption of the 9-1-1 complex sensitizes cells to commonly used DNA-damaging anticancer agents. In addition, the 9-1-1 complex plays an apical role in the DDR and a direct role in several DNA repair pathways. Therefore, the 9-1-1 complex may be a rational target for novel treatment strategies that would inhibit multiple pathways involved in survival and resistance to genotoxic stress. Indeed, it has been shown that knockdown of Hus1 in H1299 non-small cell lung carcinoma cells enhanced the cytotoxicity of cisplatin (Kinzel

et al., 2002). However, inhibition of the multitude of cellular functions that are regulated by the 9-1-1 complex could also result in undesirable effects. Ideally, the agent used to disrupt the 9-1-1 complex would suppress the pro-survival functions of the 9-1-1 complex (e.g. DNA repair) while avoiding unwanted results from inhibition of desired functions (e.g. apoptosis). Our results indicate that knockout of *Hus1* not only disrupts the binding of Rad9 to chromatin (producing desired effects), but also upregulates BH3-only protein expression and enhances the binding of Rad9 to Bcl-2 to augment the apoptotic response (avoiding undesirable effects). Taken together, these results suggest that Hus1 may be an optimal target for disruption of the 9-1-1 complex. Alternatively, agents could be designed that would promote the translocation of Rad9 from the nucleus to the cytosol, thereby attenuating the nuclear functions of Rad9, such as in the regulation of DNA repair, and potentiating the cytosolic function of Rad9 as an inducer of apoptosis. However, the precise mechanisms that regulate the translocation of Rad9 have yet to be determined, but may involve post-translational modifications, such as phosphorylation of Rad9 by c-Abl or protein kinase Cδ or cleavage of Rad9 by caspase-3. In addition, agents could be designed that would inhibit the interaction between members of the 9-1-1 complex. However, the effect of using this method to disrupt the 9-1-1 complex (without decreasing the expression of any of the members) on sensitivity to DNA damaging agents has yet to be examined. Therefore, additional studies are necessary to explore this possibility, as this strategy may also be therapeutically beneficial.

Our results indicate that targeting the 9-1-1 complex would sensitize cells to both caspase-dependent and caspase-independent cell death in response to treatment with

DNA damaging agents. Therefore, disruption of the 9-1-1 complex could efficiently sensitize cells with impaired apoptotic machinery to commonly used chemotherapeutic agents. One of the hallmarks of cancer is the ability to evade apoptosis (Hanahan and Weinberg, 2000). This resistance to apoptotic cell death is an important aspect not only of tumorigenesis, but also the development of resistance to anticancer drugs (Green and Evan, 2002; Hanahan and Weinberg, 2000). As such, a large area of research focuses its efforts on the restoration of apoptosis in cancer cells (Reed, 2006; Wang and El-Deiry, 2008). Importantly, a growing body of evidence indicates that autophagy may act as a cell death mechanism in cells with impaired apoptotic machinery (Shimizu *et al.*, 2004; Yu *et al.*, 2004). Therefore, rather than trying to restore apoptotic machinery, a more lucrative approach may be to disrupt the 9-1-1 complex and activate autophagy in these cells in order to manipulate the cell's ability to induce alternate forms of cell death. Indeed, several commonly used chemotherapeutic agents have been shown to induce autophagy (Kondo *et al.*, 2005) and could potentially be used in combination with disruption of the 9-1-1 complex to sensitize cells that are resistant to apoptosis. However, this strategy may only be feasible in apoptosis-deficient cells, as our results and those of other laboratories suggest that autophagy can promote survival in cells that are apoptosis-competent (Kroemer and Levine, 2008). In apoptosis-competent cells, an alternate approach could be to inhibit autophagy and disrupt the 9-1-1 complex in order to enhance apoptotic cell death in response to chemotherapy. While recent studies have provided insight into the interplay between the autophagic and apoptotic pathways and how this may affect sensitivity to chemotherapy, future studies are needed to further define the crosstalk between these two cell death mechanisms. Once this knowledge is obtained,

controlling the balance between apoptotic and autophagic cell death could lead to enhanced tumor cell killing and thus provide more efficient cancer treatments.

Another strategy for enhancing the cytotoxicity of anticancer agents would be to inhibit certain functions of Bif-1, in combination with disruption of the 9-1-1 complex. In this report, we demonstrate that knockdown of Bif-1 significantly enhances camptothecin-induced cell death in *Hus1*-deficient cells. However, our results indicate that knockdown of Bif-1 expression suppresses, but does not completely block the induction of autophagy. These results suggest that while Bif-1-mediated autophagy may protect cells from DNA damage-induced cell death, other functions of Bif-1 may also play a role in the suppression of cell death. In this study, we describe a novel role for Bif-1 in the regulation of endocytic vesicle trafficking and receptor degradation. While knockdown of Bif-1 did not affect the internalization of either a fluid phase marker or EGF, it accelerated the co-localization of EGF with late endosomes/lysosomes and enhanced the degradation of activated EGFR. As knockdown Bif-1 enhances the degradation of EGFR, it can be assumed that inhibition of Bif-1 function could result in the rapid attenuation of EGFR signaling. Therefore, suppression of Bif-1 function in cancer cells with hyperactivated EGFR signaling may be a potentially beneficial therapeutic strategy. However, as loss of *Bif-1* has been shown to significantly enhance spontaneous tumorigenesis in mice (Takahashi *et al.*, 2007), care should be taken when designing therapeutics that target Bif-1. In addition to the newly described role of Bif-1 in endocytic vesicle trafficking, our laboratory has also shown that Bif-1 is involved in the regulation of Bax-mediated apoptosis and the induction of autophagy (Cuddeback *et al.*, 2001; Takahashi *et al.*, 2007; Takahashi *et al.*, 2005). Therefore, in order to properly

target Bif-1 for anticancer therapy, the specific functions of Bif-1 that are responsible for its tumor suppressive capabilities and the functions that are required for regulating sensitivity to DNA damage-induced cell death must first be defined. This knowledge could then be used for the rational design of anticancer agents that would specifically target the pro-survival functions of Bif-1 while leaving the anti-tumor functions intact. Given that both autophagy and EGFR signaling have been shown to promote survival, the role of Bif-1 in these processes may be the most likely candidates for conferring resistance to DNA damage-induced cell death.

Interestingly, it has been suggested that in addition to the various gain-of-function mutations that occur in the EGFR, deregulation or inefficient EGFR degradation may also play a significant role in the aberrant activation of EGFR signaling thereby enhancing tumor development (Grandal and Madshus, 2008; Kirisits *et al.*, 2007). As EGFR signaling has been shown to persist even within endosomes (Miaczynska *et al.*, 2004; Sorkin and Von Zastrow, 2002), enhanced EGFR degradation, by inhibition of Bif-1 function, could abrogate growth factor-mediated survival signaling through EGFR-mediated pathways. In addition, it has been shown that EGFR signaling can be induced in response to chemotherapy and exposure to ionizing radiation, even in the absence of ligand binding (Rodemann *et al.*, 2007). EGFR that is activated in such a manner has been shown to confer resistance to DNA damaging chemotherapeutic agents by activating various mechanisms that efficiently repair damaged DNA, thereby counteracting the cytotoxic effects of the chemotherapy. Therefore, targeting Bif-1 functions in endocytic vesicle trafficking may have similar effects to current therapeutic approaches that inhibit EGFR signaling. Indeed, it has been shown that inhibiting EGFR

signaling in combination with chemotherapy or radiotherapy results in increased sensitivity both in preclinical and clinical studies (Nyati *et al.*, 2006).

The results presented in this report suggest that inhibiting Bif-1 function could potentially target two survival pathways: autophagy and EGFR signaling. In addition, our results indicate that the combination of inhibition of Bif-1 and disruption of the 9-1-1 complex, through loss of *Hus1*, enhances the cytotoxicity of camptothecin. These studies provide evidence supporting the concept that targeting Bif-1 and Hus1 would inhibit several survival signaling pathways and thus may sensitize otherwise resistant cancer cells to commonly used chemotherapeutic agents.

In response to genotoxic stress, a complex network of signaling pathways act in concert to activate the DNA damage response. It is therefore important to determine the molecular mechanisms that regulate of these signaling pathways in order to improve the efficacy of cancer treatments. Importantly, the crosstalk between the autophagic and apoptotic pathways must also be deciphered in order to optimize therapeutic benefits. In addition to manipulating the interplay between these pathways, other factors such as those that regulate EGFR endocytic trafficking and degradation, may also prove to be lucrative targets for sensitizing cancer cells to chemotherapy. Significantly, the results described in this report offer insight into some of the mechanisms that both sensitize and provide resistance to DNA damaging agents (Figure 54). Further research will lead to a better understanding of the molecular mechanisms are regulated by the 9-1-1 complex and Bif-1 that affect sensitivity to DNA damage and will provide valuable knowledge that can be used for the rational design of novel chemotherapeutic strategies that will offer more effective treatments for cancer.

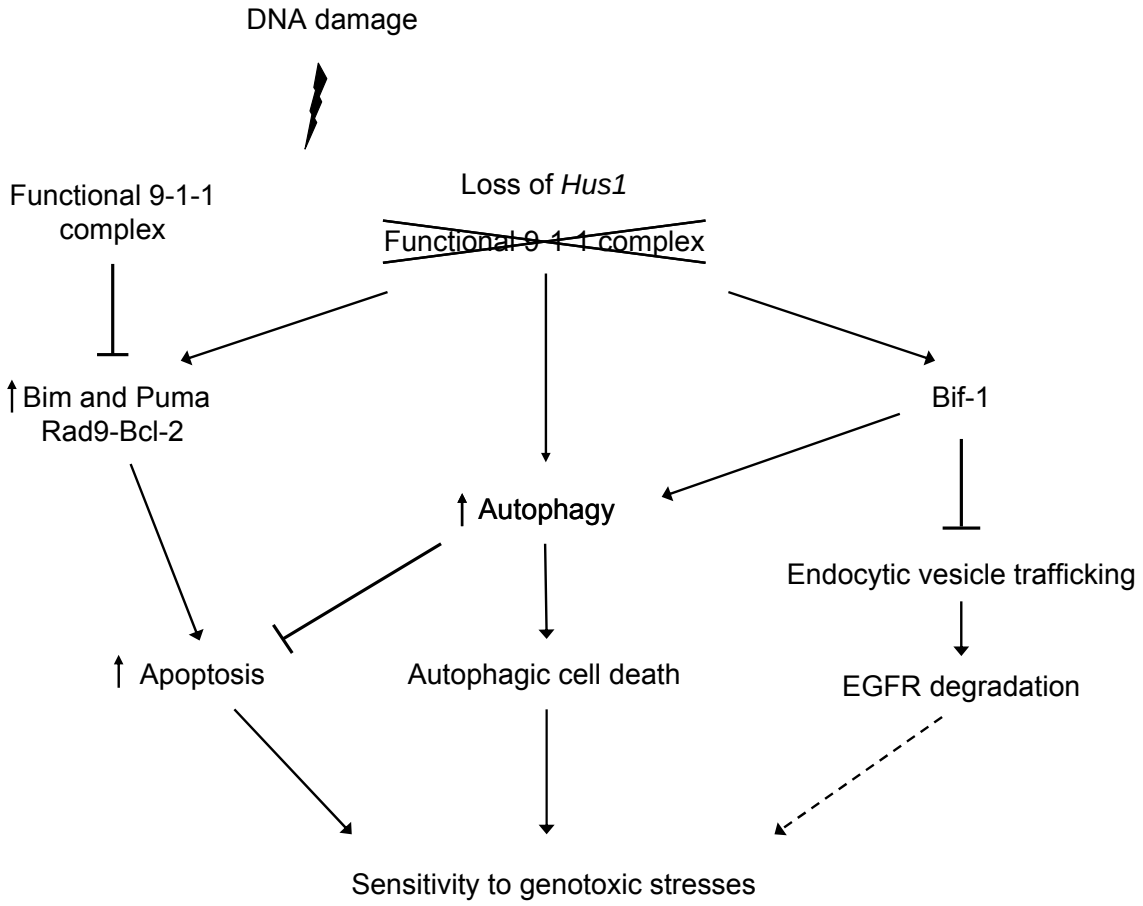

Figure 54. Proposed model for the role of the Rad9-Rad1-Hus1 complex and Bif-1 in the regulation of sensitivity to DNA damage. A functional 9-1-1 complex confers resistance to genotoxic stresses by suppressing apoptosis. Loss of *Hus1* sensitizes cells to DNA damage-induced apoptosis through the upregulation of the BH3-only proteins, Bim and Puma. Moreover, loss of *Hus1* enhances the interaction of Rad9 with Bcl-2 to potentiate the apoptotic response. Loss of *Hus1* also enhances DNA damage-induced autophagy, which promotes survival in apoptosis-competent cells. Inhibiting autophagy in apoptosis-competent cells may enhance DNA damage-induced apoptosis. Conversely, inducing autophagy in cells that are apoptosis-impaired may increase the cytotoxicity of DNA-damaging chemotherapeutic agents. In addition, Bif-1 promotes survival in *Hus1*-deficient cells, perhaps through its regulation of autophagy and/or endocytic vesicle trafficking (EGFR signaling). Notably, loss of *Hus1* sensitizes cells to both caspase-dependent and caspase-independent cell death in response to DNA damage.

References Cited

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

Abraham RT (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev* **15:** 2177-96.

Adams JM, Cory S (2007). The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* **26:** 1324-37.

Ahmed S, Hodgkin J (2000). MRT-2 checkpoint protein is required for germline immortality and telomere replication in C. elegans. *Nature* **403:** 159-64.

Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B (1999). Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics* **59:** 59-65.

Alwan HA, van Zoelen EJ, van Leeuwen JE (2003). Ligand-induced lysosomal epidermal growth factor receptor (EGFR) degradation is preceded by proteasome-dependent EGFR de-ubiquitination. *J Biol Chem* **278:** 35781-90.

Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB (2007). Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest* **117:** 326-36.

Ashkenazi A (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* **2:** 420-30.

Backer JM (2008). The regulation and function of Class III PI3Ks: novel roles for Vps34. *Biochem J* **410:** 1-17.

Baehrecke EH (2002). How death shapes life during development. *Nat Rev Mol Cell Biol* **3:** 779-87.

Baehrecke EH (2005). Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol* **6:** 505-10.

Bakkenist CJ, Kastan MB (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421:** 499-506.

Balakrishnan A, von Neuhoff N, Rudolph C, Kamphues K, Schraders M, Groenen P, van Krieken JH, Callet-Bauchu E, Schlegelberger B, Steinemann D (2006). Quantitative microsatellite analysis to delineate the commonly deleted region 1p22.3 in mantle cell lymphomas. *Genes Chromosomes Cancer* **45:** 883-92.

Bao S, Lu T, Wang X, Zheng H, Wang LE, Wei Q, Hittelman WN, Li L (2004). Disruption of the Rad9/Rad1/Hus1 (9-1-1) complex leads to checkpoint signaling and replication defects. *Oncogene* **23:** 5586-93.

Bartek J, Lukas J (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* **3:** 421-9.

Berkovich E, Monnat RJ, Jr., Kastan MB (2007). Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. *Nat Cell Biol* **9:** 683-90.

Bermudez VP, Lindsey-Boltz LA, Cesare AJ, Maniwa Y, Griffith JD, Hurwitz J, Sancar A (2003). Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro. *Proc Natl Acad Sci U S A* **100:** 1633-8.

Bessho T, Sancar A (2000). Human DNA damage checkpoint protein hRAD9 is a 3' to 5' exonuclease. *J Biol Chem* **275:** 7451-4.

Boder E (1985). Ataxia-telangiectasia: an overview. *Kroc Found Ser* **19:** 1-63.

Botti J, Djavaheri-Mergny M, Pilatte Y, Codogno P (2006). Autophagy signaling and the cogwheels of cancer. *Autophagy* **2:** 67-73.

Brandt PD, Helt CE, Keng PC, Bambara RA (2006). The Rad9 protein enhances survival and promotes DNA repair following exposure to ionizing radiation. *Biochem Biophys Res Commun* **347:** 232-7.

Brown EJ, Baltimore D (2000). ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev* **14:** 397-402.

Burns TF, El-Deiry WS (2003). Microarray analysis of p53 target gene expression patterns in the spleen and thymus in response to ionizing radiation. *Cancer Biol Ther* **2:** 431-43.

Burtelow MA, Kaufmann SH, Karnitz LM (2000). Retention of the human Rad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage. *J Biol Chem* **275:** 26343-8.

Burtelow MA, Roos-Mattjus PM, Rauen M, Babendure JR, Karnitz LM (2001). Reconstitution and molecular analysis of the hRad9-hHus1-hRad1 (9-1-1) DNA damage-responsive checkpoint complex. *J Biol Chem* **276:** 25903-25909.

Caporali S, Levati L, Starace G, Ragone G, Bonmassar E, Alvino E, D'Atri S (2008). AKT is activated in an ataxia-telangiectasia and Rad3-related-dependent manner in response to temozolomide and confers protection against drug-induced cell growth inhibition. *Mol Pharmacol* **74:** 173-83.

Carew JS, Nawrocki ST, Kahue CN, Zhang H, Yang C, Chung L, Houghton JA, Huang P, Giles FJ, Cleveland JL (2007). Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. *Blood* **110:** 313-22.

Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA, Letai A (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9:** 351-65.

Chang DY, Lu AL (2005). Interaction of checkpoint proteins Hus1/Rad1/Rad9 with DNA base excision repair enzyme MutY homolog in fission yeast, Schizosaccharomyces pombe. *J Biol Chem* **280:** 408-17.

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* **17:** 393-403.

Chen MJ, Lin YT, Lieberman HB, Chen G, Lee EY (2001). ATM-dependent phosphorylation of human Rad9 is required for ionizing radiation-induced checkpoint activation. *J Biol Chem* **276:** 16580-6.

Cheng CK, Chow LW, Loo WT, Chan TK, Chan V (2005). The cell cycle checkpoint gene Rad9 is a novel oncogene activated by 11q13 amplification and DNA methylation in breast cancer. *Cancer Res* **65:** 8646-54.

Chini CC, Chen J (2003). Human claspin is required for replication checkpoint control. *J Biol Chem* **278:** 30057-62.

Chipuk JE, Green DR (2005). Do inducers of apoptosis trigger caspase-independent cell death? *Nat Rev Mol Cell Biol* **6:** 268-75.

Cimprich KA, Cortez D (2008). ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* **9:** 616-27.

Citri A, Yarden Y (2006). EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol* **7:** 505-16.

Coppola D, Khalil F, Eschrich SA, Boulware D, Yeatman T, Wang HG (2008a). Down-regulation of Bax-interacting factor-1 in colorectal adenocarcinoma. *Cancer* **113:** 2665-70.

Coppola D, Oliveri C, Sayegh Z, Boulware D, Takahashi Y, Pow-Sang J, Djeu JY, Wang HG (2008b). Bax-interacting factor-1 expression in prostate cancer. *Clin Genitourin Cancer* **6:** 117-21.

Cortez D, Guntuku S, Qin J, Elledge SJ (2001). ATR and ATRIP: partners in checkpoint signaling. *Science* **294:** 1713-6.

Cory S, Huang DC, Adams JM (2003). The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* **22:** 8590-607.

Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell* **126:** 121-34.

Cuddeback SM, Yamaguchi H, Komatsu K, Miyashita T, Yamada M, Wu C, Singh S, Wang HG (2001). Molecular cloning and characterization of Bif-1: A novel SH3 domain- containing protein that associates with Bax. *J Biol Chem* **276:** 20559-20565.

Cuervo AM (2004). Autophagy: in sickness and in health. *Trends Cell Biol* **14:** 70-7.

Daido S, Yamamoto A, Fujiwara K, Sawaya R, Kondo S, Kondo Y (2005). Inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes malignant glioma cells by inducing autophagy. *Cancer Res* **65:** 4368-75.

Dang T, Bao S, Wang XF (2005). Human Rad9 is required for the activation of S-phase checkpoint and the maintenance of chromosomal stability. *Genes Cells* **10:** 287-95.

de la Torre J, Gil-Moreno A, Garcia A, Rojo F, Xercavins J, Salido E, Freire R (2008). Expression of DNA damage checkpoint protein Hus1 in epithelial ovarian tumors correlates with prognostic markers. *Int J Gynecol Pathol* **27:** 24-32.

Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S, White E (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* **10:** 51-64.

Degterev A, Yuan J (2008). Expansion and evolution of cell death programmes. *Nat Rev Mol Cell Biol* **9:** 378-90.

Delacroix S, Wagner JM, Kobayashi M, Yamamoto K, Karnitz LM (2007). The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. *Genes Dev* **21:** 1472-7.

Di Guglielmo GM, Baass PC, Ou WJ, Posner BI, Bergeron JJ (1994). Compartmentalization of SHC, GRB2 and mSOS, and hyperphosphorylation of Raf-1 by EGF but not insulin in liver parenchyma. *Embo J* **13:** 4269-77.

Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* **10:** 1201-4.

Edinger AL, Thompson CB (2003). Defective autophagy leads to cancer. *Cancer Cell* **4:** 422-4.

Edinger AL, Thompson CB (2004). Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* **16:** 663-9.

Egle A, Harris AW, Bouillet P, Cory S (2004). Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc Natl Acad Sci U S A* **101:** 6164-9.

Ettenberg SA, Magnifico A, Cuello M, Nau MM, Rubinstein YR, Yarden Y, Weissman AM, Lipkowitz S (2001). Cbl-b-dependent coordinated degradation of the epidermal growth factor receptor signaling complex. *J Biol Chem* **276:** 27677-84.

Fader CM, Colombo MI (2008). Autophagy and multivesicular bodies: two closely related partners. *Cell Death Differ*.

Farsad K, Ringstad N, Takei K, Floyd SR, Rose K, De Camilli P (2001). Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J Cell Biol* **155:** 193-200.

Francia S, Weiss RS, Hande MP, Freire R, d'Adda di Fagagna F (2006). Telomere and telomerase modulation by the mammalian Rad9/Rad1/Hus1 DNA-damage-checkpoint complex. *Curr Biol* **16:** 1551-8.

Friedrich-Heineken E, Toueille M, Tannler B, Burki C, Ferrari E, Hottiger MO, Hubscher U (2005). The two DNA clamps Rad9/Rad1/Hus1 complex and proliferating cell nuclear antigen differentially regulate flap endonuclease 1 activity. *J Mol Biol* **353:** 980-9.

Futter CE, Collinson LM, Backer JM, Hopkins CR (2001). Human VPS34 is required for internal vesicle formation within multivesicular endosomes. *J Cell Biol* **155:** 1251-64.

Futter CE, Pearse A, Hewlett LJ, Hopkins CR (1996). Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. *J Cell Biol* **132:** 1011-23.

Galonek HL, Hardwick JM (2006). Upgrading the BCL-2 network. *Nat Cell Biol* **8:** 1317-9.

Gembka A, Toueille M, Smirnova E, Poltz R, Ferrari E, Villani G, Hubscher U (2007). The checkpoint clamp, Rad9-Rad1-Hus1 complex, preferentially stimulates the activity of apurinic/apyrimidinic endonuclease 1 and DNA polymerase beta in long patch base excision repair. *Nucleic Acids Res* **35:** 2596-608.

Gorden P, Carpentier JL, Cohen S, Orci L (1978). Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. *Proc Natl Acad Sci U S A* **75:** 5025-9.

Gordon PB, Hoyvik H, Seglen PO (1992). Prelysosomal and lysosomal connections between autophagy and endocytosis. *Biochem J* **283 (Pt 2):** 361-9.

Gordon PB, Seglen PO (1988). Prelysosomal convergence of autophagic and endocytic pathways. *Biochem Biophys Res Commun* **151:** 40-7.

Grandal MV, Madshus IH (2008). Epidermal growth factor receptor and cancer: control of oncogenic signalling by endocytosis. *J Cell Mol Med* **12:** 1527-34.

Green DR, Evan GI (2002). A matter of life and death. *Cancer Cell* **1:** 19-30.

Haigler HT, McKanna JA, Cohen S (1979). Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J Cell Biol* **81:** 382-95.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. *Cell* **100:** 57-70.

Hang H, Lieberman HB (2000). Physical interactions among human checkpoint control proteins HUS1p, RAD1p and RAD9p, and implications for the regulation of cell cycle progression. *Genomics* **65:** 24-33.

Harper JW, Elledge SJ (2007). The DNA damage response: ten years after. *Mol Cell* **28:** 739-45.

Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA (2008). DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* **8:** 193-204.

Helt CE, Wang W, Keng PC, Bambara RA (2005). Evidence that DNA damage detection machinery participates in DNA repair. *Cell Cycle* **4:** 529-32.

Hershko T, Ginsberg D (2004). Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. *J Biol Chem* **279:** 8627-34.

Hirai I, Wang HG (2002). A role of the C-terminal region of human Rad9 (hRad9) in nuclear transport of the hRad9 checkpoint complex. *J Biol Chem* **277:** 25722-7.

Hofmann ER, Milstein S, Boulton SJ, Ye M, Hofmann JJ, Stergiou L, Gartner A, Vidal M, Hengartner MO (2002). Caenorhabditis elegans HUS-1 is a DNA damage checkpoint protein required for genome stability and EGL-1-mediated apoptosis. *Curr Biol* **12:** 1908-18.

Hopkins KM, Auerbach W, Wang XY, Hande MP, Hang H, Wolgemuth DJ, Joyner AL, Lieberman HB (2004). Deletion of mouse rad9 causes abnormal cellular responses to DNA damage, genomic instability, and embryonic lethality. *Mol Cell Biol* **24:** 7235-48.

Huang DC, Strasser A (2000). BH3-Only proteins-essential initiators of apoptotic cell death. *Cell* **103:** 839-42.

Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, Ohsumi Y (2000). A ubiquitin-like system mediates protein lipidation. *Nature* **408:** 488-92.

Inbal B, Bialik S, Sabanay I, Shani G, Kimchi A (2002). DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol* **157:** 455-68.

Ionov Y, Nowak N, Perucho M, Markowitz S, Cowell JK (2004). Manipulation of nonsense mediated decay identifies gene mutations in colon cancer Cells with microsatellite instability. *Oncogene* **23:** 639-45.

Ishii H, Inageta T, Mimori K, Saito T, Sasaki H, Isobe M, Mori M, Croce CM, Huebner K, Ozawa K, Furukawa Y (2005). Frag1, a homolog of alternative replication factor C subunits, links replication stress surveillance with apoptosis. *Proc Natl Acad Sci U S A* **102:** 9655-60.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ (2008). Cancer statistics, 2008. *CA Cancer J Clin* **58:** 71-96.

Jin HO, Park IC, An S, Lee HC, Woo SH, Hong YJ, Lee SJ, Park MJ, Yoo DH, Rhee CH, Hong SI (2006). Up-regulation of Bak and Bim via JNK downstream pathway in the response to nitric oxide in human glioblastoma cells. *J Cell Physiol* **206:** 477-86.

Jin Z, El-Deiry WS (2005). Overview of cell death signaling pathways. *Cancer Biol Ther* **4:** 139-63.

Johnson EE, Overmeyer JH, Gunning WT, Maltese WA (2006). Gene silencing reveals a specific function of hVps34 phosphatidylinositol 3-kinase in late versus early endosomes. *J Cell Sci* **119:** 1219-32.

Jones EV, Dickman MJ, Whitmarsh AJ (2007). Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase. *Biochem J* **405:** 617-23.

Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *Embo J* **19:** 5720-8.

Kai M, Wang TS (2003). Checkpoint activation regulates mutagenic translesion synthesis. *Genes Dev* **17:** 64-76.

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

Kastan MB, Bartek J (2004). Cell-cycle checkpoints and cancer. *Nature* **432:** 316-23.

Katayama M, Kawaguchi T, Berger MS, Pieper RO (2007). DNA damaging agent-induced autophagy produces a cytoprotective adenosine triphosphate surge in malignant glioma cells. *Cell Death Differ* **14:** 548-58.

Katzmann DJ, Odorizzi G, Emr SD (2002). Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol* **3:** 893-905.

Kaufmann SH (1998). Cell death induced by topoisomerase-targeted drugs: more questions than answers. *Biochim Biophys Acta* **1400:** 195-211.

Kerr JF, Wyllie AH, Currie AR (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26:** 239-57.

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

Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH (2006). Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol* **8:** 1348-58.

Kim ST, Lim DS, Canman CE, Kastan MB (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem* **274:** 37538-43.

Kim SY, Oh YL, Kim KM, Jeong EG, Kim MS, Yoo NJ, Lee SH (2008). Decreased expression of Bax-interacting factor-1 (Bif-1) in invasive urinary bladder and gallbladder cancers. *Pathology* **40:** 553-7.

Kinzel B, Hall J, Natt F, Weiler J, Cohen D (2002). Downregulation of Hus1 by antisense oligonucleotides enhances the sensitivity of human lung carcinoma cells to cisplatin. *Cancer* **94:** 1808-14.

Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T, Noda T, Ohsumi Y (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol* **147:** 435-46.

Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, Ohsumi M, Takao T, Noda T, Ohsumi Y (2000). The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol* **151:** 263-76.

Kirisits A, Pils D, Krainer M (2007). Epidermal growth factor receptor degradation: an alternative view of oncogenic pathways. *Int J Biochem Cell Biol* **39:** 2173-82.

Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, Clarke PG, Clark RS, Clarke SG, Clave C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA, Deretic V, Devenish RJ, Di Sano F, Dice JF, Difiglia M, Dinesh-Kumar S, Distelhorst CW, Djavaheri-Mergny M, Dorsey FC, Droge W, Dron M, Dunn WA, Jr., Duszenko M, Eissa NT, Elazar Z, Esclatine A, Eskelinen EL, Fesus L, Finley KD, Fuentes JM, Fueyo J, Fujisaki K, Galliot B, Gao FB, Gewirtz DA, Gibson SB, Gohla A, Goldberg AL, Gonzalez R, Gonzalez-Estevez C, Gorski S, Gottlieb RA, Haussinger D, He YW, Heidenreich K, Hill JA, Hoyer-Hansen M, Hu X, Huang WP, Iwasaki A, Jaattela M, Jackson WT, Jiang X, Jin S, Johansen T, Jung JU, Kadowaki M, Kang C, Kelekar A, Kessel DH, Kiel JA, Kim HP, Kimchi A, Kinsella TJ, Kiselyov K, Kitamoto K, Knecht E, Komatsu M, Kominami E, Kondo S, Kovacs AL, Kroemer G, Kuan CY, Kumar R, Kundu M, Landry J, Laporte M, Le W, Lei HY, Lenardo MJ, Levine B, Lieberman A, Lim KL, Lin FC, Liou W, Liu LF, Lopez-Berestein G, Lopez-Otin C, Lu B, Macleod KF, Malorni W, Martinet W, Matsuoka K, Mautner J, Meijer AJ, Melendez A, Michels P, Miotto G, Mistiaen WP, Mizushima N, Mograbi B, Monastyrska I, Moore MN, Moreira PI, Moriyasu Y, Motyl T, Munz C, Murphy LO, Naqvi NI, Neufeld TP, Nishino I, Nixon RA, Noda T, Nurnberg B, Ogawa M, Oleinick NL, Olsen LJ, Ozpolat B, Paglin S, Palmer GE, Papassideri I, Parkes M, Perlmutter DH, Perry G, Piacentini M, Pinkas-Kramarski R, Prescott M, Proikas-Cezanne T, Raben N, Rami A, Reggiori F, Rohrer B, Rubinsztein DC, Ryan KM, Sadoshima J, Sakagami H, Sakai Y, Sandri M, Sasakawa C, Sass M, Schneider C, Seglen PO, Seleverstov O, Settleman J, Shacka JJ, Shapiro IM, Sibirny A, Silva-Zacarin EC, Simon HU, Simone C, Simonsen A, Smith MA, Spanel-Borowski K, Srinivas V, Steeves M, Stenmark H, Stromhaug PE, Subauste CS, Sugimoto S, Sulzer D, Suzuki T, Swanson MS, Tabas I, Takeshita F, Talbot NJ, Talloczy Z, Tanaka K, Tanaka K, Tanida I, Taylor GS, Taylor JP, Terman A, Tettamanti G, Thompson CB, Thumm M, Tolkovsky AM, Tooze SA, Truant R, Tumanovska LV, Uchiyama Y, Ueno T, Uzcategui NL, van der Klei I, Vaquero EC, Vellai T, Vogel MW, Wang HG, Webster P, Wiley JW, Xi Z, Xiao G, Yahalom J, Yang JM, Yap G, Yin XM, Yoshimori T, Yu L, Yue Z, Yuzaki M, Zabirnyk O, Zheng X, Zhu X, Deter RL (2008). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4:** 151-75.

Klionsky DJ, Cregg JM, Dunn WA, Jr., Emr SD, Sakai Y, Sandoval IV, Sibirny A, Subramani S, Thumm M, Veenhuis M, Ohsumi Y (2003). A unified nomenclature for yeast autophagy-related genes. *Dev Cell* **5:** 539-45.

Klionsky DJ, Cuervo AM, Seglen PO (2007). Methods for monitoring autophagy from yeast to human. *Autophagy* **3:** 181-206.

Komatsu K, Hopkins KM, Lieberman HB, Wang H-G (2000a). Schizosaccharomyces pombe rad9 contains a BH3-like region and interacts with the anti-apoptotic protein bcl-2. *FEBS Lett* **481:** 122-6.

Komatsu K, Miyashita T, Hang H, Hopkins KM, Zheng W, Cuddeback S, Yamada M, Lieberman HB, Wang HG (2000b). Human homologue of S. pombe Rad9 interacts with BCL-2/BCL-xL and promotes apoptosis. *Nat Cell Biol* **2:** 1-6.

Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* **169:** 425-34.

Kondo Y, Kanzawa T, Sawaya R, Kondo S (2005). The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* **5:** 726-34.

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* **16:** 3-11.

Kroemer G, Levine B (2008). Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol* **9:** 1004-10.

Kumagai A, Lee J, Yoo HY, Dunphy WG (2006). TopBP1 activates the ATR-ATRIP complex. *Cell* **124:** 943-55.

Kundu M, Thompson CB (2008). Autophagy: basic principles and relevance to disease. *Annu Rev Pathol* **3:** 427-55.

Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* **17:** 525-35.

Lavin MF (2008). Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. *Nat Rev Mol Cell Biol* **9:** 759-69.

Lee DH, Goldberg AL (1998). Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* **8:** 397-403.

Lee J, Kumagai A, Dunphy WG (2007). The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR. *J Biol Chem* **282:** 28036-44.

Lee JW, Jeong EG, Soung YH, Nam SW, Lee JY, Yoo NJ, Lee SH (2006). Decreased expression of tumour suppressor Bax-interacting factor-1 (Bif-1), a Bax activator, in gastric carcinomas. *Pathology* **38:** 312-5.

Lee MW, Hirai I, Wang H-G (2003). Caspase-3-mediated cleavage of Rad9 during apoptosis. *Oncogene* **22:** 6340-6346.

Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2:** 183-92.

Levine B, Klionsky DJ (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* **6:** 463-77.

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

Levine B, Kroemer G (2009). Autophagy in aging, disease and death: the true identity of a cell death impostor. *Cell Death Differ* **16:** 1-2.

Levine B, Yuan J (2005). Autophagy in cell death: an innocent convict? *J Clin Invest* **115:** 2679-88.

Levitt PS, Zhu M, Cassano A, Yazinski SA, Liu H, Darfler J, Peters RM, Weiss RS (2007). Genome maintenance defects in cultured cells and mice following partial inactivation of the essential cell cycle checkpoint gene Hus1. *Mol Cell Biol* **27:** 2189-201.

Levkowitz G, Waterman H, Ettenberg SA, Katz M, Tsygankov AY, Alroy I, Lavi S, Iwai K, Reiss Y, Ciechanover A, Lipkowitz S, Yarden Y (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol Cell* **4:** 1029-40.

Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, Beguinot L, Geiger B, Yarden Y (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* **12:** 3663-74.

Li H, Zhu H, Xu CJ, Yuan J (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94:** 491-501.

Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, Jung JU (2006). Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol* **8:** 688-99.

Liang C, Feng P, Ku B, Oh BH, Jung JU (2007). UVRAG: a new player in autophagy and tumor cell growth. *Autophagy* **3:** 69-71.

Liang C, Lee JS, Inn KS, Gack MU, Li Q, Roberts EA, Vergne I, Deretic V, Feng P, Akazawa C, Jung JU (2008). Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nat Cell Biol* **10:** 776-87.

Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* **402:** 672-6.

Lieberman HB (2006). Rad9, an evolutionarily conserved gene with multiple functions for preserving genomic integrity. *J Cell Biochem* **97:** 690-7.

Lindsey-Boltz LA, Bermudez VP, Hurwitz J, Sancar A (2001). Purification and characterization of human DNA damage checkpoint Rad complexes. *Proc Natl Acad Sci U S A* **98:** 11236-41.

Liou W, Geuze HJ, Geelen MJ, Slot JW (1997). The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J Cell Biol* **136:** 61-70.

Lockshin RA, Zakeri Z (2004). Apoptosis, autophagy, and more. *Int J Biochem Cell Biol* **36:** 2405-19.

Loegering D, Arlander SJ, Hackbarth J, Vroman BT, Roos-Mattjus P, Hopkins KM, Lieberman HB, Karnitz LM, Kaufmann SH (2004). Rad9 protects cells from topoisomerase poison-induced cell death. *J Biol Chem* **279:** 18641-7.

Longva KE, Blystad FD, Stang E, Larsen AM, Johannessen LE, Madshus IH (2002). Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. *J Cell Biol* **156:** 843-54.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94:** 481-90.

Luthi AU, Martin SJ (2007). The CASBAH: a searchable database of caspase substrates. *Cell Death Differ* **14:** 641-50.

Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, Juin P, Tasdemir E, Pierron G, Troulinaki K, Tavernarakis N, Hickman JA, Geneste O, Kroemer G (2007a). Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *Embo J* **26:** 2527-39.

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

Maniwa Y, Yoshimura M, Bermudez VP, Yuki T, Okada K, Kanomata N, Ohbayashi C, Hayashi Y, Hurwitz J, Okita Y (2005). Accumulation of hRad9 protein in the nuclei of nonsmall cell lung carcinoma cells. *Cancer* **103:** 126-32.

Marino G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, Lopez-Otin C (2007). Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. *J Biol Chem* **282:** 18573-83.

Masuda M, Takeda S, Sone M, Ohki T, Mori H, Kamioka Y, Mochizuki N (2006). Endophilin BAR domain drives membrane curvature by two newly identified structure-based mechanisms. *Embo J* **25:** 2889-97.

Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, 3rd, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316:** 1160-6.

McNiven MA, Cao H, Pitts KR, Yoon Y (2000). The dynamin family of mechanoenzymes: pinching in new places. *Trends Biochem Sci* **25:** 115-20.

Melo J, Toczyski D (2002). A unified view of the DNA-damage checkpoint. *Curr Opin Cell Biol* **14:** 237-45.

Mendez J, Stillman B (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Mol Cell Biol* **20:** 8602-12.

Meyerkord CL, Takahashi Y, Araya R, Takada N, Weiss RS, Wang HG (2008). Loss of Hus1 sensitizes cells to etoposide-induced apoptosis by regulating BH3-only proteins. *Oncogene* **27:** 7248-59.

Miaczynska M, Pelkmans L, Zerial M (2004). Not just a sink: endosomes in control of signal transduction. *Curr Opin Cell Biol* **16:** 400-6.

Mijaljica D, Prescott M, Devenish RJ (2007). Different fates of mitochondria: alternative ways for degradation? *Autophagy* **3:** 4-9.

Mizushima N (2005). The pleiotropic role of autophagy: from protein metabolism to bactericide. *Cell Death Differ* **12 Suppl 2:** 1535-41.

Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, Natsume T, Ohsumi Y, Yoshimori T (2003). Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci* **116:** 1679-88.

Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008). Autophagy fights disease through cellular self-digestion. *Nature* **451:** 1069-75.

Mizushima N, Noda T, Ohsumi Y (1999). Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. *Embo J* **18:** 3888-96.

Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M, Ohsumi Y (1998). A protein conjugation system essential for autophagy. *Nature* **395:** 395-8.

Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y, Yoshimori T (2001). Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* **152:** 657-68.

Mizushima N, Yoshimori T (2007). How to interpret LC3 immunoblotting. *Autophagy* **3:** 542-5.

Montecucco A, Biamonti G (2007). Cellular response to etoposide treatment. *Cancer Lett* **252:** 9-18.

Mordes DA, Glick GG, Zhao R, Cortez D (2008). TopBP1 activates ATR through ATRIP and a PIKK regulatory domain. *Genes Dev* **22:** 1478-89.

Nabetani A, Yokoyama O, Ishikawa F (2004). Localization of hRad9, hHus1, hRad1, and hRad17 and caffeine-sensitive DNA replication at the alternative lengthening of telomeres-associated promyelocytic leukemia body. *J Biol Chem* **279:** 25849-57.

Nakano K, Vousden KH (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7:** 683-94.

Nicholson DW (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* **6:** 1028-42.

Niida H, Nakanishi M (2006). DNA damage checkpoints in mammals. *Mutagenesis* **21:** 3-9.

Noda T, Kim J, Huang WP, Baba M, Tokunaga C, Ohsumi Y, Klionsky DJ (2000). Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. *J Cell Biol* **148:** 465-80.

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

Nyati MK, Morgan MA, Feng FY, Lawrence TS (2006). Integration of EGFR inhibitors with radiochemotherapy. *Nat Rev Cancer* **6:** 876-85.

O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA, Goodship JA (2003). A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. *Nat Genet* **33:** 497-501.

Ohsumi Y (2001). Molecular dissection of autophagy: two ubiquitin-like systems. *Nat Rev Mol Cell Biol* **2:** 211-6.

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

Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435:** 677-81.

Oltvai ZN, Korsmeyer SJ (1994). Checkpoints of dueling dimers foil death wishes. *Cell* **79:** 189-192.

Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J (2001). A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* **61:** 439-44.

Pandita RK, Sharma GG, Laszlo A, Hopkins KM, Davey S, Chakhparonian M, Gupta A, Wellinger RJ, Zhang J, Powell SN, Roti Roti JL, Lieberman HB, Pandita TK (2006). Mammalian Rad9 plays a role in telomere stability, S- and G2-phase-specific cell survival, and homologous recombinational repair. *Mol Cell Biol* **26:** 1850-64.

Parker AE, Van de Weyer I, Laus MC, Oostveen I, Yon J, Verhasselt P, Luyten WH (1998). A human homologue of the Schizosaccharomyces pombe rad1+ checkpoint gene encodes an exonuclease. *J Biol Chem* **273:** 18332-9.

Parrilla-Castellar ER, Arlander SJ, Karnitz L (2004). Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex. *DNA Repair (Amst)* **3:** 1009-14.

Peterson MR, Emr SD (2001). The class C Vps complex functions at multiple stages of the vacuolar transport pathway. *Traffic* **2:** 476-86.

Pierrat B, Simonen M, Cueto M, Mestan J, Ferrigno P, Heim J (2001). SH3GLB, a new endophilin-related protein family featuring an SH3 domain. *Genomics* **71:** 222-34.

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

Pommier Y (2006). Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer* **6:** 789-802.

Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson EM, Jr. (2003). JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. *Neuron* **38:** 899-914.

Puthalakath H, Strasser A (2002). Keeping killers on a tight leash: transcriptional and post- translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* **9:** 505-12.

Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* **112:** 1809-20.

Rauen M, Burtelow MA, Dufault VM, Karnitz LM (2000). The human checkpoint protein hRad17 interacts with the PCNA-like proteins hRad1, hHus1, and hRad9. *J Biol Chem* **275:** 29767-71.

Reed JC (2003). Apoptosis-targeted therapies for cancer. *Cancer Cell* **3:** 17-22.

Reed JC (2006). Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. *Nat Clin Pract Oncol* **3:** 388-98.

Reggiori F, Shintani T, Nair U, Klionsky DJ (2005). Atg9 cycles between mitochondria and the pre-autophagosomal structure in yeasts. *Autophagy* **1:** 101-9.

Ren Y, Meng S, Mei L, Zhao ZJ, Jove R, Wu J (2004). Roles of Gab1 and SHP2 in paxillin tyrosine dephosphorylation and Src activation in response to epidermal growth factor. *J Biol Chem* **279:** 8497-505.

Rhind N, Russell P (1998). Mitotic DNA damage and replication checkpoints in yeast. *Curr Opin Cell Biol* **10:** 749-58.

Rodemann HP, Dittmann K, Toulany M (2007). Radiation-induced EGFR-signaling and control of DNA-damage repair. *Int J Radiat Biol* **83:** 781-91.

Roos-Mattjus P, Hopkins KM, Oestreich AJ, Vroman BT, Johnson KL, Naylor S, Lieberman HB, Karnitz LM (2003). Phosphorylation of human Rad9 is required for genotoxin-activated checkpoint signaling. *J Biol Chem* **278:** 24428-37.

Roos-Mattjus P, Vroman BT, Burtelow MA, Rauen M, Eapen AK, Karnitz LM (2002). Genotoxin-induced Rad9-Hus1-Rad1 (9-1-1) chromatin association is an early checkpoint signaling event. *J Biol Chem* **277:** 43809-12.

Sabbioneda S, Minesinger BK, Giannattasio M, Plevani P, Muzi-Falconi M, Jinks-Robertson S (2005). The 9-1-1 checkpoint clamp physically interacts with polzeta and is partially required for spontaneous polzeta-dependent mutagenesis in Saccharomyces cerevisiae. *J Biol Chem* **280:** 38657-65.

Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P (2004). Toxic proteins released from mitochondria in cell death. *Oncogene* **23:** 2861-74.

Scarlatti F, Granata R, Meijer AJ, Codogno P (2009). Does autophagy have a license to kill mammalian cells? *Cell Death Differ* **16:** 12-20.

Shi G, Chang DY, Cheng CC, Guan X, Venclovas C, Lu AL (2006). Physical and functional interactions between MutY glycosylase homologue (MYH) and checkpoint proteins Rad9-Rad1-Hus1. *Biochem J* **400:** 53-62.

Shibue T, Taniguchi T (2006). BH3-only proteins: integrated control point of apoptosis. *Int J Cancer* **119:** 2036-43.

Shiloh Y, Kastan MB (2001). ATM: genome stability, neuronal development, and cancer cross paths. *Adv Cancer Res* **83:** 209-54.

Shimizu S, Kanaseki T, Mizushima N, Mizuta T, Arakawa-Kobayashi S, Thompson CB, Tsujimoto Y (2004). Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol* **6:** 1221-8.

Shintani T, Mizushima N, Ogawa Y, Matsuura A, Noda T, Ohsumi Y (1999). Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *Embo J* **18:** 5234-41.

Smirnova E, Toueille M, Markkanen E, Hubscher U (2005). The human checkpoint sensor and alternative DNA clamp Rad9-Rad1-Hus1 modulates the activity of DNA ligase I, a component of the long-patch base excision repair machinery. *Biochem J* **389:** 13-7.

Sorkin A, Von Zastrow M (2002). Signal transduction and endocytosis: close encounters of many kinds. *Nat Rev Mol Cell Biol* **3:** 600-14.

Soubeyran P, Kowanetz K, Szymkiewicz I, Langdon WY, Dikic I (2002). Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors. *Nature* **416:** 183-7.

St Onge RP, Besley BD, Park M, Casselman R, Davey S (2001). DNA damage-dependent and -independent phosphorylation of the hRad9 checkpoint protein. *J Biol Chem* **276:** 41898-905.

St Onge RP, Besley BD, Pelley JL, Davey S (2003). A role for the phosphorylation of hRad9 in checkpoint signaling. *J Biol Chem* **278:** 26620-8.

St. Onge RP, Udell CM, Casselman R, Davey S (1999). The human G2 checkpoint control protein hRAD9 is a nuclear phosphoprotein that forms complexes with hRAD1 and hHUS1. *Mol Biol Cell* **10:** 1985-1995.

Strasser A (2005). The role of BH3-only proteins in the immune system. *Nat Rev Immunol* **5:** 189-200.

Stucki M, Jackson SP (2006). gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst)* **5:** 534-43.

Sunters A, Fernandez de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA, Coffer PJ, Medema RH, Coombes RC, Lam EW (2003). FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* **278:** 49795-805.

Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, Ohsumi Y (2001). The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *Embo J* **20:** 5971-81.

Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, Liang C, Jung JU, Cheng JQ, Mul JJ, Pledger WJ, Wang HG (2007). Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* **9:** 1142-51.

Takahashi Y, Karbowski M, Yamaguchi H, Kazi A, Wu J, Sebti SM, Youle RJ, Wang HG (2005). Loss of Bif-1 suppresses Bax/Bak conformational change and mitochondrial apoptosis. *Mol Cell Biol* **25:** 9369-82.

Takahashi Y, Meyerkord CL, Wang HG (2008). BARgaining membranes for autophagosome formation: Regulation of autophagy and tumorigenesis by Bif-1/Endophilin B1. *Autophagy* **4:** 121-4.

Tanida I, Mizushima N, Kiyooka M, Ohsumi M, Ueno T, Ohsumi Y, Kominami E (1999). Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. *Mol Biol Cell* **10:** 1367-79.

Tasdemir E, Galluzzi L, Maiuri MC, Criollo A, Vitale I, Hangen E, Modjtahedi N, Kroemer G (2008). Methods for assessing autophagy and autophagic cell death. *Methods Mol Biol* **445:** 29-76.

Taylor RC, Cullen SP, Martin SJ (2008). Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* **9:** 231-41.

Thompson CB (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* **267:** 1456-1462.

Toth S, Nagy K, Palfia Z, Rez G (2002). Cellular autophagic capacity changes during azaserine-induced tumour progression in the rat pancreas. Up-regulation in all premalignant stages and down-regulation with loss of cycloheximide sensitivity of segregation along with malignant transformation. *Cell Tissue Res* **309:** 409-16.

Toueille M, El-Andaloussi N, Frouin I, Freire R, Funk D, Shevelev I, Friedrich-Heineken E, Villani G, Hottiger MO, Hubscher U (2004). The human Rad9/Rad1/Hus1 damage sensor clamp interacts with DNA polymerase beta and increases its DNA substrate utilisation efficiency: implications for DNA repair. *Nucleic Acids Res* **32:** 3316-24.

Tsujimoto Y, Shimizu S (2005). Another way to die: autophagic programmed cell death. *Cell Death Differ* **12 Suppl 2:** 1528-34.

Tsurimoto T (1999). PCNA binding proteins. *Front Biosci* **4:** D849-58.

van den Bosch M, Bree RT, Lowndes NF (2003). The MRN complex: coordinating and mediating the response to broken chromosomes. *EMBO Rep* **4:** 844-9.

Venclovas C, Thelen MP (2000). Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes. *Nucleic Acids Res* **28:** 2481-2493.

Vieira AV, Lamaze C, Schmid SL (1996). Control of EGF receptor signaling by clathrin-mediated endocytosis. *Science* **274:** 2086-9.

Volinia S, Dhand R, Vanhaesebroeck B, MacDougall LK, Stein R, Zvelebil MJ, Domin J, Panaretou C, Waterfield MD (1995). A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. *Embo J* **14:** 3339-48.

Volkmer E, Karnitz LM (1999). Human homologs of *Schizosaccharomyces pombe* rad1, hus1, and rad9 form a DNA damage-responsive protein complex. *J Biol Chem* **274:** 567-570.

Wan J, Cheung AY, Fu WY, Wu C, Zhang M, Mobley WC, Cheung ZH, Ip NY (2008). Endophilin B1 as a novel regulator of nerve growth factor/ TrkA trafficking and neurite outgrowth. *J Neurosci* **28:** 9002-12.

Wang H-G, Rapp UR, Reed JC (1996). Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell* **87:** 629-38.

Wang JC (2002). Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* **3:** 430-40.

Wang W, Brandt P, Rossi ML, Lindsey-Boltz L, Podust V, Fanning E, Sancar A, Bambara RA (2004a). The human Rad9-Rad1-Hus1 checkpoint complex stimulates flap endonuclease 1. *Proc Natl Acad Sci U S A* **101:** 16762-7.

Wang W, El-Deiry WS (2008). Restoration of p53 to limit tumor growth. *Curr Opin Oncol* **20:** 90-6.

Wang W, Lindsey-Boltz LA, Sancar A, Bambara RA (2006a). Mechanism of stimulation of human DNA ligase I by the Rad9-rad1-Hus1 checkpoint complex. *J Biol Chem* **281:** 20865-72.

Wang X, Guan J, Hu B, Weiss RS, Iliakis G, Wang Y (2004b). Involvement of Hus1 in the chain elongation step of DNA replication after exposure to camptothecin or ionizing radiation. *Nucleic Acids Res* **32:** 767-75.

Wang X, Hu B, Weiss RS, Wang Y (2006b). The effect of Hus1 on ionizing radiation sensitivity is associated with homologous recombination repair but is independent of nonhomologous end-joining. *Oncogene* **25:** 1980-3.

Wang X, Zou L, Zheng H, Wei Q, Elledge SJ, Li L (2003). Genomic instability and endoreduplication triggered by RAD17 deletion. *Genes Dev* **17:** 965-70.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292:** 727-30.

Weiss RS, Enoch T, Leder P (2000). Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress. *Genes Dev* **14:** 1886-98.

Weiss RS, Leder P, Vaziri C (2003). Critical role for mouse Hus1 in an S-phase DNA damage cell cycle checkpoint. *Mol Cell Biol* **23:** 791-803.

Weiss RS, Matsuoka S, Elledge SJ, Leder P (2002). Hus1 acts upstream of chk1 in a mammalian DNA damage response pathway. *Curr Biol* **12:** 73-7.

Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J (2001). Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* **29:** 629-43.

Wiley HS (2003). Trafficking of the ErbB receptors and its influence on signaling. *Exp Cell Res* **284:** 78-88.

Willis SN, Adams JM (2005). Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* **17:** 617-25.

Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM, Huang DC (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* **315:** 856-9.

Wong HK, Fricker M, Wyttenbach A, Villunger A, Michalak EM, Strasser A, Tolkovsky AM (2005). Mutually exclusive subsets of BH3-only proteins are activated by the p53 and c-Jun N-terminal kinase/c-Jun signaling pathways during cortical neuron apoptosis induced by arsenite. *Mol Cell Biol* **25:** 8732-47.

Xie Z, Klionsky DJ (2007). Autophagosome formation: core machinery and adaptations. *Nat Cell Biol* **9:** 1102-9.

Yamada T, Carson AR, Caniggia I, Umebayashi K, Yoshimori T, Nakabayashi K, Scherer SW (2005). Endothelial nitric-oxide synthase antisense (NOS3AS) gene encodes an autophagy-related protein (APG9-like2) highly expressed in trophoblast. *J Biol Chem* **280:** 18283-90.

Yang JY, Xia W, Hu MC (2006). Ionizing radiation activates expression of FOXO3a, Fas ligand, and Bim, and induces cell apoptosis. *Int J Oncol* **29:** 643-8.

Yarden Y, Sliwkowski MX (2001). Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2:** 127-37.

Yin Y, Zhu A, Jin YJ, Liu YX, Zhang X, Hopkins KM, Lieberman HB (2004). Human RAD9 checkpoint control/proapoptotic protein can activate transcription of p21. *Proc Natl Acad Sci U S A* **101:** 8864-9.

Yoshida K, Komatsu K, Wang HG, Kufe D (2002). c-Abl tyrosine kinase regulates the human Rad9 checkpoint protein in response to DNA damage. *Mol. Cell. Biol.* **22:** 3292-3300.

Yoshida K, Wang HG, Miki Y, Kufe D (2003). Protein kinase Cdelta is responsible for constitutive and DNA damage- induced phosphorylation of Rad9. *Embo J* **22:** 1431-41.

Yoshimori T (2004). Autophagy: a regulated bulk degradation process inside cells. *Biochem Biophys Res Commun* **313:** 453-8.

Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y (1991). Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem* **266:** 17707-12.

Young AR, Chan EY, Hu XW, Kochl R, Crawshaw SG, High S, Hailey DW, Lippincott-Schwartz J, Tooze SA (2006). Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. *J Cell Sci* **119:** 3888-900.

Yousefi S, Perozzo R, Schmid I, Ziemiecki A, Schaffner T, Scapozza L, Brunner T, Simon HU (2006). Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol* **8:** 1124-32.

Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, Baehrecke EH, Lenardo MJ (2004). Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* **304:** 1500-2.

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

Zeng X, Overmeyer JH, Maltese WA (2006). Functional specificity of the mammalian Beclin-Vps34 PI 3-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme trafficking. *J Cell Sci* **119:** 259-70.

Zhou BB, Elledge SJ (2000). The DNA damage response: putting checkpoints in perspective. *Nature* **408:** 433-9.

Zhu A, Zhang CX, Lieberman HB (2008). Rad9 has a functional role in human prostate carcinogenesis. *Cancer Res* **68:** 1267-74.

Zhu M, Weiss RS (2007). Increased common fragile site expression, cell proliferation defects, and apoptosis following conditional inactivation of mouse Hus1 in primary cultured cells. *Mol Biol Cell* **18:** 1044-55.

Zimmermann KC, Bonzon C, Green DR (2001). The machinery of programmed cell death. *Pharmacol Ther* **92:** 57-70.

Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev* **15:** 1481-6.

Zou L, Cortez D, Elledge SJ (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. *Genes Dev* **16:** 198-208.

Zou L, Elledge SJ (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300:** 1542-8.

About the Author

Cheryl Meyerkord attended Wittenberg University, where she earned a Bachelor of Science Degree in Biology in May 2003. After graduation, she joined the Cancer Biology Ph.D. program at the H. Lee Moffitt Cancer Center and Research Institute. She conducted her doctoral research under the mentorship of Dr. Hong-Gang Wang. Cheryl received funding for her graduate research from the Department of Defense Breast Cancer Research Program. During her time in the Cancer Biology Program, Cheryl contributed to several studies, which led to the publication of three papers. The focus of her research was on investigating various mechanisms that are regulated by the Rad9-Rad1-Hus1 complex and Bif-1 that affect sensitivity to DNA damage.