p53 and Beclin 1 in *Caenorhabditis elegans* are involved in cell death and DNA damage repair

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2013

This manuscript has been read and accepted for the
Graduate Faculty in Biochemistry in satisfaction of the
dissertation requirement for the degree of Doctor of Philosophy

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Abstract

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p53 is a key regulator of growth arrest, DNA repair, apoptosis, and autophagy in response to cellular stress in animals as diverse as *Caenorhabditis elegans* and humans. In mammals, p53 functions as a key tumor suppressor protein that promotes removal of potentially tumorigenic cells. *p53* is mutated or deleted in over 50% of all mammalian tumors and these tumors are highly resistant to available therapies. Therefore, identifying therapeutic agents and cell death pathways that promote p53-independent cell death is vital to the future of cancer therapy. We are interested in identifying how the alkylating agent, 10-decarbomyl mitomycin C (DMC) promotes p53-indpendent cell death. To answer this question, we used a *C. elegans* as a model system to identify known and novel cell death genes involved in DMC-induced death. *C. elegans* have an orthologue of *p53, cep-1*, that functions in germline cell death in a similar way to p53 in apoptosis. The germline in *C. elegans* has both mitotic and meiotic cells and displays CEP-1/p53-dependent cell death in response to UV. We examined both germline cell death and transcript levels of CEP-1 target genes in *C. elegans*. We found that DMC increased CED-1::GFP positive cells and DNA lesions in the absence of CEP-1 while UV required CEP-1 for germline cell death but not nuclear lesion formation. More lesions were seen over time in *cep-1(gk138)* mutant worms leading to the idea that CEP-1 is involved in DNA repair. Additionally, we examined the role of autophagy in cell death and DNA damage and saw that initially the knockdown of *bec-1* required CEP-1 for increases in germ cell death. However in the F1

generation of *bec-1* RNAi knockdown animals, the observed increase in cell death was due to a lack of clearance. Furthermore, *bec-1* knockdown resulted in the increase of DNA lesions in worms with UV damage.

Acknowledgements

I began my graduate experience with limited research background and am grateful for the science education Dr. Jill Bargonetti gave me. Through Dr. Bargonetti, I was able to develop as a scientist and grow as a person. Her never ending patience, ideas and motivation helped me throughout my graduate studies and I will forever be thankful to her.

I am very much thankful to the members of the Bargonetti lab for their ideas, thoughts and support my time in the lab. I am extremely appreciative to Dr. Ryan Doonan for his help in developing the project and constant support. And to Danielle Okoro for keeping me sane.

I am grateful to my committee members and advisors for their valuable comments and tremendous support throughout my graduate work. To Dr. Alicia Melendez and her laboratory for allowing me to use their supplies and microscopes, and most importantly, for my *C. elegans* training. I am thankful to Dr. Xinxhu Deng for her assistance in germline cell death and Dr. Joel Meyer and Dr. Amanda Bess for helping us set up the Lesion assay in our laboratory.

And finally to my parents, who never let me give up my dream.

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Commonly Used Abbreviations

AO: Acridine orange
CDK: Cyclin dependent kinase
CED: Cell death abnormal
CEP: *C. elegans* p53-1
CPDs: Cyclobutane-pyrimidine dimers
CS: Cockayne Syndrome
DMC: 10-decarbomyl mitomycin C
DRAM: Damage-regulated autophagy modulator
DTC: Distal tip cell
EGL: Egg laying abnormal
ERCC: Excision repair cross-complementing rodent repair deficiency
GGR: Global genome repair
GLP: Germline proliferation defective
IR: Ionizing Radiation
MOMP: Mitochondrial outer membrane permeabilization
mTOR: Mammalian target of Rapamycin
NER: Nucleotide excision repair
NGM: Nematode growth media
QPCR: Quantitative polymerase chain reaction
q-RTPCR: Quantitative reverse transcription polymerase chain reaction
TCR: Transcription-coupled repair
TMRE: Tetramethylrhodamine ethyl ester
UV: Ultraviolet
XP: Xeroderma pigmentosum
6-4PP: 6-4 photoproducts

CHAPTER 1:

Introduction

1.1 p53

The p53 protein is a tumor suppressor and the key regulator of cellular responses to stress (Lu and Abrams, 2006). p53 was first discovered as a protein associated with the simian virus 40 (SV40) T-antigen (Gartel et al., 1996; Hainaut and Vahakangas, 1997). Because *p53* is often mutated in cancers, *p53* was originally classified as an oncogene (Ashkenazi, 2002; Farmer et al., 1996; Jeffrey et al., 1995; Montes de Oca Luna et al., 1995; Nigro et al., 1989; Smart et al., 2008; Vassilev et al., 2004). Later on, p53 was determined to be a tumor suppressor that is targeted for inactivation by many tumor inducing viruses. p53 is inactivated by viruses in different ways. The large T-antigen of SV40 binds to p53 inhibiting the transactivation of wild-type p53 (Mietz et al., 1992). The Friend virus-induced erythroleukemia causes rearrangements of the DNA binding domain in the *p53* gene rendering it inactive (Alberts et al., 2008; Gudkov and Komarova, 2003; Vassilev, 2004; Zong and Thompson, 2006). The Human papillomaviruses (HPVs) encode an oncoprotein, termed E6, which targets p53 for degradation (Janicke et al., 2008; Kano and Fujiwara, 1981; Wozniak and Ross, 1983). The loss of p53 wild type activity is often detected in tumors and functional p53 inhibits oncogene induced transformation of tissue culture cell lines (Lee et al., 2008; Stokes et al., 2007). *p53* null mice are normal at birth but develop tumors by six months of age (Honda et al., 1997; Hussain and Harris, 2000; Levine et al., 2008; Michael and Oren, 2003; Roemer, 1999). Mice born heterozygous for *p53* develop tumors after a loss of heterozygosity (Feng et al., 2012; Hori et al., 2002; Levine and Yuan, 2005). More than 50% of human tumors have mutant *p53* (Castedo et al., 2004a; Kinyamu and Archer, 2003; Lu and Abrams, 2006; Zauberman et al., 1993). This section reviews the structure and functions of p53 in the mammalian cells.

1.1.1 p53 structure

The *Tp53* gene is found on human chromosome 17p13.1 and is approximately 20kb in length (Castedo et al., 2004b; Karantza-Wadsworth et al., 2007; Yue et al., 2003). The gene encodes a 53 kDa protein with 393 amino acids containing the following domains: an acidic N-terminus transactivation domain, a proline rich domain, a central hydrophobic DNA-binding domain, a tetramerization domain and a regulatory domain at the basic C-terminus (Figure 1.1) (Bullock and Fersht, 2001; Ding et al., 2006; Sur et al., 2009; Venot et al., 1998). The N-terminal transactivation domain actively recruits the transcription machinery to promoter regions (Sur et al., 2009). The proline rich domain is involved in the activation of apoptosis (Durocher and Jackson, 2001). The most frequently mutated residues in *p53* are found within the DNA binding domain, termed "hot spot mutations" which is required for p53 to bind to the DNA (Figure 1.1) (Bullock and Fersht, 2001; Mordes and Cortez, 2008; Zambetti et al., 1992). These missense mutations can be contact or conformation mutations inhibiting p53 from binding to the DNA properly (Bullock et al., 2000; Cho et al., 1994; Joerger et al., 2006). The hot spot residues are conserved in homologues of p53, including p63 and p73 (Yang and McKeon, 2000). Without this residue, p53 cannot bind to the DNA at the sequence specific locations and promote the activation of its target genes (Farmer et al., 1992; Zambetti et al., 1992). The C-terminus includes the domains involved in proper localization, regulation and oligomerization of p53 (Shiloh, 2001). p53 forms a tetramer and the quaternary structure is vital for p53 to interact with DNA and activate gene transcription (Jeffrey et al., 1995; Shiloh and Kastan, 2001).

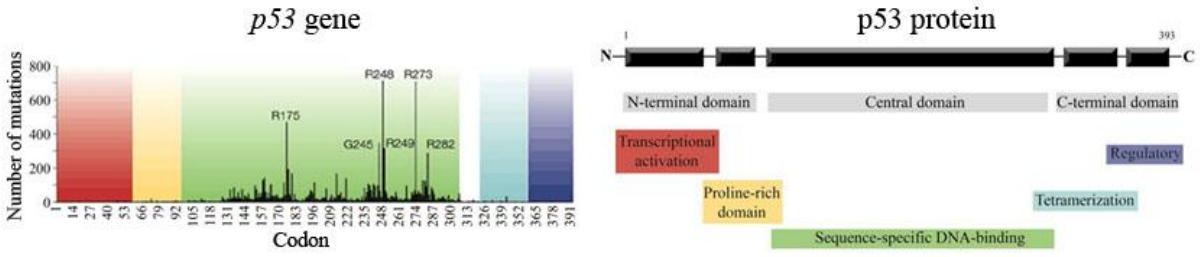

Figure 1.1: *p53* gene and protein domains: *p53* gene is most frequently mutated in the DNA binding domain causing the protein to not bind to the DNA correctly. The protein consists of a transcriptional activation, a proline-rich, a DNA-binding, the tetrazmerizaion and regulatory domains. (Bullock and Fersht, 2001)

1.1.2 p53 regulation by Mdm2

After cellular stress, p53 is post-translationally modified and stabilized (Lu and Abrams, 2006). p53 is kept at low levels in non-stressed normal cells by its downstream target and key regulator Mdm2. The *mdm2* gene is a downstream target of p53 and Mdm2 is an E3 ubiquitin ligase that targets p53 for degradation by the ubiquitin-proteasome pathway (Barak et al., 1993; Carr, 2002). The hydrophobic pocket at the N-terminus of Mdm2 binds to the N-terminus of p53 (Poyurovsky et al., 2010). Mdm2 ubiquitinates p53 at the C-terminus targeting p53 for proteasomal degradation (Frey and Gandhi; Kubbutat et al., 1998). Additionally, Mdm2 can co-localize with p53 at the chromatin inhibiting p53 transcriptional activation (Arva et al., 2005; White et al., 2006). Phosphorylation of the N-terminus of p53 within the Mdm2 binding domain disrupts the Mdm2-p53 complex and activates p53 (Sakaguchi et al., 2000; Shieh et al., 1997; Unger et al., 1999). Phosphorylation, acetylation, and sumolation are post-translational modifications of p53 and are found throughout the protein (Gu and Roeder, 1997; van Gent et al., 2001; Vousden and Prives, 2009). These modifications stabilize p53 and assist in keeping p53 in the nucleus to activate the transcription of its downstream targets (Chehab et al., 1999; van Gent et al., 2001). Once p53 is stabilized, the half-life and levels of p53 increase (Bouska

and Eischen, 2009). Furthermore, these modifications prevent Mdm2 from binding to the activated p53. Tumors with wild-type *p53* can have mutations in the *mdm2* gene, to block wild type p53 activity. For example, *mdm2* gene amplification or a single nucleotide polymorphism in the promoter, and these often increase Mdm2 levels to inhibit wild type p53 activity (Arva et al., 2005; Fakharzadeh et al., 1991; Michael and Oren, 2003; Oren et al., 2002).

1.1.3 p53 signaling pathways

Human and Murine cells use p53 as a first line of defense against transformation by inducing cell cycle arrest, DNA repair or apoptosis (Vousden and Prives, 2009). Once p53 is stabilized, p53 associates with consensus response elements at the promoters of target genes. Then p53 will activate the transcription of over 200 known targets and (Vousden and Prives, 2009). These target genes include regulators of cell cycle arrest, autophagy, DNA repair and apoptosis. *p21* is a key target gene of p53 which induces cell cycle arrest (Iwakuma and Lozano, 2003; Janicke et al., 2008; Shangary and Wang, 2009). p21 is a cyclin-dependent kinase (CDK) inhibitor and promotes G_1 arrest. p21 binds to CDK4/6/cyclin D and CDK2/cyclin E complexes inhibiting their ability to enter S phase (Juven et al., 1993; Shangary and Wang, 2009).

Additionally, p53 works in the nucleus and in the cytoplasm to activate apoptosis (Baptiste and Prives, 2004; Vousden, 2000). The *noxa* and *puma* genes are pro-apoptotic targets upregulated by p53 (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). NOXA and PUMA are BH3-only proteins and inhibit the Bcl-2 anti-apoptotic family members (Vousden and Lu, 2002). The Bcl-2 family members inhibit, Bax and Bak, BH123 proteins. The *bax* and *bak* genes are also targets of p53 (Miyashita and Reed, 1995; Pohl et al., 1999). Once the Bcl-2

family members are inhibited by NOXA and PUMA, Bax and Bak can oligomerize on the outer mitochondria membrane to form pores, termed MOMP for mitochondria outer membrane permeabilization (Ashkenazi, 2002). MOMP promotes the release of cytochrome C into the cytoplasm and cytochrome C will bind to Apaf1, a procaspase-activating factor, to form an apoptosome. This complex recruits and activates procaspase-9 by cleavage and caspase-9 turns on the executioner, caspase-3. Once caspase-3 is activated, it cleaves cellular proteins and activates apoptosis in cells (Alberts et al., 2008; Ashkenazi, 2002).

In addition to the nuclear activity of p53, p53 works in the cytoplasm to activate apoptosis. p53 can interact with the Bcl-2 family members directly (Tomita et al., 2006). Once *puma* is upregulated and translated, PUMA binds to Bcl-2 family members releasing p53 and allowing p53 to directly activate Bax/Bid and induce MOMP (Baptiste and Prives, 2004; Chipuk et al., 2005; Moll et al., 2005). Here, the nuclear and cytoplasmic activities of p53 work hand in hand to activate the intrinsic caspase pathway.

Furthermore, human p53 can activate autophagy transcriptionally (Crighton et al., 2006). After DNA damage, nuclear p53 activates the transcription of proautophagic genes, such as *dram* (damage-regulated autophagy modulator), a lysosomal protein that is involved in turning on macroautophagy and is also involved in p53-mediated apoptosis (Crighton et al., 2006). DRAM works to promote apoptosis via the induction of autophagy.

1.1.4 p53 and the nucleotide excision repair pathway

Cells have DNA damage response pathways to initiate DNA repair, cell cycle arrest, and/or cell death (Kastan, 2008). These pathways are critical in thwarting genomic instability and potential cancer cell development. Genes that are involved in DNA damage sensing and repair are often compromised in cancer and lead to further genomic instability and inappropriate cell growth (Kastan, 2008). The sensors of DNA damage typically activate kinase cascades leading to the stabilization and activation of p53 (Stokes et al., 2007).

The nucleotide excision repair (NER) pathway is required for the repair of DNA damage induced by Ultraviolet (UV) and alklyating agents (Prakash and Prakash, 2000). UV induces DNA damage by forming cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) distorting the double helix and activates p53 (Barak et al., 1993; Maltzman and Czyzyk, 1984). Alkylating agents that can form adducts, interstrand crosslinks and intrastrand crosslinks activate p53 as well (Fritsche et al., 1993). These forms of DNA damage require the NER pathway and p53 for full DNA repair (Wang et al., 2001). The NER pathway includes proteins that recognize the damage, helicases that unwind the DNA strands and endonucleases to cleave the damage (Evans et al., 1997). The NER pathway can be activated by lesions that are found in regions of the DNA not being actively transcribed, termed global genome repair (GGR), or by stalled replication forks, termed transcription-coupled repair (TCR) (Hoeijmakers, 2001). Early responders to GGR include the xeroderma pigmentosum (XP) family members XPC, XPA, XPE, the human homologue of yeast Rad23, hHR23b, and replication protein A (RPA) (McHugh et al., 2001). After the early responders recognize the damage, helicases, XPB and XPD, are recruited and function with the transcription factor TFIIH to unwind the DNA (Evans et al.,

1997). Next, the endonucleases ERCC1-XPF (Excision repair cross-complementing rodent repair deficiency, complementation group 1) complex and XPG are recruited to cleave the 5' and 3' ends, respectively, removing 27-30 nucleotides containing the lesion (De Silva et al., 2000; van Vuuren et al., 1993). The new strand is synthesized by DNA polymerase ε or δ working with proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). Finally, the newly synthesized strand is connected to the parental strand by DNA ligase I (Lane et al., 2010b). TCR is repaired quickly using Cockayne syndrome (CS) factors A and B, TFIIH and XPG assisting to recruit the NER repair family proteins and replace the stalled polymerase (Lane et al., 2010a).

p53 works with GGR pathway to repair UV induced DNA damage. Cells without functional p53 cannot fully repair UV-mediated DNA damage (Oliner et al., 1992). After UV, p53 activates the transcription of *xpc*, *DDB2* and *gadd45* (Oliner et al., 1992; Oliner et al., 1993). XPC and DDB2 (DNA damage binding protein) are early responders in GGR. GADD45 (growth arrest and DNA damage) causes cell cycle arrest at G2/M phase by inhibiting Cdc2-cyclin B1 complex and GADD45 binds to chromatin during DNA repair after UV treatment (Jin et al., 2000; Smith et al., 2000). p53 can regulate the helicase activities of XPB and XPD by physically binding to them (Wang et al., 1995). p53 also assists ATR in the nuclear import of XPA (Li et al., 2011).

1.1.5 p53 family members

p53 has two family members in the mammalian system, p63 and p73 (Allocati et al., 2012). Similar to p53, p63 and p73 can regulate cell cycle progression and cell death. The three proteins all have DNA binding domains, oligomerization domains and transactivation domains. In addition, p63 and p73 have a Sterile alpha motif (SAM) domain at their C-terminus which is crucial for protein/protein interactions. Similarly, the *p63* and *p73* genes have alternative splicing giving rise to different protein isoforms with different functions (Allocati et al., 2012). All three family members form tetramers but p63 and p73 can form mixed tetramers while p53 does not (Joerger et al., 2009).

p63 and p73 regulate the cell cycle and apoptosis in similar fashions to p53. The transactivation domains of both p63 and p73 activate the transcription of *p21* to promote G1 arrest (Allocati et al., 2012). The two proteins can also repress cyclins required for G2/M progression. In order to promote cell death, p63 and p73 upregulate Bax and PUMA and promote the translocation of Bax. p63 and p73 also activate the death receptor pathway by inducing the expression of different Trail proteins and CD95, respectively (Allocati et al., 2012).

While *p53* null mice develop tumors at early ages (Honda et al., 1997; Hussain and Harris, 2000; Levine et al., 2008; Michael and Oren, 2003; Roemer, 1999), *p63* and *p73* null mice have different phenotypes. *p73* null mice die early from abnormally developed hippocampus and have increased levels of apoptosis in sympathetic neurons (Allocati et al., 2012). In addition, *p73* null mice have increased chromosomal instability and will develop spontaneous tumors, but due to their shortened lifespan, tumor growth is not always observed (Allocati et al., 2012).

p63 null mice have different phenotypes; these mice have irregular ectodermal development and will have abnormal skin, salivary glands, teeth and hair follicles (Allocati et al., 2012). These animals die young and have truncated limbs. p63 plays a key role in oocyte death during meiosis in mice (Suh et al., 2006). A p63 isoform is always on during meiotic arrest in female germ cells and promotes DNA damage induced oocyte cell death in a p53 independent pathway. p63 monitors the integrity of the female germline in mice, which we have shown to be a similar feature to the p53-family homologue, CEP-1, in *C. elegans* (Suh et al., 2006). Furthermore, p63 plays a role in programmed cell death in developing testis in male mice (Petre-Lazar et al., 2007). These data give p63 a novel role in maintaining the integrity of the germline.

1.2 *Caenorhabditis elegans*

Caenorhabditis elegans are free living nematodes with a largely conserved genome to humans (Brenner, 1974). Included in these conserved genes are the core apoptotic regulators and apoptosis was first described in *C. elegans* (Sulston and Horvitz, 1977). Furthermore, DNA damage repair genes and autophagy are conserved in *C. elegans*. In an adult worm, only the germline is actively dividing and the somatic cells are post-mitotic. This mimics a human adult with a proliferating tumor. We are using *C. elegans* as a model system to observe the differences in DNA damage and cell death in a whole animal system with and without *p53*.

1.2.1 *C. elegans* and their germline

C. elegans grow to about 1 mm and, when grown at room temperature, have a reproductive cycle of around three days (Brenner, 1974). The life cycle begins as an egg which

completes embryogenesis after being laid. The worm hatches and undergoes four larval stages (L1 through L4). After completing the fourth larval (L4) stage, the worm becomes an adult. As an adult, only the germline cells are actively dividing and the somatic cells are fully differentiated and are post-mitotic. *C. elegans* have 6 chromosomes and about 40% of the worm genome is conserved to humans (Lai et al., 2000). *C. elegans* can be either hermaphrodites or males. Males are formed from nondisjunction of the X chromosome, normally from heat shock, yielding an XO genotype. In the laboratory, *C. elegans* can be grown on agar plates with bacteria (Brenner, 1974) .

The *C. elegans* germline development starts during the larval stages (Hubbard and Greenstein, 2005). The germline begins as four cells in early L1 and develops into 12 cells by the end of L1. During the L2 phase, the somatic gonad is formed and the germ cells are polarized to the anterior and posterior of the gonad. As the worm develops into an early L3, the gonad arms are extended and there is an increase in germ cell proliferation. These changes are induced by signaling from the distal tip cell and the developing gonad sheath cells. Once the worm becomes an early L4, the germ cells begin to transition and enter meiosis. Spermatogenesis occurs during L4 while oogenesis is restricted to adulthood (Hubbard and Greenstein, 2005).

The adult hermaphrodite germline is divided up into different domains found within a syncytium (Conradt and Xue, 2005; Kimble and Crittenden, 2005). The mitotic region is at the distal end and is controlled by the distal tip cell (DTC) (Figure 1.2) (Boxem, 2006). The DTC controls proliferation via notch signaling. The DTC release a ligand (LAG-2) which is received by the mitotic cells via their LAG-1 (germline proliferative defective) receptor. This signaling promotes mitosis and not meiosis (Kimble and Crittenden, 2005). As the nuclei move away from

the DTC, GLP-1 levels decrease and allow the nuclei to transition into meiosis. At the start of meiosis, nuclei enter early prophase (pachytene) and can undergo physiological or DNA damage induced cell death (Figure 1.2, boxed region). This death is caspase dependent. Cells that survive move proximally, they develop into oocytes, pass through the spermatheca to get fertilized and will move into the uterus to be expelled out the vulva (Kimble and Crittenden, 2005).

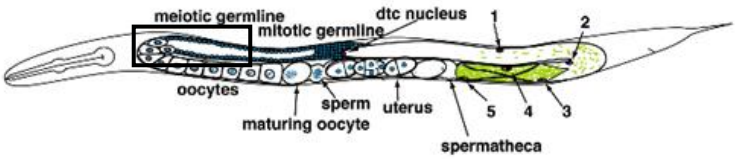

Figure 1.2: Germline in adult *C. elegans*. Schematic drawing of the germline in an adult hermaphrodite (Greenstein, 2005). The left hand side depicts the actively proliferating germline. The boxed area is where germline cell death is observed. The right hand side shows the somatic cells of the gonad arm. Five pairs of sheath cells cover the gonad arm. Shown is one member of each pair (1-5).

The somatic gonad regulates the germline and its development (Greenstein, 2005). There are five pairs of somatic gonad cells that are interspersed throughout the gonad (Figure 1.2). The first pair covers the entire beginning of the gonad and forms an irregular meshwork between germ cells via extended filopida. These cells regulate the movement of mitotic and early meiotic cells. The second pair lies over the bend and has intermediate properties between the first pair and the third, fourth and fifth pairs. Pairs three, four and five have muscle components that organize into dense networks and are involved in contraction to drive ovulation (Greenstein, 2005).

Localized notch signaling from the DTC and the somatic gonad control the developing gonad (Kimble and Crittenden, 2005). When key germline developmental genes are mutated, the gonad can be altered. An example of a mutant gonad is when the *glp-1* gene is lost or mutated. *glp-1* null animals have no GLP-1 receptor and do not develop a germline. On the other hand,

glp-1 gain of function mutants have a constitutive GLP-1 and form tumors, a population of solely mitotic cells (Figure 1.3) (Kimble and Crittenden, 2005; Pepper et al., 2003). The mitotic cells of the tumor mutants are highly resilient to IR (Ionizing Radiation) (Deng, X. unpublished data). These proliferating cells maintain CEP-1 activity that can be activated by UV-mediated DNA damage (Doonan, R. and Martin, D., unpublished data).

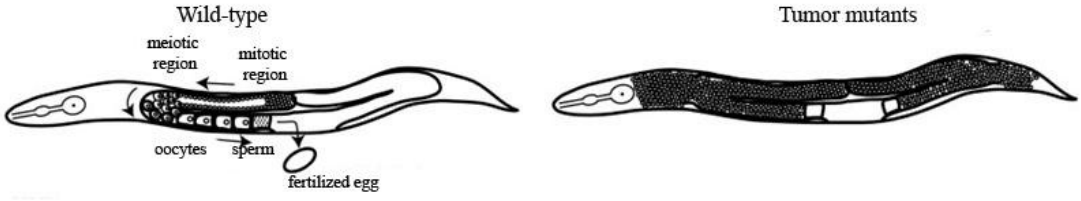

Figure 1.3: Comparison of wild-type germline to a tumor mutant. Schematic drawing of germline in an adult hermaphrodite. The wild-type worm has differentiated regions with different nuclei while the tumor mutant worm has only mitotic cells (Doonan, R.).

1.2.2 Programmed cell death

C. elegans undergo conserved programmed cell death during both embryonic and post-embryonic development, called developmental cell death (Conradt and Xue, 2005). Programmed cell death also occurs in the gonad of hermaphrodites, recognized as germline cell death. Developmental cell death has been well studied. Cells sentenced to death have been identified as well as their exact time of death. The key players in worm developmental cell death include CED-3 and CED-4 (cell death abnormal). During hermaphrodite development, 131 cells out of the 1090 somatic cells die religiously (Conradt and Xue, 2005). However, germ cell death is not fixed; rather, each cell is individually regulated and, under normal conditions, around 50% of germ cells are fated to die (Gumienny et al., 1999). These cells are normally engulfed by the somatic gonad quickly and are used as "nurse cells" to fuel oocyte development (Gartner et al., 2008). Germline cell death is carried out via CED-4 and CED-3, similar to developmental cell

death, and can be activated by DNA damage through the CEP-1/p53 pathway (Conradt and Xue, 2005; Gartner et al., 2008).

Both physiological and DNA damage induced germline cell deaths occur in the early pachytene region (Figure 1.2, boxed region (Gartner et al., 2008)). The majority of programmed cell death requires the core apoptotic machinery. This includes: the BH3 homologues, *egl-1* and *ced-13*, the Apaf-1 gene, *ced-4* and the sole worm caspase, *ced-3*. EGL-1 (egg laying abnormal) and CED-13 bind the anti-apoptotic Bcl-2 homologue, CED-9; this allows CED-4 to be released from CED-9 at the mitochondria. CED-4 moves to the perinuclear membranes and dimerizes with itself. Two CED-3 proenzymes are brought near the CED-4 complex and CED-3 is activated by autoproteolysis; after which, cell death is activated and nuclear fragmentation occurs followed by engulfment of cellular corpses (Conradt and Xue, 2005; Hubbard and Greenstein, 2005). This pathway occurs during programmed cell death and germline cell death.

1.2.3 CEP-1 and DNA damage response pathways

In 2001, two independent groups, using two different methods, identified the p53 homologue in *C. elegans*, termed CEP-1 for *C. elegans* p53-1 on chromosome I (Derry et al., 2001; Schumacher et al., 2001). CEP-1 is a transcription factor that promotes apoptosis upon DNA damage in the germline. CEP-1 plays a role in repressing cell proliferation after UV damage and activates apoptosis in the germline after worms are treated with a variety of DNA damaging agents (ENU, IR, UV) (Derry et al., 2007). The CEP-1 pathway is required for normal meiotic segregation in the germline and is activated by cellular and genotoxic stress (Derry et al., 2001; Schumacher et al., 2001). Additionally, CEP-1::GFP is found ubiquitously during

development, but after hatching, is restricted to the nuclei of pharynx cells and germline. Therefore any p53 activity detected in an adult worm comes from either the pharynx or the germline. The expression was done by fusing the 4.5 kb upstream sequence and the *cep-1* gene in-frame to a GFP marker. The construct was co-injected with a dominant *rol-6(su1006)* that is robust in the zygotes. Therefore, the exact location of CEP-1 in the somatic cells in the adult worm is not clear. Apoptosis in somatic cells is unaffected by CEP-1, therefore, the CEP-1-dependent cell death pathway in adult worms is primarily observed in the germline (Derry et al., 2001).

CEP-1 has similar domains to mammalian p53 including: a transactivation domain at the N-terminus, a DNA binding domain in the core and an oligomerization domain at the C-terminus (Figure 1.4) (Derry et al., 2007; Ou et al., 2007). CEP-1 is an 83 kDa protein, made up of 644 amino acids. The DNA binding domain has 15% sequence homology to the human p53 including the DNA binding arginines (Ou et al., 2007). Similar to p63 and p73, CEP-1 has a SAM (Sterile Alpha Motif) domain (Ou et al., 2007). CEP-1 binds to the p53 responsive elements *in vivo* and *in vitro* (Huyen et al., 2004; Schumacher et al., 2001). The primary downstream targets of CEP-1 include two proapoptotic genes, *egl-1* and *ced-13* (*puma* and *noxa* homologues, respectively), (Hofmann et al., 2002; Schumacher et al., 2005b).

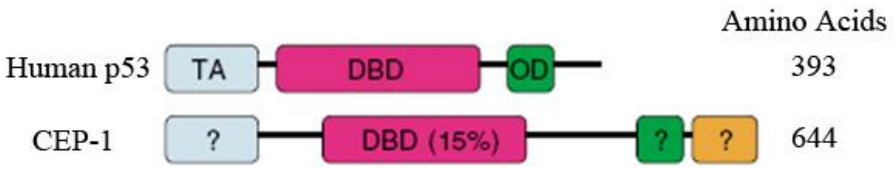

Figure 1.4: p53 and CEP-1 homology. CEP-1 and p53 have the most homology within their DNA binding domain (Adapted from (Ou et al., 2007)). This conservation includes the hot-spot mutations frequently mutated in cancer. There is low sequence homology to the transactivation and oligomerization domains in p53. The Yellow box indicates a low sequence homology to the SAM motif found in p63 (Ou et al., 2007).

Arrays have been carried out to identify additional CEP-1 downstream targets after DNA damage (Derry et al., 2007; Greiss et al., 2008). The activation of *egl-1* and *ced-13* were both confirmed in the different arrays. Other CEP-1 dependent genes were found. After UV induced DNA damage, 1394 genes are activated and 942 are repressed by CEP-1, many of which contain p53-binding sites (Derry et al., 2007). Of those, *phg-1*, a negative regulator of germline proliferation after UV treatment, was identified to be a CEP-1 target. PHG-1, the Gas1 (growth arrest specific 1) homologue, is required for the cell cycle arrest that occurs after UV but not for germline cell death (Derry et al., 2007; Stergiou et al., 2007).

The DNA damage response to apoptotic genes are conserved between mammalian and C. elegans; thus allowing us to investigate CEP-1 activation in worms similar to p53 activity in mammalian tissue culture. DNA damage response genes are conserved between mammalian cells and C. elegans (O'Neil and Rose, 2006). Figure 1.5 illustrates, in part, the pathway and its conservation. Induced germline cell death requires EGL-1, CED-13, CED-4 and CED-3, similar to physiological death, but also involves DNA damage sensor genes and CEP-1. The upstream activators of CEP-1 include the 9-1-1 complex consisting of HPR-9 (Homolog of *S. Pombe* Rad), MRT-2 (MoRTal germline), and HUS-1 (Human HUS1 related), the ATM and ATR

homologues, ATM-1 (ataxia telangectasia mutated) and ATL-1 (ataxia telangectasia mutated-like), and CHK-1 (CHeckpoint Kinase) and CHK-2 (O'Neil and Rose, 2006). The NER pathway is also highly conserved in *C. elegans* and is required for proper DNA repair and cell death, these include: XPA-1 (Xeroderma pigmentosum), XPB-1, XPC-1, XPD-1, XPE-1, XPF-1, XPG-1, ERCC-1 (Excision-Repair Cross-Complementing) and CSB-1 (Cockayne Syndrome B) (O'Neil and Rose, 2006; Stergiou et al., 2011). The DNA damage response genes in the worms have similar roles to the mammalian system and signal to CEP-1. The phenotype from worms mutated in the DNA damage response genes varies. There are mutants with increased germline cell death, such as: RAD-54 (RADiation sensitivity abnormal) and RAD-51, proteins involved in homologous recombination (Stergiou et al., 2011). Other DNA damage repair mutants have reduction in their DNA damage induced cell death, for example, NER repair proteins (Stergiou et al., 2011). These genes are required for genome integrity and therefore mutants can have embryonic lethality, reduced brood size and a Him (male) phenotype (O'Neil and Rose, 2006).

The primary role of CEP-1 is the activation of cell death after DNA damage, but CEP-1 has been reported to be required for numerous other stress responses with a *w40* allele (Derry et al., 2001). DNA damage, such as IR and UV, induced germline cell death is not observed after the loss of *cep-1* (Quevedo et al., 2007; Schumacher et al., 2001; Stergiou et al., 2007). *cep-1(w40)* mutants have a chromosomal rearrangement of one *cep-1* allele yielding a dominant negative effect. The *cep-1(w40)* mutants show low levels of embryonic lethality under normal conditions, but under hypoxic conditions, *cep-1(w40)* animals had elevated levels of embryonic lethality (Derry et al., 2001). Wild-type starved larval worms had better survival rates when compared to *cep-1(w40)* mutant worms. Finally, similar to DNA damage mutants, *cep-1(w40)*

mutant worms and *cep-1* RNAi fed worms had elevates levels of a Him phenotype (Derry et al., 2001).

The upstream DNA damage response pathway leads to the phosphorylation of CEP-1 in response to DNA damage (O'Neil and Rose, 2006). CEP-1 is stable but activated by posttranslational modifications (Quevedo et al., 2007). CEP-1 is negatively regulated through a few different proteins that attenuate activation of CEP-1. For example, ABL-1, the homologue to c-Abl, is an upstream inhibitor of CEP-1. ABL-1 inhibits the activation of the check point genes after DNA damage preventing CEP-1 from getting posttranslationally activated (Deng et al., 2004). SCF^{FSN-1} (Skp1/cullin/F-Box), an E3 Ubiquitin Ligase, inhibits CEP-1 activity in response to ENU (N-ethyl-N-nitrosourea), a DNA alkylating agent (Gao et al., 2008). Additionally, AKT-1 and AKT-2 prevent posttranslational phosphorylation of CEP-1 and therefore limit *egl-1* and *ced-13* fold induction and CEP-1-dependent germline cell death but by regulating upstream of CEP-1 (Quevedo et al., 2007). GLD-1 (germline defective-1) regulates CEP-1 levels in the early meiotic region of the germline. GLD-1 inhibits *cep-1* translation by binding to the 3' untranslated region of the *cep-1* mRNA keeping CEP-1 protein levels low under normal conditions. *gld-1* mutant worms have an increased level of CEP-1 activity in the germline (Schumacher et al., 2005a). No Mdm2 homologue has been found in *C. elegans*, while other lower organisms do have Mdm2 homologues, for example, *Xenopus* (Lane et al., 2010a; Lane et al., 2010b). There may be a potential Mdm2-like protein because the p53 residues that Mdm2 binds to are conserved in the N-terminus of CEP-1 (Derry et al., 2001).

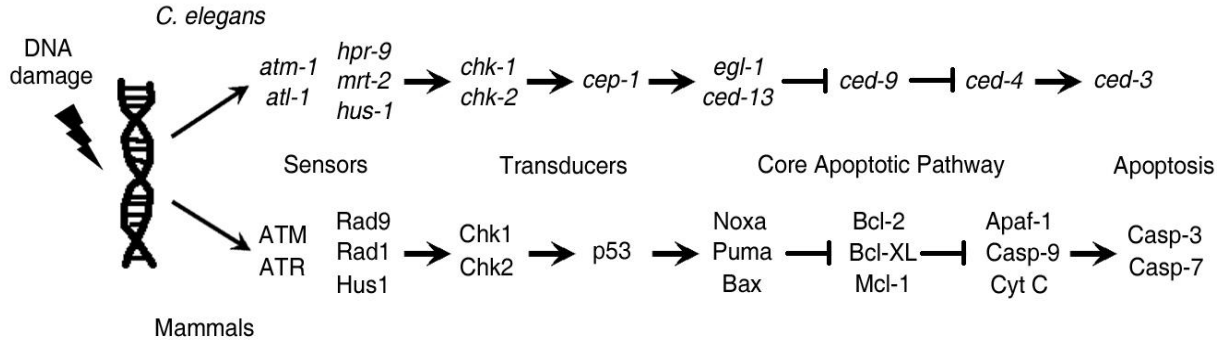

Figure 1.5: Conserved DNA damage induced cell death pathway. The DNA damage cell death pathway is conserved. The sensors, transducers and core apoptotic pathway after DNA damage are all similar between *C. elegans* and the mammalian systems. (Adapted from (Ito et al., 2010))

Pharmacological compounds have a hard time entering the worm and reaching their target (Burns et al., 2010). Therefore, *C. elegans* have only been used as a preclinical pharmacological model with a very limited number of compounds. Gleevec, a c-Abl inhibitor often used in human cancer therapy, has been used to induce apoptosis in *C. elegans* germline along with IR. This allowed them to characterize the c-Abl-dependent cell death pathway in *C. elegans* with the use of chemotherapeutics (Deng et al., 2004). They fortified the idea that *C. elegans* can be a preclinical model for testing drugs and determining their molecular pathway. However the promise of this method has been difficult to repeat due to permeability problems.

1.2.4 Identifying germline cell death

Physiological germline cell death occurs after specific kinase signals, starvation or aging (Conradt and Xue, 2005). After DNA damage from IR, UV or ENU in wild-type worms, there is a significant increase in visible germ cell corpses above the basal levels (Conradt and Xue, 2005;

Gao et al., 2008; Stergiou et al., 2007). Therefore, DNA damage in *C. elegans* can be assessed by scoring for increases in germline cell death. Scoring for germline cell death can be done in a few different ways. One way to score for germline cell death is by using Nomarski optics or differential interference contrast (DIC). Dying cells can be identified by their resemblance to a flat round disk, a unique morphology, that disappear under an hour (Gumienny et al., 1999).

Other assays are through dyes, such as acridine orange (AO) or SYTO 12 (Gao et al., 2008; Gumienny et al., 1999). AO stains dying cells that are being engulfed and binds to acidic compartments. AO does not work well in engulfment defective mutants (Shaham, 2006). SYTO 12 stains all DNA in the cells, and cells undergoing apoptosis have condensed chromosomes and will stain brighter than healthy cells. SYTO 12 can stain dying cells that are observed by Nomarski and early stage germ cell corpses Nomarski optics cannot detect (Shaham, 2006).

In addition to the dyes, CED-1::GFP, a transgenic strain, can be used to detect dying germ cells that are being engulfed by the somatic gonad. *ced-1::gfp* is under control of the *lim-7* promoter and is expressed in the sheath cells around the gonad that are responsible for engulfing dying germ cells (Shaham, 2006; Zhou et al., 2001). CED-1 is a transmembrane protein in the phagocytotic cells. CED-1 will cluster around an apoptotic cell forming a ring. This reporter makes it easier to score germ cell death objectively and without staining. Similar to SYTO 12, early apoptotic cells that are not visible under DIC can be detected by scoring for CED-1::GFP positive cells (Shaham, 2006).

1.3 Autophagy

Autophagy is a process where large portions of cells, such as old organelles, are degraded (Hansen et al., 2008; Mizushima and Klionsky, 2007; Ryter et al., 2012). This process occurs for cellular remodeling of large proteins and in response to starvation. Vesicles are formed around organelles and substrates that are targeted for degradation forming autophagasomes. Autophagasomes will then fuse with lysosomes, and their content gets degraded (Melendez and Neufeld, 2008). Autophagy in cancer is paradoxical because it can be used for cell survival or an alternative method of programmed cell death that is caspase independent (Mathew et al., 2007). In this section we will look at the role of autophagy in *C. elegans* and mammalian systems and cancer.

1.3.1 Beclin 1 and autophagy

Beclin 1 is a vital mediator of autophagy in mammals (Samara and Tavernarakis, 2008). Beclin 1 is the mammalian homologue to *atg6/vps30* (autophagy related gene 6 and vacuolar protein sorting 30), which controls autophagy. The *beclin 1* gene is found on the human chromosome 17q21, which is often mono-allelically deleted in ovarian and breast cancers, similar to the *p53*, which is found on the same chromosome (Liang et al., 1999). *beclin 1 -/-* mice have early embryonic lethality (Sinha and Levine, 2008). Beclin 1 is part of the Class III PI3Kinase complex that helps to localize autophagic proteins to the preautophagosomal membrane and interacts with Vps34 (Furuya et al., 2005). Beclin 1 was initially isolated as a Bcl-2 interacting protein (Liang et al., 1998). Bcl-2 and its family members have been shown to inhibit Beclin 1-dependent autophagy in yeast and mammalian cells. Beclin 1 has a BH3 domain,

similar to the proapoptotic proteins that Bcl-2 family members bind to as well. The BH3-analog ABT-737, used to activate apoptosis by inhibiting Bcl-2, also releases Beclin 1 from Bcl-2 and activates autophagy (Maiuri et al., 2007; Oltersdorf et al., 2005). Bcl-2 and its family members play an important role in the inhibition of both autophagy and apoptosis in cells, preventing two key forms of cell death and can have an important role in Bcl-2 mediated oncogenesis (Sinha and Levine, 2008).

Autophagy and Beclin 1 also play important roles in the response to metabolic stress and genomic integrity. *beclin 1* heterozygosity has been published to impair autophagy and increase cells' sensitivity to metabolic stress, independent of caspases (Karantza-Wadsworth et al., 2007). Cells that have lost one copy of *beclin 1* are more susceptible to DNA damage and produce larger tumors in nude mice. These cells are resistant to inhibitors of gene amplification. Therefore, autophagy and Beclin 1 play an important role in limiting metabolic stress in order to prevent DNA damage and tumor growth (Karantza-Wadsworth et al., 2007).

1.3.2 Autophagy and BEC-1 in *C. elegans*

C. elegans have a *beclin 1* homologue termed *bec-1* on chromosome IV (Melendez et al., 2003). BEC-1 is required for viability and fertility as well as for normal dauer development and survival. Dauer worms form under starvation or stressful conditions after the L3 stage. BEC-1 is also necessary for normal growth, movement and vulval development. *bec-1* null mutants do not develop into adults and show high levels of embryonic lethality early on in embryogenesis. Additionally, *bec-1* null worms have increases in vacuolization, have uncoordinated phenotypes,

and molting defects (Melendez and Levine, 2009; Melendez et al., 2003; Takacs-Vellai et al., 2005).

Similar to Beclin 1, BEC-1 forms a complex with CED-9/Bcl-2 (Takacs-Vellai et al., 2005). CED-9 inhibits autophagy and apoptosis in *C. elegans* similar to the mammalian system. The role of BEC-1 in apoptosis has been studied as well. In *bec-1(RNAi)* worms, a significant increase in cell corpses is observed. While the observed increase in cell death is CED-3/caspase dependent, the increased death seems to be due to a lack of clearance of cell corpses (Ruck et al., 2011; Takacs-Vellai et al., 2005). Whether or not BEC-1 plays a direct role CEP-1-dependent cell death is still being investigated.

There is a relationship between autophagy and apoptosis during *C. elegans* development after DNA damage (Erdelyi et al., 2011). Using the incorporation of Uracil into DNA after a depletion of Thymidine from the knockdown of deoxyuridine triphosphate nucleotidohydrolase (dUTPase), an enzyme that converts dUTP to dUMP allowing the production of dTMP as DNA damage, cell death was scored. The damage causes an increase in embryonic cell death that was CEP-1-independent but CED-3-dependent. In addition to an increase in TUNEL positive cells, there was an increase in autophagy and autophagy reporters, including a robust increase in BEC-1::GFP in developing embryos. Subsequently, the treatment of worms with 5-fluorouracil, which also blocks the synthesis of thymidine, caused an increase in autophagy (Erdelyi et al., 2011). Autophagy is also involved in the removal of DNA damaged mitochondrial in *C. elegans* (Bess et al., 2012). After UV induced DNA damage, autophagy is increased to remove the damaged mitochondria. The loss of *bec-1* and other autophagy genes prevents the clearance of old mitochondria and the DNA damage is retained (Bess et al., 2012). Therefore, in addition to

apoptosis, worms use autophagy after specific stresses, such as DNA damage, to ensure development occurs properly (Erdelyi et al., 2011). This shows that BEC-1 and autophagy have many functions in *C. elegans.*

1.3.3 p53 and autophagy

p53 plays a dual role in autophagy, by activating and suppressing autophagy. After DNA damage, p53 activates the transcription of *dram*, (Crighton et al., 2006), *sestrin1* and *sestrin2* (Budanov and Karin, 2008). DRAM is a lysosomal protein that promotes autophagy (Crighton et al., 2006). The Sestrins are involved in inhibiting the mammalian target of Rapamycin (mTOR) after DNA damage promoting autophagy (Budanov and Karin, 2008). Conversely p53 can inhibit autophagy in by activating *tigar* under ischemic conditions. This shuts off autophagy and the production of reactive oxygen species to promote cell death (Hoshino et al., 2012; Tasdemir et al., 2008). Cytosolic p53 can also inhibit autophagy. p53 prevents autophagy by inhibiting AMP-dependent kinase (an activator of autophagy) and activating mTOR (Tasdemir et al., 2008). This occurs to block autophagy in order to activate cell death (Green and Kroemer, 2009). The removal or inhibition of cytoplasmic p53 in mammalian cells and *C. elegans* promotes autophagy (Tasdemir et al., 2008). The block on autophagy by cytoplasmic p53 may be to promote MOMP and to prevent the clearing of permeabilized mitochondria by autophagy (Green and Kroemer, 2009). Furthermore, Bcl-2 can inhibit both Beclin 1 and p53 preventing both autophagy and apoptosis (Abraham, 2001; Levine et al., 2008; Lu et al., 2005).

1.3.4 Autophagy in cancer and cell death

The role of autophagy in tumor suppression and growth is paradoxical. This is highlighted by the fact that autophagic genes are often deleted in cancers, and when added back to the cancer, cancer cell growth is inhibited (Liang et al., 1999). This suggests that autophagy genes are tumor suppressors. PTEN, a known activator of autophagy, is also highly mutated in cancers (Li et al., 1997). Typically, autophagy works as a tumor suppressor early on in cancer development or in normal cells. Once the tumor is formed, the tumor will have an increase in metabolic needs and can become "addicted" to autophagy (Mizushima and Komatsu, 2011). In these cases, autophagy can be repressed and tumor growth is inhibited (Amaravadi et al., 2007). Therefore in the well developed cancer, autophagy activation is an oncogenic property.

In support of autophagy as a gain-of-function for the tumor, inhibition of autophagy can lead to cell death (Maiuri et al., 2009). The inhibition of autophagy sensitizes cancer cells to death induced by alkylating agents (Amaravadi et al., 2007). Autophagy protects cells by inhibiting necrosis in apoptotic-deficient cells by acting as a buffer to metabolic stress (Degenhardt et al., 2006). Once the cells are made sensitive to metabolic stress by AKT activation, the combined impairment of apoptosis and autophagy promotes necrotic cell death (Degenhardt et al., 2006). Additionally, using chloroquine and other inhibitors of autophagy, prevents tumor development in mice and hydroxycholoroquine, an inhibitor of autophagy, is currently in clinical trials (Amaravadi et al., 2011; Mathew et al., 2007).

1.4 Introduction to this thesis and rationale

p53 is a tumor suppressor that promotes responses to different cellular stresses including DNA damage, starvation and oncogenic activation (Lu and Abrams, 2006; Vousden and Prives, 2009). These p53-dependent responses vary from cell cycle arrest, DNA repair, autophagy, to cell death. Functional p53 is lost in over 50% of all cancers (Lu and Abrams, 2006) and therefore identifying p53-independent cell death pathways is crucial for the treatment of tumors. *C. elegans* have a homologue of p53, termed CEP-1 that promotes cell cycle arrest and cell death after UV treatment (Stergiou et al., 2007). While the DNA repair machinery activates CEP-1, it is not known if CEP-1 works with the repair machinery to assist in DNA repair, for CEP-1 was not transcriptionally active in the absence of HUS-1 (Hofmann et al., 2002; Stergiou et al., 2007). This work followed UV induced DNA damage in wild-type and *cep-1(gk138)* mutant worms, and it became evident that CEP-1, like p53, played a role in the removal of UV DNA lesions. This correlation would further the conservation of the multiple functions p53 in other organisms.

C. elegans already have known CEP-1/p53-independent cell death pathways. CEP-1 is not required for developmental cell death and physiological cell death, but these cell deaths do rely on the core apoptotic cell death pathway (Conradt and Xue, 2005). Additionally, linker cell death in male worms occurs independently of the core apoptotic pathway. This cell dies cell autonomously and required LIN-29, but not the core apoptotic pathway (Blum et al., 2008). Here we see potential cell death pathways that signal independently of CEP-1/p53. Therefore we can use *C. elegans* as a model organism to begin to look for agents that induce CEP-1-independent cell death.

We would like to administer DNA damaging agents to *C. elegans* to induced germline cell death. Treating *C. elegans* with compounds to promote cell death has been done using N-ethyl-N-nitrosourea (ENU), Gleevec and ceramide. ENU and Gleevec require CEP-1 to induce cell death, while ceramide does not (Deng et al., 2004; Deng et al., 2008; Quevedo et al., 2007). We attempted to administer different DNA damaging agents and small molecules to the worms to observe for germline cell death. However, due to a thick outer cuticle and efficient xenobiotic pumps, the worms prevent the uptake of the DNA damaging agents (Burns et al., 2010). These properties make drug administration tough. In order to determine if our agent was taken up by the worm, we scored for cell death and the ability for an agent to form DNA lesions, using UV treatment as a positive control. This way we could properly asses the ability of chemicals to access the worm, target the DNA, induce DNA damage and ultimately induce cell death.

BEC-1 is involved in DNA damage and cell death (Erdelyi et al., 2011; Takacs-Vellai et al., 2005) but little was known of the involvement of Beclin 1/BEC-1 in p53/CEP-1 signaling. In mammalian cells and *C. elegans*, p53 blocks autophagy while DNA damage promotes autophagy (Bess et al., 2012; Erdelyi et al., 2011; Tasdemir et al., 2008). Therefore, further studies on the activities of BEC-1 and its involvement with CEP-1 and apoptosis were required. We were interested in determining if there is any crosstalk between the two pathways. Herein, we saw that partial loss of *bec-1* promoted CEP-1-dependent cell death while the full loss of *bec-1* blocked clearance of the dead cells. Furthermore, the loss of *bec-1* with additional DNA damage gave elevated levels of DNA lesions. Thus it was proposed that BEC-1 may lie upstream of CEP-1 to mediate germline cell death with DNA damage and potentially physiological cell death. Additionally, CEP-1 and BEC-1 may not regulate each other directly but the two proteins may

regulate a similar target and alter the activity of this target depending on the cell's preferred outcome.

CHAPTER 2:

Materials and Methods

2.1 *C. elegans* experiments

Growth media and strains

Worms were grown on nematode growth media (NGM) at either 15º, 20º or 25ºC (Brenner, 1974). One liter of NGM includes: 3 g NaCl (Fisher), 17 g agar (Fisher), 2.5 g bactopeptone (Becton, Dickinson and Company), 1 mL cholesterol (Sigma, 5mg/mL in 95% Ethanol), 1 mL 1 M $CaCl_2$, 1 mL 1 M $MgSO_4$, 25 mL 1 M potassium phosphate pH 6. The plates were dried and the *E. coli* strain OP50 was spread for worms to eat. Seeded plates were left overnight at room temperature and could be used for three weeks. M9 buffer was prepared using 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 L. Sterilize by autoclaving. The wild type strain used was *C. elegans* variety Bristol (N2). The following mutant strains were used as well: *bcIs39[P(lim-7)ced-1::GFP + lin-15(+)]* (expression of functional CED-1::GFP fusion protein in the sheath cells, MD701), *bec-1(ok691)/nT1[qIs51]* (2100 bp deletion kept over a balancer expressing GFP in the pharynx, VC517), *cep-1(gk138)* (1660 bp deletion, TJ1), *glp-1(q224)* (base substitution G1043E, JK1107), and *glp-1(ar202)* (base substitution G529E, GC833). Strains were provided by the Caenorhabditis Genetics Center. *cep-1(gk138); bcIs39[P(lim-7)ced-1::GFP + lin-15(+)]* (JBC1), *cep-1(gk138); bec-1(ok691)/nT1[qIs51]* (JBC3) and *cep-1(gk138); glp-1(ar202)* (JBC2) were prepared by the Bargonetti Laboratory. The *bec-1::rfp* strain was a gift from the Melendez laboratory. The *glp-1(ar202)* and *cep-1(gk138); glp-1(ar202)* were maintained at 15ºC and moved to 25ºC as eggs to promote a tumor phenotype.

RNAi feeding

RNAi plates were prepared using the normal NGM method with a final concentration of 2 mM (*bec-1*) or 6 mM (*cep-1*) of IPTG (Fermentas) and 50 μg/mL Carbenicillin (Sigma). L4 worms were placed on RNAi seeded plates with bacteria containing the L4440 control plasmid or *cep-1* RNAi plasmid a generation before treatment (generous gifts from the Gartner lab). L4 worms were placed on RNAi seeded plates with bacteria containing the L4440 control plasmid or *bec-1* RNAi plasmid (generous gifts from the Melendez lab). Parental worms were used 24 or 48 hours post L4 as indicated. F1 worms were laid and grown on RNAi plates and used 24 or 48 hours post L4 as indicated.

UV treatments

UV treatments were done with a SpectroLinker. Worms were rinsed off plates with M9 and added to plates without bacteria. Worms were then exposed to 50 or 100 J/m^2 of UV or left untreated as negative controls. For RNA extraction, the worms were rinsed off and placed back onto seeded NGM plates and allowed to recover for four hours, after which the worms were washed, collected, and frozen at -80°C. For germline cell death analysis, worms were placed back onto NGM plates for 24 hours and then scored. For Lesion analysis, the worms were placed in lysis buffer after treatment. For repair analysis, worms were frozen four, eight, sixteen or twenty-four hours after treatment. All worms were frozen at -80°C until processing.

RNA isolation from *C. elegans* and quantitative reverse transcription PCR (q-RTPCR)

Worms were centrifuged at 1,500 rpm for 4 minutes at 4°C, washed twice with M9, and the RNA was isolated using QIAshredder columns and the RNeasy Mini Kit (Qiagen) following manufacturer's protocol. 60 µL of beta mercaptoethanol (BME, Sigma) was added to 6 mL of RNeasy Lysis Buffer (RLT) and 600 µL was added to the worm pellets. Worms were incubated for 10 minutes and then spun for 2 minutes at 13,000 RPM in the QIAshredder. 600 µL of 70% ethanol was added to the flow through and spun on a collecting column. The collecting column was washed with 350 µL of RNeasy Wash Buffer (RW1) and then incubated with 10 µL DNase and 70 µL of RNeasy DNA Digest Buffer (RDD) for 15 minutes. The collecting column was washed again with 350 µL of RW1 and then 500 µL of RNeasy Clean Up Buffer (RPE), each spun for 30 seconds. A last spin of 500 µL of RPE was done for 2 minutes and the membrane was dried. The collecting column was placed on a fresh 1.5 mL eppendorf collecting tube and the RNA was eluted with 30 µL of RNase free water, and spun at 10,000 RPM for 1 minute. The RNA was only used if the yield was greater than 100 ng/µL. RNA was stored at -80°C. 300 ng of RNA in 25 µL of volume was used for cDNA synthesis using the High Capacity cDNA Archive Kit reagents and the protocol from the manufacturer (Applied Biosystems). 25 µL of the reverse transcription (RT) master mix contained 1x RT buffer, 1x dNTP's, 1x random primers and 1 U/µL of multiscribe Reverse Transcriptase. The reaction was incubated at 25°C for 10 minutes and then at 37°C for 2.5 hours. The cDNA was stored at 4°C for short term and -20°C for long term. Amplification of gene transcripts by quantitative PCR with 0.125 µL of primers for *egl-1*, *ced-13* and *tbg-1* (Operon and Applied Biosystems, see sequences below, final primer concentration of 0.05 pmol/µL) (Schumacher et al., 2005b) combined with the 3 µL of cDNA

and 12.5 µL of SYBR Green PCR Master Mix (volume brought up to 25 µL with nuclease free water) (Applied Biosystems) was carried out following the program: one cycle, 2 minutes, 50°C; one cycle, 10 minutes, 94°C; and 40 cycles or 15 seconds at 94°C and 1 minute at 60°C for *egl-1* and *tbg-1* or 54.6°C for *ced-13* in a 7500 Sequence Detection System (Operon and Applied Biosystems).

Target Gene	Primer Sequence
tbg-1	5`-CGTCATCAGCCTGGTAGAACA-3` 5`-TGATGACTGTCCACGTTGGA-3`
egl-1	5`-TACTCCTCGTCTCAGGACTT-3' 5`-CATCGAAGTCATCGCACAT-3`
ced-13	5`-ACGGTGTTTGAGTTGCAAGC-3` 5`-GTCGTACAAGCGTGATGGAT-3`

Induction of germ-cell apoptosis using DMC

10-decarbamyl-mitomycin C (DMC) was a gift from Dr. Maria Tomasz. 2 mM was diluted to desired concentration in 30% methanol. Except for the concentration curve, all DMC experiments carried out using 1 mM DMC. 45 µL DMC or 45 µL 30% Methanol were placed onto the bacteria of seeded NGM plates. After three hours, young adult worms were moved to treated plates and left either over night or for five hours. Germline cell death was analyzed at the end of treatments and worms were frozen in lysis buffer for analysis using the lesion assay.

Germline apoptosis scoring

Studies of germline apoptosis were done using CED-1::GFP engulfment (Shaham, 2006) by mounting treated worms after a 24 hour recovery after UV or immediately after DMC feeding. Slides were prepared with 2% agarose and 5 μL of 3 mM levamisole diluted in M9 Buffer was added to anesthetize worms. Normarski and green fluorescent images were taken using an ApoTome Zeiss microscope taking pictures of each plane of the germline (1 μm thick with an average of 25 slices per worm). Images were saved using a generic label and later scored blindly by two independent scientists. Each scientist counted CED-1::GFP positive cells from the green images however exact counts of CED-1::GFP positive cells did not always match. The average trends of CED-1::GFP positive cells per treatment were always consistent for the two scientists.

Lesion assay

Five worms were placed in 150 μL of lysis buffer (1x r*Tth* XL DNA polymerase buffer and 1 mg/mL proteinase K (Applied Biosystems and Sigma)) after treatment and frozen for at least 10 minutes at -80°C. The lysis step included 1 hour at 65°C, with a vortex after 5 minutes, and 15 minutes at 95°C. The 50 μL QPCR (quantitative polymerase chain reactions) mixtures contained the following: 9.6 μL sterile de-ionized water, 15 μL 3.3× r*Tth* XL DNA polymerase buffer, 5 μL of 1 mg/mL bovine serum albumin, 4 μL dNTPs (2.5 mmol/L of each), 2.4 μL 25 mM $MgO(Ac)_2$, 2 μL of each primer, and 5 μL of 2 ng/μL genomic DNA template. All primers were used at 10 μmol/L except the small mitochondrial primers, which were used at 7.5 μmol/L (final concentrations 0.4 and 0.3 μmol/L). r*Tth* XL DNA polymerase was diluted in 1× buffer, and 5 μL was added in a hot start procedure. The 3.3× buffer, $MgO(Ac)_2$, and r*Tth* XL DNA

polymerase were from the GeneAmp XL PCR kit (Applied Biosystems). The cycling conditions for the small mitochondrial and small nuclear targets were as follows: 1 cycle of 75°C for 2 minutes; 1 cycle of 94°C for 1 min minutes; mitochondria 25 cycles, nuclear 29 cycles of 94°C for 15 seconds, 63°C for 45 seconds, and 72°C for 30 seconds, and 1 cycle of 72°C for 5 minutes. The cycling conditions for the large mitochondrial and small nuclear targets were: 1 cycle of 75°C for 2 minutes; 1 cycle of 94°C for 1 minutes; mitochondria 30 cycles, nuclear 31 cycles of 94°C for 15 seconds and 66°C for mitochondria or 68°C for nuclear for 12 minutes, and 1 cycle of 72°C for 10 minutes (Meyer et al., 2007). The primers used were as following (IDT):

Target Site	Primer Sequence
Small mitochondria (one extreme of Large mitochondria target)	5′ CAC ACC GGT GAG GTC TTT GGT TC 3′ 5′ TGT CCT CAA GGC TAC CAC CTT CTT CA 3′
Large mitochondria (10.9 kb of mitochondrial genome, excludes the AT-rich regions)	5′ CCA TCA ATT GCC CAA AGG GGA GT 3′ 5′ TGT CCT CAA GGC TAC CAC CTT CTT CA 3′
Small Nuclear (polymerase ε target)	5′ TCC CGT CTA TTG CAG GTC TTT CCA 3′ 5′ GAC GCG CAC GAT ATC TCG ATT TTC 3′
Large Nuclear (*unc-2*)	5′ TGG CTG GAA CGA ACC GAA CCA T 3′ 5′ GGC GGT TGT GGA GTG TGG GAA G 3′

DNA quantity was measured after QPCR using PicoGreen dye (Invitrogen). Each sample was measured in duplicate. 90 μL of 1xTE buffer, 10 μL of DNA and 100 μL of a solution of PicoGreen reagent (5 μL of reagent per milliliter of 1x TE) were added to each well. The sample

was mixed and incubated at room temperature for 10 minutes in the dark. The fluorescence was read using 485nm of excitation and 530nm for emission using a Spectra Max Gemini EM detector (Molecular probes). Values were normalized to untreated samples using a Poisson distribution (Santos et al., 2006). Blanks were subtracted from all values. Small mitochondria and nuclear numbers where divided by the average of the small values in their experiment, termed "factor". The large mitochondria and nuclear values were divided by the factor. The result was divided by the untreated value, and yielded a relative amplification value. The –LN of the relative amplification value was then done and this value was multiplied by 10 and divided by 10.939 to determine the lesions/10kb value.

Analysis of mitochondria membrane potential

Synchronized worms were placed on NGM plates with 0.1 μM of tetramethylrhodamine ethyl ester (TMRE, Sigma) for 12 hours before imaging. Worms were treated with 100 J/m^2 of UV at the end of the 12 hour incubation. Worms were treatment with 1 mM of DMC for the last five hours of the 12 hour TMRE feeding. Live worms were then imaged for their red florescence under a Nikon microscope (Zuryn et al., 2008). Ten worms were analyzed per experiment. The amount of dye in the mitochondrial matrix was quantified using Image J to give the mean red channel intensity of each worm from images takes at 10x.

Egg lay and embryonic viability counts

L4 worms were treated with 100 J/m^2 of UV or left on NGM plates or fed 1 mM DMC for five hours. After treatments, worms were allowed to recover for 24 hours at 20°C. Single worms were

placed on fresh plates and allowed to lay eggs overnight. Eggs were scored the next morning and adults were removed. The eggs were then scored the next day to determine the percentage of eggs that hatched (Gao et al., 2008).

2.2 Tissue culture experiments

Cell culture

The cancer culture cells used in this study will be MCF-7.Control and MCF-7.*beclin 1*. The MCF-7.Control and MCF-7.*beclin 1* clone were generous gifts from Beth Levine. They were grown in DMEM media (Mediatech) containing 10% FBS, 50 U/mL of penicillin, 50 μg/mL of streptomycin, 1 mg/mL G418 (CellGro), 10 μg/mL insulin (Sigma) and 200 μg/mL Hygromycin B (Calbiochem) in the absence of 2 μg/mL tetracycline (Sigma) to induce Beclin 1 expression for five days (Furuya et al., 2005). All cells were incubated with 5% CO_2 at 37°C.

Treatments

Cells were treated with 10 μM DMC (initial stock of 2 mM dissolved in 30% methanol) for 6, 24 or 48 hours, with cells maintained at 37°C in 5% CO_2 (Boamah et al., 2007). Cells were harvested for 5 minutes at 2000 RPM at 4°C, washed in 1xPBS three times and stored at -80°C until RNA or protein extraction.

Tissue culture q-RTPCR

RNA was isolated using QIAshredder columns and RNeasy Mini Kit (Qiagen). 60 µL of BME was added to 6 mL of RLT buffer and 600 µL was added to the cell pellets. Cells were spun for 2 minutes at 13,000 RPM in QIAshredder. 600 µL of 70% ethanol was added to the flow through and spun for 30 seconds on a collecting column. The column was washed with 350 µL of RW1 Buffer and then incubated with 10 µL DNase and 70 µL of RDD buffer for 15 minutes. The column was washed again with 350 µL of RW1 and then 500 µL of RPE Buffer, each spin was 30 seconds. A last spin of 500 µL of RPE was done for 2 minutes and the membrane was dried. The column was added to a fresh 1.5 mL eppindorf collecting tube and the RNA was eluted with 30 µL of RNase free water for 1 minute at 10,000 RPM. 5 µg of RNA was used for cDNA synthesis using High Capacity cDNA Archive Kit reagents (Applied Biosystems). 3 µL of cDNA were combined with 12.5 µL of Taqman Universal Master Mix and 1.25 µL of Applied Biosystems Assays on Demand primers/probes for *puma* (Hs00248075), *p21* (Hs00355782), *beclin-1* (Hs00186838) and *actin* (4352935E) were added. The PCR reaction was carried out in 7500 Sequence Detection System with the following program: one cycle, 2 minutes, 50°C; one cycle, 10 minutes, 94°C; and 40 cycles or 15 seconds at 94°C and 1 minute at 60°C (Applied Biosystems). Samples were normalized to *actin* and untreated cells (Boamah et al., 2007).

Whole cell protein extract and Western blot

Cells were harvested and washed in 1xPBS. Cells were resuspended in three volumes of RIPA buffer (0.1% SDS, 1% NP-40, 0.5% Deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-Cl pH8, 1 mM PMSF, 8.5 µg/ml Aprotinin and 2 µg/ml Leupeptin). The

tubes were vortexed and incubated on ice for 20 to 30 minutes and vortexed every five minutes. After debris formed, the lysate was spun at 13,000 RPM for 10 minutes and the supernatant was transferred to a fresh tube. 35 μg of protein samples were size fractionated by electrophoresis in 10% denaturing poly-acrylamide gels and then electrotransferred to nitrocellulose membranes (Amersham). The resulting blots were probed for Beclin 1 in 1% milk at 1:4000 (Novus). Actin was used as a loading control, 1:2000 (Sigma). The membranes were then incubated in anti-mouse or anti-rabbit (Sigma) secondary antibodies and the signals were visualized using standard chemiluminescence reagents.

CHAPTER 3:

CEP-1 was involved in the removal of UV and DMC induced DNA lesions

3.1 Introduction

After ultraviolet (UV) light induces DNA damage, mammalian p53 activates genes for DNA repair and apoptosis (Oliner et al., 1992; Oliner et al., 1993; Smith et al., 2000). UV damage forms cyclobutane pyrimidine dimers (CPDs) or 6-4 photoproducts (6-4PPs); both distort the double helix and activate the nucleotide exchange repair (NER) pathway (Barak et al., 1993; Maltzman and Czyzyk, 1984). Repair of the UV induced lesions without functional p53 is compromised (Smith et al., 2000). p53 works with the NER pathway to assist in the removal of UV induced lesions in two ways. p53 activates the transcription of *xpc*, *gadd45a* and *DDB2* (Nelson and Kastan, 1994; Oliner et al., 1993). These three proteins play a role during NER and specifically induce global genomic repair (GGR) after UV treatment. In addition, p53 works with the NER pathway to promote the removal of lesions after UV damage. p53, with ATR, mediates the nuclear import of XPA after UV damage during S-phase. While the relocation of XPA does not require p53 to be transcriptionally active, p53 must be phosphorylated at Serine 15 (Li et al., 2011).

In addition to the NER pathway, p53 activates cell death after UV damage (Ford and Hanawalt, 1997). The p53 protein activates *puma*, *noxa* and *bax* after UV damage (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000; Pohl et al., 1999; Yu et al., 2001). Once translated, these proteins are involved in the inhibition of Bcl-2 to activate caspases and promote apoptosis (Alberts et al., 2008; Ashkenazi, 2002). Furthermore, p53 binds to Bcl-xL in the cytoplasm, and once PUMA levels rise, p53 can be replaced by PUMA and will induce mitochondrial outer membrane permeabilization (MOMP) by binding to BAX (Baptiste and Prives, 2004; Chipuk et al., 2005).

In *C. elegans*, the role of its p53 is conserved in the activation of cell death after UV exposure by the *C. elegans* p53-1 (CEP-1) (Stergiou et al., 2007). After UV exposure, germ cells undergo cell death if CEP-1 is present. This activation of cell death is mediated by the upregulation of *egl-1* and *ced-13*, the two BH3-only genes in *C. elegans*. In addition to CEP-1, the NER pathway in worms is required for UV-induced cell death (Stergiou et al., 2007). The NER pathway is upstream of the homologous recombination (HR) machinery and these pathways signal to the apoptotic machinery to remove damaged cells (Stergiou et al., 2007; Stergiou et al., 2011). Furthermore, after UV exposure, the NER pathway is necessary for the normal life span of adult worms (Meyer et al., 2007). After chronic exposure to UV, NER deficient worms have a significantly larger buildup in DNA lesions when compared to wild-type worms (Meyer et al., 2007).

We investigated the role of CEP-1 in UV mediated DNA damage and repair in worms. CEP-1 acts downstream of the NER signaling pathway in *C. elegans* (Stergiou et al., 2007) but how it regulates the removal of DNA lesions has not been reported. While the pro-apoptotic functions of CEP-1 are conserved in *C. elegans*, little research has been carried out to explain whether CEP-1 plays a role in the NER pathway. We discovered that CEP-1 was involved in the removal of lesions after UV exposure. Additionally, in the absence of CEP-1, worms increased sensitivity to cell death and DNA damage induced by the alkylating agent 10-decarbomyl-mitomycin C (DMC). Moreover the loss of CEP-1 in *glp-1(ar202)* tumors caused the germ cells to be more susceptible to immediate nuclear DNA damage.

3.2 Results

3.2.1 Wild-type and *cep-1(gk138)* mutant worms have similar levels of nuclear DNA damage after UV treatment.

UV-C has been reported to activate DNA damage, CEP-1 transcriptional activity, and CEP-1-dependent cell death (Meyer et al., 2007; Stergiou et al., 2007). In order to determine if we could reproduce this previously published work, we treated young adult wild-type and *cep-1(gk138)* mutant worms with 100 J/m^2 of UV. We analyzed the RNA levels of *egl-1* and *ced-13* four hours after UV exposure using quantitative reverse transcription polymerase chain reaction (q-RTPCR). We detected the transcriptional activation of these two CEP-1 target genes after UV exposure in wild-type worms but not in *cep-1(gk138)* mutants (Figure 3.1A).

In order to quantify germline cell death objectively, we used a CED-1::GFP marker. Dying germ cells are engulfed by the somatic gonad and CED-1 is localized to the outer membrane of the engulfing somatic cell (Shaham, 2006; Zhou et al., 2001). The CED-1::GFP construct allows visualization of the engulfment by a green circle around the dying cell (Figure 3.1B). We imaged the germlines using an ApoTome microscope that allowed the worms to be photographed three-dimensionally, capturing 1 µm slices of the germline over 25 µm (25 slices per worm, on average). This way the scoring could be done blindly at a later date and all planes of the germline were scored objectively without knowledge of the DNA damage treatment. We observed that the wild-type worms had a significant increase in CED-1::GFP positive cells after UV treatment while there was no significant increase in CED-1::GFP positive cells in the *cep-1(gk138)* mutant worms twenty-four hours post exposure (Figure 3.1C).

The requirement of CEP-1 for UV mediated cell death is well documented (Stergiou et al., 2007). However how CEP-1 influences the presence of UV-induced lesions has not been examined in *C. elegans.* We therefore asked if CEP-1 was involved in nuclear lesion formation or removal. DNA damage caused by UV exposure results in both mitochondrial and nuclear DNA damage in wild-type worms (Meyer et al., 2007). To determine if there was an increase in nuclear DNA lesions without CEP-1, we used a quantitative polymerase chain reaction (QPCR) based assay to measure the levels of lesions after UV exposure (Boyd et al., 2010; Meyer et al., 2007). This assay uses two PCR reactions to determine the presence of lesions: a small one yielding a product of about 150 base pairs and a large one, with a product of 10 kilo-base pairs. The small PCR reaction gives you a baseline of the amount of DNA you have in your PCR reaction. The large PCR reaction will give a significantly decreased product if there is DNA damage due to a blocked or inhibition polymerase activity (Meyer, 2010; Meyer et al., 2007). Young adult wild-type and *cep-1(gk138)* mutant worms were exposed to 50 and 100 J/m^2 of UV. After treatment, the worms were immediately frozen in the lysis buffer so little to no repair could occur. The wild-type and *cep-1(gk138)* mutant worms both demonstrated increased formation of nuclear DNA lesions. After treatment, we observed no differences in the levels of nuclear lesions after UV exposure in the two strains (Figure 3.1D). Therefore the UV induced nuclear DNA damage was the same between the two strains and required CEP-1 for the death of damaged cells. Interestingly, in the absence of any damage, *cep-1(gk138)* mutant worms had 0.2 lesions/10kb when normalized to untreated wild-type worms. Thus the loss of *cep-1* induced genomic instability in the mutant worms.

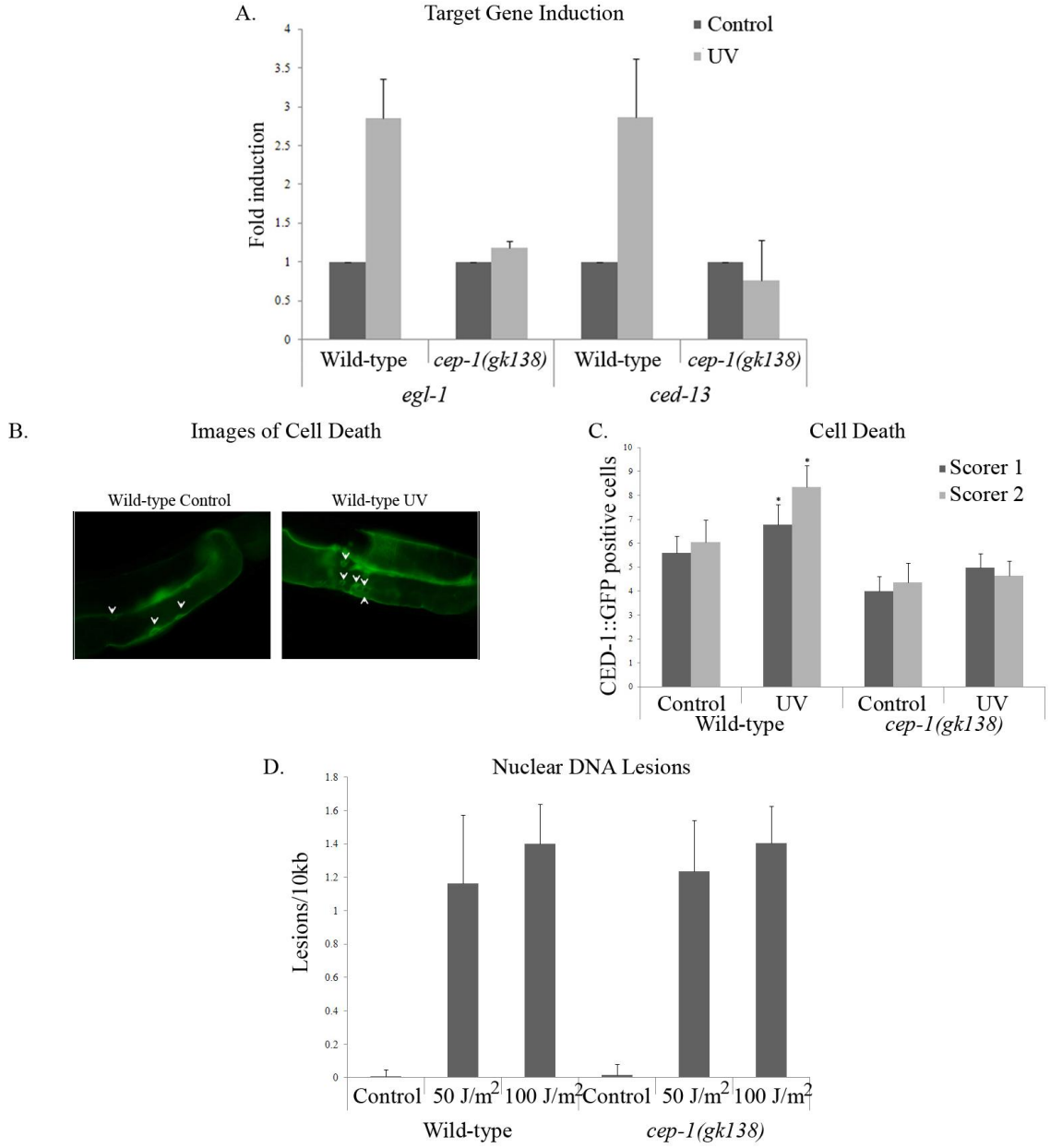

Figure 3.1: Wild-type and *cep-1(gk138)* mutant worms had similar amounts of nuclear DNA damage after UV exposure. A) Q-RTPCR of *egl-1* and *ced-13* mRNA levels four hours post UV treatment of 100 J/m^2. Fold induction compared to cDNAs amplified from untreated worms. Average of three representative experiments is shown. Error bars indicate standard error. Normalized to *tbg-1*. Done by Dr. Ryan Doonan. B) Images of CED-1::GFP positive cells of wild-type worms either untreated or 24 hours after 100 J/m^2 UV. Arrows point to CED-1::GFP positive cells. Images taken at 40x. C) Worms were treated 24 hours post L4 and imaged 24 hours later. Counts were done blindly of CED-1::GFP positive cells and scored by two independent people. Error bars indicate standard error and the number of worms scored was 20. D) Lesions of nuclear DNA were done on 24 hour post L4 worms. Worms were exposed to 50 or 100 J/m^2 of UV, placed in the lysis buffer and frozen. Average of three representative experiments is shown. Error bars indicate standard error.

3.2.2 *cep-1(gk138)* mutant worms had less mitochondrial DNA damage after UV.

After UV treatment, wild-type worms have an increase in lesions in both their nuclear and mitochondrial DNA (Meyer et al., 2007). We observed no difference between wild-type and *cep-1(gk138)* mutants in their UV induced nuclear DNA damage, and we hypothesized the same would be true for UV induced mitochondrial DNA damage. Using the lesion assay and primers for mitochondrial DNA, we measured lesions after 50 and 100 J/m^2 of UV exposure. Unlike the nuclear damage, the mitochondrial DNA damage was significantly different between the two strains (Figure 3.2A). The wild-type worms had a large increase in mitochondrial DNA damage after UV, while the *cep-1(gk138)* mutants had considerably less mitochondrial DNA damage (Figure 3.2A). This was a surprising read out and therefore we compared the ratio of small mitochondria detected values to small nuclear to determine if *cep-1(gk138)* mutants had less mitochondrial DNA. The averaged ratio in the wild-type worms was 2.3 and was below that of the *cep-1(gk138)* mutants, 3.5 (average of five independent experiments). The *cep-1(gk138)* mutants had 1.5 times the amount of mitochondrial DNA when compared to the wild-type worms. This indicated that the *cep-1(gk138)* mutants did not have less mitochondrial DNA but had fewer mitochondrial DNA lesions after UV exposure.

Additionally we looked at the levels of mitochondria with and without UV exposure in the two strains. We used tetramethylrhodamine ethyl ester (TMRE) to measure the levels of intact mitochondria. TMRE is a lipophilic cation whose mitochondrial uptake depends on the strength of the mitochondrial membrane potential (Zuryn et al., 2008). Wild-type and *cep-1(gk138)* mutant worms were fed NGM plates with TMRE for twelve hours and then exposed to 100 J/m^2 of UV. When comparing untreated wild-type and *cep-1(gk138)* mutant worms under

the 10x objective, the *cep-1(gk138)* mutant worms had less stained mitochondrion than the wild-type worms (Figure 3.2B, left panel and C). The difference was not due to a change in the areas scored; both worms had similar sized areas. After UV treatment, the wild-type worms had a significant decrease in TMRE uptake when compared to the untreated worms (Figure 3.2B, upper panel and C). On the other hand the untreated and treated *cep-1(gk138)* mutant worms did not have much of a difference in their TMRE uptake (Figure 3.2B, lower panel and C). The decrease in TMRE intensity in wild-type worms was consistent with the increase in mitochondrial DNA lesions, leading to an increase in faulty or defected mitochondria. On the other hand, the UV exposure did not alter mitochondrial DNA lesions or intact mitochondria in *cep-1(gk138)* mutants. The mitochondria of the *cep-1(gk138)* mutant worms seemed to be more resistant to UV induced DNA damage.

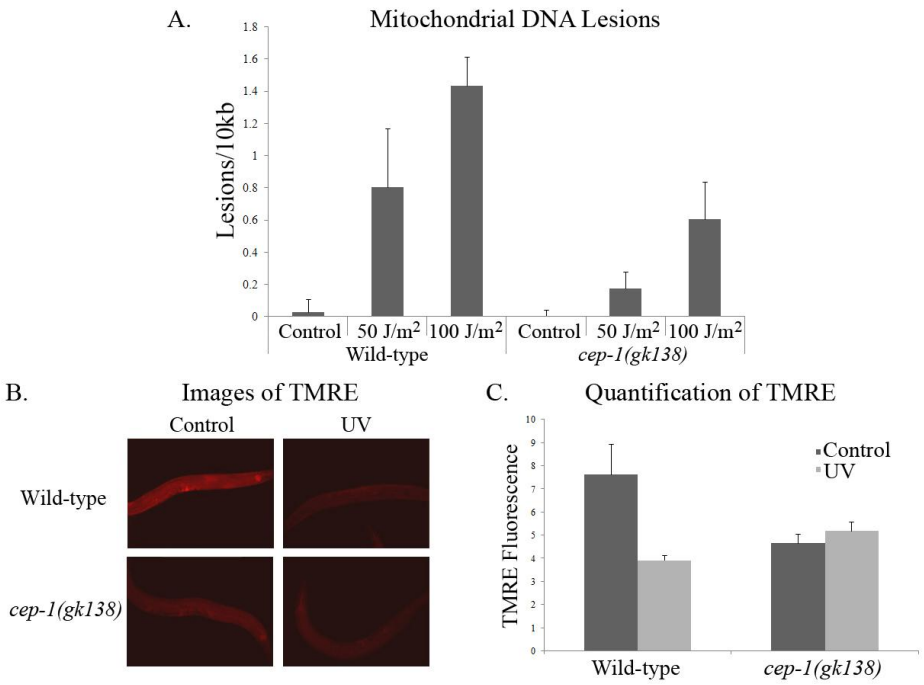

Figure 3.2: *cep-1(gk138)* mutant worms had less mitochondrial DNA damage after UV. A) Lesions in mitochondrial DNA were done on 24 hour post L4 worms. Worms were exposed to 50 or 100 J/m^2 of UV, placed in the lysis buffer and frozen. Average of three representative experiments is shown. Error bars indicate standard error. B) Synchronized worms were fed tetramethylrhodamine ethyl ester (TMRE) on NGM plates for 12 hours and then were treated with 100 J/m^2 of UV and imaged. Representative images of wild-type and *cep-1(gk138)* mutant worms taken at 10x and C) the average TMRE intensity of worms is shown. Average of two experiments is shown, number of worms scored per bar was 20. Error bars indicate standard error.

3.2.3 CEP-1 is involved in DNA repair of UV induced lesions.

p53 plays a role in assisting the NER pathway to repair UV induced lesions (Li et al., 2011; Oliner et al., 1992; Oliner et al., 1993; Smith et al., 2000). This feature has not been reported yet for CEP-1. However, the fact that CEP-1 is found predominantly in the worm germline suggests that it is important for maintaining genomic integrity. If CEP-1 plays a role in DNA repair, we hypothesized that *cep-1(gk138)* mutant worms would have a decrease in the removal of DNA lesions when compared to wild-type worms after UV exposure. We allowed the

worms to recover for four or eight hours after exposure to 100 J/m^2 of UV and then measured their nuclear and mitochondrial lesions. The amount of nuclear lesions in wild-type worms decreased by the eight hour time point close to zero while the *cep-1(gk138)* mutant worms retained a high degree of DNA lesions (Figure 3.3A). Additionally the wild-type worms removed all mitochondrial lesions by four hours while after eight hours the *cep-1(gk138)* mutant worms retained almost all mitochondrial DNA lesions (Figure 3.3B). Therefore CEP-1 plays a role in the removal of UV induced lesions in the nuclear and mitochondrial genomes in wild-type worms.

To further examine the influence of CEP-1 on genomic integrity we asked if CEP-1, following UV exposure, assisted in egg viability. If CEP-1 is involved in genomic integrity, we hypothesized that there would an increase in the production of non-viable eggs in the absence of functional CEP-1. Worms lacking DNA damage repair genes have a decrease in viable eggs after DNA damage (Boulton et al., 2004; Garcia-Muse and Boulton, 2005; Stergiou et al., 2007). We exposed L4 wild-type and *cep-1(gk138)* mutant worms to 100 J/m^2 of UV and allowed them to recover for 24 hours. The worms laid eggs overnight on fresh plates and the number of eggs produced was scored. Both wild-type and *cep-1(gk138)* mutant worms had a decrease in the number of eggs produced when comparing to untreated controls (Figure 3.3C). On the second day, the plates were counted for remaining eggs to calculate the percentage of eggs that hatched. A significant decrease in the survival of the eggs was observed in the two strains after UV exposure (Figure 3.3D). After UV exposure, 68% of the wild-type eggs hatched while only 36% of *cep-1(gk138)* mutant eggs hatched (Figure 3.3D). This was a significant difference between

the two strains and can be attributed to the lack of lesion removal and cell death. Therefore the *cep-1(gk138)* mutant worms laid more damaged eggs that did not survive.

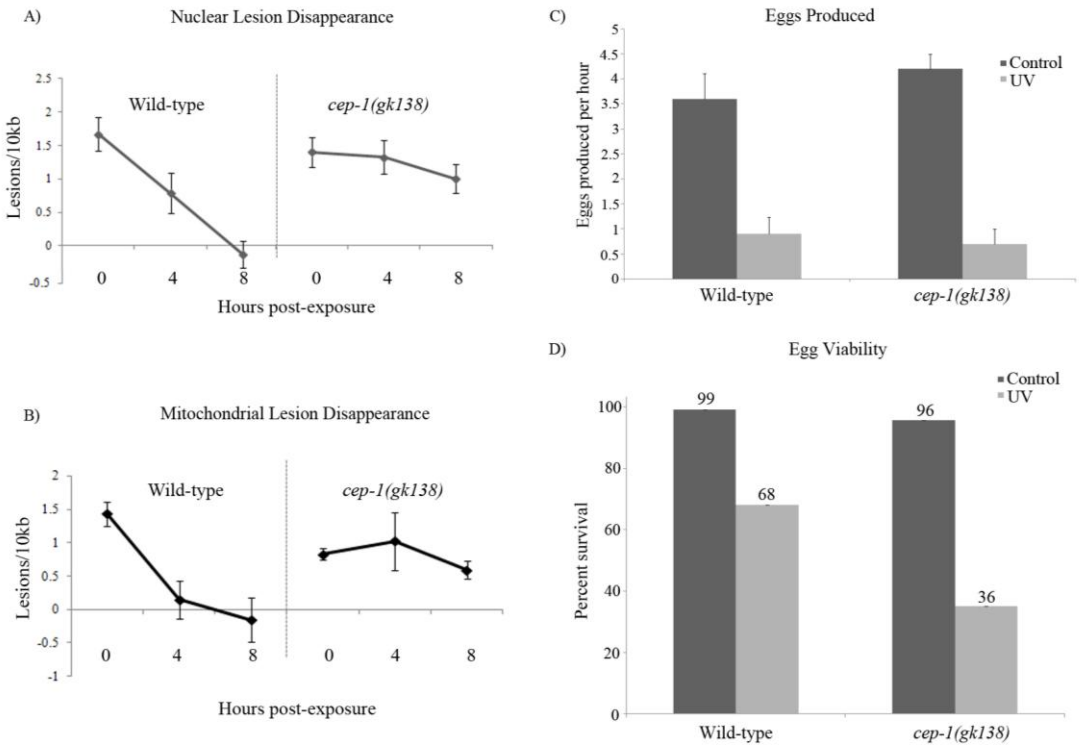

Figure 3.3: *cep-1(gk138)* mutants had less repair and decreased egg viability than wild-type worms after UV treatment. Lesions of both A) nuclear and B) mitochondrial DNA were done on 24 hour post L4 worms. Worms were exposed to 100 J/m^2 of UV, placed in the lysis buffer immediately or four or eight hours after treatment and frozen. Average of three representative experiments is shown. Error bars indicate standard error. C) L4 worms were treated with UV and allowed to recover for 24 hours. Worms were then transferred to fresh plates and allowed to lay eggs for 12 hours at 20°C. Unhatched eggs and survived animals were counted 1 and D) 2 days later to assess survival. Egg laying values represent number of eggs laid per hour per adult hermaphrodite. Average of 18 worms shown. Error bars indicate standard error.

3.2.4 *cep-1(gk138)* mutant worms were sensitive to DMC treatment.

The mitomycin C derivative 10-decarbomyl-mitomycin C (DMC) induces p53-independent cell death via the depletion of Chk1 (Boamah et al., 2007). Additionally, DMC forms DNA lesions in MCF-7 cells in both the mitochondria and nuclear DNA (Boamah et al.,

2010). Therefore we examined if DMC would had an effect on *C. elegans* germline cell death and DNA damage in the presence and absence of CEP-1. Introduction of drugs/agents to *C. elegans* has previously been done by soaking, injecting or feeding on plates although the introduction of drugs into *C. elegans* is extremely difficult (Deng et al., 2008; Gao et al., 2008; Ved et al., 2005). Few agents can penetrate the thick cuticle and reach their target site (Burns et al., 2010). Furthermore, worms have extensive xenobiotic efflux pumps preventing the drugs from accumulating into cells. We experimented with the three methods of drug administration to determine if we could get a change in DNA lesions and germline cell death after DMC treatment. We began with soaking the worms but concluded that this method was not reproducible due to induced starvation with its own negative side effects (data not shown). Next we injected DMC into worms similar to what had been carried out using ceramide to induce germline cell death (Deng et al., 2008). Again we found no significant increase in germline cell death (data not shown). Finally, we added varying concentrations of DMC to the bacteria of seeded NGM plates and allowed the young adult worms to eat the bacteria overnight. Interestingly, *cep-1(gk138)* mutants had a significant increase in CED-1::GFP positive germ cells when 1 mM of DMC was added to the bacteria but the wild-type worms did not show an increase (Figure 3.4).

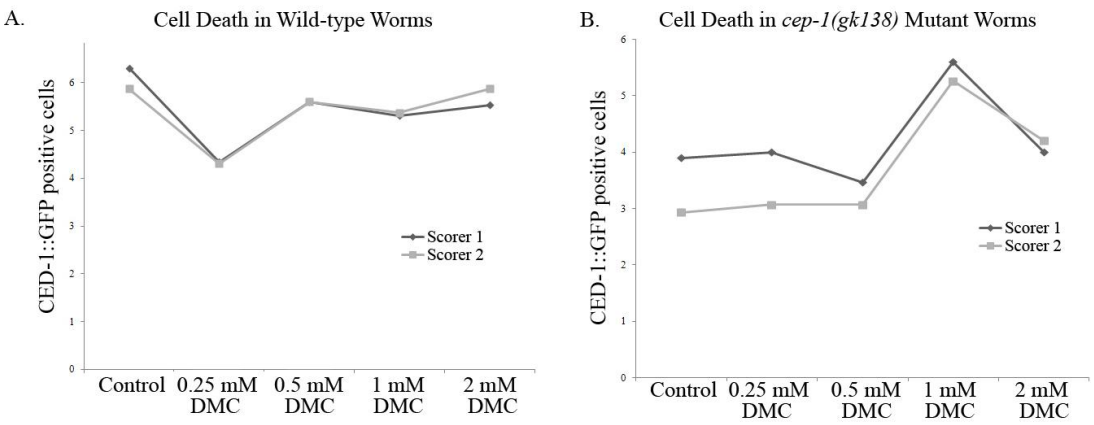

Figure 3.4: DMC increased germline cell death in *cep-1(gk138)* mutant worms but not in wild-type worms. Concentration curves of synchronized young adult A) *ced-1::gfp* worms and B) *cep-1(gk138); ced-1::gfp* worms fed DMC or 30% methanol overnight. Counts were done blindly of CED-1::GFP positive cells and scored by two independent people. The number of worms scored per point was 20.

3.2.5 DMC caused lesions in *cep-1(gk138)* mutant worms.

In order to determine that DMC had entered the worm cells and caused DNA damage, we measured DNA lesions. Using the Lesion assay, we measured lesions after an overnight treatment of 1 mM DMC and detected no lesions in either strain (data not shown). Even though there was an increase in death seen in the *cep-1(gk138)* mutant worms, no damage was detected. This could be due to DNA repair in both strains, or excessive cell death that removed all of the DNA lesions, therefore we treated the worms for five hours and immediately processed the DNA to prevent repair. The mitochondrial and nuclear DNA lesions were then quantified. DNA lesions were detected in the wild-type worms but more DNA lesions were detected in the *cep-1(gk138)* mutant worms (Figure 3.5A). After five hours of treatment, neither strain had a significant increase in germline cell death (data not shown). The wild-type worms showed significantly less lesions than the *cep-1(gk138)* mutants and this could be due to their ability to detect and repair the damage faster than the *cep-1(gk138)* mutants (Figure 3.5A). In addition, as

opposed to UV exposure, DMC treatment increased mitochondrial DNA lesions in the *cep-1(gk138)* mutants (Figures 3.2A and 3.5A). Perhaps DMC is working solely in a CEP-1-independent pathway that might work through the mitochondria increasing its DNA damage.

Similar to what we observed after UV treatment, if DMC was causing DNA damage, there could be a decrease in egg survival due to a lack of CEP-1. DMC treatment did not decrease the number of eggs produced strains, but there was a decrease in egg survival after DMC in both strains (data not shown, Figure 3.5B). This decrease was greater in the *cep-1(gk138)* mutant worms, where only 73% of the eggs hatched while 87% of the wild-type eggs hatched (Figure 3.5B).

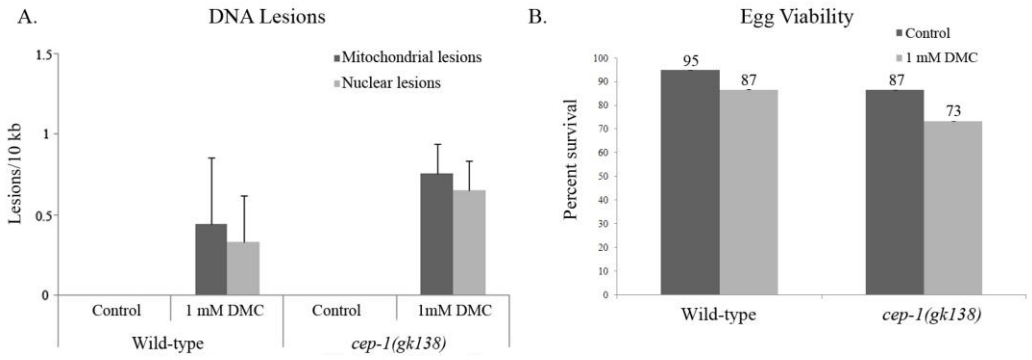

Figure 3.5: DMC caused increased lesions and decreased egg survival in *cep-1(gk138)* mutant worms. A) Lesions of both mitochondrial and nuclear DNA were done on worms after five hours of treatment. Worms were fed 1mM DMC or 30% methanol for five hours, placed in the lysis buffer and frozen. Average of three representative experiments is shown. Error bars indicate standard error. B) L4 worms were treated with 1 mM DMC for five hours and allowed to recover for 24 hours. Worms were then transferred to fresh plates and laid eggs for 12 hours at 20°C. Eggs and surviving animals were counted on day 1 and unhatched eggs were counted on day 2 to assess survival. Percentage survival indicates the number of eggs unhatched on the second day after DMC treatment. Average of 18 worms shown. Error bars indicate standard error.

3.2.6 *cep-1(gk138); glp-1(ar202)* tumor mutant worms had increased nuclear lesions after DNA damage.

We were interested in determining if the germline tumor mutant worms could initiate CEP-1 signaling and if CEP-1 played a role in their apparent resistance to DNA damage. Investigating highly proliferative germ cells and DNA damage resistant cells can help us understand mammalian tumors and potentially how they block cell cycle arrest, apoptotic signals and DNA damage signaling, in the presence and absence of p53/CEP-1. Localized notch signaling from the DTC and the somatic gonad control the developing gonad (Kimble and Crittenden, 2005). The GLP-1 receptor is expressed on mitotic cells and receives the LAG-2 ligand from the DTC instructing them to remain mitotic. As these cells move away from the DTC, they lose the signaling and transition into meiosis (Kimble and Crittenden, 2005). When *glp-1* is either deleted or constitutively active, germline developmental is altered. *glp-1(q224)* (loss of function) animals have no GLP-1 receptor and do not develop a gonad at 25°C. On the other hand, *glp-1(ar202)* (gain of function) mutants have a constitutive GLP-1 and form tumors, a population of solely mitotic cells when grown at 25°C (Kimble and Crittenden, 2005). These cells seem to be resistant to DNA damage due to hyperactive homologous recombination DNA repair (Deng, X., unpublished data).

We measured DNA damage in *glp-1(ar202)* and *cep-1(gk138); glp-1(ar202)* tumor worms and saw that immediately after 50 and 100 J/m^2 of UV treatment, the *glp-1(ar202)* mutant worms had mitochondrial DNA damage while the *cep-1(gk138); glp-1(ar202)* worms had both mitochondrial and nuclear DNA damage (Figure 3.6A). The differences in nuclear DNA damage between the two strains could be due to the lack of CEP-1 mediated DNA repair or the lack of

death or the lack of cells with damaged nuclei. DMC treatment of these worms showed increased nuclear DNA damage the *cep-1(gk138); glp-1(ar202)* worms (Figure 3.6B). DMC induced little mitochondrial DNA damage in the *glp-1(ar202)* worms and no detectable damage in the *cep-1(gk138); glp-1(ar202)* worms (data not shown). We treated *glp-1(ar202)* and *cep-1(gk138); glp-1(ar202)* worms with 100 J/m^2 of UV and measured *egl-1* fold induction four hours later. *glp-1(ar202)* did have induction of *egl-1* while the *cep-1(gk138); glp-1(ar202)* worms did not (Figure 3.6C). These indicated that the CEP-1 in the *glp-1(ar202)* mutant worms was transcriptionally functional.

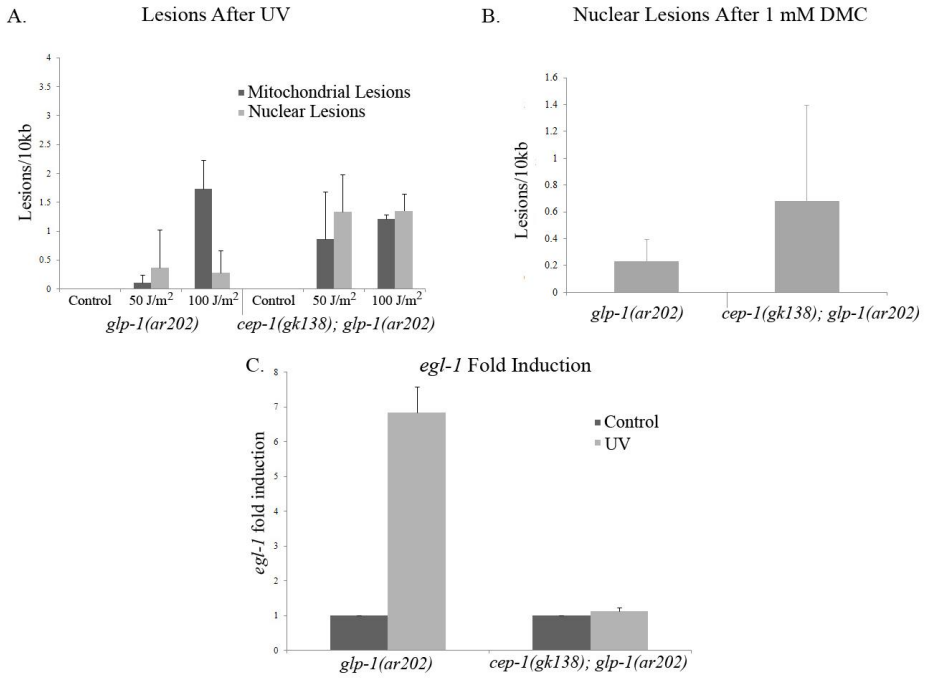

Figure 3.6: CEP-1 was activated in *glp-1(ar202)* tumor mutant worms. A) Lesions of both mitochondrial and nuclear DNA were assessed 24 hour post L4 worms immediately following UV exposure. Worms were exposed to 50 or 100 J/m^2 of UV, placed in the lysis buffer and frozen. Average of three representative experiments is shown. Error bars indicate standard error. B) Lesions in nuclear DNA were done on worms right after five hours of treatment. Worms were fed 1 mM DMC or 30% methanol for five hours, placed in the lysis buffer and frozen. Average of four representative experiments is shown. Error bars indicate standard error. C) Q-RTPCR of *egl-1* mRNA levels four hours post UV treatment of 100 J/m^2. Fold induction compared to cDNAs amplified from untreated worms. Average of six representative experiments is shown. Error bars indicate standard error. Normalized to *tbg-1*. Done by Dr. Ryan Doonan and Daniel Martin.

3.2.7 CEP-1 has a potential role in DNA damage repair in somatic cells

The primary published role for CEP-1 is the induction of germline cell death after DNA damage (Derry et al., 2001). When exogenous CEP-1::GFP is added into worms with its upstream promoter, CEP-1 is only localized to the pharynx and germline of adult worms (Derry et al., 2001). While this suggests that CEP-1 may not play a role in the somatic cells of adult worms. Two-thirds of the cells in an adult hermaphrodite are somatic cells therefore the DNA damage response we observe may be from the somatic cells. In support of CEP-1 being active in only the germline, *egl-1* induction is significantly lower in *glp-4(bn2)* mutants, worms that lack a germline at 25°C, when compared to wild-type worms after IR treatment (Hofmann et al., 2002). This experiment was repeated using the *glp-1(q224)* mutant worms, the germline deficient animals were exposed to 100 J/m^2 of UV and measured for *egl-1* expression four hours after treatment. Unlike the previously published work, the *glp-1(q224)* mutant worms had *egl-1* induction (Doonan, R., Figure 3.7A). Therefore the somatic cells did have a robust increase in *egl-1* expression after UV induced DNA damage. We next asked if CEP-1 would play a role in the removal of UV induced lesions in the *glp-1(q224)* mutant worms. Nuclear lesions were measured immediately, four and eight hours after exposure to 100 J/m^2 of UV and *cep-1* was knocked down using RNAi. Immediately after treatment, there was no difference in the amount of lesions UV induced in the presence and absence of CEP-1 (Figure 3.7B). Over time, similar to what was seen in Figure 3.3A, the *glp-1(q224)* mutant worms with *cep-1* had full nuclear lesion removal while the worms without *cep-1* retained their lesions at the eight hour mark (Figure 3.7B). These data suggest that CEP-1 plays a role in the somatic cell DNA repair after UV induced damage.

In order to address the role of CEP-1 in UV induced DNA damage repair in the germline, we followed the UV induced lesions over time in the tumor mutant and observed that only initially did the *glp-1(ar202)* tumor mutants not have nuclear DNA damage (Figure 3.7C). After four hours, the *glp-1(ar202)* tumor mutants had increases in nuclear lesions and both strains had full removal by twenty-four hours (Figure 3.7C). CEP-1 was not the only player in the tumor mutants DNA repair pathway. Even though the damage was repaired at similar rates in both tumor mutants, the *cep-1(gk138); glp-1(ar202)* tumor worms had larger tumors four days after UV treatment while the *glp-1(ar202)* tumor mutants had decreased tumor size (Martin, D., data not shown). The initial difference in nuclear DNA damage (Figure 3.6A) could have increased mutations in the *cep-1(gk138); glp-1(ar202)* tumors driving tumorigenesis.

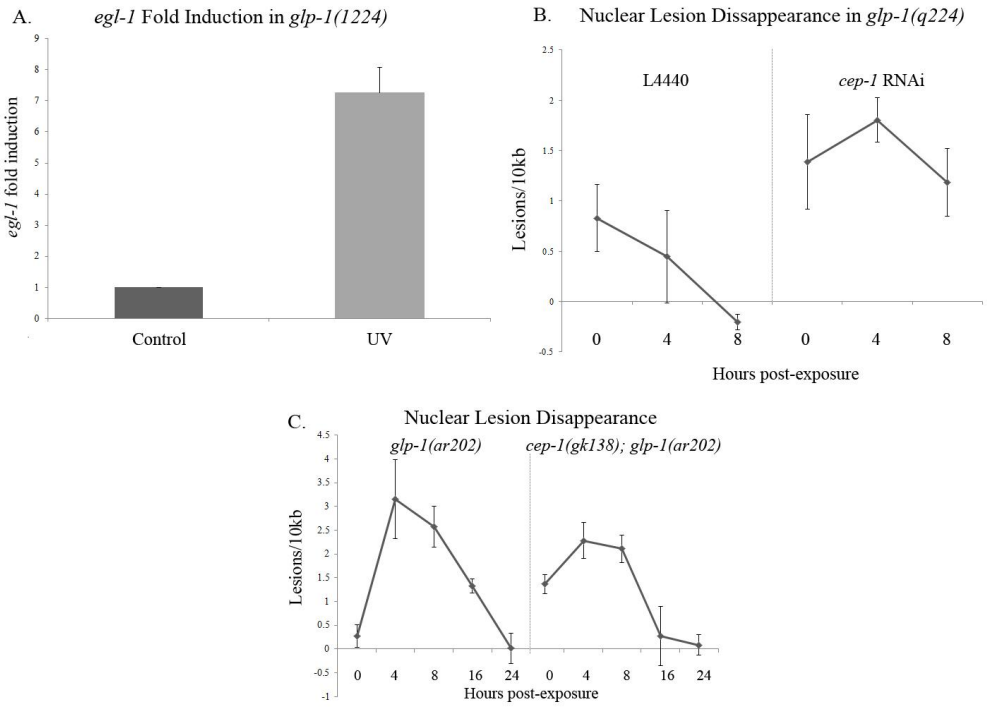

Figure 3.7: CEP-1 functioned to remove UV induced lesions in somatic cells but not in germline tumors. A) Q-RTPCR of *egl-1* mRNA levels four hours post UV treatment of 100 J/m^2. Fold induction compared to cDNAs amplified from untreated worms. Average of two representative experiments is shown. Error bars indicate standard error. Normalized to *tbg-1*. Done by Dr. Ryan Doonan. B) Nuclear lesions were measured immediately, four and eight hours after 100 J/m^2 of UV in *glp-1(q224)* worms grown on either L4440 or *cep-1* RNAi plates at 25°C. Average of three representative experiments is shown. Error bars indicate standard error. C) Nuclear lesions were quantified immediately, four, eight, sixteen and twenty-four hours after UV exposure. Average of three representative experiments is shown. Error bars indicate standard error.

3.3 Discussion

Our data produced that the activation of CEP-1 after UV is clearly observed by the upregulation of *egl-1* and *ced-13* (Figure 3.1A). Additionally, CEP-1 was required for cell death after UV but not for UV induced cell cycle arrest (Figure 3.2C). Importantly we found that the *cep-1(gk138)* mutant worms had equal amounts of nuclear DNA damage after UV exposure when compared to wild-type worms. CEP-1 and its signaling pathways were required for the

proper response to the damage (Figure 3.1D). Interestingly we found that CEP-1 was required for the clearance of the UV induced DNA lesions (Figure 3.3A,B).

p53 is involved in the NER pathway (Boulikas, 1996) and we demonstrated for the first time that CEP-1 had a significant role in the NER pathway in *C. elegans* as well. The NER pathway, like the CEP-1 pathway, is required for germline cell death after UV treatment (Stergiou et al., 2007). We found that clearance of nuclear DNA lesions induced by UV required CEP-1 (Figure 3.3A). While the loss of the nuclear lesions could be due to NER repair, apoptosis and autophagy, it could also be from the actively replicating germline which dilute the damage through replication coupled repair (Meyer et al., 2007; Stergiou et al., 2007). Worms lacking proper DNA repair, remove the UV damaged DNA at a slower rate, similar to the *cep-1(gk138)* mutants (Stergiou et al., 2007). While the wild-type and *cep-1(gk138)* mutants have similar levels of nuclear DNA damage after UV exposure, the wild-type worms can repair or remove the damage while the *cep-1(gk138)* mutants cannot. This would result in increased genomic instability which is a phenotype associated with mutation in the mammalian p53 (Livingstone et al., 1992; Yin et al., 1992).

The lesion assay we used to measure DNA damage detects the inhibition of the long PCR product while using the short PCR product as a normalizer for the levels of DNA present (Meyer, 2010; Van Houten et al., 2000). This loss corresponds to DNA lesions that inhibit replication directly or recombination events that would prevent an increase in the levels of PCR product. However there is no way to determine the type of DNA lesion because all forms of DNA damage block the polymerase from amplifying the long product (Meyer, 2010). Additionally, every cell has different rates of repair after DNA damage and the targeted PCR

regions may be repaired faster or slower than others. When measuring the lesions, we take an average of five worms per experiment, understanding that each cell in each worm can react uniquely to the DNA damage. Although, we consistently saw that the *cep-1(gk138)* mutant worms did not efficiently remove the UV induced lesions when compared to the wild-type worms (Figure 3.3A,B), the pathway by which the wild-type worms repair their DNA may differ therefore yielding a difference in measurable lesions. Our data indicated a strong difference in removal of lesions between the wild-type and *cep-1(gk138)* mutant worms, but we cannot determine the mechanism of lesion clearance.

Mitochondria lack the NER pathway and therefore most of their DNA repair will come from mitochondria fission, fusion and autophagy (Bess et al., 2012). The mitochondrial damage is also diluted out due to actively diving germ cells and is retained in worms lacking a germline, similar to nuclear DNA lesions (Meyer et al., 2007). The *cep-1(gk138)* mutants had less intact or healthy mitochondria to begin with and did not have a lot of mitochondrial DNA lesions after UV treatment (Figure 3.2). This occurred even though the two strains had similar amounts of mitochondrial DNA. Despite the differences seen in the initial damage, the repair or loss of the mitochondrial lesions in the *cep-1(gk138)* mutants was still compromised (Figure 3.3B). p53 has been shown to prevent the removal of sick mitochondria in order to promote apoptosis and block autophagy (Hoshino et al., 2012; Tasdemir et al., 2008), thus if CEP-1 is acting similarly, the loss of CEP-1 would promote the removal of sick mitochondria preventing cell death. Due to the lack of observable healthy mitochondria while the mitochondrial DNA is present in the *cep-1(gk138)* mutants, we can hypothesize that the mitochondria are there but in autophagosomes. Therefore, if the mitochondria are in autophagosomes they would be unstainable and perhaps

their DNA is condensed preventing robust increases in mitochondrial DNA lesions. In the wild-type worms, we saw increases in mitochondrial DNA lesions after UV treatment along with a decrease in stainable mitochondria (Figure 3.2). Both strains could be repairing their damaged mitochondria using fusion, fission and autophagy (Bess et al., 2012), but lack of observable mitochondrial repair in the *cep-1(gk138)* mutants is potentially due to the lack of clearance of the cells containing the damaged mitochondria.

p53 has cytoplasmic roles including the promotion of MOMP by binding to BAX (Green and Kroemer, 2009). Perhaps CEP-1 can act similarly and promote the release of CED-4 from CED-9 once CEP-1 is post-translationally modified, thus additionally promoting germline cell death and the removal of damaged cells. When CEP-1 is not functional, the removal of these cells does not occur and the damage is retained. Therefore the loss of CEP-1 activity can cause further genomic instability, similar to what occurs in cancers.

Over 50% of human cancers have lost functional p53 and this in turn promotes further genomic instability to apoptotic resistant cells (Lu and Abrams, 2006; Yin et al., 2005). The identification of agents that could induce p53-independent cell death is crucial to fighting cancer. 10-decarbomyl-mitomycin C (DMC) can activate p53-independent cell death and DNA damage in human cancer cells (Boamah et al., 2010; Boamah et al., 2007). Few pharmacological agents have effects in *C. elegans* because of their thick cuticle and extensive xenobiotic efflux pumps (Burns et al., 2010). We began with blindly scoring for germline cell death over a range of DMC concentrations to observe for any changes. In *C. elegans*, DMC promoted germline cell death only in the *cep-1(gk138)* mutants but not in the wild-type worms (Figure 3.4A). Furthermore, the wild-type worms had significantly less DNA damage from DMC than the *cep-1(gk138)* mutants

after five hours of treatment (Figure 3.5A). We concluded that DMC was taken up by the worms when administered on plates at high concentrations but after a short treatment, wild-type worms were more efficient at removing the DNA lesions. Both strains were able to remove the damage after overnight treatments. The sensitivity of the *cep-1(gk138)* germline to DMC was probably due to their reduced capability of repair in both the nucleus and mitochondria.

We examined the sensitivity of the germline to UV inducted DNA damage in *cep-1(gk138)* mutants in the *glp-1(ar202)* tumor mutant worms. These worms had a tumorous mitotic germline. We discovered that CEP-1 was transcriptionally functional in the *glp-1(ar202)* tumor mutant worms and very little nuclear damage was observed after immediate DNA damage from UV and five hours of DMC treatments (Figure 3.6). These tumors are quickly dividing and have high levels of DNA replication possibly allowing them to quickly remove their damage by replication coupled repair machinery. In the *cep-1(gk138); glp-1(ar202)* mutant worms, UV and DMC treatments were able to cause significantly more nuclear DNA lesions than the wild-type worms (Figure 3.6A,B). Thus further confirming the involvement of CEP-1 in nuclear damage repair and the susceptibility of *cep-1(gk138)* mutants to maintain DNA damage. On the other hand, the mitochondrial lesions in the *glp-1(ar202)* tumor mutant worms were greater after UV and DMC treatments than the *cep-1(gk138); glp-1(ar202)* double mutant worms (Figure 3.6A,B). Perhaps the mitochondria in the *glp-1(ar202)* tumor mutant worms were not being repaired, removed or duplicated as fast as the mitochondria in the germline of the wild-type worms.

While the PCR reactions are primer specific, allowing us to compare the amplicons between the nuclear and mitochondrial genomes, the PCR reaction is not cell type specific

(Meyer, 2010). In wild-type worms, two-thirds of the cells are somatic cells and one-third is the actively dividing germline. It was then postulated that the source of DNA damage could be from the somatic cells in addition to the germline. Using *glp-1(q224)* mutant worms, we saw that CEP-1 was activated in the somatic cells and the knockdown of *cep-1* did impair nuclear DNA damage repair (Figure 3.7). Previously it was shown that *glp-1(q224)* mutant worms have nuclear DNA repair after UV at slower rates than wild-type (Meyer et al., 2007) and germline null animals have no *egl-1* activation after IR (Hofmann et al., 2002). Our data does not support the previously published work using a different germline null animal with IR to induce downstream target activation (Hofmann et al., 2002). The difference in our results could be due to the differences in lesion types and the type of CEP-1 activation. Furthermore, our repaired damage occurred at faster rates than the Meyer paper in wild-type worms, but we do see a clear decrease in the rate of removal of lesions when *cep-1* was knocked down ((Meyer et al., 2007) and Figure 3.7). Our data indicates a DNA repair role for CEP-1 in the soma that has not been previously reported.

CEP-1 is the only known p53 homolog in *C. elegans* but CEP-1 could contain all p53 family member properties (Derry et al., 2001; Ou et al., 2007; Schumacher et al., 2001). While there are no development issues in *cep-1(gk138)* mutant worms, these mutants lack DNA damage induced germline cell death. This is similar to *p63* null mice which are deficient in proper germ cell development (Petre-Lazar et al., 2007; Suh et al., 2006). CEP-1 and p63 both function in the germline to maintain proper development of offspring. Thus the primary role of CEP-1/p53 could be to protect the germline and then the different family members broke off and p53 evolved into a tumor suppressor. The loss of *cep-1* did not alter germline tumor growth, but

after UV damage, the tumors in the *cep-1(gk138); glp-1(ar202)* mutants increased indicating a buildup of mutations and loss of proper cell cycle controls (data not shown).

In summary, we have seen that CEP-1 has a novel role in *C. elegans* germline integrity genomic integrity and the loss of CEP-1 sensitizes genomes towards nuclear DNA damage (Figure 3.8). The sensitivity of *cep-1(gk138)* mutant germlines to DNA damage translates into decreased embryonic viability following UV and DMC induced genomic damage. CEP-1 can potentially be working with the NER pathway to remove UV induced lesions and promote cells survival (Figure 3.8, green arrows). Furthermore, the loss of CEP-1 causes germlines to be susceptible to more immediate DNA damage and increases tumor size (Figure 3.8, red arrow). It is unclear if this will cause *cep-1(gk138)* mutant germlines to become more easily transformed and maintain multiple mutations. These data conclude that CEP-1, like p53, was involved in DNA repair.

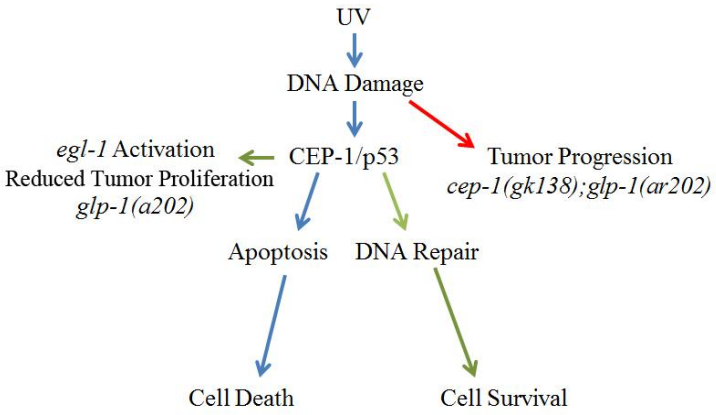

Figure 3.8: Model for CEP-1/p53 function in *C. elegans*. CEP-1 is known to promote cell death after UV induced damage (blue arrows). Here we reported that CEP-1 was also involved in DNA repair and reduced tumor proliferation (green arrows). Furthermore, the loss of *cep-1* in tumor mutants caused tumor progression (red arrow).

CHAPTER 4:

BEC-1 cross-talk with CEP-1 in response to UV induced DNA damage

4.1 Introduction

Autophagy is a paradoxical pathway which can promote cell survival and cell death (Mathew et al., 2007). The survival pathway requires the removal of old organelles and portions of the cytoplasm to help maintain the bioenergetics of the cell (Ryter et al., 2012). Secondly, autophagy is considered a form of programmed cell death (Galluzzi et al., 2012; Kang et al., 2011; Maiuri et al., 2008). Cells require proper levels of autophagy; high levels of autophagy promote cell death and compromised autophagy activates apoptosis (Abedin et al., 2007; Kroemer and Levine, 2008). In cancer, tumors with functional autophagy use it as a survival mechanism and such cells undergo cell death when autophagy is blocked (Amaravadi et al., 2011; Amaravadi et al., 2007). On the other hand, some tumors mutate autophagy genes in order to proliferate. These tumors lose their tumorgenecity when functional autophagy is reintroduced (Liang et al., 1999).

Autophagy, like apoptosis, is conserved between mammals and *C. elegans* (Jia and Levine, 2010). Beclin 1 is a key regulator of autophagy in mammalian cells and has an homologue, BEC-1, in *C. elegans* (Liang et al., 1998; Melendez et al., 2003). Beclin 1 and BEC-1 are involved in the early stages of the formation of the autophagosome (Ryter et al., 2012). Without Beclin 1 and BEC-1, proper autophagy cannot occur (Liang et al., 1998; Melendez et al., 2003). Additionally, Beclin 1 and BEC-1 play a role in genomic instability. Human cancer cells that have deficient apoptosis and a loss of one copy of *beclin 1* have increased DNA damage, indicated by increased γ-H2AX staining (Karantza-Wadsworth et al., 2007). Furthermore, when worms have *bec-1* knocked down, they have increased TUNEL staining, demonstrating increased DNA damage and cell death (Takacs-Vellai et al., 2005).

The relationship between autophagy, cell death and DNA damage has been investigated in worms. The knockdown of *bec-1* in worms causes increases in CED-3-dependent cell death (Takacs-Vellai et al., 2005). This cell death has been seen in the F1 knockdown animals and is attributed to a lack of clearance of the dead cells (Ruck et al., 2011). However, the relationship between CEP-1 and BEC-1 and germline cell death has not been clearly studied in worms. We carried out studies to see if CEP-1 was responsible for the cell death in *bec-1* knockdown animals. We observed that partial knockdown of *bec-1* caused CEP-1-dependent germline cell death. Full knockdown of *bec-1* increased cell death independently of CEP-1 due to a lack of clearance of dead germ cells. Furthermore, the loss of *bec-1*, upon the addition of UV DNA damage, produced more lesions than UV in the presence of BEC-1. These data indicated a complex connection between BEC-1, the clearance of cell death and DNA damage.

4.2 Results

4.2.1 Increased cell death by the knockdown of *bec-1* in parental worms required CEP-1.

bec-1/bec-1 mutant worms are embryonic lethal (Takacs-Vellai et al., 2005). If the *bec-1* mutant worm has maternal *bec-1*, it can develop into a young adult but dies early with a poorly developed germline (Takacs-Vellai et al., 2005). RNAi has been used to study the role of BEC-1 and full knockdown of *bec-1* increases germline cell death, due to a lack a clearance (Ruck et al., 2011; Takacs-Vellai et al., 2005). We investigated the role BEC-1 played in UV mediated cell death and DNA damage using RNAi to reduce the *bec-1* levels. We first looked at partial loss of *bec-1* in worms, termed parental worms. To ensure proper knockdown of *bec-1*, we fed L4 BEC-1::RFP worms either L4440 (empty plasmid) or *bec-1* RNAi and allowed them to eat the bacteria

for 24 or 48 hours. After 24 hours, the BEC-1::RFP was still there (as can be seen by the red in the image); by 48 hours, all traces of the BEC-1::RFP were gone (Figure 4.1A, 24 hours and 48 hours control). Therefore we concluded that *bec-1* RNAi did knockdown *bec-1* partially in the parental generation. Interestingly, UV treatment increased BEC-1::RFP levels slightly in the L4440 worms and in the *bec-1* knockdown worms (Figure 4.1A, 48 hours).

Next we placed L4 CED-1::GFP wild-type and *cep-1(gk138)* worms onto either L4440 or *bec-1* RNAi plates. After 24 hours, a portion of the worms were treated with 100 J/m^2 of UV. 24 hours later, worms were scored for germline cell death. Wild-type worms had high basal levels of CED-1::GFP positive cells after *bec-1* knockdown while *cep-1(gk138)* mutant worms did not have increased germ cell death (Figure 4.1B). Additionally, after UV treatment, there was an increase in dead germ cells in *bec-1* RNAi fed worms while there was no difference in the *cep-1(gk138)* mutant worms (Figure 4.1C). This suggested that the *bec-1* knockdown induced germline cell death in a CEP-1-dependent manner. Surprisingly, the L4440 animals were sick and showed a slight increase in CEP-1-independent UV induced cell death. To determine if the increase in *bec-1* knockdown CEP-1-dependent cell death was due to a lack of clearance, we waited and watched corpses over an hour to see if clearance was repressed. In the parental worms, there was clearance of CED-1::GFP corpses in less than an hour in L4440 and *bec-1* RNAi fed worms. Therefore the increase in cell death in parental worms was not due to a lack of corpse clearance. The loss of *bec-1* activated a CEP-1-dependent increase of germline cell death.

We reasoned that the increase of CEP-1-dependent germline cell death could have been from increased CEP-1 activation in the absence of BEC-1. If this were true, RNAi fed animals would have increased levels of CEP-1 downstream target gene activation. After treatment with

100 J/m^2 of UV, worms were allowed to recover for four hours and then prepared for RNA extraction. *egl-1* mRNA was increased by UV but knockdown of *bec-1* did not increase *egl-1* activation (Figure 4.1D). Therefore, the increase in CED-1::GFP positive germ cells was not due to a transcriptionally hyper-activated CEP-1.

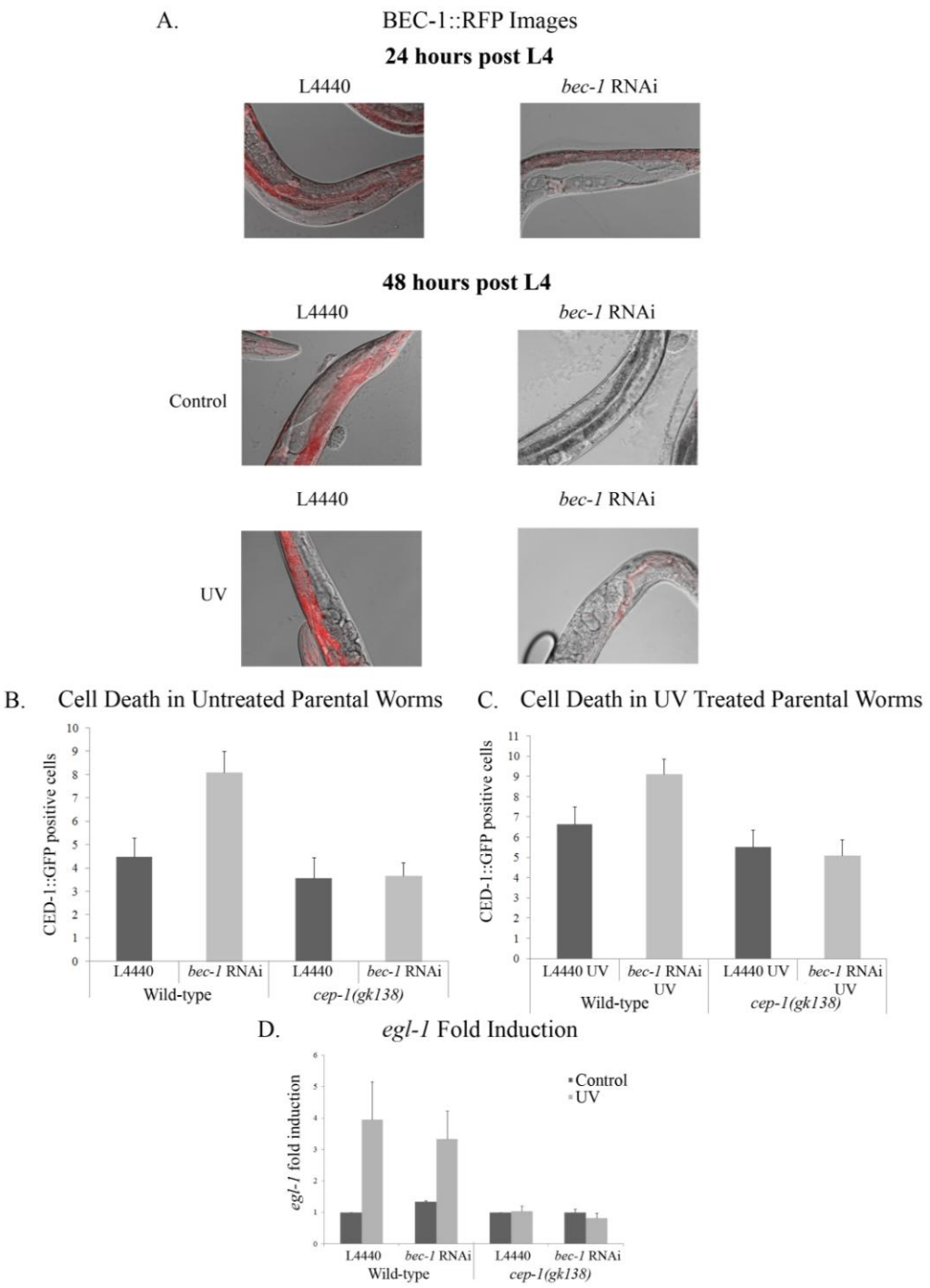
A.
BEC-1::RFP Images
24 hours post L4
L4440
bec-1 RNAi
48 hours post L4
L4440
bec-1 RNAi
Control
L4440
bec-1 RNAi
UV
B.
Cell Death in Untreated Parental Worms
CED-1::GFP positive cells
L4440
bec-1 RNAi
L4440
bec-1 RNAi
Wild-type
cep-1(gk138)
C.
Cell Death in UV Treated Parental Worms
CED-1::GFP positive cells
L4440 UV
bec-1 RNAi UV
L4440 UV
bec-1 RNAi UV
Wild-type
cep-1(gk138)
D.
egl-1 Fold Induction
egl-1 fold induction
Control
UV
L4440
bec-1 RNAi
L4440
bec-1 RNAi
Wild-type
cep-1(gk138)

Figure 4.1

Figure 4.1: In parental worms, increased death from *bec-1* knockdown required CEP-1. A) Images of BEC-1::RFP worms 24 or 48 hours after L4 worms were placed on L4440 or *bec-1* RNAi plates. 100 J/m^2 of UV exposure was done 24 hours after L4 and observed 24 hours after that. The Nomarski and red channels were placed on top of each other. B) CED-1::GFP worms were fed L4440 or *bec-1* RNAi and scored for germline cell death in the absence or C) presence of 100 J/m^2 of UV. Counts were done blindly of CED-1::GFP positive cells by two independent people. Error bars indicate standard error and the number of worms scored per bar was 20. D) Q-RTPCR of *egl-1* mRNA levels four hours post 100 J/m^2 of UV treatment. Fold induction compared to cDNAs amplified from untreated worms. The average of three representative experiments is shown. Error bars indicate standard error. Normalized to *tbg-1*.

4.2.2 DNA lesions after UV treatment were increased following partial *bec-1* knockdown.

We investigated if there was an increase in DNA lesions caused by the loss of *bec-1*. It has been shown that the loss of Beclin 1 induces genomic instability (Karantza-Wadsworth et al., 2007) and therefore we hypothesized that we would detect increased lesions in the absence of *bec-1*. DNA damage increases BEC-1 levels and autophagy (Bess et al., 2012; Erdelyi et al., 2011; Karantza-Wadsworth et al., 2007). After worms were subjected to 100 J/m^2 of UV, we quantified mitochondrial and nuclear DNA lesions. The addition of *bec-1* knockdown significantly increased UV induced DNA lesions (Figure 4.2A). Importantly, UV-induced nuclear DNA lesions in *cep-1(gk138)* mutant worms were also increased by *bec-1* knockdown (Figure 4.2B). On the other hand, UV-induced mitochondrial DNA lesions did not increase after *bec-1* knockdown (Figure 4.2B).

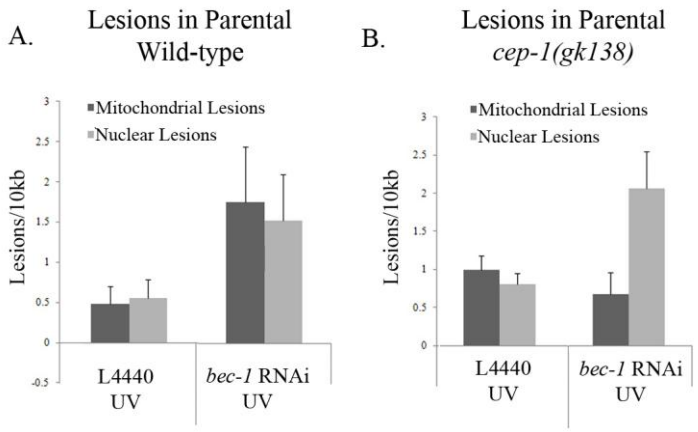

Figure 4.2: UV treatment after *bec-1* knockdown increased the number of DNA lesions in the P0 animals. A) Wild-type and B) *cep-1(gk138)* mutant worms were exposed to 100 J/m^2 of UV, placed in the lysis buffer and frozen. Lesions of both mitochondrial and nuclear DNA were done. Average of three representative experiments is shown. Error bars indicate standard error.

4.2.3 Knockdown of *bec-1* in F1 worms caused increased germline cell death due to a lack of corpse clearance.

After studying the parental worms that only had a partial loss of *bec-1* during their life, we went on to study their offspring (F1). These worms were hatched onto RNAi plates and had not been exposed to BEC-1 during their life span. As can be observed in the BEC-1::RFP images, no detectable BEC-1 was present in these worms grown on *bec-1* RNAi plates (Figure 4.3A, upper panel). As was documented by others, UV damage induced BEC-1 increase and autophagy (Bess et al., 2012; Erdelyi et al., 2011) (Figure 4.3A, lower panel). This was observed in the parental worms, but not to the same magnitude (Figure 4.1A). It has been published that BEC-1::GFP levels and autophagy increase after DNA damage, and here we observed the BEC-1::RFP increase (Bess et al., 2012; Erdelyi et al., 2011).

Partial *bec-1* knockdown required CEP-1 for the increases in cell corpses (Figure 4.1B). We scored for the CED-1::GFP in the next generation we observed a CEP-1 independent increase in cell death (Figure 4.3B). To determine if the increased observable germline cell death was due to an actual increase in cell death or because of faulty clearance, we watched CED-1::GFP corpses for over an hour. After an hour, some of the corpses in the L4440 fed worms disappeared while the *bec-1* RNAi fed worms did not have any corpses fade. This confirmed that the observable increase of CED-1::GFP positive cells in the F1 generation was due to a loss of clearance (Ruck et al., 2011). Additionally, this decrease in clearance occurred independently of CEP-1.

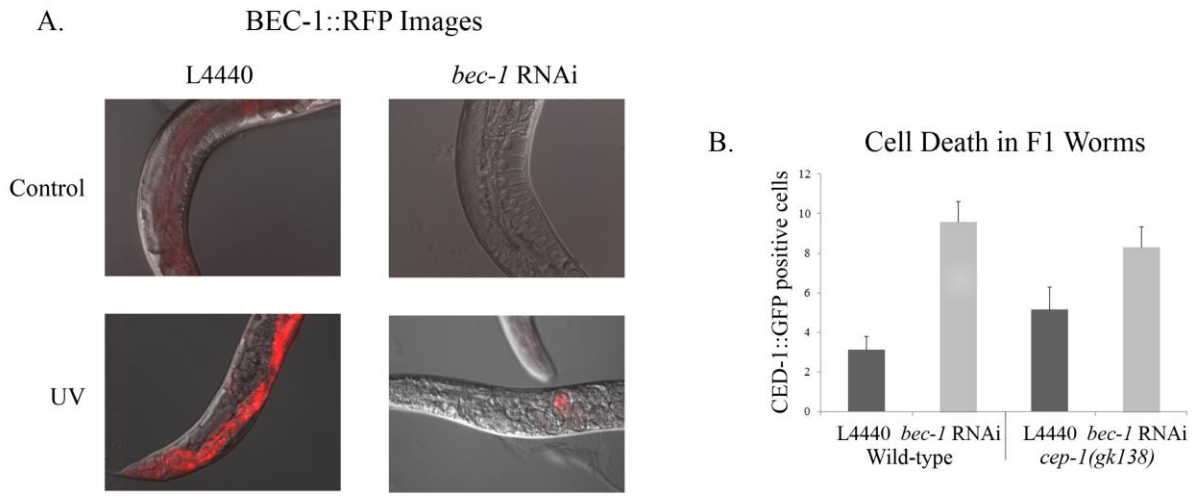

Figure 4.3: In F1 worms, *bec-1* knockdown increased CED-1::GFP cells independent of CEP-1. A) Images of BEC-1::RFP worms 48 hours post L4. Worms were grown up on RNAi plates and UV treatments were done 24 hours after L4. The Nomarski and red channels were placed on top of each other. B) CED-1::GFP worms were fed L4440 or *bec-1* RNAi and scored for germline cell death. Counts were done blindly of CED-1::GFP positive cells by two independent people. Error bars indicate standard error and the number of worms scored per bar was 20.

4.2.4 UV treatment after *bec-1* RNAi in the F1 worms resulted in an increase in DNA lesions.

We examined if the loss of *bec-1* caused an increase in DNA damage in the F1 generation. In wild-type worms, there was a significant increase in both mitochondria and nuclear DNA lesions caused by the loss of *bec-1* (Figure 4.4A). However, the addition of *bec-1* knockdown and 100 J/m^2 of UV caused a robust increase in mitochondria DNA lesions (Figure 4.4A). This could be due to the lack of proper mitophagy and the increase in mitochondria instability that the loss of BEC-1 induced. Surprisingly the loss of *bec-1* in conjunction with UV in the F1 worms did not increase the nuclear lesions (Figure 4.4A). Perhaps this is from CEP-1 mediated NER. On the other hand, the *cep-1(gk138)* mutant worms had the opposite response. The knockdown of *bec-1* slightly increased mitochondria damage (Figure 4.4B). However, the additive effect of the *bec-1* knockdown and UV exposure significantly increased the nuclear DNA lesions (Figure 4.4B). Again, this hints to the possible role of CEP-1 in immediate response to DNA damage in the nucleus.

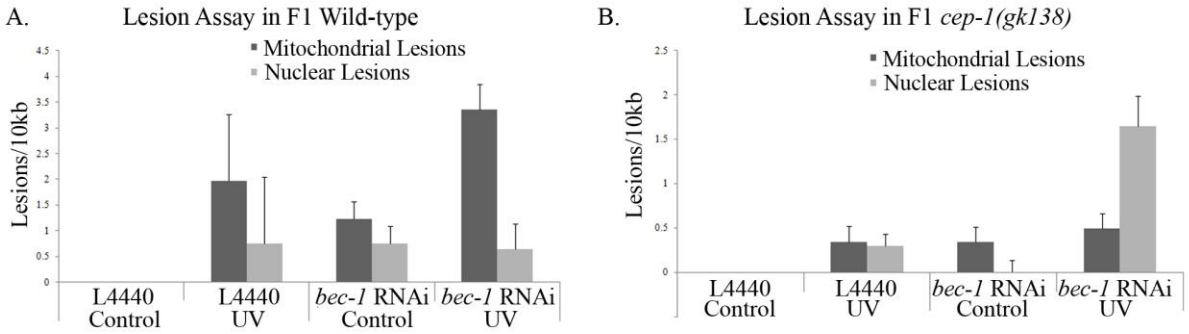

Figure 4.4: Wild-type worms had increased mitochondrial DNA lesion after *bec-1* knockdown with UV treatment while *cep-1(gk138)* mutants had increased nuclear lesions. A) Wild-type and B) *cep-1(gk138)* mutant worms were exposed to 100 J/m^2 of UV, placed in the lysis buffer and frozen. Lesions of both mitochondrial and nuclear DNA were analyzed. Average of three representative experiments is shown. Error bars indicate standard error.

4.2.5 *bec-1* knockdown decreased egg survival when coupled with UV exposure.

UV exposure decreased egg survival and we asked whether or not the loss of *bec-1* would induce further embryonic mortality. L4 worms from the *bec-1* RNAi fed F1 generation were treated with 100 J/m^2 of UV, allowed to recover for twenty-four hours and then placed on fresh plates to lay eggs overnight. The numbers of eggs produced were counted along with the percentage of eggs that did not hatch. *bec-1* knockdown decreased the percentage of eggs that hatched in the wild-type worms (Figure 4.5). On the other hand, the *cep-1(gk138)* mutant worms did not have any change in egg viability upon *bec-1* knockdown (Figure 4.5). After UV treatment, the percentage of wild-type eggs that hatched was 50% and the *cep-1(gk138)* mutant eggs decreased to 64% (Figure 4.5). The buildup of DNA damage in these wild-type worms most likely contributed to their lack of viable eggs. The *cep-1(gk138)* mutant worms grown on L4440 plates were sickly and had decreases in egg viability before UV treatment. Therefore, we did not observe any changes in egg viability after UV treatment in the *cep-1(gk138)* mutant worms grown on L4440 plates (Figure 4.5).

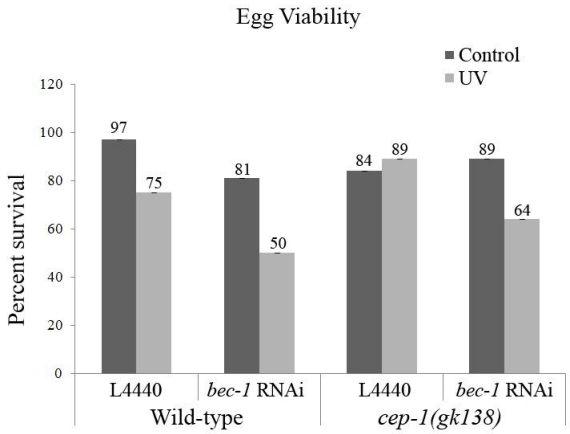

Figure 4.5: Wild-type and *cep-1(gk138)* mutant worms had a decrease in egg survival in the absence of *bec-1* after UV treatment. L4 worms were treated with UV and allowed to recover for 24 hours. Worms were then transferred to fresh plates and allowed to lay eggs for 12 hours at 20°C. Unhatched eggs and survived animals were counted 1 and 2 days later to assess survival. Average of 18 worms shown. Error bars indicate standard error.

4.3 Discussion

In order to investigate the role that BEC-1 plays in CEP-1 signaling, cell death and DNA damage, we examined worms with partial (parental worms) and full (F1 worms) *bec-1* knockdown. Parental worms with *bec-1* knockdown had increased germline cell death that was CEP-1-dependent that did not alter *egl-1* mRNA induction (Figure 4.1B,D). This CEP-1-dependent cell death was activated without the induction of DNA damage. This could be due to the removal of inhibitors of CEP-1 and the cell death pathway. For instance, the removal or inhibition of the tyrosine kinase ABL-1 increased CEP-1-dependent germline cell death (Deng et al., 2004). Additionally, the FOXO homologue DAF-16 has target genes involved in the nuclear-pore that inhibit the CEP-1-dependent cell death pathway (Pinkston-Gosse and Kenyon, 2007). Perhaps BEC-1, like ABL-1 and DAF-16, works upstream of CEP-1 to mediate cell death without altering the transcriptional activity of CEP-1. This could be occurring directly through

the CEP-1 signaling pathway. ABL-1 inhibits the activation of upstream regulators of CEP-1. CEP-1 and HUS-1, an upstream activator of CEP-1, were required for cell death in the absence of *abl-1*, but EGL-1 was not (Deng et al., 2004). This could be why we did not see a change in *egl-1* fold induction after the removal of BEC-1. Therefore, BEC-1 may function as an inhibitor of cell death by blocking the CEP-1 signaling pathway. The BEC-1 signaling pathway could be involved upstream of CEP-1 mediating the activation of CEP-1 promoting cell death.

BEC-1 is required for autophagy in *C. elegans* (Melendez et al., 2003) and BEC-1 mediated autophagy may inhibit apoptosis. In a Myc driven tumor model, the inhibition of autophagy activated p53-mediated cell death (Amaravadi et al., 2007). This is reminiscent of what was seen in *C. elegans*. If autophagy in *C. elegans* blocks the promotion of apoptosis, then the removal of BEC-1-mediated autophagy could be the reason why we observed increased cell death. The removal of BEC-1 and activation of CEP-1 can be working along with the activation of the BH3-only genes by CEP-1 after DNA damage. Furthermore, CEP-1, like p53, may have pro-apoptotic activities in the cytoplasm and could be another driver for CEP-1-dependent increased cell death after *bec-1* knockdown.

The complete knockdown of *bec-1* in the F1 worms increased CED-1::GFP cells independently of CEP-1 (Figure 4.3B). This was due to a lack of clearance of the engulfed cell corpses. The somatic gonad was able to engulf the corpses, but not clear the corpses from the gonad yielding an observable increase in germline cell death. In general, the wild-type worms were more sensitive to the loss of *bec-1*. The untreated F1 wild-type worms had increased basal levels of mitochondrial and nuclear DNA lesions (Figure 4.4). Furthermore, the loss of *bec-1* in the F1 wild-type worms decreased the percentage of eggs that hatched, 97% compared to 81%

(Figure 4.5). Once worms were exposed to UV DNA damage, the wild-type and *cep-1(gk138)* mutant worms both had increased DNA lesions and significant decreases in the percentage of eggs hatching (Figures 4.4 and 4.5). Without the addition of DNA damage, the *cep-1(gk138)* worms were less sensitive to *bec-1* knockdown than the wild-type worms. Possibly because BEC-1 and CEP-1 were functioning in the same signaling pathway and the loss of the second protein was redundant after the first was lost. If CEP-1 was present, then the loss of BEC-1 promoted the CEP-1-mediated cell death pathway. Although in the absence of CEP-1, the loss of BEC-1 did not induce any changes without DNA damage.

We initially hypothesized that BEC-1 could be a negative inhibitor of the CEP-1 cell death pathway similar to the inhibition Mdm2 has on p53. Mdm2 promotes the degradation of p53 via the proteasome and blocks p53 transcriptionally (Arva et al., 2008). *mdm2* -/- mice with wild-type *p53* are embryonic lethal (Hollstein et al., 1991; Kussie et al., 1996). Similarly, *beclin 1* -/- mice have early embryonic lethality as do *bec-1/bec-1* mutant worms (Sinha and Levine, 2008; Takacs-Vellai et al., 2005). The removal of *p53* rescues the *mdm2* -/- mice (Montes de Oca Luna et al., 1995) but in the *cep-1(gk138); bec-1(ok691)* double mutant, the *bec-1(ok691)* phenotype was not reversed (data not shown). There were high levels of embryonic lethality in the double mutants and few worms developed into adults. Therefore, while the loss of *bec-1* increased CEP-1-dependent cell death, the removal of CEP-1 did not rescue the *bec-1* knockout phenotype, indicating that CEP-1 activation is not the deciding factor for *bec-1/bec-1* embryonic lethality. It suggest that a lack of clearance in the end is deleterious even if you inhibit the early onset of CEP-1 induced apoptosis.

There may not be a direct relationship between CEP-1 and BEC-1 but the two proteins may alter the same target in different ways in their pathways. p53, Beclin 1 and BEC-1 have been shown to bind to the anti-apoptotic CED-9/Bcl-2 family members (Chipuk et al., 2005; Maiuri et al., 2007; Oltersdorf et al., 2005; Takacs-Vellai et al., 2005; Tomita et al., 2006). Perhaps CEP-1, like p53, can bind to CED-9 and mediate the control CED-9 has on autophagy and apoptosis. Furthermore, there may be another target that both CEP-1/p53 and BEC-1/ Beclin 1 regulate yielding different results. Both proteins do contain the ability to bind to pro-apoptotic Bcl-2 family members and contain BH3 only binding capability, there might be another protein that they both regulate in different ways to promote cell death or autophagy (Chipuk et al., 2005; Park et al., 2012; Sinha and Levine, 2008). NOXA, PUMA, and Bax are all potential targets that can be regulated by both Beclin 1 and p53 to promote autophagy or apoptosis (Elgendy et al., 2011; Park et al., 2012). In *C. elegans,* possibly the most likely targets are EGL-1 and CED-13 that can bind differently to CED-9 depending if CEP-1 or BEC-1 are active.

Here we have demonstrated a novel role that BEC-1 played in cell death and the clearance of DNA damage. While the increase in cell corpses by *bec-1* knockdown has already been observed, the dependency on CEP-1 after a partial knockdown was novel. Furthermore, the full loss of *bec-1* sensitized worms to DNA damage similar to the loss of *beclin 1* (Karantza-Wadsworth et al., 2007). We have also observed that *cep-1* is potentially acting in a similar pathway to *bec-1* because the loss of both genes did not alter viability or survival of worms more than the loss of *bec-1* alone. While the exact role of BEC-1 on the CEP-1 cell death pathway is unknown, we observed that they worked in similar pathways. Figure 4.6 depicts the possible connection between the pathways, where there may be a physical inhibition that BEC-1/Beclin 1

play on CEP-1/p53 and apoptosis (Figure 4.6A, red arrows). BEC-1/Beclin 1 does participate in DNA repair potentially leading to cell survival (Figure 4.6A, green arrows). Furthermore, there could be another target CEP-1/p53 and BEC-1/Beclin 1 regulate to direct the pathway. For instance, they both could both inhibit CED-9/Bcl-2 activating apoptosis or autophagy. On the other hand, they could activate pro-apoptotic BH3-only members to activate their targeted pathway (Figure 4.6B, purple arrows).

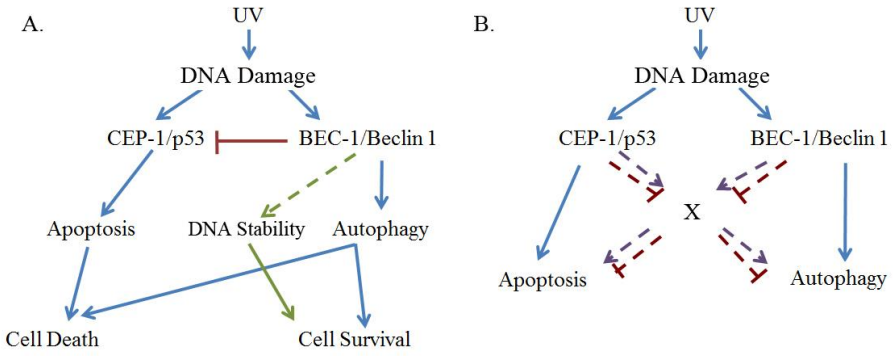

Figure 4.6: Model for BEC-1/Beclin 1 function in *C. elegans.* A) BEC-1 is known to promote autophagy after UV induced damage (blue arrows). Here we reported that BEC-1 is also involved in DNA repair (green arrows) and could inhibit CEP-1/p53 signaling and apoptosis (red block). B) CEP-1/p53 and BEC-1/Beclin 1 could interact through a downstream target that could promote or inhibit apoptosis or autophagy (purple arrows and red blocks).

CHAPTER 5:

Discussion, Future Directions and Perspectives

5.1 Novel roles for the p53 homologue in *C. elegans*.

Mammalian p53 has multiple roles in cells to thwart tumorigenesis including inducing cell cycle arrest, apoptosis, DNA repair and autophagy (Vousden and Prives, 2009). To date, the p53 homologue in *C. elegans* has been shown to be required for DNA damage induced germline cell death and UV mediated cell cycle arrest (Stergiou et al., 2007). Additionally, the knockdown or deletion of *cep-1* leads to increases in autophagy (Tasdemir et al., 2008). We observed that CEP-1 played a role in UV mediate DNA damage and repair. The loss of *cep-1* decreased the removal of UV induced lesions and sensitized the *glp-1(ar202)* tumor mutants for UV and DMC induced nuclear DNA damage. Furthermore, *cep-1(gk138)* mutant worms had fewer mitochondrial DNA lesions after UV treatment and less detectable mitochondria when using the TMRE stain. These differences occurred even though there was no variation in amount of mitochondrial DNA isolated. Therefore there may be a potential inhibition CEP-1 has on the susceptibility of mitochondria to DNA damage. We have also observed DNA damage mediated CEP-1-independent germline cell death. This confirmed the usage of *C. elegans* as a model system to look for pharmacological agents to induce DNA damage and germline cell death in the absence of p53. While these conclusions were made from the data collected, they could be extended by further experiments. This section describes such potential future experiments.

5.1.1 The involvement of CEP-1 in the NER pathway.

Preliminary data have shown that CEP-1 was involved in DNA repair after UV exposure. We have observed that *cep-1(gk138)* mutant worms did not remove the UV-induced lesions at a similar rate to the wild-type worms (Figure 3.3A,B). Furthermore, UV exposure decreased the

viability of eggs more in the *cep-1(gk138)* mutant worms than in wild-type worms potentially due to the lack of germline cell death, DNA repair or increase in genomic instability (Figure 3.3D).

To further confirm the involvement of CEP-1 in the UV induced lesion repair pathway, we can look for the removal of cyclobutane pyrimidine dimers (CPDs) by immunofluorescence (Stergiou et al., 2011). CPDs are a major form of lesions caused by UV exposure which require the NER and the homologous recombination pathways for their removal in *C. elegans*. Without the NER pathway, CPDs are not removed and can be observed by immunofluorescence in the germline of adult worms after UV. If CEP-1 is required for the NER pathway, the same trend would be seen. We can use the *xpa-1(ok698)* or *rad-54(ok615)* mutant strains as positive controls for the lack of CPD removal (Stergiou et al., 2011). If CEP-1 is involved and assists in NER pathway, the removal of CPDs would be hindered but not abolished completely.

Preliminary data did indicate a possible role of CEP-1 in somatic cells. The involvement of CEP-1 in the NER pathway in somatic cells can be confirmed using the lesion assay on starved L1 worms. Arrested L1 worms do not undergo cell division and any loss of lesions is due to DNA repair (Meyer et al., 2007). *xpa-1(rb864)* mutant worms have no repair after UV exposure as L1 starved worms while wild-type worms have repair (Meyer et al., 2007). Using *cep-1(gk138)* arrested L1 worms, we can detect if these worms have retained or lost their DNA lesions after UV exposure. Similar to the loss of CPDs, we can measure the changes in lesions over time and see if there are any differences between the wild-type and *cep-1(gk138)* mutant worms. If the repair in the *cep-1(gk138)* mutant worms mimic or is similar to the *xpa-1(rb864)* mutant worms, then we know CEP-1 is involved in the NER pathway in somatic cells.

5.1.2 How p53/CEP-1 mediates mitochondrial stability.

In *cep-1(gk138)* mutant worms, less mitochondrial DNA lesions were quantified after UV exposure and fewer intact mitochondria were observed (Figure 3.2). The loss of *cep-1* has been shown to cause increases in LGG-1::GFP and the inhibition of cytoplasmic p53 also increases autophagy (Tasdemir et al., 2008). The loss of *p53* increases mitophagy after ischemia which prevents the buildup of damaged mitochondria, a usual promoter of cell death (Hoshino et al., 2012). CEP-1 may also prevent the removal of old or damaged mitochondria via autophagy to promote cell death. Even though there were no differences in the levels of mitochondrial DNA between wild-type and *cep-1(gk138)* mutants, the number of intact mitochondria was different. Furthermore, only the wild-type worms had an increase in mitochondrial DNA lesions and detectable mitochondria after UV treatment (Figure 3.2). Therefore, CEP-1 may be involved in the inhibition of mitophagy and the activation of MOMP and cell death.

Using *glp-1(q224)* mutant worms that lack a germline and mitochondrial DNA repair after UV, we can look for the loss of mitochondrial DNA lesions after knocking down *cep-1* (Bess et al., 2012; Meyer et al., 2007). If CEP-1 is preventing the removal of old mitochondria, knocking down *cep-1* would promote the removal of mitochondrial DNA lesions in the *glp-1(q224)* mutant worms (Bess et al., 2012).

p53 works to activate apoptosis in the cytoplasm by binding to Bax/Bid to promote MOMP (Moll et al., 2005). This activation is inhibited by the Bcl-2 family member proteins and promoted by elevated levels of PUMA (Baptiste and Prives, 2004; Chipuk et al., 2005). It would be interesting to investigate if CEP-1, like p53, can bind to the Bcl-2 homologue in worms, CED-9. Like Bcl-2, CED-9 inhibits both autophagy and apoptosis in *C. elegans* and can directly

interact with BEC-1 (Hengartner et al., 1992; Hengartner and Horvitz, 1994; Takacs-Vellai et al., 2005). If CEP-1 has cytoplasmic activities, including activating the release of CED-4 from CED-9 or the regulation of CED-9 on BEC-1, CED-9 may directly bind to CEP-1. This would contribute transcriptionally independent roles of CEP-1 in the promotion of apoptosis. To determine if there is a CED-9::CEP-1 complex, immunoprecipitations can be done to isolate CEP-1 and probe for CED-9 to verify if they do bind to each other from adult worms (Takacs-Vellai et al., 2005).

5.1.3 Characterizing the CEP-1-independent DMC induced cell death pathway.

DMC was able to induce germline cell death in *cep-1(gk138)* mutant worms and increase their mitochondria and nuclear DNA lesions (Figures 3.4B and 3.5A). The mode by which DMC is acting in these worms needs to be further explored. There are CEP-1-independent cell death pathways that have been identified and converge to the activate CED-4 and CED-3, similar to the CEP-1-dependent cell death pathway.

One example is the ceramide signaling pathway that has been shown to be involved in apoptosis and is an example of CEP-1-independent cell death (Deng et al., 2008; Gulbins and Li, 2006). The ceramide biosynthesis pathway (*lagr-1* and *hyl-1,* two ceramide synthases) is activated by DNA damage and is essential for DNA damage induced germline cell death. The addition of ceramide, can slightly rescue germline cell death in a *cep-1* mutant background. But ceramide does require the presence of EGL-1, CED-4 and CED-3 for germline cell death (Deng et al., 2008).

Perhaps the ceramide pathway is the CEP-1-independent cell death pathway that is involved in DMC induced cell death. Feeding the *lagr-1(gk327); hyl-1(ok976)* double mutant worms *cep-1* RNAi, we can see if DMC will still cause increases in germline cell death. If there is a loss of germline cell death, then we know that DMC is working to activate ceramide and its inhibition on CED-9. If there is a significant increase in cell death after the removal of the ceramide pathway, then perhaps the ceramide pathway is blocking the cell death somehow by not allowing CED-9 to be inhibited.

5.2 BEC-1 is involved in DNA damage and cell death.

Autophagy and Beclin 1 are involved in genomic stability and cell death (Elgendy et al., 2011; Karantza-Wadsworth et al., 2007). The partial knockdown of *bec-1* increased cell death in a CEP-1 dependent manner and caused increases in DNA damage with the addition of UV treatment (Figures 4.1B and 4.2). The full knockdown of *bec-1* causes increases in cell death due to a lack of clearance ((Ruck et al., 2011; Takacs-Vellai et al., 2005), Figure 4.3B). Moreover, the full knockdown of *bec-1* increased DNA damage in wild-type worms with and without UV (Figure 4.4A). We observed that BEC-1 played a role in CEP-1 mediated cell death and UV induced DNA damage. Additional experiments are required to fully delineate the involvement of BEC-1/Beclin 1 on the CEP-1/p53 signaling pathway. Furthermore, if BEC-1 is involved in DNA damage and sensitizing worms towards UV induced lesions, the changes in DNA damage on *glp-1(ar202)* tumor mutant worms could be investigated in the absence of *bec-1*.

5.2.1 The regulation of BEC-1/Beclin 1 on CEP-1/p53.

p53 has been shown to inhibit autophagy in the cytoplasm (Tasdemir et al., 2008), but little has been shown on whether or not autophagy and Beclin 1 alter the activity of p53. An inhibitor of autophagy, termed "spautin-1", causes the reduction of both Beclin 1 and p53 and *BECN1+/-* newborn mice had reduced levels of p53 (Liu et al., 2011). Additionally, the MCF-7 breast cancer cell line has low detectable levels of endogenous Beclin 1 and compromised p53 activity. When *beclin 1* is exogenously expressed in MCF-7 cells, they detected fewer colonies formed in soft agar and no tumor formation in nude mice (Liang et al., 1999). There are fewer cells after Beclin 1 was expressed but there is no decrease in viability. Perhaps, in the MCF-7.*beclin 1* cells, the addition of both Beclin 1 and DNA damage could increase the p53 activity causing increased cell death and a decrease in tumorigenesis.

Using cell lines from Dr. Beth Levine's Laboratory at University of Texas Southwestern that contain either an empty vector (MCF-7.Control) or a wild type copy of *beclin 1* gene (MCF-7.*beclin 1*), we were able to induce Beclin 1 protein and mRNA levels in MCF-7 after five days of tetracycline removal (Liang et al., 1999) (Figure 5.1). Using these cell lines, we compared the mRNA levels of the downstream targets of p53 after two hours of treatment with 10 μM DMC. No significant change was seen after the addition of Beclin 1 and DMC in *p21* and *puma* levels (Figure 5.1B). When *bec-1* was knocked down in *C. elegans*, no transcriptional changes were seen in the CEP-1 target gene *egl-1* (Figure 4.1D). Therefore there may not be any transcriptional changes on p53 with and without Beclin 1 at this time point.

To properly study the relationship between p53 and Beclin 1 in the MCF-7 cells after DNA damage, longer treatment with DMC should be done. Perhaps, two hours of treatment was

not sufficient to stabilize p53 and allow p53 to activate transcription of its downstream targets, if any changes will be observed. Western blots for p53 will be done with and without Beclin 1 in the presence of DMC or UV to determine if p53 stability is affected by Beclin 1. Q-RTPCR will be done to measure any differences in *p21* and *puma* levels with and without Beclin 1 in the presence of DMC or UV after six hours of treatment. Therefore we can detect if p53 stability and activity is altered with exogenous *beclin 1*.

Additionally, after treatments, induction of autophagy and apoptosis should be assessed to see which way the cells preferentially die once autophagy is functional again. The addition of 8-amino-adenosine, a nucleoside analog, with Beclin 1, the MCF-7.*beclin 1* cells did not die via apoptosis (Krett et al., 2004; Polotskaia et al., 2012). This could be done as well to determine if the addition of Beclin 1 pushes the MCF-7 cells to undergo apoptosis or autophagy after UV and DMC treatments. DMC causes increased cell death in MCF-7 cells, and even though there is an increase in stabilized p53, p53 is not transcriptionally active (Boamah et al., 2010). Perhaps Beclin 1 will assist p53 to activate apoptosis after UV or DMC or p53-independent cell death via autophagy will occur. Activation of autophagy could be measured by detecting the LC3-II isoform or the degradation of p62 via Western blot analysis (Kroemer et al., 2009). While increases in apoptosis will be detected by PARP cleavage, phosphatyidylserine flipping and MOMP activation using TMRE.

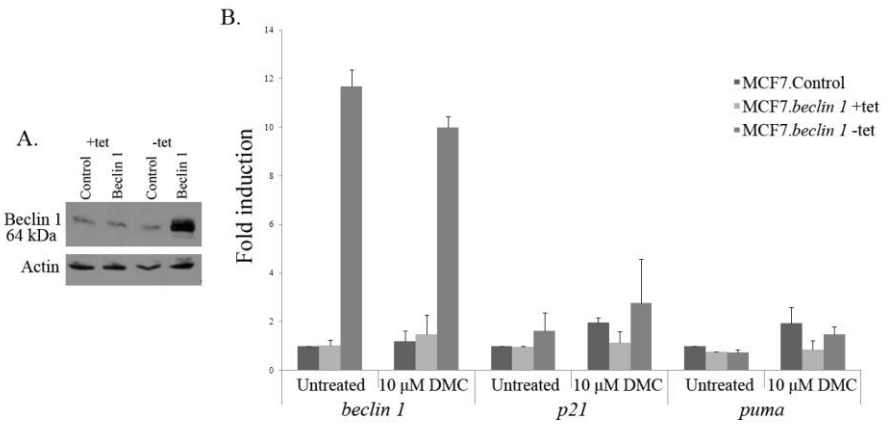

Figure 5.1: Beclin 1 did not alter activation of p53 target genes after DMC treatment. A) Western blot of Beclin 1 and Actin levels in MCF-7.Control and MCF-7.*beclin 1* cells with and without tetracycline (tet) for five days. B) *beclin 1*, *p21* and *puma* mRNA fold induction in MCF-7.Control and MCF-7.*beclin 1* cells with and without tetracycline and treatment with 10 μM of DMC for two hours. Average of two representative experiments is shown. Error bars indicate standard error. Normalized to *actin* and untreated cells.

Perhaps BEC-1/Beclin 1 and CEP-1/p53 mediate a downstream target forcing the cell to undergo autophagy or apoptosis, depending on the regulator. If CEP-1 can interact with CED-9, we can look for CED-9 interacting preferentially with either CEP-1 or BEC-1, depending on the background. After UV, wild-type cells may prefer to undergo apoptosis, releasing CEP-1 from CED-9, but not BEC-1. The opposite may occur under starvation conditions where BEC-1 would be released allowed autophagy to occur. Although there is a strong possibility that CED-9 will not interact with CEP-1. If this is so, BEC-1 and CEP-1 could activate or alter the localization of EGL-1/CED-13 promoting autophagy or apoptosis (Maiuri et al., 2007). We can compare if CED-4 gets translocated, indicating cell death or LGG-1::GFP levels increase which represents autophagy.

5.2.2 The role of BEC-1 in DNA damage in *glp-1(ar202)* tumor mutants.

The *glp-1(ar202)* mutant worms have a tumor of mitotic cells that seem to be resistant to DNA damage induced cell death when grown at 25°C. It has been shown that BEC-1 played a role in their proliferation and inhibited GLP-1 signaling in tumor mutant worms (Ames, K. unpublished data). We would like to further investigate the roles of BEC-1 and CEP-1 in the tumor mutant worms. The *glp-1(ar202)* tumor mutants had an increase in *egl-1* activation after UV treatment (Figure 3.6A). Would the knockdown of *bec-1* alter this activation of *egl-1*? Even though we did not see any changes in the *egl-1* levels after *bec-1* knockdown in the wild-type worms (Figure 4.1D), the mitotic tumor cells may have different regulatory mechanisms that BEC-1 could be involved with. Furthermore, the loss of *bec-1* could alter the progression of the germline tumor after UV treatment. We can follow the tumor four days after UV exposure and measure the tumor size in the presence and absence of *bec-1*.

In addition, perhaps BEC-1 and autophagy play a survival role in the developing tumors, similar to mammalian tumors that use autophagy to maintain their bioenergetics. Therefore after the knockdown of *bec-1*, possibly they will be more vulnerable to DNA damage and death after UV treatment. The knockdown of DNA repair genes in the tumor mutants made them susceptible to death after DNA damage (Deng, X., unpublished data). BEC-1 has been shown to be involved in genome integrity and perhaps after *bec-1* knockdown, we will see the same response to damage as was seen after the knockdown of the DNA repair genes. Also, if the cells are dying in the tumor normally but are quickly cleared, the loss of *bec-1* will stop their engulfment and the dead cells will become visible.

5.3 Perspectives

The loss of functional p53 often occurs in human tumors thus allowing for the survival of cells which lack proper DNA damage checkpoints, cell cycle arrest and cell death (Vousden and Prives, 2009). p53 is conserved in its activation of cell death in different lower organisms including *C. elegans* and *Drosophila* (Derry et al., 2001; Ollmann et al., 2000; Schumacher et al., 2001). Additionally, p53 in *C. elegans* is involved in UV mediated germline cell cycle arrest and the regulation of autophagy (Stergiou et al., 2007; Tasdemir et al., 2008). Herein, is evidence that p53 in *C. elegans* was involved in DNA repair and mitochondrial stability, therefore furthering the conservation of p53 in *C. elegans*. The loss of *p53* in tumor mutant worms promoted UV induced nuclear DNA damage indicating that p53 in *C. elegans* helped maintain genomic integrity. We have also identified the ability of a DNA alkylating agent to promote p53-independent cell death and form lesions in *p53* mutant worms. Classifying the p53-independent cell death pathway in *C. elegans* can assist in identifying cell death pathways to activate in human tumor cells which lack functional p53. Moving forward, it would be interesting to further confirm the involvement of p53 in *C. elegans* in DNA repair and mitochondria stability.

Furthermore, we have observed that Beclin 1 in *C. elegans* was also involved in maintaining genomic integrity after UV induced DNA damage. Partial loss of *beclin 1* in *C. elegans* increased cell death via the p53 cell death pathway. The complete loss of *beclin 1* in *C. elegans* increased DNA lesions in wild-type worms and decreased the viability of their offspring. The exact role Beclin 1 plays in *C. elegans* in the inhibition of p53 and cell death still needs to be investigated. In addition, the mechanism through which Beclin 1 decreases genomic stability in

wild-type and tumor worms needs to be examined further to classify Beclin 1 in the DNA damage.

CHAPTER 6:

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