C. elegans CEP-1/p53 and BEC-1 Are Involved in DNA Repair

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Abstract

p53 is a transcription factor that regulates the response to cellular stress. Mammalian p53 functions as a tumor suppressor. The C. elegans cep-1, cep-1, regulates DNA-damage induced germline cell death by activating the transcription of egl-1 and ced-13. We used the C. elegans model to investigate how, in the whole animal, different forms of DNA damage can induce p53-dependent versus p53-independent cell death and DNA repair. DNA damage was induced by ultraviolet type C (UVC) radiation, or 10-decarbamoyl mitomycin C (DMC, an agent known to induce mammalian p53-independent cell death). Wild-type or cep-1 loss-of-function mutant animals were assayed for germline cell death and DNA lesions. Wild-type animals displayed greater removal of UVC-lesions over time, whereas cep-1 mutant animals displayed increased UVC-lesion retention. The cep-1 mutation increased UVC-lesion retention directly correlated with a reduction of progeny viability. Consistent with DMC inducing p53-independent cell death in mammalian cells DMC induced a C. elegans p53-independent germline cell death pathway. To examine the influence of wild-type CEP-1 and DNA damage on C. elegans tumors we used glp-1(ar202gf)/Notch germline tumor mutants. UVC treatment of glp-1 mutant animals activated the CEP-1 target gene egl-1 and reduced tumor size. In cep-1(gk138b);glp-1(ar202gf) animals, UVC treatment resulted in increased susceptibility to lesions and larger tumorous germlines. Interestingly, the partial knockdown of bec-1 in adults resulted in a CEP-1-dependent increase in germline cell death and an increase in DNA damage. These results strongly support cross-talk between BECT-1 and CEP-1 to protect the C. elegans genome.

Introduction

The tumor suppressor protein p53 is a transcription factor involved in activating cell cycle arrest, apoptosis, autophagy, and DNA repair [1]. The Caenorhabditis elegans p53-1, CEP-1, is an ancient ortholog of p53 with a conserved DNA binding domain that includes the residues often mutated in human cancers [2,3]. In human cells, wild-type p53 is maintained at low levels, however, following DNA damage, p53 is stabilized and the increased levels result in the activation of downstream target genes [4]. In C. elegans, CEP-1 is activated by DNA damage, initiated by ultraviolet C (UVC) light, ionizing radiation (IR) and N-ethyl-N-nitrosourea (ENU) [5]. CEP-1 induces germline cell death by activating the transcription of the target genes egl-1 and ced-13 [5,6]. EGL-1 and CED-13 are orthologs to the human BH3 domain proteins Puma and Noxa [7,8]. Activation of egl-1 and ced-13 results in germline, but not somatic, cell death [3]. UVC induced cell death in C. elegans also requires the nucleotide excision repair (NER) pathway [9]. The involvement of CEP-1 has been examined for apoptotic cell death but if CEP-1 or how CEP-1 participates in autophagic cell death has not been examined.

Autophagy is a self-eating signaling cascade that is used for both cell survival and cell death [10]. A critical regulator of autophagy in mammals is the protein Beclin 1 [11,12]. BEC-1 is the C. elegans ortholog of mammalian Beclin 1, and is required for viability, fertility, growth, dauer development and survival [12,13,14]. BEC-1 interacts directly with, and regulates, CED-9/Bcl-2 [14]. There is a complex connection between BEC-1 and the induction of cell death. High levels of TUNEL staining, indicative of an accumulation of DNA damage, occur in bec-1 null animals [14]. Furthermore, an increase in germline cell death has been noted in bec-1 null mutants and bec-1 RNAi fed animals, and this increase occurs at least in part due to a delay in apoptotic cell corpse degradation [14,15].

The C. elegans germline cell death is increasingly used as a model system to study human cancer associated signaling events [16,17,18]. The cross-talk between CEP-1, BEC-1, and DNA repair is an under-studied area. Investigating the highly proliferative germ cells in C. elegans, and resistance to DNA damage in germline-tumor phenotype animals, can help us understand how mammalian tumors respond to DNA damage signaling. Localized GLP-1/Notch signaling controls the mitotic proliferation that occurs in the distal part of the gonad, even during adulthood [19]. The GLP-1/Notch receptor is activated by the LAG-2 ligand, which is produced by the Distal Tip Cell (DTC) to keep cells in the distal region of the gonad in mitosis. As germ cells move away
from the DTC signal, they lose the signal and transition into meiosis. Thus, the GLP-1/Notch receptor controls mitosis and allows for the inhibition of meiosis or differentiation. GLP-1/Notch constitutive gain-of-function mutants result in a germline-tumor phenotype, where germ cells persist in mitosis and do not enter meiosis [19,20]. This gain-of-function causes hyper-proliferation of the germ line which presented us with a source of actively proliferating cells in a multicellular model. We have used the C. elegans multicellular eukaryote as a model to evaluate the requirement for CEP-1 on DNA repair, and signaling for cell death, in wild-type and germline-tumor animals.

UVC radiation causes dose dependent DNA lesions in wild-type C. elegans [21]. Using wild-type and cep-1(gk138) mutant animals, we compared the activation of p53 target genes and the efficiency of DNA-lesion removal, following DNA damage, initiated by UVC and the chemotherapeutic 10-decarbamoyl mitomycin C (DMC). DMC is a mitomycin analogue that initiates a robust mammalian p53-independent cell death signal [22,23]. We measured DNA damage and repair in wild type and cep-1(gk138) mutant animals after UVC or DMC treatment. We hypothesized a genetic dependency for CEP-1 to repair bulky DNA-lesions, and therefore, expected that in the absence of CEP-1, we would observe an increase in DNA-lesions. Indeed, in the absence of CEP-1, we observed a reduction in the DNA repair of UVC induced lesions. Interestingly UVC exposure induced only CEP-1/p53-dependent cell death, while DMC treatment induced cell death that did not require CEP-1/p53. Moreover, we found that a partial loss of bec-1 induced CEP-1/p53-dependent germline cell death while only slightly activating CEP-1/p53 target genes, and depletion of bec-1 in cep-1 mutant animals throughout development resulted in exacerbated UVC induced lesions. These data indicate that CEP-1 plays a role in removing DNA damage and that the loss of BEC-1 sensitizes worms to increased DNA damage. This suggests that CEP-1 and BEC-1 cross-talk to facilitate robust DNA repair to protect the C. elegans genome.

Results

cep-1(gk138) Mutants Exhibit UVC-induced Nuclear DNA Damage Lesions

While the influence of UVC radiation on DNA lesions and cell death has been examined in wild-type animals, the influence on cep-1(gk138) mutant animals is an under-studied area [24]. It is well established that UVC damage induces germ cell death in wild-type C. elegans, activates CEP-1 to initiate the transcription of egl-1 and ced-13, and uses the nucleotide excision repair pathway in this process [7,8,24]. In keeping with the published literature, only wild-type animals displayed a significant UVC-induced increase in germline cell death (Fig. 1B) [24]. The previously published work used Nomarski optics to score cell corpses per gonad arm. We examined how the loss of CEP-1 influenced UVC induced DNA damage and cell death by exposing young adult animals to UVC radiation and using a CED-1::GFP reporter to score for germline cell death (Fig. 1A). To eliminate any possible bias, we had two different individuals blindly score the number of CED1::GFP engulfed cell corpses. Using CED1::GFP positive cells we observed a significant increase in cell death with a P value of 0.002; however the values of corpses per gonad arm were lower than those previously published that were obtained with Nomarski optics [24]. To examine the influence of CEP-1 on UVC-induced DNA damage, we used the PCR-based lesion detection method (introduced and validated by the Meyer laboratory [21]). This lesion assay measures DNA damage via the inhibition of PCR amplification of specific mitochondrial and nuclear genes. UVC treatment causes a drastic increase in the lesions detected per 10 kb, when compared to untreated animals [21,25]. We predicted that UVC treated wild-type, and cep-1(gk138) mutant animals would sustain similar levels of UVC-induced DNA lesions. As expected, both wild-type and cep-1(gk138) young adult animals displayed a robust increase in nuclear DNA lesions (Fig. 1C).

DNA damage lesions are the initial stimulus that activates the p53-pathway. We confirmed that UVC-induced DNA lesions activated CEP-1 target genes in wild-type animals, with the egl-1 and ced-13 induction levels increased in young adults, four hours post UVC treatment. As previously reported [24], no increase in either egl-1 or ced-13 was observed following UVC treatment of the cep-1(gk138) mutants (Fig. 1D).

CEP-1 Allows for the Removal of UVC-induced DNA-lesions thus Facilitating DNA Repair

Mammalian p53 is involved in DNA repair by activating downstream targets that assist in the nucleotide excision repair (NER) pathway [26,27,28,29]. p53 deficient mouse embryonic fibroblast cells(MEFs) have less DNA repair when compared to cells with wild-type p53 after UVC treatment [28,30]. However, the role of CEP-1 in DNA repair has yet to be well characterized. UVC DNA damage induces DNA lesions in C. elegans that are measurable by PCR analysis, and, in wild-type animals, these lesions are rapidly repaired [21]. We examined the influence of CEP-1 on C. elegans DNA repair, by measuring nuclear DNA lesions four and eight hours after UVC treatment. We observed repair in wild-type worms, since after eight hours very few lesions remained (Fig. 2A). The cep-1(gk138) mutants, on the other hand, demonstrated very minor removal of the UVC-induced DNA lesions (Fig. 2A). This indicated that CEP-1 was required for efficient nuclear DNA repair.

C. elegans with defects in DNA damage repair display a reduction in the number of viable eggs laid after DNA damage [24,31,32]. A decrease in egg laying, or egg survival, can indicate DNA damage that is not repaired. To further confirm that CEP-1 played a role in DNA repair, we scored the number of eggs laid and percent egg survival in UVC exposed wild-type and cep-1(gk138) mutant animals. Both, wild-type and cep-1(gk138) mutant animals, treated with UVC, produced less eggs (Fig. 2B), and the viability of eggs laid by both strains decreased after UVC treatment (Fig. 2C). Importantly, while wild-type worms had 68% egg survival following DNA damage, cep-1(gk138) mutants had only 36% egg survival (Fig. 2C). Thus, low egg survival for CEP-1 mutant animals correlated with a decreased capacity to repair UVC-induced lesions.

DMC Induces DNA Damage and CEP-1-independent Cell Death in cep-1(gk138) Mutant Animals

DMC is an alkylating agent that results in a high frequency of bulky DNA-adducts in human cells and causes p53-dependent and p53-independent cell death [22,23]. We asked if, in C. elegans, DMC could induce nuclear DNA damage and germline cell death. 1 mM DMC was placed on plates and fed to animals for five hours. DMC treatment induced DNA damage and germline cell death. We confirmed that DMC induced nuclear DNA damage and germline cell death by examining DNA damage lesions (Fig. 3A) and measuring germline cell death (Fig. 3B). Importantly, only CEP-1 and BEC-1 Are Involved in DNA Repair

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increased to slightly above 0.5 lesions per 10 kb. In both cases the lesions per 10 kb dropped lower when followed over time (data not shown). After an overnight treatment with varying DMC concentrations, CED-1::GFP positive cells were scored in wild-type and cep-1(gk138) animals to determine if there was an increase in cell death. Wild-type animals did not show an increase in CED-1::GFP positive cells, but with 1 mM DMC treatment the cep-1(gk138) mutants displayed a reproducible increase in cell death (Fig. 3C). This cell death was reduced at 2 mM DMC, potentially due to activation of xenobiotic efflux pumps [33]. DMC treatment did not affect the number of eggs laid by either of the two strains. The cep-1(gk138) mutants displayed a decrease in egg survival after DMC treatment (data not shown). Taken together these data suggest that the very few lesions induced by DMC resulted in more of a synthetic lethality when there is no CEP-1 expressed in the cep-1(gk138) mutant animals. The molecular pathway for DMC induced p53-independent cell death remains under investigation.

Figure 1. Wild-type and cep-1(gk138) mutant worms had similar amounts of nuclear DNA damage after UVC exposure. A) Images of CED-1::GFP positive cells of wild-type worms either untreated or 24 hours after 100 J/m² UVC. Arrows point to CED-1::GFP positive cells. Magnification is 400x. B) Worms were treated 24 hours post L4 and imaged 24 hours later. The number of CED-1::GFP positive cells in the germ line was scored blindly by two independent people. Error bars indicate standard error and the number of worms scored was 20. The difference between wild-type untreated and UVC treated animals had a P value were all equal to or less than 0.002. C) Analysis of nuclear DNA lesions on 24 hour post L4 wild-type or cep-1(gk138) mutant animals, after exposure to 50 or 100 J/m² of UVC. Average of three representative experiments is shown. Error bars indicate standard error. The wild-type P values were 0.0001 and 0.003 for a comparison to untreated at 50 or 100 J/m² respectively. The cep-1(gk138) mutant P values were 0.012 and 0.02 for a comparison to untreated at 50 or 100 J/m² respectively. D) Q-RT-PCR of egl-1 and ced-13 mRNA levels four hours post UVC treatment of 100 J/m². Fold induction compared to cDNAs amplified from untreated worms. Average of three representative experiments is shown. Error bars indicate standard error. Normalized to act-1. The P values for wild-type animals were less than or equal to 0.02 for both egl-1 and ced-13. No significant change was detected in cep-1(gk138) mutant animals.

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CEP-1 can be Activated in glp-1(ar202gf) Tumor Mutant Animals and cep-1(gk138); glp-1(ar202gf) Tumor Mutant Worms have Increased Nuclear Lesions after DNA Damage

We were interested in determining if CEP-1 played a role in the apparent resistance to DNA damage of glp-1/Notch germline-tumor mutants (maintained at 25°C). To address this, we examined the gain of function glp-1(ar202gf) mutant animals and constructed cep-1(gk138); glp-1(ar202gf) double mutant animals. Both genotypes produced almost 100% offspring with a Tumorous (Tum) germline at 25°C. We measured nuclear DNA damage in glp-1(ar202gf) and cep-1(gk138); glp-1(ar202gf) double mutant animals that displayed the Tum phenotype. Immediately after 50 and 100 J/m² of UVC treatment, the glp-1(ar202gf) mutant animals had fewer nuclear DNA lesions than the cep-1(gk138); glp-1(ar202gf) animals (Fig. 4A). To determine if this increase in DNA lesions correlated with the expected decrease in CEP-1 activity we scored for egl-1 fold induction. We treated glp-1(ar202gf) and cep-1(gk138); glp-1(ar202gf) animals with 100 J/m² of UVC and...
measured egl-1 fold induction four hours later. UVC treatment of glp-1(ar202gf) induced egl-1 expression, while the same treatment of cep-1(gk138); glp-1(ar202gf) double mutant animals did not (Fig. 4B). Therefore, just as in wild-type worms, glp-1(ar202gf) worms contained CEP-1 protein that was transcriptionally active and that participated in DNA repair. Clearance of the DNA lesions for the treated glp-1(ar202gf) and cep-1(gk138); glp-1(ar202gf) animals showed an intriguing result that supports a previous report of a UVC-mediated coordination between the nucleotide excision repair pathway (NER) and the homologous repair pathway (HR) [34]. When we examined lesion clearance in UVC treated glp-1(ar202gf) mutants and cep-1(gk138); glp-1(ar202gf) double mutants we observed an increase in lesions four hours after treatment with a subsequent decrease by 24 hours in both cases. In cep-1(gk138); glp-1(ar202gf) double mutants we documented that UVC treatment caused a substantial increase in lesions to above 1.5 per 10 kb (Fig. 4A); we observed an increase in lesions per 10 kb at four hours after UVC treatment and all the lesions were absent after 24 hours (Fig. 4C). We reasoned that the high rate of DNA replication in the cep-1(gk138); glp-1(ar202gf) double mutant mitotic germine allowed for increased homologous recombination repair in these animals. This is supported by Figure 5, which demonstrated that the absence of a germline yields animals that show efficient UVC lesion clearance in the presence of CEP-1 but not its absence (see below).

CEP-1 has a Role in DNA Damage Repair in Germlineless Animals

The primary published role for CEP-1 is the induction of germ cell death after DNA damage [3]. When CEP-1::GFP is exogenously expressed, CEP-1 is only localized to the pharynx and the germ line of adult animals [3]. While this suggests that CEP-1 may not play a role in the somatic cells of adult worms, one-third of the total number of cells in the adult hermaphrodite, are somatic cells. To investigate the UVC DNA damage response in somatic cells, we investigated animals that lacked a germline. We used the glp-1(q224lf) mutant animals that are deficient in the germ line and exposed them to 100 J/m² of UVC and measured for egl-1 expression. We observed that the glp-1(q224lf) mutant animals displayed egl-1 induction (Fig. 5A), suggesting that egl-1 expression, after UVC induced DNA damage, also occurred in somatic cells. We next asked if CEP-1 played a role in the removal of UVC induced lesions in the glp-1(q224lf) mutant animals.
Following exposure to 100 J/m² of UVC, and cep-1 depletion by RNAi, nuclear lesions were measured, either immediately, or with a clearance time of four or eight hours. Immediately after treatment, there was no difference in the amount of lesions induced by UVC in the presence or absence of CEP-1 activity (Fig. 5B). The glp-1(q224lf) mutant worms, expressing a functional cep-1 gene, displayed high nuclear lesion removal eight hours after UVC treatment. The animals without cep-1 expression retained their nuclear DNA lesions (Fig. 5B). These data suggest that CEP-1 plays a role in DNA repair in somatic cells.

To address the role of CEP-1 in UVC induced DNA damage repair in germline tumor animals, we asked how the UVC induced lesions would influence the size of the germline-tumor in glp-1(ar202gf) mutant animals. We were unable to detect a robust increase in UVC-induced lesions in these animals. The tumor size of glp-1(ar202gf) mutants and cep-1(gk138); glp-1(ar202gf) double mutants was measured by how far the tumor had invaded the mouth [35]. The larger the tumor size observed, the smaller the distance between the tumor and the mouth. Interestingly, four days after UVC treatment the cep-1(gk138); glp-1(ar202gf) double mutants had larger tumors while the glp-1(ar202gf) tumor mutants had decreased tumor size (Fig. 6). We examined the induction of cell death by SYTO-12 staining of glp-1(ar202gf) mutants and cep-1(gk138); glp-1(ar202gf) double mutant animals four hours after UVC treatment and did not detect a significant difference of cell death in the two populations. This suggests that the variable tumor size could not be explained by increased cell death in the single mutant glp-1(ar202gf) animals.

Partial Knockdown of become-1 did not Increase the Number of Apoptotic Corpses in the Absence of CEP-1

The knockdown of become-1, throughout development, results in an increase in embryonic and germline cell death that has been shown to be due to a defect in apoptotic cell clearance [14,15,36,37]. Partial knockdown by become-1 RNAi treatment of L4 larvae for 24 hours, and exposure to UVC, resulted in an increase in apoptotic germ cells that were diligently degraded and thus not due to a defect in clearance (Figs. 7, A and B). The increase in apoptotic germ cells after become-1 partial knockdown (in wild-type L4 larvae treated with RNAi against become-1), was dependent on CEP-1 because the cep-1(gk138) mutant animals treated with become-1 RNAi as L4 larvae for 24 hours had no increase in cell death (Fig. 7A). Additionally, wild-type animals had an increase in CED-1::GFP positive cells in become-1 RNAi fed animals after UVC treatment while cep-1(gk138) mutant worms did not (Fig. 7B). This suggested that the partial become-1 knockdown-induced germline cell death was dependent on the presence of CEP-1. The partial knockdown of become-1 only slightly increased the transcriptional activity of CEP-1, from 1 to 1.3 before UVC treatment (Fig. 7C). Figure 7D shows the knockdown of BEC-1::RFP by feeding L4 worms become-1 RNAi for 48 hours. We then knocked down become-1 throughout development by also feeding become-1 RNAi to their F1 progeny. To determine if the increased observable germline cell death was due to an actual increase in cell death or because of faulty clearance, we followed CED-1::GFP corpses for over an hour. After an hour, some of the corpses in the control (empty vector, L4440) fed worms disappeared, while the apoptotic cells in become-1 RNAi fed animals were not degraded. This confirmed that the observable
increase of CED-1::GFP positive cells in animals RNAi treated against bec-1 throughout development was due to a defect in apoptotic cell clearance [15]. Additionally, this detectable increase in cell death occurred independently of CEP-1.

bec-1 Knockdown in Treated Adults and Progeny Increases DNA Damage

It has been shown that Beclin 1 in mammals is involved in genomic instability and that its levels increase after DNA damage [38,39]. Embryos with bec-1(ok691) and bec-1 RNAi have an increase in TUNEL-positive cells, which could reflect an increase in DNA damage and cell death [38]. After bec-1 knockdown for 24 hours, lesions in nuclear DNA were measured with and without UVC treatment to determine if bec-1 knockdown influenced the amount of DNA damage. After UVC treatment a significant increase in lesions were detected in L4440 fed and bec-1 RNAi fed animals. The partial RNAi knockdown of bec-1 (in L4 larvae) resulted in an increase in the number of lesions in wild-type animals but the change had a P value of 0.19 (Fig. 8A). The cep-1(gk138) mutants had increases in nuclear DNA lesions after UVC and bec-1 knockdown that were similar to each other (Fig. 8A).

After studying the treated adult worms that only had knockdown of bec-1 for 24 hours, we went on to study their progeny. These worms were treated with RNAi against bec-1 throughout development (Fig. 8B). In cep-1(gk138) mutant worms significant lesions were detected with the P values equal or less than 0.02 for time points four, eight and sixteen hours after UVC treatment when compared to untreated animals. In cep-1(gk138); bec-1 RNAi mutant worms significant lesions were detected with the P values equal or less than 0.004 for time points zero, four and eight hours after UVC treatment when compared to untreated animals.

Figure 4. CEP-1 was activated in glp-1(ar202gf) tumor mutant worms. A) Nuclear DNA lesions were measured in glp-1(ar202gf) or cep-1(gk138); glp-1(ar202gf) double mutants, immediately following UVC exposure. Animals were exposed to 50 or 100 J/m² of UVC, as adults (24 h post L4 stage). Average of three representative experiments is shown. Error bars indicate standard error. Only cep-1(gk138); glp-1(ar202gf) double mutants had a statistically significant increase in lesions with P value of 0.003 after 100 J/m². B) Expression of egl-1 mRNA determined by Q-RTPCR in glp-1(ar202gf) or cep-1(gk138); glp-1(ar202gf) double mutant animals, 4 hours post UVC treatment of 100 J/m². Data are reported as fold induction compared to expression in untreated worms and normalized to tbg-1. Average of six representative experiments is shown. Error bars indicate standard error. The egl-1 fold change had a P value in glp-1(ar202gf) of 0.008 and the P value in cep-1(gk138); glp-1(ar202gf) double mutants was 0.03. C) Nuclear lesions were quantified immediately, four, eight, sixteen and twenty-four hours after UV exposure in glp-1(ar202gf) and cep-1(gk138); glp-1(ar202gf) worms. Average of three representative experiments is shown. Error bars indicate standard error. In glp-1(ar202gf) mutant worms significant lesions were detected with the P values equal or less than 0.02 for time points four, eight and sixteen hours after UVC treatment when compared to untreated animals. In cep-1(gk138); glp-1(ar202gf) mutant worms significant lesions were detected with the P values equal or less than 0.004 for time points zero, four and eight hours after UVC treatment when compared to untreated animals.
knockdown and UVC exposure significantly increased the nuclear DNA lesions from less than 1 to more than 1.5 lesions per 10 kb with a P value of 0.025 (Fig. 8B). This suggests a cross-talk between CEP-1 and BEC-1 in DNA repair to improve genomic stability.

Discussion

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 [40]. p53 is conserved in its activation of cell death in different lower organisms including C. elegans and Drosophila [2,3,41]. Additionally, p53 in C. elegans is involved in UVC mediated germline cell cycle arrest and the regulation of autophagy [24,42]. The loss of BEC-1 in C. elegans causes increased apoptosis and DNA damage [14] and DNA damage increases BEC-1::GFP levels [39]. Herein, is evidence that CEP-1/p53 and BEC-1 in C. elegans were involved in maintaining genomic integrity, therefore furthering the conservation of CEP-1/p53 and BEC-1 in C. elegans.

p53, in mammals, has been well described as responding to DNA damage to assist in DNA repair and to initiate signal transduction cascades for the activation of cell cycle arrest and cell death [40]. We have reported here, for the first time, that after UVC irradiation and DMC chemotherapeutic treatment, CEP-1/p53 is involved in DNA repair. UVC exposure causes thymine dimers and, if not repaired, can eventually cause DNA double strand breaks. DMC is an alkylating agent which causes DNA adducts and DNA crosslinks, at high frequency and also p53-independent cell death [22,23]. While cep-1(gk138) mutant animals do not have increased cell death after UVC exposure, we observed a significant increase in UVC induced nuclear DNA damage that was not effectively repaired (Fig. 2). The lack of DNA repair

Figure 5. CEP-1 acts to remove UVC induced lesions in somatic cells. A) egl-1 expression measure by Q-RTPCR in glp-1(q224lf) animals that lack a germline, 4 hours post UVC treatment of 100 J/m² or control animals without treatment. Data are reported as fold induction compared to expression in untreated worms and normalized to tbg-1. Average of two representative experiments is shown. Error bars indicate standard error, P value of 0.008. B) Nuclear DNA lesions were measured immediately, four and eight hours after 100 J/m² of UVC in glp-1(q224lf) 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. Wild-type animals at 0 hours had significant lesions with a P value of 0.03 and no significant lesions at 4 and 8 hours. cep-1 depleted animals had significant lesions at 0, 4, and 8 hours after UVC treatment with P values of equal to or less than 0.05. doi:10.1371/journal.pone.0088828.g005

Figure 6. glp-1(ar202gf) tumor mutants displayed an increase in tumor size in the absence of cep-1, four days after UVC treatment. The size of the germ line tumors was determined in glp-1(ar202gf) or cep-1(gk138); glp-1(ar202gf) double mutant animals grown at the restrictive temperature after treatment with 100 J/m² of UVC, and allowed to recover for four days. The size of the tumor is inversely proportional to the distance between the mouth and the tumor. Median distances of 18 worms are shown. glp-1(ar202gf) or cep-1(gk138); glp-1(ar202gf) double mutant animals had P values of 0.04 and 0.009 respectively for their change in tumor size versus untreated controls. doi:10.1371/journal.pone.0088828.g006
provoked a decrease in egg viability in cep-1(gk138) animals (Fig. 3). Therefore CEP-1/p53 in C. elegans, similar to p53 in mammals, participates in DNA repair.

We previously showed that DMC induces p53-independent cell death and DNA damage in human cancer cells [22,23]. Very few pharmacological agents effectively enter into C. elegans, because of the thick cuticle of the animal, coupled with the animal’s extensive xenobiotic efflux pumps [33]. We scored for germline cell death over a range of DMC concentrations. In C. elegans, DMC promoted germline cell death in the cep-1(gk138) mutants and not in the wild-type animals (Figs. 3B and 3C). Furthermore, after five hours of treatment, the wild-type animals had less DNA damage from DMC, than the cep-1(gk138) mutants but neither population had lesions detected in the significance range detected for UVC (Fig. 3A). This induced death indicated that DMC was taken up by the animals, when administered on plates at high concentrations, for short treatments, and the low level of lesions detected suggested that active DNA repair was ongoing. The increased sensitivity of the cep-1(gk138) mutant germline to DMC treatment was probably due to the reduced capability of DNA repair. Moreover, the increase in drug concentration appeared to cause an increase in the xenobiotic efflux pumps as a reduction in cell death was seen when the DMC concentration was increased from 1 mM to 2 mM. Overall C. elegans are excellent at evading the toxicity of chemotherapeutic DNA damaging drugs.

Animals with germline tumors were subjected to UVC treatment to examine the influence of CEP-1/p53 activity on developing tumors. We discovered that CEP-1/p53 was transcriptionally functional in the glp-1(ar202gf) tumor animals with very little nuclear damage observed after immediate UVC-induced DNA damage (Fig. 4). This was in striking contrast to the increased nuclear DNA damage lesions observed after UVC treatment of the cep-1(gk138); glp-1(ar202gf) mutants (Fig. 4A). The germ line tumors are quickly dividing with high levels of DNA replication and this possibly couples with CEP-1 activity in glp-1(ar202gf) animals to repair the actively replicating genome directly after UVC-mediated DNA damage. Interestingly, 24 hours after UVC-mediated damage we observed complete lesion removal in both populations (Fig. 4C). UVC treatment of glp-1(ar202gf) animals with functional CEP-1/p53, had tumors that...
decreased in size. However, the UVC treatment of cep-1(gk138); glp-1(q224lf) mutant animals resulted in an increase in tumor size, presumably due to the animals sustaining an increase in genomic instability following lesion removal (Fig. 6). This confirmed the involvement of CEP-1/p53 in nuclear damage repair and the susceptibility of cep-1(gk138) mutant tumors to sustain more genomic instability, which resulted in increased tumor sizes. No increase in apoptosis was detected in glp-1(q224lf) as compared to cep-1(gk138); glp-1(q224lf) mutants.

In wild-type *C. elegans*, one-third of all cells are somatic, and the remaining two-thirds, are actively dividing germ cells. Therefore the source of DNA damage, we observed after UVC treatment, was potentially in the somatic cells, as well as in the germ line. The PCR reactions to measure DNA lesions do not distinguish whether the DNA lesions occur in somatic or germ cells, but rather are primer specific, allowing us to compare the amplitcons between the nuclear genomes [25]. Using a glp-1(q224lf) mutant strain that had no germ cells at 25°C, we observed that CEP-1/p53 was activated in the somatic cells (Fig. 5). Furthermore, when there was no functional CEP-1/p53 present, in glp-1(q224lf) loss of function mutant animals, they had reduced DNA repair (Fig. 5). Our data did not support the previously published work, using a different germline null animal and exposure to ionizing radiation (IR) to induce CEP-1 downstream target gene activation [7]. Previously, it was shown that glp-1(q224lf) mutant animals have nuclear DNA repair after UVC at slower rates than wild-type [21] and germline null animals have no egl-1 activation after IR [7]. The difference in our results could be due to the differences in lesion types and the type of CEP-1 activation. Our data indicate a previously unreported role for CEP-1 in DNA repair of UVC lesions in the soma, as well as in the germ line.

BEC-1 in *C. elegans* has important roles in autophagy, cell death and DNA damage, but its relationship with p53 has yet to be clearly defined. We observed that adult worms with bec-1 knockdown, for a short period of time, had increased cell death in the germ line that was CEP-1-dependent, and the depletion of bec-1 only slightly increased egl-1 mRNA induction (Fig. 7). While we observed CEP-1-dependent cell death, caused by bec-1 knockdown in adult animals, we did not observe increased DNA damage lesions in these animals (Fig. 8A). Therefore, there may be cross-talk between CEP-1 and BEC-1 activities that regulates CEP-1 DNA repair functions. In mammals, Beclin 1 associates with the phosphatidylinositol 3-Kinase/Vps34 to activate the formation of the pre-autophagosomal membrane [43]. The reduction of BEC-1 activity in the RNAi depleted animals might function to activate a CEP-1 dependent death pathway in a way that has not yet been elucidated. In a Myc driven tumor model, the inhibition of autophagy activates p53-mediated cell death [44]. This is reminiscent of what we observed in *C. elegans*. On the other hand, RNAi depletion of bec-1 in the progeny increased nuclear DNA-damage lesions in UVC treated, animals with a more profound outcome observed in cep-1(gk138) mutant animals (Fig. 8B). This suggests that in the absence of CEP-1 and BEC-1 there is less DNA repair and less DNA repair clearance.

Here, we have demonstrated novel roles for CEP-1 and BEC-1 in cell death, and the removal of DNA damage. The increased sensitivity of the cep-1(gk138) mutant germ line to DNA damage, following UVC treatment, or DMC chemotherapy treatment, resulted in decreased embryonic viability. This strongly suggests that CEP-1 participates in improving the germ line genomic integrity to increase fecundity. This is not surprising as p53, p63, and p73 have all been shown to be guardians of female reproduction in mammals [45,46]. Autophagy mitigates DNA damage in mammary tumorigenesis and beclin 1 controls this process [38]. Our data suggest a cooperative role for BEC-1 in DNA damage repair and apoptotic corpse clearance. An increase in the number of cell corpses has been reported to occur in animals depleted of bec-1 activity throughout development [15], but the dependency of cell death on CEP-1 after a partial knockdown of bec-1 resulting from the RNAi depletion of adults (starting at the L4 stage), is a new finding. While the exact role of BEC-1 on CEP-1, and vice versa, is unknown we observed that both participated in DNA repair, cell death, and cross-talk in the *C. elegans* germ line.
Materials and Methods

Growth Media and Strains

All strains were maintained and constructed using standard methods and were grown on nematode growth media (NGM) at either 15°C, 20° or 25°C [47]. One liter of NGM includes: 3 g NaCl (Fisher), 17 g agar (Fisher), 2.5 g bactopeptone (Becton, Dickinson and Company), 1 mL 0.1 M MgSO₄, H₂O to 1 L. Sterilize by autoclaving. Overnight at room temperature and could be used for three weeks. Strain OP50 was spread for worms to eat. Seeded plates were left until 90% covered with E. coli and rinsed off plates with M9 and added to plates without bacteria. The following mutant strains used were as wildtype strains Bristol (N2). The following mutant strains used were as well: bcI39/P(lim-7):ced-1::GFP fusion protein in the sheath cells, MD701 [48], cep-1 (JBC1), (base substitution G529E, GC833). Strains were stored at −80°C until use. Worms were frozen at 25°C for long term. Amplification of gene transcripts by quantitative PCR with 0.125 μL of primers for egl-1, ced-13 and hkg-1 (Operon and Applied Biosystems, see sequences below, final primer concentration of 0.05 pmol/μL) [8] combined with the Thermocycler (Perkin Elmer) 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 hkg-1 or 54.6°C for ced-13 in a 7500 Sequence Detection System (Operon and Applied Biosystems). Primers for egl-1 were forward: 5'-gctatcactggtaggaac-3' and reverse: 5'-gctagtcgacttgga-3'. Primers for ced-13 were forward: 5'-acgctgatcactggta-3' and reverse: 5'-gctgctgatcactggta-3'. Primers for hkg-1 were forward: 5'-gctactgctgatcactgg-3' and reverse: 5'-gctgctgatcactggg-3'.
and green fluorescent images were taken using an ApoTome Zeiss microscope taking pictures of each plane of the germ line (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 images, and although the exact counts of CED-1::GFP positive cells did not always match, the average trend in the number of CED-1::GFP positive cells, per treatment, were always consistent for the two scientists.

DNA Lesion Assay

Five worms were placed in 1.5 mL of lysis buffer (1x rTth XL DNA polymerase buffer and 1 mg/mL protease K (Applied Biosystems and Sigma) after treatment and frozen for at least 10 minutes at −80°C. The lysis step included 1 hour at 5°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× rTth 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. rTth 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 rTth XL DNA polymerase were from the GeneAmp XL PCR kit (Applied Biosystems). The cycling conditions for the small nuclear target was as follows: 1 cycle of 75°C for 2 minutes; 1 cycle of 94°C for 1 minute; 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 nuclear targets was: 1 cycle of 75°C for 2 minutes; 1 cycle of 94°C for 1 minute; 31 cycles of 94°C for 15 seconds and 68°C for 12 minutes, and 1 cycle of 72°C for 10 minutes [21]. The primers used were as following (IDT): 1) Small Nuclear (polymerase ε target): forward 5′tcccgtctttgctggtttccc3′ and reverse 5′gaccgagccgacatctcggc3′; and 2) Large Nuclear (unc-2): forward 5′tggctcggacgaaacc3′ and reverse 5′ gcctggtttggaaggg3′. The DNA quantity was measured after QPCR using PicoGreen dye (Invitrogen). Each sample was measured in duplicate. 90 μL of 1×TE buffer, 10 μL of DNA and 100 μL of a solution of PicoGreen reagent (5 μL of reagent per milliliter of 1× 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 485 nm of excitation and 530 nm for emission using a Spectra Max Gemini EM detector (Molecular probes). Values were normalized to untreated samples using a Poisson distribution [51]. Blanks were subtracted from all values. Small nuclear numbers where divided by the average of the small values in their experiment, termed “factor”. The large 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 calculated and this value was multiplied by 10 and divided by 10.939 to determine the lesions/10 kb value.

Tumor Growth

Synchronized adult worms were placed on plates and allowed to lay eggs. After about 100 eggs were laid the adults were picked off the plate, and the egg-lay sync plates were placed at 25°C. Each day until the worms reach the L4 stage, the egg-lay synchronized plates were looked through, and any worms that were obviously younger or older than the majority of the worms were picked off the plates. Three days after the egg-lay sync (when the worms have reached the L4 stage), the worms were moved to an unseeded plate and each strain was treated with 100 J/m² of UVC, and placed back at 25°C. The worms were allowed to recover for four days after UVC treatment. Four days after UVC treatment, the worms were washed off the plates, fixed with ethanol, and stained with DAPI (Vectashield). Under the microscope, pictures were taken of any intact worms (worms that have not exploded due to the size of their tumor and/or the fixing and staining process), and the gonad tumor progression was determined. The gonad tumor progression was determined by measuring the distance between the anterior-most point of the tumor, and the anterior-most point of the mouth. Cell death was analyzed four hours after UVC treatment and corpses were identified by SYTO-12 staining.

Statistics

The Graph Pad Prism software version 6.0 d was used to analyze all data. The significance was computed by unpaired, two-tailed student t test. The P values are presented in the figure legends.

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Author Contributions

Conceived and designed the experiments: SH AM JB. Performed the experiments: SH DM JB. Analyzed the data: SH DM AM JB. Contributed reagents/materials/analysis tools: AM JB. Wrote the paper: SH JB. Edited the manuscript: SH AM JB.

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