The Caenorhabditis elegans Werner Syndrome Protein Functions Upstream of ATR and ATM in Response to DNA Replication Inhibition and Double-Strand DNA Breaks

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Abstract

WRN-1 is the Caenorhabditis elegans homolog of the human Werner syndrome protein, a RecQ helicase, mutations of which are associated with premature aging and increased genome instability. Relatively little is known as to how WRN-1 functions in DNA repair and DNA damage signaling. Here, we take advantage of the genetic and cytological approaches in C. elegans to dissect the epistatic relationship of WRN-1 in various DNA damage checkpoint pathways. We found that WRN-1 is required for Chk1 phosphorylation induced by DNA replication inhibition, but not by UV radiation. Furthermore, WRN-1 influences the RPA-1 focus formation, suggesting that WRN-1 functions in the same step or upstream of RPA-1 in the DNA replication checkpoint pathway. In response to ionizing radiation, RPA-1 focus formation and nuclear localization of ATM depend on WRN-1 and MRE-11. We conclude that C. elegans WRN-1 participates in the initial stages of checkpoint activation induced by DNA replication inhibition and ionizing radiation. These functions of WRN-1 in upstream DNA damage signaling are likely to be conserved, but might be cryptic in human systems due to functional redundancy.

Introduction

Werner syndrome (WS) is associated with rapid acceleration of aging, and is caused by mutations in the RecQ family DNA helicase gene, WRN [1]. Clinical symptoms of WS include short stature, hair-graying, cataract formation, and neoplasm of mesenchymal origins [2–5]. The role of WRN in premature aging may be linked to telomere regulation. WS fibroblasts have a reduced replicative life span, which is alleviated by the forced expression of Terc and H-telomerase [8]. WRN-1 is the C. elegans homolog of the human Werner syndrome protein, a RecQ helicase, mutations of which are associated with rapid aging and increased genome instability. Relatively little is known as to how WRN-1 functions in DNA repair and DNA damage signaling. Here, we take advantage of the genetic and cytological approaches in C. elegans to dissect the epistatic relationship of WRN-1 in various DNA damage checkpoint pathways. We found that WRN-1 is required for Chk1 phosphorylation induced by DNA replication inhibition, but not by UV radiation. Furthermore, WRN-1 influences the RPA-1 focus formation, suggesting that WRN-1 functions in the same step or upstream of RPA-1 in the DNA replication checkpoint pathway. In response to ionizing radiation, RPA-1 focus formation and nuclear localization of ATM depend on WRN-1 and MRE-11. We conclude that C. elegans WRN-1 participates in the initial stages of checkpoint activation induced by DNA replication inhibition and ionizing radiation. These functions of WRN-1 in upstream DNA damage signaling are likely to be conserved, but might be cryptic in human systems due to functional redundancy.

Nevertheless, relatively little is known about the cellular functions of WRN in DNA repair and in DNA damage signalling. Furthermore, it is not known if any of the in vitro biochemical activities reported for WRN are required for its function in vivo. Human WRN was implicated in a G2 cell cycle checkpoint, in response to the inhibition of chromosomal decatenation [15]. In addition, human WRN is required for full ATM activation and for slowing down S-phase progression in response to DNA interstrand crosslinks or in response to the inhibition of DNA replication [16]. Here we exploit the Caenorhabditis elegans germ line which is the only proliferative tissue in adult worms, as an experimental system to analyse the functions of WRN in DNA damage signalling. The gonad contains various germ cell types that are arranged in an ordered distal to proximal gradient of differentiation [17; Figure 1A]. The distal end of the gonad is comprised of a mitotic stem cell compartment, followed by a ‘transition zone’ where entry into meiotic prophase occurs. DNA replication failure and DNA double strand breaks lead to a prolonged cell cycle arrest of mitotic germ cells [18].
Author Summary

Werner syndrome is a premature aging syndrome associated with genomic instability. The protein linked to Werner syndrome, WRN, has both helicase and exonuclease activities and is thought to be involved in DNA repair, including the resolution of replication fork arrest as well as in telomere maintenance. However, no definite and detailed role of the protein has been elucidated in vivo. We take advantage of the Caenorhabditis elegans germ cell system to explore DNA damage response defects associated with WRN, and we focus particularly on the role of \( wrn \) in the cell cycle checkpoint in response to DNA replication blockage and ionizing radiation (IR). We show that WRN functions together with RPA upstream of C. elegans ATM and RPA in the cell cycle arrest pathway triggered by IR–induced double-strand DNA breaks. These functions of WRN in upstream DNA damage signaling are likely to be conserved, but not obvious in human systems due to functional redundancy.

The C. elegans genome encodes four worm RecQ family proteins that correspond to their human orthologs. C. elegans WRN-1 is most closely related to human WRN, but has no exonuclease domain [19]. We previously showed that WRN-1 depletion by RNA leads to reduced lifespan. We also observed an increased incidence of diverse developmental defects whose frequency was further accentuated by \( \gamma \)-irradiation [19]; a phenotype likely associated with DNA repair defects occurring during development [20]. In addition, we provided evidence for an abnormal checkpoint response to DNA replication blockage.

In this study, we further explore DNA damage response defects associated with WRN-1 in the C. elegans germ cell system, and particularly focus on the role of \( wrn \) in the cell cycle checkpoint in response to DNA replication blockage and ionizing radiation. We show that WRN-1 functions together with RPA-1 upstream of C. elegans ATM in the intra S-phase checkpoint pathway, and upstream of C. elegans ATM and RPA to trigger cell cycle arrest in response to IR-induced double-strand DNA breaks.

Results

**CHK-1 Phosphorylation in Proliferating Germ Cells of C. elegans**

To probe activation of the DNA damage checkpoint in mitotic C. elegans germ cells and also to examine the relationship between WRN-1 and CHK-1, we used a commercially available antibody against the conserved Ser345 phosphoepitope of human CHK1 corresponding to C. elegans Ser344. In humans, CHK1 is phosphorylated by ATR on Ser317 (which is not conserved in C. elegans) and/or Ser345 when DNA replication is inhibited or upon DNA damage induced by UV, ionizing radiation, or genotoxic chemicals [21]. As shown in Figure 1B, CHK-1 phosphorylation was absent before induction of DNA damage, but became apparent in the nuclei of enlarged germ cells that result from inhibition of DNA replication by hydroxyurea (HU). It had been previously shown that treating C. elegans germ lines with the deoxyribonucleotide-depleting drug hydroxyurea leads to transient germ cell cycle arrest [22]. Germ cell cycle arrest results in enlarged cells and nuclei due to cessation of cell division while cellular growth continues unabated. CHK-1 phosphorylation was also observed in the majority of germ cell nuclei after UV radiation (Figure 1B). One hour after \( \gamma \)-irradiation (IR; ionizing radiation), phosphorylation of CHK-1 was observed in germ cell nuclei at the pachytenic and transition stages but not in those of the mitotically proliferating region (Figure 1B).

To ask why phosphorylation of CHK1 Ser345 is detected in some cells of the proliferating region after UV treatment but not after IR treatment, we labeled S-phase cells with 5-bromodeoxyuridine...
WRN-1 Is Required for CHK-1 Phosphorylation after DNA Replication Blockage, but Not after UV Radiation

Our previous RNA-based results hinted that *wrn-1* might be involved in the S-phase DNA replication checkpoint [19]. To confirm this result and to further examine the function of *wrn-1* in the DNA replication checkpoint, we obtained *wrn-1* deletion mutants and backcrossed them six times to eliminate unlinked mutations (Figure S1A). The 196 bp deletion mutation of *wrn-1* (gk99) eliminates the start codon of *wrn-1* and results in the complete absence of WRN-1 protein as determined by immunostaining germ cells with the specific WRN-1 antibodies that we generated (Figure S1B) and by Western blotting (data not shown).

We next wished to determine the role of WRN-1 in relation to the previously established roles of ATL-1 (worm ATR), and CHK-1 in the DNA replication checkpoint by analyzing the morphology of DAPI-stained nuclei of germ cells after HU treatment (Figure S2A). In contrast to the wild type where germ cell nuclei were enlarged and had a diffuse distribution of chromatin in response to HU treatment, the majority of nuclei in *wrn-1*(gk99), *atl-1* (RNAi), or *chk-1* (RNAi) worms were small and apparently continued to divide. These nuclei had unusually compact chromatin and showed signs of extensive chromatin fragmentation, a phenotype likely to reflect escape from S-phase arrest and subsequent abnormal mitosis (Figure S2A). Inhibition by *chk-1* and *atl-1* RNAi was not complete but extensive, as determined by reverse transcription of mRNAs followed by quantitative PCR amplification (Figure S3A). In contrast, *atm-1*(gk186) cells, the nuclei were uniformly enlarged as in wild type cells, suggesting that ATM-1 is not required for the DNA replication checkpoint, in agreement with Garcia-Muse and Boulton [26]. The convergence of the *wrn-1*(gk99) and the *atl-1* and *chk-1* RNAi phenotypes suggested that *wrn-1*, *atl-1*, and *chk-1* might act in the same genetic pathway needed for HU-mediated cell cycle arrest. To test this hypothesis we asked if the phenotypes resulting from *atl-1* or *chk-1* RNAi were aggravated in the *wrn-1*(gk99) background and found that this was not the case (Figure S2A). Thus it is likely that *wrn-1* indeed functions in the same linear genetic pathway as *atl-1* and *chk-1* to mediate HU-dependent cell cycle arrest.

Having established that *wrn-1*, *atl-1*, and *chk-1* are required for activation of the intra S-phase checkpoint, we next tested if CHK-1 phosphorylation after HU treatment depends on WRN-1 (Figure 2A). We found that CHK-1 phosphorylation in germ cells was greatly reduced in a *wrn-1*(gk99) mutant (and also after RNAi-mediated *wrn-1* knockdown, data not shown) and after *atl-1* RNAi (Figure 2A). The dependence of CHK-1 phosphorylation on WRN-1 and ATL-1 after DNA replication inhibition was also confirmed by Western blotting of worm extracts with the pSer345 CHK1 antibody (Figure 2B), indicating that CHK-1 phosphorylation is reduced in *wrn-1* and *atl-1* in the extracts. This reduction is not as extensive as the reduction observed by the immunostaining of germ cells but nevertheless appears to reflect a reduction in CHK-1 phosphorylation. CHK-1 protein levels are likely to be the same in wild-type, *wrn-1* or *atl-1* worms, given that we did not observe a difference in *chk-1* mRNA levels in those strains (Figure S3B). We could not directly confirm CHK-1 proteins levels due to the absence of a specific antibody (data not shown). Unlike the cases of *wrn-1* and *atl-1* deficiencies, the CHK1 phosphorylation was not affected by knockout of a *C. elegans* ATM homolog, ATM-1 (Figure 2A and 2B).

In contrast to the effect of DNA replication inhibition, CHK-1 phosphorylation caused by UV-radiation was not affected by the *wrn-1* mutation (Figure 3A), whereas it was greatly attenuated by *atl-1* RNAi. This observation in germ cells was confirmed by Western analysis of worm extracts (Figure 3B). In summary, our data suggest that WRN-1 functions upstream of CHK-1 in the DNA replication checkpoint but not in the DNA damage checkpoint activated by UV radiation. Either deficiency of *atm-1* or *wrn-1* was not detrimental enough to induce CHK-1 phosphorylation, in the absence of UV-radiation (Figure S4).

WRN-1 Is Required for the Efficient Formation of RPA-1 Foci in Response to DNA Replication Inhibition

Since WRN-1 controls CHK-1 phosphorylation after DNA replication inhibition, we asked whether RPA focus formation, which occurs upstream of CHK1 and ATR in mammalian cells, is also influenced by WRN-1. After HU treatment the nuclei of wild type cells were enlarged and contained RPA-1 foci, as demonstrated by Garcia-Muse and Boulton [26] (Figure 4A). RPA-1 foci disappeared, and RPA-1 protein (Figure S5C) in worm extracts was greatly reduced by *rpa-1* RNAi as well as the mRNA level (Figure S3A), (this also confirms efficient RPA-1 depletion and the specificity of our antibody). In *wrn-1*(gk99) germ cells, RPA-1 foci were not as abundant or intensely fluorescent as in wild type cells (Figure 4A and Figure S5A with and without HU treatment, respectively). However, RPA-1 focus formation was not affected by *atl-1* knockdown, as shown by Garcia-Muse and Boulton [26], and in agreement with the fact that in mammalian cells RPA binds to single-stranded DNA and then recruits ATR by binding to ATRIP [27]. In line with the results of Figure 2, *rpa-1* RNAi also largely eliminated the phosphorylation of CHK1-Ser345 induced by HU (Figure S2B). The observation that the *wrn-1* mutation affects RPA-1 focus formation suggested that WRN-1 either functions upstream of RPA-1 or in the same step as RPA-1 focus formation. To distinguish between these possibilities, we tested whether nuclear localization of WRN-1 was affected by RPA-1. WRN-1 was present in the nuclei of all control germ cells and its abundance increased significantly after HU treatment (Figure 4B). In addition, the distribution of WRN-1 in the nucleoplasm became uneven, and WRN-1 aggregated into spots or lumps that were less abundant than RPA-1 foci in response to HU treatment. In *rpa-1* knockdown cells, neither the increase in WRN-1 abundance nor its aggregation into spots or lumps occurred upon HU treatment (Figure 4B and Figure S3B with and without HU treatment, respectively). At present, we can not completely rule out that the failure of CHK1-Ser345 phosphorylation and WRN-1 spot formation upon RPA-1 depletion could be due to abolished
DNA replication predicted to occur when RPA-1 is fully depleted. We consider that this possibility is unlikely, as we only partially depleted RPA-1 allowing a continued germ cell proliferation and DNA replication. Furthermore, we found that almost all WRN-1 nuclear spots colocalized with RPA-1 foci, but not vice versa (Figure 4C), further supporting the notion that RPA-1 may acts prior to or in the same step as WRN-1 at stalled replication forks.

**WRN-1 Participates Upstream of ATM-1 and RPA-1, but Downstream of MRE-11, in the Checkpoint Activation Induced by Ionizing Radiation**

We next wished to test if wrn-1 functions in the checkpoint pathway that leads to cell cycle arrest of γ-irradiated proliferating germ cells. After γ-irradiation, the number of germ cells in the mitotic region of gonads is greatly reduced in wild type worms (Figure 5A) due to cell cycle arrest. Arrested cells, however, enlarge due to continued cellular growth in the absence of cell division [18]. We found that in response to IR the number of mitotic germ cells was much less reduced in *wrn-1*(gk99), *atl-1*(RNAi), *atm-1*(gk186), and *chk-1*(RNAi) gonads as compared to wild type gonads, suggesting that WRN-1 and the three other well-known checkpoint proteins play significant roles in IR-dependent cell cycle arrest (Figure 5A). To determine the epistatic relationships of WRN-1 in this checkpoint signaling pathway, we examined whether the nuclear localization of WRN-1 was altered in response to IR and if so whether this depended on various known DNA damage checkpoint and repair proteins. We found that WRN-1 accumulated on the chromatin of irradiated wild type cells and that this accumulation was greatly attenuated by *mre-11*(RNAi), but was unaffected by *rpa-1*(RNAi), *atl-1*(RNAi), and the *atm-1*(gk186) mutation (Figure 5B and Figure S6A with and without IR, respectively). Since *C. elegans* RPA-1 was reported to form nuclear foci in response IR [26], we tested whether their formation was affected by WRN-1. In fact, IR-induced RPA-1 focus formation was significantly reduced by the *wrn-1*(gk99) mutation (Figure 5C). In contrast, RPA-1 focus formation was more evident in the *atm-1* mutant than in wild type cells, indicating that *atm-1* is not required for RPA-1 focus formation, a result in agreement with the previous report by Garcia-Muse and Boulton [26]. We next asked if ATM accumulates on chromatin in response to IR and if so whether this depends on wrn-1. For this purpose, we generated a specific ATM-1 antibody and found that ATM accumulated on the chromatin of wild type cells in response to IR treatment (Figure 5D). However, it did not accumulate significantly upon IR treatment of *mre-11*(RNAi), *wrn-1*(gk99), or *rpa-1*(RNAi) cells, implying that the IR-dependent accumulation of ATM on chromatin requires the products of all three genes (Figure 5D). In contrast, *atl-1* knockdown did not affect ATM-1 localization (Figure 5D), suggesting that ATM-1 acts in a parallel or independent pathway. Our combined results therefore suggest that sequential action of MRE-11 and WRN-1 is needed for efficient RPA-1 focus formation, and that this in turn leads to the nuclear accumulation of ATM-1 in response to IR.
Discussion

We have demonstrated that WRN-1 regulates the DNA replication checkpoint upstream of CHK-1, and probably in the same step as RPA-1, but that the checkpoint activation induced by UV is not affected by WRN-1. It is an intriguing question whether the helicase activity of WRN-1 is required for the DNA replication checkpoint. We have observed normal cell cycle regulation after HU- or IR-treatment of \textit{wrn-1} (tm764) mutants, which have a deletion from the fifth exon to the following intron (Figure S1A, S1B, S1C, S1D). Since this deletion eliminates the helicase motif, it is likely to abolish the helicase activity of wild type WRN-1 that was measured by Hyun et al. [28] in vitro. Therefore, the helicase activity of WRN-1 seems not to be essential for checkpoint function. However, this activity is probably essential for the function of WRN-1 in DNA repair, since the \textit{wrn-1} (tm764) mutation resulted in increased frequency of developmental defects in response to IR (Figure S1E, S1F). A similar observation was made for SGS1, the only RecQ helicase in \textit{S. cerevisiae} [30–32]; its helicase activity was not required for activation of RAD53, a CHK2 homolog [29], after inhibition of DNA replication. Among five RecQ homologs in humans, only limited checkpoint activity has been observed for WRN, namely upon inhibition of chromosomal decatenation [15] and in the activation of ATM induced by interstrand DNA crosslinking and DNA replication inhibition [16]. We propose that WRN-1 affects CHK-1, and probably also ATL-1/ATR activation, by increasing the stability of RPA on the single stranded DNA (ssDNA) at arrested DNA replication forks (Figure 6A). While the components of the so-called 9-1-1 complex, implicated in recognizing arrested replication forks, are conserved in \textit{C. elegans} [30–32], the gene coding for ATRIP, which recruits ATR to RPA on ssDNA, has not been identified in \textit{C. elegans}. WRN-1 may be able to substitute for ATRIP and recruit ATL-1/ATR to the fork, a hypothesis supported by the finding that RPA physically interacts with WRN [28,33] and by the colocalization of WRN-1 and RPA-1 (Figure 4C). However, at present we do not know if \textit{C. elegans} WRN-1 physically interacts with RPA and ATL-1/ATR in vivo. The positioning of WRN-1 in the same step or upstream of RPA-1 in our model differs from the situation in human cells, where depletion of WRN does not affect fork recovery but impairs fork progression after replication inhibition [34]. Another possible function of WRN-1 is to promote the uncoupling of DNA polymerase and helicase activities at stalled replication forks, thereby increasing the length of ssDNA and the concomitant coating of the ssDNA with RPA-1 [35]. Although we place WRN-1 in the same step as RPA-1, upstream of ATR and CHK1, WRN-1 is not as essential for worm survival under normal conditions as these three checkpoint proteins. A possible reason for this difference is that WRN-1 may only respond to exogenously induced stalls of replication forks, but not to those formed spontaneously. In agreement with this idea, RAD-51 foci, indicative of replication fork collapse, were not observed in \textit{wrn-1} (gk99) cells in the absence of exogenously-induced DNA damage (data not shown), whereas they were observed in the \textit{atl-1} (tm853) mutant [26]. Unlike the case of DNA replication inhibition, WRN-1 is not needed for the checkpoint activation induced by UV; this could be due to the ability of ATR to bind to...
UV-damaged DNA [36] or due to the presence of another protein that recruits ATR to UV-damaged DNA.

We found that in contrast to wild type, the cell cycle arrest was significantly alleviated in wrn-1 mutants by treatment with 75 Gy of IR, as well as in atl-1, atm-1, and chk-1 deficient animals (Figure 5A). Stergiou et al. [37] found that the atm-1 mutation did not affect the cell cycle arrest induced by high dose IR (120 Gy), but they noted a moderate effect on the apoptosis of germ cells after low dose IR (20–40 Gy). However, another study based on atm-1 RNAi reported that ATM-1 is needed for IR-dependent cell cycle arrest upon treatment with 75 Gy of IR [26].

Our results suggest that WRN-1 is an upstream component of the signaling pathway that mediates IR-dependent cell cycle arrest. We found that in response to IR the nuclear localization of WRN-1 depended on the presence of MRE-11 to a significant extent (Figure 5B), and that RPA-1 focus formation was promoted by WRN-1 (Figure 5C). In human cells, WRN interacts with the MRN complex via the NBS1 subunit of MRN, and WRN focus formation depends on NBS1 [13,14]. Nevertheless, it is likely that the accumulation of C. elegans WRN-1 is regulated differently, as there is no NBS1 homolog in the worm genome. However, the fact that WRN translocates to double-strand DNA breaks (DSBs) within a few minutes, with the same kinetics as NBS1, also agrees with the proposed role of WRN-1 at the initial stage of the checkpoint pathway [38].

We propose that WRN-1 functions downstream of MRE-11 and upstream of RPA-1 in the DSB signaling pathway leading to...
Figure 5. WRN-1 influences cell cycle arrest and the nuclear localization of ATM-1 and RPA-1 after γ-irradiation. (A) L4-stage wild-type N2, wrn-1(gk99), atl-1(RNAi), atm-1(gk186) and chk-1(RNAi) worms were irradiated with γ-rays (75 Gy), and cultured for 12 h before scoring germ cells. Germ cells in the mitotically proliferating region of gonads (within 75 μm of the tip cell) were stained with DAPI and counted under a fluorescence microscope. Error bars indicate standard errors of means of 12 worms of each genotype. (B–D) One-day-old adult worms of wild-type N2, wrn-1(gk99), atl-1(RNAi), atm-1(gk186), chk-1(RNAi), rpa-1(RNAi), and mre-11(RNAi) strains were irradiated with γ-rays (75 Gy) and cultured for 1 h before immunostaining. Mitotic germ cells were stained using antibody against (B) WRN-1, (C) RPA-1, and (D) ATM-1. Knockdown of atl-1, chk-1, mre-11, and rpa-1 were carried out from (A) the L1 and (B–D) L4 stages. Magnification bars, (A) 25 μm and (B–D) 10 μm.

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WRN activates ATR and ATM

(A) After DNA replication inhibition by hydroxyurea (HU), ATM-CHK2 activation is induced in the downstream of ATR in mammals, and in addition to ATR-CHK1 activation [51]. In C. elegans, the nuclear focus formation of RPA-1 and WRN-1 are interdependent, however RPA-1 knockdown may have indirectly affected WRN-1 foci by reducing the number of replication forks. ATR/ATL-1 is inserted between RPA-1 and CHK-1, because it was positioned below RPA-1 by Garcia-Muse and Boulton [26]. (B) Ionizing radiation (IR) induces ATR-CHK1 activation in the downstream of ATM, as proposed by Jazayeri et al. [44] in mammals. In C. elegans, the nuclear localization of WRN-1 in response to IR is affected by MRE-11 and is a prerequisite for efficient RPA-1 focus formation. It needs to be determined whether WRN-1 conversely affects the nuclear localization or function of MRE-11, as labeled by a question mark. The nuclear accumulation of ATM requires WRN-1 and RPA-1, as well as MRE-11. ATR/ATL-1 is positioned below RPA-1, as proposed by Garcia-Muse and Boulton [26], and is required for effective cell cycle arrest (Figure 5A). CHK-1 is located below ATR, because it was shown to be essential for cell cycle arrest in response to IR by Kalogeropoulos et al. [52] and also in Figure 5A. doi:10.1371/journal.pgen.1000801.g006

Figure 6. Comparison of DNA replication and damage checkpoint pathways between mammals and C. elegans, and roles of the C. elegans WRN homolog in these pathways. (A) After DNA replication inhibition by hydroxyurea (HU), ATM-CHK2 activation is induced in the downstream of ATR in mammals, and in addition to ATR-CHK1 activation [51]. In C. elegans, the nuclear focus formation of RPA-1 and WRN-1 are interdependent, however RPA-1 knockdown may have indirectly affected WRN-1 foci by reducing the number of replication forks. ATR/ATL-1 is inserted between RPA-1 and CHK-1, because it was positioned below RPA-1 by Garcia-Muse and Boulton [26]. (B) Ionizing radiation (IR) induces ATR-CHK1 activation in the downstream of ATM, as proposed by Jazayeri et al. [44] in mammals. In C. elegans, the nuclear localization of WRN-1 in response to IR is affected by MRE-11 and is a prerequisite for efficient RPA-1 focus formation. It needs to be determined whether WRN-1 conversely affects the nuclear localization or function of MRE-11, as labeled by a question mark. The nuclear accumulation of ATM requires WRN-1 and RPA-1, as well as MRE-11. ATR/ATL-1 is positioned below RPA-1, as proposed by Garcia-Muse and Boulton [26], and is required for effective cell cycle arrest (Figure 5A). CHK-1 is located below ATR, because it was shown to be essential for cell cycle arrest in response to IR by Kalogeropoulos et al. [52] and also in Figure 5A. doi:10.1371/journal.pgen.1000801.g006

Materials and Methods

Strains and EST Clones

C. elegans strain Bristol N2 was maintained as described [46] at 20°C unless noted. The wrn-1(ky99) and atm-1(ky186) strains were obtained from the C. elegans Genetics Center (St Paul, MN, USA), which was generated as part of the National Bioresource Project (Japan), was obtained from Dr. Shohei Mitani (Tokyo Women’s Medical University School of Medicine). The mutant strains were outcrossed six times with N2 to remove possible unrelated mutations. The EST clones of all-1 (yk1218d05), chk-1 (yk1302e07), rpa-1 (yk873c12), and mre-11 (yk133i9) were provided by Dr. Y. Kohara (National Institute of Genetics, Japan).

Bacteria-Mediated RNAi

Bacteria-mediated RNAi of chk-1, atl-1, rpa-1, and mre-11 was performed as described, with minor modifications [47]. An approximately 1.2 kb cDNA fragment of the open reading frame (ORF) of Y39H10A.7 (CHK-1) was derived from the EST clone yk1302e07 after being digested with Xhol. The EST clone yk1218d05 of all-1 was digested using BamHI and PstI to produce a 1.1 kb cDNA fragment. The EST clone yk873c12 of rpa-1 was digested using Xhol to produce a 0.64 kb cDNA fragment, and
yk133b9 of 
mre-11 using XhoI and XhoI to produce a 1.6 kb fragment.

The cDNA fragments were cloned into pPD129.36(L4440) plasmid and transformed into Escherichia coli strain HT115(DE3). Ten N2 worms were allowed to lay embryos on plates covered with E. coli cells producing double-stranded RNA of targeted genes for 2 h. F1 worms were grown on RNAi feeding plates to the L4 stage or 1-day-old adults at 25°C before being treated with DNA damaging agents. In the experiments shown in Figure 4 and Figure 5B–5D, RNAi was performed for 16 h from the L4 stage (instead of the L1 stage) before treatment with HU or IR.

Hydroxyurea Treatment

To observe pCHK1 expression or nuclear morphology, worms were grown from L1 to L4 larvae on RNAi feeding plates at 25°C. L4 stage worms were transferred to new RNAi feeding plates containing 25 mM hydroxyurea. After 16 h, their gonads were dissected out and immunostained using rabbit-anti-phospho-CHK1(Ser345) antibody. To visualize WRN-1 and RPA-1 expression, worms were grown from L4 larvae to 1-day-old adults (for 16 h) on RNAi feeding plates at 25°C, and then transferred to RNAi feeding plates containing 25 mM hydroxyurea for 0 h. Dissected gonads were immunostained with anti-WRN-1 and anti-RPA1 antibodies.

γ- and UV Radiation

Worms were grown from L1 larvae to 1-day-old adults at 25°C on RNAi feeding plates (except for Figure 5B–5D, where RNAi was performed from the L4 stage for 16 h). They were γ-irradiated (75 Gy) using a 137Cs source (IBL 437C, CIS Biointernational) or UV-system (Invitrogen). pDEST15 recombinants containing the cloned into pENTR/D/TOPO (Invitrogen). The constructs were prepared from EST clone yk444h6 by PCR with forward primer fragment containing 748–1950 nucleotides of the ORF was amplified by PCR from EST clone yk787c12 with forward primer 5’-TACGTACGGTGTAACCATTGA. The amplicons were sub-transformed into E. coli MG1695 cells. After 1 h, gonads were dissected and immunostained.

Antibody Preparation

Polyclonal antiserum to WRN-1 protein was generated by immunizing mice and rabbits with an amino-terminal WRN-1 fragment corresponding to amino acids 1–209, as described in Lee et al. [19]. The mouse and rabbit anti-WRN-1 polyclonal antibodies were used for immunostaining and Western blot analysis, respectively. The 1–699 bases of the rpa-1 open reading frame (ORF) were amplified by PCR from EST clone yk787c12 with forward primer 5’-CACCAGGGCGCAATCTCAATCAC and reverse primer 5’-TACGTACGGTGTAACCATTGA. An att-L cDNA fragment containing 748–1950 nucleotides of the ORF was prepared from EST clone yk444h6 by PCR with forward primer 5’-CAGCCGAAATGCAATGGCTAG and reverse primer 5’-GTCAAAAACGGCAGATCC. The amplicons were sub-cloned into pENTR/D/TOPO (Invitrogen). The constructs were subsequently cloned into pDEST15 using the Gateway cloning system (Invitrogen). pDEST15 recombinants containing the rpa-1 or att-L fragment were transformed into E. coli BL21AI. The E. coli cells were grown at 37°C to an OD600 nm of 0.5 in LB medium containing 100 µg/ml ampicillin. L-arabinose (Sigma-Aldrich) was added to a final concentration of 0.2% (w/v), and the cells were grown for an additional 4 h at 37°C. The overexpressed proteins were used to generate antibodies in rats. Rabbit anti-CHK1(pSer345), mouse anti-α-tubulin, and anti-BrdU antibodies were purchased from Cell Signaling Technology, Developmental Studies Hybridoma Bank, and Becton-Dickinson, respectively.

Immunostaining

Gonads were extruded by decapitating adult worms, fixed in 3% paraformaldehyde, and immunostained as described [46]. However, to enhance immunostaining of phospho-CHK1 and WRN-1, we used a tyramide signal amplification system (Invitrogen). Gonads were reacted with rabbit anti-CHK1(pSer345) or mouse anti-WRN-1 (1:50 dilution), followed by horseradish peroxidase (HRP)-goat anti-rabbit (or anti-mouse) IgG at 1:100 dilution. Alexa Fluor 488 tyramide at 1:100 dilution was used to detect HRP. Anti-RPA1 and anti-ATM-1 antisera (1:50 dilution) were incubated with gonads, which were then reacted with Fluor 488-conjugated goat anti-rabbit secondary antibodies (Molecular Probes, 1:1000 dilution). When mouse anti-BrdU antiserum (1:10 dilution) was used as a primary antibody, Alexa Fluor 555-conjugated goat anti-mouse antibody (Molecular Probes) was used as the secondary antibody. After staining with DAPI (4,6-diamidino-2-phenylindole, 1 mg/ml), specimens were observed with a fluorescence microscope (DMR HC, Leica).

BrdU Labeling and Immunostaining

To incorporate BrdU into E. coli chromosomal DNA, E. coli MG1695 cells, which are thymidine auxotrophs (from E. coli Genetic Stock Center), were grown overnight in LB containing 10 µg/ml trimethoprim, 0.5 µM thymidine, and 10 µM BrdU, as described by Ito and McGhee [49]. C. elegans chromosomal DNA was labeled with BrdU by culturing hermaphrodites for 30 min on NGM plates seeded with BrdU-labeled E. coli cells. Adult worms were treated with γ- or UV radiation and transferred to plates containing unlabeled E. coli OP50 cells. After 1 h, gonads were extruded, fixed, and washed, and then soaked in 2 N HCl for 15 min at room temperature to denature DNA and expose the BrdU epitope. This was followed by neutralization in 0.1 M sodium tetraborate solution for 15 min at room temperature and double immunostaining for pCHK-1 and BrdU.

Western Blot Analysis

Adult worms were washed off ten NGM plates (dia. 55 mm) with 1×PTW (PBS with 0.1% Tween 20). The pellets were mixed with 2 volumes of 2× sample loading buffer (200 mM Tris·Cl, pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 0.4 mM PMSF) and kept in boiling water for 10 min. The lysates were electrophoresed on 8% SDS polyacrylamide gels and electroblotted onto nitrocellulose membranes (Schleicher & Schuell BioScience). The blots were treated with 5% BSA in PBS with 0.1% Tween 20, and then washed with 1× TBST. The primary antibodies were used and detected using ECL (Amersham Sciences). Luminescence was captured with a LAS-3000 imaging system (Fujifilm).

Assessment of Cell Cycle Arrest

L4 larvae were treated with 75 Gy of γ-rays, and their gonads were dissected out 12 h later. After staining with DAPI, the gonads were observed using a fluorescence microscope. The cell cycle arrest phenotype was assessed by counting the number of mitotic nuclei present in one focal plane within 75 µm of the distal tip cell.

Measurement of mRNA by quantitative RT-PCR

To determine RNAi efficiency targeting chk-1, mre-11, rpa-1, and att-L, worms were grown from L4 larvae to 1-day-old adults (for 16 h) on RNAi feeding plates at 25°C (Figure S3A). To test whether att-L RNAi indirectly affected the level of chk-1 mRNA, worms were grown from L1 to L4 larvae on the feeding plates at 25°C (Figure S3B). L4 stage worms were transferred to new RNAi feeding plates containing 25 mM hydroxyurea and grown for 16 h (Figure S3B). The worms were washed three times in 1× PTW (PBS with 0.1% Tween 20) to free them of bacteria. RNA
Figure S1 Structures of wrn alleles, and comparison of their responses to hydroxyurea and ionizing radiation. (A) Schematic representation of the structure of the C. elegans wrn-1 gene and of the deletions in the gk99 and tm764 alleles. (B) Representative images of the mitotic regions of wild-type, wrn-1(gk99), and wrn-1(tm764) gonads from one-day-old adult worms immunostained with WRN-1 antibody before and after hydroxyurea (HU, 25 mM) treatment for 8 h. In the nuclei of wrn-1(tm764) gonads, WRN-1 is significantly increased by HU treatment, but not to the same level as in wild-type gonads. (C) Morphological changes of DAPI-stained nuclei in mitotic germ cells after HU treatment. After HU treatment, the premeiotic nuclei in wild-type and wrn-1(tm764) gonads were substantially enlarged and reduced in number. In contrast, much smaller nuclei, some of which were condensed, were observed in the wrn-1(gk99) and wrn-1(RNAi) gonads. Magnification bars are (B) 10 μm and (C) 25 μm. (D) L1-stage worms were irradiated with γ-rays (20 Gy) and their growth was measured after 48 h at 20°C. (E) Developmental abnormalities were scored 3 days after IR (60 Gy) at the L1 stage. Small, small body; Ruptured, ruptured body; Transp, transparent; Small, small body; Ruptured, ruptured body; Transp, transparent; Unc, uncoordinated movement. (F) Hatching rates were measured for F1 embryos collected 0–24, 24–48, and 48–72 h periods after feeding RNAi for 16 h. Reverse transcription after random priming and real-time PCR using gene-specific primers were followed. (A) The mRNA levels of target genes (chk-1, mre-11, rpa-1, and atl-1) were estimated by performing qRT-PCR using primers specific for the corresponding gene of the wild-type. The error bars indicate standard error of the mean. The amplified cDNA fragments were separated on 0.7% agarose gels after 25 PCR cycles. (B) No significant effects of wrn-1 or atl-1 deficiency on the mRNA expression of chk-1 (p values of t test >0.5). The chk-1 mRNA levels were estimated as in (A) before and after HU treatment. Found at: doi:10.1371/journal.pgen.1000801.s003 (0.32 MB TIF)

Figure S2 Epistatic relationships of WRN-1 with checkpoint proteins in the cell cycle arrest induced by hydroxyurea. (A) Knockdown of wrn-1, atl-1, and chk-1 were performed from the L1 stage. Images are DAPI-stained nuclei in the gonads of worms, untreated (−HU) or exposed to 25 mM hydroxyurea (+HU) from the L4 stage for 16 h. After HU treatment, premeiotic nuclei in wild-type and atm-1(gk186) gonads were substantially enlarged and reduced in number. In contrast, much smaller nuclei, some of which were condensed, were observed in the mitotic regions of wrn-1(RNAi), atl-1(RNAi), and chk-1(RNAi) gonads. Double deficiencies of atl-1 or chk-1 in the wrn-1(gk99) background did not increase the nuclear phenotype, compared with the single deficiencies of atl-1, chk-1, and wrn-1(RNAi) in the background of wrn-1(gk99) did not change the nuclear phenotype, supporting that wrn-1(gk99) is a null mutation. (B) Knockdown of rpa-1 was carried out from the L4 stage for 16 h before HU treatment, and premeiotic germ cells were probed with phospho-CHK1(S345) antibody. rpa-1 knockdown induced the nuclear phenotype as for the knockdown of atl-1 or chk-1, and abolished phosphorylation of CHK-1(S345). Magnification bars are 25 μm. Found at: doi:10.1371/journal.pgen.1000801.s002 (1.23 MB TIF)

Figure S3 Efficient knockdown of target mRNA expressions and no significant effects of other deficiencies on the chk-1 mRNA expression. Total RNA was isolated form worms after performing feeding RNAi for 16 h. Reverse transcription after random priming and real-time PCR using gene-specific primers were followed. (A) The mRNA levels of target genes (chk-1, mre-11, rpa-1, and atl-1) were estimated by performing qRT-PCR using primers specific for the corresponding gene of the wild-type. The error bars indicate standard error of the mean. The amplified cDNA fragments were separated on 0.7% agarose gels after 25 PCR cycles. (B) No significant effects of wrn-1 or atl-1 deficiency on the mRNA expression of chk-1 (p values of t test >0.5). The chk-1 mRNA levels were estimated as in (A) before and after HU treatment. Found at: doi:10.1371/journal.pgen.1000801.s004 (0.97 MB TIF)

Figure S4 Absence of CHK-1(S345) phosphorylation in untreated germ cells deficient in WRN-1 or checkpoint proteins. (A) Phosphorylation of CHK-1(S345) in mitotic germ cells probed with phospho-CHK1(S345) antibody. Magnification bar, 10 μm. (B) Worm extracts analyzed by western blotting using antibodies to phospho-CHK1(S345), and α-tubulin as a control. Found at: doi:10.1371/journal.pgen.1000801.s004 (0.97 MB TIF)

Figure S5 Absence of RPA-1 and WRN-1 focus formation in untreated germ cells, and specificity of RPA-1 antibody. Absence of (A) RPA-1 focus formation and (B) WRN-1 spot formation in germ lines deficient in rpa-1, wrn-1, or atl-1 before hydroxyurea (HU) treatment. (C) Western analysis of RPA-1 in worm extracts after the knockdown, and of α-tubulin as a control. Magnification bar, 10 μm. Found at: doi:10.1371/journal.pgen.1000801.s005 (1.34 MB TIF)

Figure S6 No significant effects of checkpoint proteins on the nuclear localization of WRN-1 and ATM-1 in untreated gonads. (A) Lack of significant effects of mre-11 or atm-1 deficiency on the nuclear localization of WRN-1, and (B) of mre-11, wrn-1, or rpa-1 deficiency on the nuclear localization of ATM-1 in untreated gonads. However, atm-1 deficiency slightly induced the nuclear localization of ATM-1 in untreated gonads, but the level reached was much lower than after IR. Worms were irradiated as one-day-old adults with IR (75 Gy) and cultured for 1 h before immunostaining. Magnification bars, 10 μm.

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Author Contributions

Conceived and designed the experiments: SJL AG BA HSK. Performed the experiments: SJL MH. Analyzed the data: SJL AG HSK. Contributed reagents/materials/analysis tools: HSK. Wrote the paper: AG HSK.

References

1. Yu CE, Oshima J, Fu YH, Wijman EM, Hisama F, et al. (1996) Positional cloning of the Werner’s syndrome gene. Science 272: 258–262.

2. Shen J, Loeb LA (2001) Unwinding the molecular basis of the Werner syndrome. Mech Ageing Dev 122: 921–944.
24. Moser SC, von Elsner S, Bussing I, Alpi A, Schnabel R, et al. (2009) Functional
23. Gatei M, Sloper K, Sorensen C, Syljuasen R, Falck J, et al. (2003) Ataxia-
22. MacQueen AJ, Villeneuve AM (2001) Nuclear reorganization and homologous
21. Zhao H, Piwnica-Worms H (2001) ATR-mediated checkpoint pathways regulate
20. Clejan I, Boerckel J, Ahmed S (2006) Developmental modulation of
19. Wubbels S, Stinchcomb M, Hengartner MO, Gartner A (2001) Exonuclease-1 deletion
18. Gartner A, Boag PR, Blackwell TK (2008) Germline survival and apoptosis.
17. Kimble J, Crittenden SL (2007) Controls of germline stem cells, entry into
16. Cheng WH, Muftioglu M, Muftuoglu M, Dawut L, Morris C, et al. (2008) WRN is
15. Franchitto A, Oshima J, Pichierri P (2003) The G2-phase decatenation
14. Jazayeri A, Falck J, Lukas C, Bartek J, Smith GC, et al. (2006) ATM- and cell
13. Schneitz FC, von Kobbe C, Opresko PL, Arthur LM, Komatsu K, et al. (2004) Linkage between Werner syndrome protein and the Mre11 complex including Nbs1.
12. Suzuki N, Shiratani M, Goto M, Furuchi Y (1999) Werner syndrome helicase contains a 5'-3' exonuclease activity that digests DNA and RNA strands in DNA/DNA and RNA/DNA duplexes dependent on unwinding. Nucleic Acids Res 27: 2361-2369.
11. Shen JC, Gray MD, Oshima J, Kamath-Loeb AS, Fry M, et al. (1998) Werner syndrome protein. I. DNA helicase and dna exonuclease reside on the same polypeptide. J Biol Chem 273: 54139-54144.
10. Moser MJ, Holley WR, Chatterjee A, Mian IS (1997) The proofreading domain
9. Opresko PL, Otterlei M, Graakjaer J, Bruheim P, Dawut L, et al. (2004) The
8. Chang S, Multani AS, Cabrera NG, Naylor ML, Laud P, et al. (2004) Essential
7. Chang S, Multani AS, Cabrera NG, Naylor ML, Laud P, et al. (2004) Essential
6. Wyllie FS, Jones CJ, Skinner JW, Haughton MF, Wallis C, et al. (2000) Mammalian telomerase protects the newborn baby during an S-M checkpoint during early embryogenesis. Cell Cycle 3: 1196–1200.
5. Bohr VA (2008) Rising from the RecQ-age: the role of human RecQ helicases in ageing syndrome protein, WRN, is a 3'-5' exonuclease activity that digests DNA and RNA exonuclease domains. Nucleic Acids Res 25: 5110–5118.
4. Cheng WH, Muftioglu M, Bohr VA (2007) Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2. Mol Cell 14: 763-774.
3. Cheng WH, Muftioglu M, Bohr VA (2007) Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2. Mol Cell 14: 763-774.
2. Cheng WH, Muftioglu M, Ahn B (2008) Biochemical characterization of the WRN-1 DNA polymerase I and other DNA and/or RNA exonuclease domains. Nucleic Acids Res 25: 5110–5118.
1. Cheng WH, Muftioglu M, Bohr VA (2007) Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2. Mol Cell 14: 763-774.