Master Thesis

A study for genes interacting with the RecQ helicase *wrn-1* gene

University of Ulsan

Department of Biological Science

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Pham Thi Thanh Binh

ABSTRACT

Werner's syndrome (WS) is an autosomal recessive disorder in humans characterized by the premature development of age-associated pathologies. WRN, the product of *WRN* gene which is defective in WS, plays a key role in protecting genome. The cell derived from WS patients show genomic instabilities such as telomere shortening. *wrn-1*, a *Caenorhabditis elegans* (*C. elegans*) homolog of the *WRN,* was identified and its mutation also exhibited a shorter life span. Although many biochemical studies of WRN protein showed its role in DNA metabolism of DNA damage, the defect of WRN activity is not sufficient to explain several phenotypes of WS. Thus, other additional cellular deficits may better explain manifestations seen in WS. To study this unsolved question, we investigated the involvement of *wrn-1* in organismal developments by the use of gene knockout in *wrn-1* mutants. Our results showed that *wrn-1* acts with either *mre-11* or *him-6* during oocyte and embryo developments. When the expression of both *wrn-1* and *mre-11* was reduced, larval development was greatly retarded compared with a single reduction of each gene in the N2 control worms. The reduced expression of *him-6* in *wrn-1* mutant resulted in increased embryonic lethality and arrested cell divisions in embryo. In addition, *wrn-1(gk99);him-6(RNAi)* or *wrn-1(gk99);mre-11(RNAi)* worms displayed multiple chromosomal abnormalities in oocyte nuclei at the diakinesis stage. These results suggest that WRN-1 functionally interacts with MRE-11 or HIM-6 and its interaction is involved in organismal developments.

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INTRODUCTION

The RecQ helicases are highly conserved in evolution and are required for maintaining genome stability in all organisms (Croteau, Popuri et al. 2014). In humans, loss of WRN, a member of the RecQ helicase family, cause a monogenic disease characterized with premature aging including short stature, early graying and loss of hair (Oshima, Sidorova et al. 2017). WRN unwinds DNA duplexes with moderate processivity by translocating along one of the strands in 3′–5′ direction while hydrolyzing ATP and WRN alone among the human RECQ helicases contains a 3′–5′ exonuclease activity (Gray, Shen et al. 1997, Huang, Li et al. 1998). WRN displays different activities on in vitro and model physiologic substrates such as G-quadruplexes and Holliday Junctions (Mohaghegh, Karow et al. 2001). These activities have suggested that WRN is important in homology-dependent recombination (HR) and DNA replication (Croteau, Popuri et al. 2014). In addition, WRN appears to function in several other DNA repair pathways as WRN interacts physically and functionally with other DNA metabolic proteins (Croteau, Popuri et al. 2014). For example, WRN limits the exonuclease activity of MRE11 on one nascent strand at collapsed replication forks by interaction with MRE11 via NBS1 (Su, Mukherjee et al. 2014). Also, WRN interacts with BLM, one of the human RecQ helicases, which inhibits the exonuclease activity of WRN, and WRN and BLM proteins help convert Holliday junctions (von Kobbe, Karmakar et al. 2002, Machwe, Karale et al. 2011). However, the contributions of WRN and its interacting partners to the maintenance of genome stability in normal organismal development are not well studied.

In the model organism *Caenorhabditis elegans* (*C. elegans*), WRN-1, *C. elegans* WRN homolog, contains the helicase domain but not the exonuclease domain (Kusano, Berres et al. 1999). *wrn-1* mutant animals have a shortened life span, increased sensitivity to DNA damage, and accelerated aging phenotypes (Lee, Yook et al. 2004). WRN-1 unwinds a variety of DNA structures such as forked duplexes, Holliday junctions, bubble substrates, D-loops, and flap duplexes, and 3'-tailed duplex substrates (Hyun,

Bohr et al. 2008). Thus, these features confer the involvement of WRN-1 in preventing the accumulation of abnormal structures, contributing to genomic stability.

In addition to the activities of WRN-1, WRN-1 can be modulated by interaction with other proteins in different cellular pathways to display various phenotypes. Thus, it is necessary to investigate interacting proteins or genes with WRN-1. We initially screened genes showing synthetic lethality with *wrn-1* and found several genes including *mre-11* and *him-6*.

HIM-6 is the *C. elegans* homolog of the mammalian BLM protein and worms with *him-6* mutations are radiation sensitive and are predominantly male as a consequence of chromosome missegregation (Kusano, Berres et al. 1999, Wicky, Alpi et al. 2004). While the rate of meiotic crossover recombination is reduced in *him-6* mutants, the frequency of double-strand breaks (DSBs) is increased in mitotically proliferating germ cells of *him-6* mutants (Wicky, Alpi et al. 2004). Thus, it has been proposed that HIM-6 could act after the initiation of recombination and might be necessary for a late step of recombination to process meiotic or mitotic recombination intermediates. MRE-11 (meiotic recombination 11) is the *C. elegans* ortholog of yeast Mre11p and homozygous *mre-11* mutants display a Him phenotype indicative of a severe defect in meiotic chromosome segregation, consistent with an absence of chiasmata in *mre-11* mutant oocytes (Chin and Villeneuve 2001).

These reports suggest that *wrn-1*, *mre-11* and *him-6* are essential to maintain genomic stability, which is deficient affecting the development of sexually reproducing organisms. We present here the effect of *wrn-1* to embryonic and post-embryonic development by interacting either with *mre-11* or *him-6* in *C. elegans* model.

MATERIALS AND METHODS

1. *Caenorhabditis elegans* strains

The *Caenorhabditis elegans (C. elegans)* Bristol N2 was maintained at 20°C on Nematode growth media (NGM) plates. The *C. elegans* mutant strain *wrn-1(gk99)* was obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA).

2. Bacteria-mediated RNAi

Bacteria containing plasmids for inducing dsRNAs of *him-6* and *mre-11* purchased from Source BioScience (www.geneservice.co.uk) and bacteria-mediated RNAi was performed as previously described (Kamath, Martinez-Campos et al. 2001). Briefly, *E. coli* harboring plasmid DNA were streaked on LB agar plates containing 100 μg/ml ampicillin and incubated at 37ºC for overnight. Then, single colony was picked into LB media containing 100 μg/ml ampicillin at 37ºC for overnight (but no longer than 16 hours). 120 μl of grown bacteria was spread on agar plates composed of standard NGM/agar medium supplemented with 50 μg/ml carbenicillin and 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and grown overnight at room temperature. Three to five N2 and *wrn-1 (gk99)* at L4-stage worms were allowed to lay eggs on plates covered with *E. coli* cells producing double-stranded RNA of targeted genes for 24 hours. F1 worms were grown on RNAi feeding plates to the L4 stage or 1-day-old adults at 20ºC using for followed experiments.

3. Detection of double-stranded RNA (dsRNA)

Single colonies of HT115(DE3) bacteria containing cloned L4440 plasmids were grown for 16 h in LB with 100 μg/ml ampicillin at 37°C. The culture was diluted to OD600 = 0.04 and allowed to grow to OD600 = 0.5 at 37°C. T7 polymerase was induced by the addition of 1 mM IPTG, and the culture was incubated with shaking for overnight at 23°C.

E. coli containing induced dsRNA was harvested for RNA extraction. Total nucleic acid was extracted from bacteria as described with some modifiers (Timmons, Court et al. 2001).

A pellet of *E. coli* culture was resuspended in 100 µl of Tris/sucrose buffer (50 mM Tris-Cl pH 8.0, 25% sucrose), lysozyme (2 µl of 10 mg/ml) and Triton X-100 (1 µl of 10%) were added, incubated at 4ºC for 1 hour, DNase I (3 U) was added and then incubated at 4ºC for 2 h. EDTA and RNase was added and incubated at 4ºC for 1 h. The mixture was centrifuged at 14000 rpm for 30 min at 4ºC. The supernatant was transferred to a new tube and extracted with phenol: chloroform (9:1), then centrifuged at 14000 rpm for 10 min at 4ºC, and transferred top aqueous phase to new RNase-free eppendorf tubes. Next, RNAs were precipitated in 1/10 volume of 10 M ammonium acetate and 2 volumes of ethanol. RNAs were resuspended in 30 µl RNase free water.

4. Embryonic lethality and larval development assays

F1 generation worms at the L4 stage were grown on NGM (nematode growth medium)-RNAi plates for 24 hours to lay eggs and then removed. Total eggs were counted. After 24 hours, the number of unhatched eggs was counted. Embryonic lethality percentages were calculated by dividing the number of unhatched eggs by the total number of laid eggs. In order to measure larval growth at 20°C, hatched eggs of F2 generation were counted. Subsequently, worms reaching the L4 stage, as defined by vulva shape, were scored every 12 hours (Lee, Yook et al. 2004).

5. Measurement of mRNA by quantitative RT-PCR

To determine RNAi efficiency targeting mre-11, worms were grown from L4 larvae to 1-day-old adults on RNAi feeding plates at 20ºC. The process was performed as described in (Park, Lee et al. 2008, Lee, Gartner et al. 2010). The worms washed by M9 buffer (22 mM KH_2PO_4, 22 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$) to free them of bacteria. Worms were collected in 1.5 ml centrifuge tubes and dissolved in 1 ml of Trizol (Invitrogen). Tubes were vortexed, incubated at room temperature for 5 min. Then, tubes with 200 µl of chloroform was added and vortexed for 15 s and then incubated at room temperature for 5 min. Tubes were centrifuged at 12,000 g for 15

min at 4 °C and transferred top aqueous phase to new RNase-free eppendorf tube. Equal volumes of isopropanol were added, gently inverted and incubated at room temperature for 10 min and centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was carefully discarded. 100 µl of 75% ethanol was added followed by centrifugation at 4000 g for 5 min at 4°C. The supernatant was discarded and the pellet was dried. The pellet was dissolved in 30 µl RNase free water.

cDNA libraries were synthesized with QuantiTect® Reverse Transcription (QIAGEN) with 1 µg RNA per reaction and a 30 minutes extension time. cDNA was amplified using the Taq DNA Polymerase with Standard Taq Buffer (M0273, NEB) following 30 cycles: pre-denaturation at 95ºC for 30 sec, denaturation at 95ºC for 30 sec, annealing at 54ºC for 30 sec and extension at 68ºC for 1 min.

Primers used for amplification (in 5' to 3' orientation) were:

mre-11:

5'-TGCCTCTGCCTCCGATTAAC-3' (forward)

5'-ACTTCATCTTCACTGCCGCT-3' (reverse)

him-6:

5'-CCACGGACGAATTCGGATCT-3' (forward)

5'-GACCGGAGGAAAAGCCAGAA-3' (reverse)

β-actin:

5'-AGCGTGGTTACTCTTTCACC-3' (forward)

5'-AGAGGGAAGCGAGGATAGAT-3' (reverse)

Products were run on a 1% agarose gel, stained with GelRed and exposed under UV lamp.

6. Visualization of meiotic chromosomes at diakinesis

F1 adult hermarphrodies (15 to 20 worms) were microdissected in cold PBST (1x PBS, 0.1% Tween20), 0.25 mM levamisole. Worms were sliced near head and tail, and gonads were released. Samples were then fixed with cold methanol for at least 10 min at -20ºC, washed three times with PBST, stained in 4,6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml), and washed in PBST. The stained gonads were mounted on 2% agarose pads and were observed under a Carl Zeiss Axioskop2 plus fluorescence microscope. From 3 to 7 oocytes were observed for each genotype.

7. Visualization of embryonic cell division

F1 generation worms at the adult stage were grown on NGM-RNAi plates at 20ºC. The adult worms were dissected to isolate 2-cell-stage F1 embryos in M9 buffer then transferred onto the 2% agarose pad (dissolved agarose in M9 buffer). The embryos were observed under a microscope (Axio Imager A1, Carl Zeiss) to image differential interference contrast (DIC) microscopy at different time points (0, 5, 10, 15, 20, 30, 60 minutes and 20 hours later).

8. Visualization of arrested embryos

F1 generation worms at the L4 stage were grown on NGM- RNAi plates for 24 hours to let them lay eggs and then removed. The unhatched eggs were picked onto the 2% agarose pad (dissolved agarose in M9 buffer) and were observed under a microscope (Axio Imager A1, Carl Zeiss) to image differential interference contrast (DIC) microscopy.

9. Cell cycle arrest assay

To observe nuclear morphology, worms were grown at 20ºC to larval stage L4. L4 stage worms were transferred to a new nematode growth medium (NGM) plate containing 25 mM of HU for 16 hours (Jung, Lee et al. 2014). Gonads were dissected at the indicated times after DNA damage treatment and 4,6-diamidino-2-phenylindole (DAPI, 500 ng/ml) staining was performed. Three stained gonads were observed with a Carl Zeiss Axioskop2 plus fluorescence microscope.

10. Statistical analysis

Experiments were repeated three times in each case. OriginPro 8.5 (OriginLab, Northampton, Massachusetts, USA) was used to perform statistical tests and graphs. The Student's t-test tests were used. * in bar graphs indicate statistically significant p-values. ($p < 0.05$). *$p < 0.05$; **$p < 0.01$ and, ***$p < 0.001$, as stated in bar graphs. Error bars represent the standard error of the mean in every case (SEM).

RESULTS

1. Combined loss of *wrn-1* and either *mre-11* or *him-6* increased embryonic lethality in *C. elegans*

To determine whether *wrn-1* interplays with either *mre-11* or *him-6*, we conducted the lethality assay of wild type and *wrn-1(gk99)* mutant by RNA-mediated interference (RNAi) targeting genes, *mre-11* or *him-6*. Double-stranded RNAs of *mre-11* and *him-6* genes were induced and detected in *E. coli* (Figure S1). It was found that *mre-11* and *him-6* genes were knocked down in *C. elegans* (Figure S2A-S2B). The embryonic lethality was calculated by dividing the number of unhatched eggs with a total number of F2 laid eggs. F1 embryos showed no effects of the RNAi. A significant increase of embryo lethality were detected in *wrn-1;mre-11(RNAi)* and *wrn-1;him-6(RNAi)* F2 embryos compared to either the *mre-11(RNAi)* or *him-6(RNAi)* embryos (Figure 1). The wild type N2 and *wrn-1(gk99)* showed 100% embryonic viability, whereas *mre-11(RNAi)* and *him-6 (RNAi)* showed about 75% and 37% embryonic lethality, respectively. On the other hand, *RNAi* targeting *mre-11* led to a severe lethality in *wrn-1(gk99)* animals, only 3% survival. When *him-6* gene was knocked down in *wrn-1(gk99)* animals, lethality was increased almost 2 folds compared to *him-6(RNAi)* animals. This result suggests a synergistic effect of the double deficiency of *wrn-1* and either *mre-11* or *him-6*.

2. *wrn-1* and *him-6* are involved in embryo development after the gastrula stage

Since the knockdown of either *mre-11* or *him-6* in *wrn-1(gk99)* mutants resulted in a high level of embryonic lethality, we examined the developmental progress of unhatched eggs. The unhatched embryos of F2 generation were picked into M9 buffer, transferred onto 2% agarose pad, and observed for 36 hours. Eggs in hermaphrodites are normally laid during gastrulation and the laid eggs develop *ex utero* from gastrulation stage after first cleavage to first movement (comma) until three fold, rolling stage (Figure 2A) (Denich, Schierenberg et al. 1984). We observed early embryo development before laying eggs (in utero development) (Fiure 2B-C). Embryo cell divisons in *wrn-1(gk99), him-6(RNAi)*, *mre-11(RNAi), wrn-1;mre-11(RNAi)*, and *wrn-1;him-6(RNAi)* was faster than those in N2 when compared the number of nuclei at 60 mins. Unhatched eggs from *mre-*

11(RNAi) and *wrn-1;mre-11(RNAi)* were observed at the gastrula stage at 24 hours and 36 hours, indicating embryo arrest (Figure 2D-E), whereas *him-6(RNAi)* embryos arrested at gastrula, comma, and the three-fold stages (Figure 2D-E). When *him-6* was knocked down in *wrn-1(gk99),* embryos were arrested at gastrula and comma, but fewer arrest was observed at the three-fold stage (Figure 2E). These embryos were unalterably arrested at different stages of embryogenesis, remained being unhatched. These results indicate that *wrn-1* and *him-6* may function in morphogenetic processes in embryogenesis. However, it is possible that *wrn-1* with either *mre-11* or *him-6* functions in meiotic process in oocyte development because embryonic arrest could be caused by the consequence of defective oocytes.

3. Double deficiency of *wrn-1* and *mre-11* increased chromosome aberrations

Since embryonic lethality may be caused by aneuploidy gametes, we examined chromosome numbers at diakinesis in oocytes. At diakinesis in *C. elegans*, six pairs of homologous chromosomes (bivalents) are visible corresponding to desynapsed homologous chromosomes physically held by chiasmata (Schvarzstein, Wignall et al. 2010, Johnson, Lemmens et al. 2013). F1 adult worms were dissected out to release gonads, stained with DAPI, and observed under a fluorescence microscope. Six-DAPI stained bodies was observed in wild type (Figure 3A) and about 12.5% of oocytes of *wrn-1(gk99)* displayed chromosome aberrations, containing a mixture of bivalents and univalents (Figure 3A-3B). *mre-11(RNAi)* and *him-6(RNAi)* displayed 60% and 45% chromosome aberrations, respectively (Figure 3B). However, when *him-6* was knocked down in *wrn-1(gk99)*, the percentage of oocytes containing univalents were 69%, which is higher than the respective single mutants. The highest chromosome aberration was observed in *wrn-1;mre-11(RNAi),* 95% chromosome aberration (Figure 3B). The average number of chromosomes per oocytes showed that except N2, each genotype worm had more than six bivalents chromosomes, *wrn-1(gk99)* had aberrant chromosomes from 7 to 9 (average 7.5) and *mre-11(RNAi)* had 7 to 10 (average 8.8), whereas *wrn-1(gk99); mre-11(RNAi)* had 7 to 10 (average 9.5), *him-6(RNAi)* had 7 to 10 (average 8.0) and *wrn-1(gk99); him-6(RNAi)* had 7 to 9 (average 8.1) (Table 1). Therefore, it is likely that the high percentage of nonviable progeny was caused by chromosome aberrations.

4. Loss of *mre-11* retards post-embryonic development of *wrn-1 (gk99)*

Most of eggs from *wrn-1;him-6(RNAi)* and *wrn-1;mre-11(RNAi)* remained unhatched, but a few eggs were hatched, it is of interest whether hatched eggs are developed normally or not. The hatched eggs of F2 generation were observed continuously every twelve hours until the L4 stage, scoring L4 larvae. Post 36 hours hatching, about 25% of N2 eggs, about 50% of *him-6(RNAi)* eggs, but, only 9% of *mre-11(RNAi)* eggs reached the L4 stage (Figure 4A). The larvae of N2 and *him-6(RNAi)* showed a similar developing rate, but the larval development of *mre-11(RNAi)* was slightly retarded at 60 hours post-hatching and 90% of the larvae reached the L4 stage at 84 hours post-hatching (Figure 4A). In such condition, *wrn-1(gk99)* and *wrn-1;him-6(RNAi)* eggs also displayed a similar developing rate. On the other hand, *wrn-1;mre-11(RNAi)* showed a markedly retarded development because about 53% of the eggs being reached the L4 stage at 48 hours compared to 90% of *wrn-1(gk99)* alone, and the portion of the larvae developed to the L4 stage was 81% at 96 hours (Figure 4B). Thesedata suggest the depletion of *mre-11* and *wrn-1* affects the developmental process of post-embryonic stages.

5. *wrn-1, mre-11* and *him-6* are required for recovery from stalled replication forks

We examined the consequences of perturbing DNA replication in mitotic germ cells by treating worms with the ribonucleotide reductase inhibitor hydroxyurea (HU). HU depletes nucleotide pools, leading to replication fork stalling/collapse (Petermann, Orta et al. 2010). After treatment with 25 mM HU, N2 mitotic nuclei arrested in S/G2 but continued to grow, resulting in enlarged and consequently fewer nuclei. It has been shown that *C. elegans* WRN-1 participates in the initial stages of checkpoint activation in response to HU or IR (Lee, Gartner et al. 2010) resulted in a failure to arrest in response to HU of *wrn-1(gk99)* mutant. Analysis of the mitotic germ cells in *mre-11 (RNAi), wrn-1;mre-11(RNAi)* after HU revealed an intermediate morphology of nuclei containing large and small nuclei (Figure 5A). *him-6 (RNAi)* showed enlarged germ cells, similar to HU-induced cell cycle arrest in wild-type N2 worms, whereas the mitotic nuclei of the *wrn-1;him-6(RNAi)* exhibited an absence of cell cycle arrest compared with the HU-treated N2 and *him-6 (RNAi)* worms (Figure 5B).

After the release from HU for 24 hours in wild-type worms, germ cells remain arrested until repair is completed, whereas reduced recovery was observed in *him-6*. In contrast, *wrn-1(gk99)* germline nuclei challenged with HU bypassed the DNA damage checkpoint and prematurely divided, resulting in more small nuclei. *mre-11(RNAi) and wrn-1;mre-11(RNAi)* still had an intermediate large and small nuclei. *wrn-1;him-6 (RNAi)* displayed that the recovery was defective, mitotic germ cells still remained damaged. These results indicated that HIM-6 is not involved in checkpoint in response to stalled replication forks, whereas WRN-1 and MRE-11 are is involved in cell cycle checkpoint. Since stalled replication forks are recovered by nucleic acid transaction such as homologous recombination or regressing forks, WRN-1, MRE-11 and HIM-6 may be involved in these transactions.

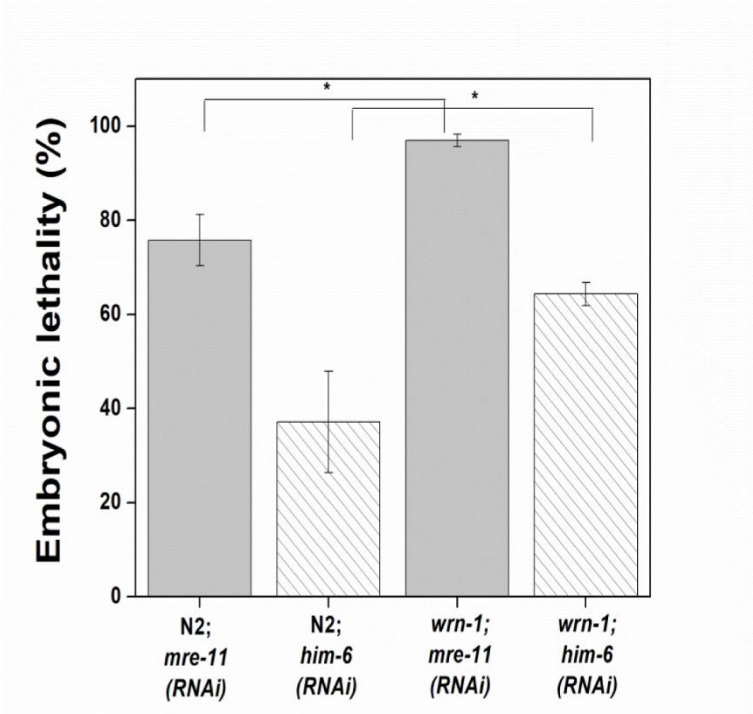
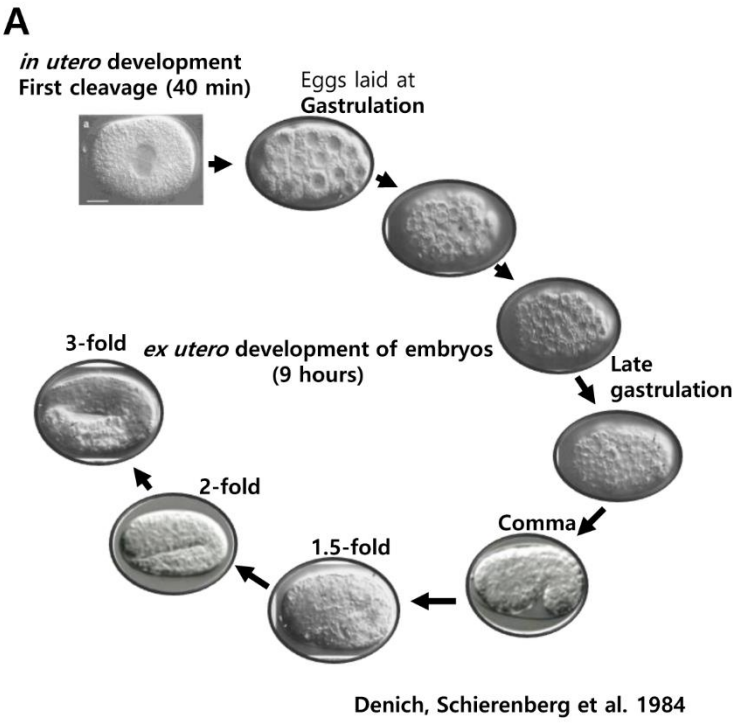
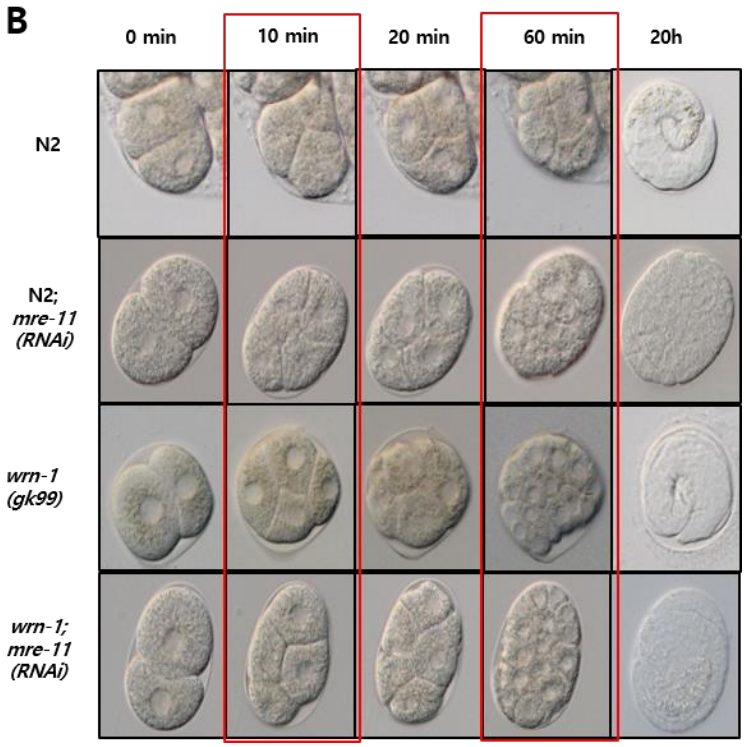
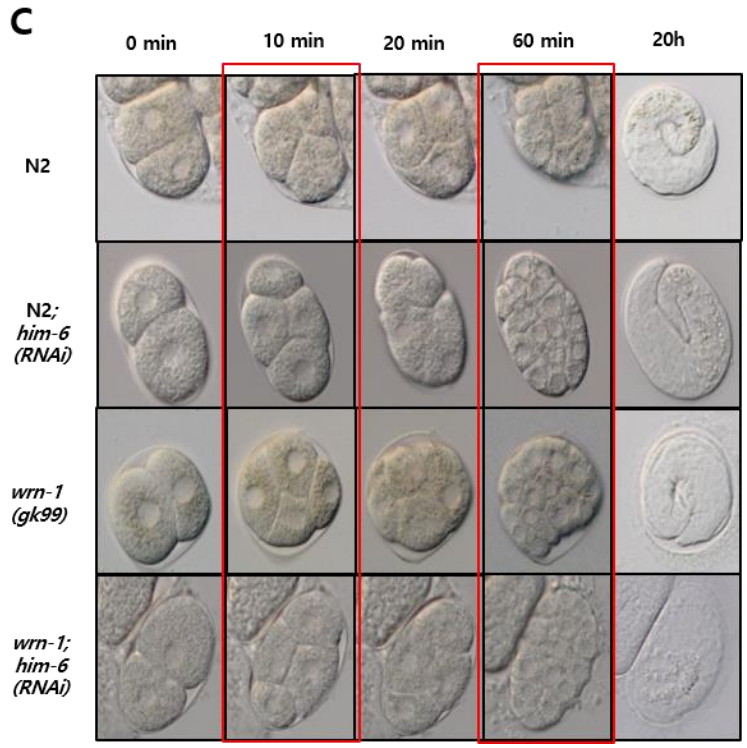


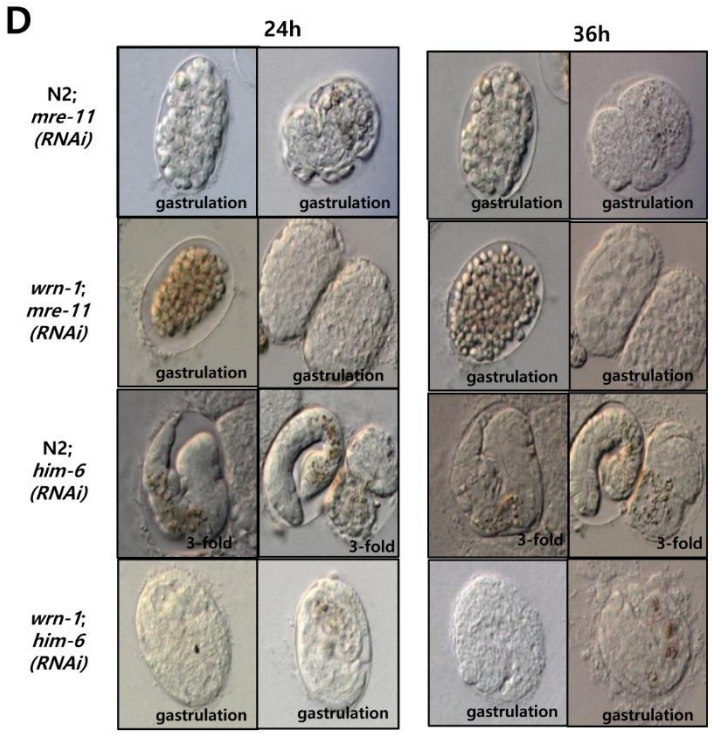
Figure 1. Combined loss of *wrn-1* and either *mre-11* or *him-6* increased embryonic lethality in *C. elegans*

Embryonic lethality of F2 generations produced by F1 worms bacteria-mediated with double-stranded RNA of *him-6* and *mre-11*. Egg-laying was conducted with three animals per genotype, among 100 and 250 eggs of F2 generations were counted per experiment and each experiment was repeated three times. (p=0.03)


A
in utero development
First cleavage (40 min)
Eggs laid at
Gastrulation
3-fold
ex utero development of embryos
(9 hours)
Late
gastrulation
2-fold
Comma
1.5-fold
Denich, Schierenberg et al. 1984


B
0 min
10 min
20 min
60 min
20h
N2
N2;
mre-11
(RNAi)
wrn-1
(gk99)
wrn-1;
mre-11
(RNAi)


C
0 min
10 min
20 min
60 min
20h
N2
N2; him-6 (RNAi)
wrn-1 (gk99)
wrn-1; him-6 (RNAi)


D
24h
36h
N2; mre-11 (RNAi)
wrn-1; mre-11 (RNAi)
N2; him-6 (RNAi)
wrn-1; him-6 (RNAi)
gastrulation
3-fold

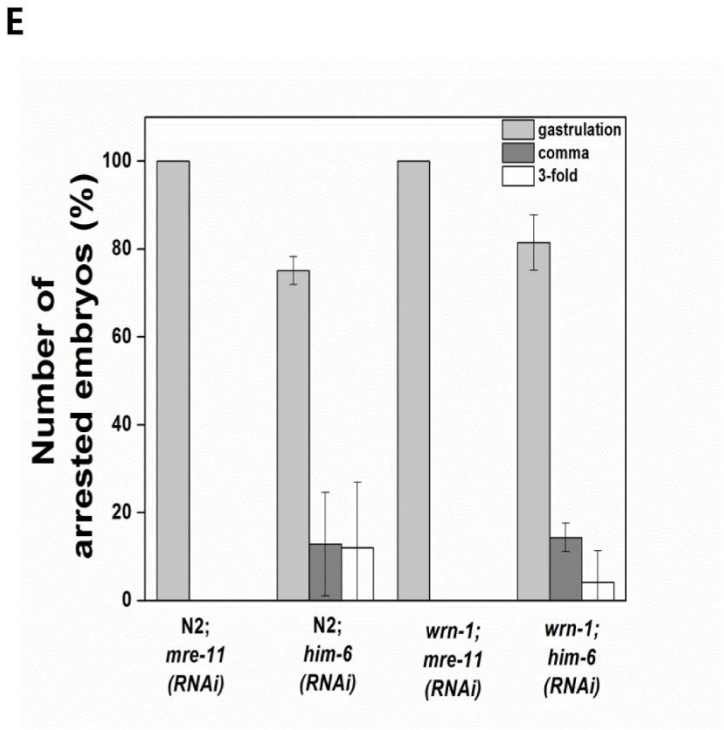
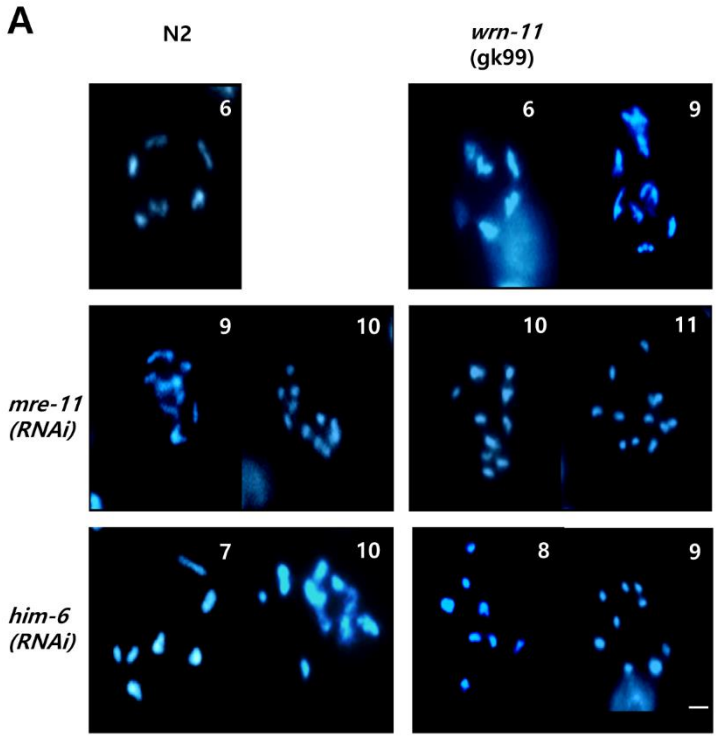


Figure 2. *wrn- 1* and *him-6* are involved in embryo development after the gastrula stage

A. Embryonic stages of development at 22ºC from first cleavage to 3-fold stage (Denich, Schierenberg et al. 1984). The early embryonic division of F1's embryos (n=10) of *him-6 (RNAi), wrn-1; him-6(RNAi)* (B) and *mre-11 (RNAi), wrn-1; mre-11 (RNAi)* (C) compare with N2 and *wrn-1 (gk99)* (n=5) at each time point. D. The lethal embryonic phenotype of F2 generations as knocked down *mre-11* or *him-6* in N2 and *wrn-1 (gk99)* after 24 h and 36 h from the point removing F1 adult worms at 20ºC. From 7 to 16 embryonic cells were observed per genotype, and each experiment was repeated three times. E. Percentage of arrested stages


A
N2
wrn-11
(gk99)
6
6
9
9
10
10
11
mre-11
(RNAi)
7
10
8
9
him-6
(RNAi)

B

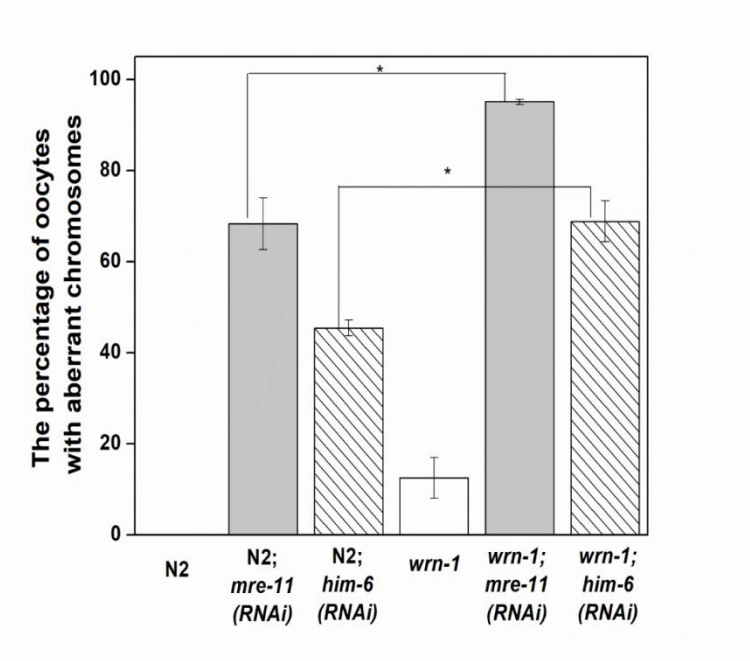


Figure 3. Double deficiency of *wrn-1* and *mre-11* increased chromosome aberrations

A. Representative DAPI-stained oocyte nuclei. B. Oocytes containing more or less than the normal 6 bivalent chromosomes per oocyte were scored between 3 and 7 oocytes for each bar graph, *mre-11* (p=0.01), *him-6* (p=0.02). Each strain worms at the adult stage were selected and gonads were dissected out, stained with DAPI and observed under fluorescent microscope. Scale bar 2 μm.

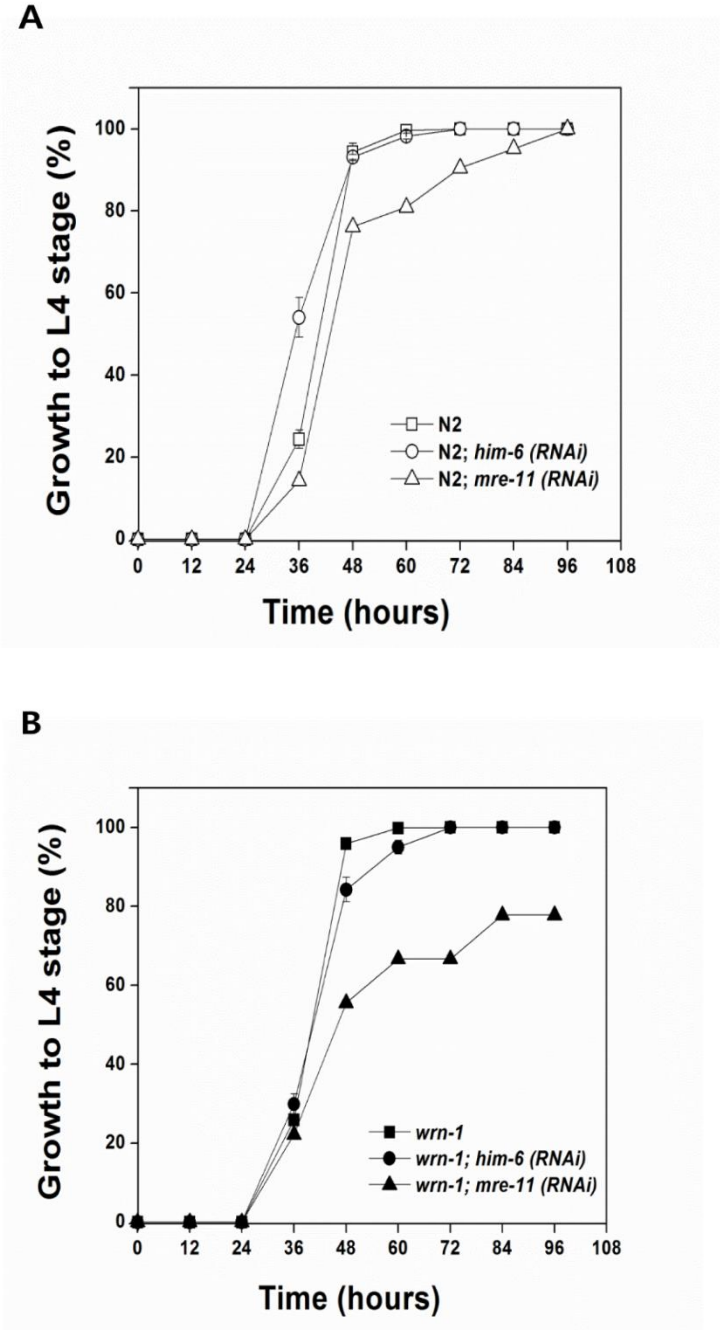
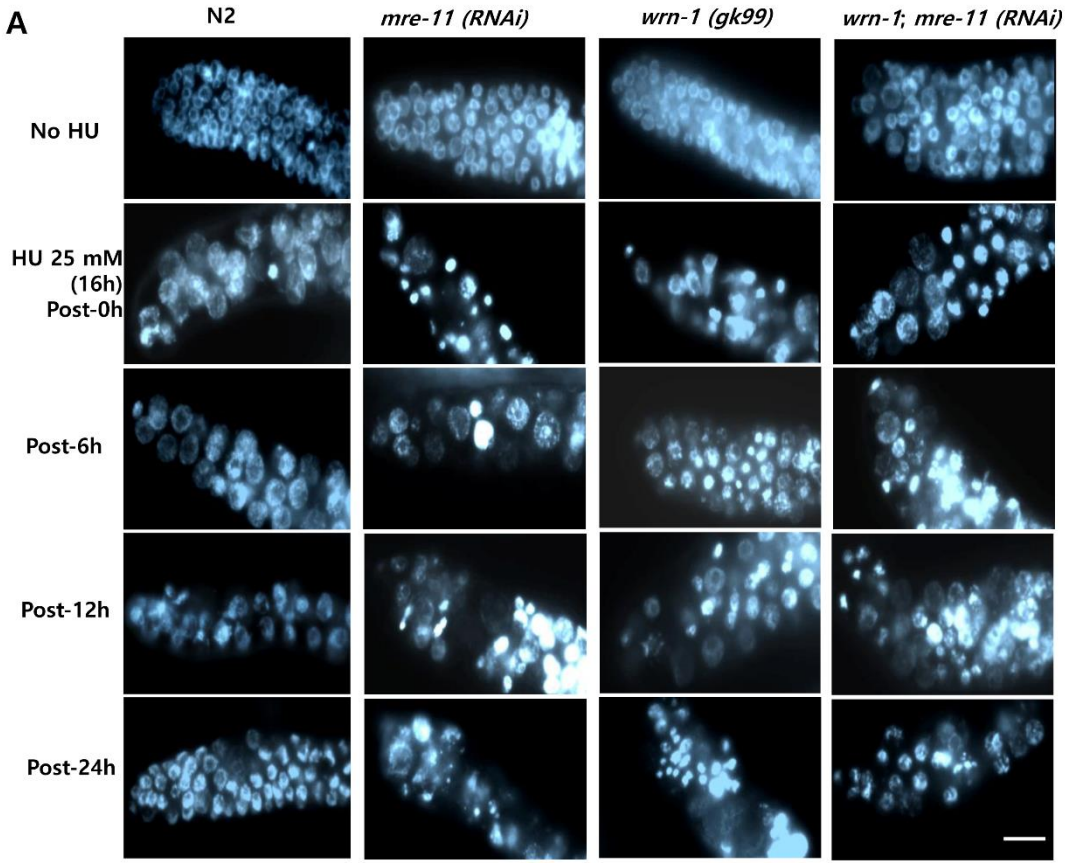


Figure 4. Loss of *mre-11* retards post-embryonic development of *wrn-1 (gk99)*

Growth of N2 (A) and *wrn-1 (gk99)* (B) larva when losing *mre-11* or *him-6*. The development to L4 stage of F2 generations were measured every twelve hours at 20°C. From 50 to 260 F2 worms were used per measurement with two additional repetitions and standard errors (s.e.m.) are shown by error bars. In case of *mre-11* depletion, the standard errors are not shown because the number of hatched eggs is low, about under 20 survival worms.


A
N2
mre-11 (RNAi)
wrn-1 (gk99)
wrn-1; mre-11 (RNAi)
No HU
HU 25 mM (16h) Post-0h
Post-6h
Post-12h
Post-24h

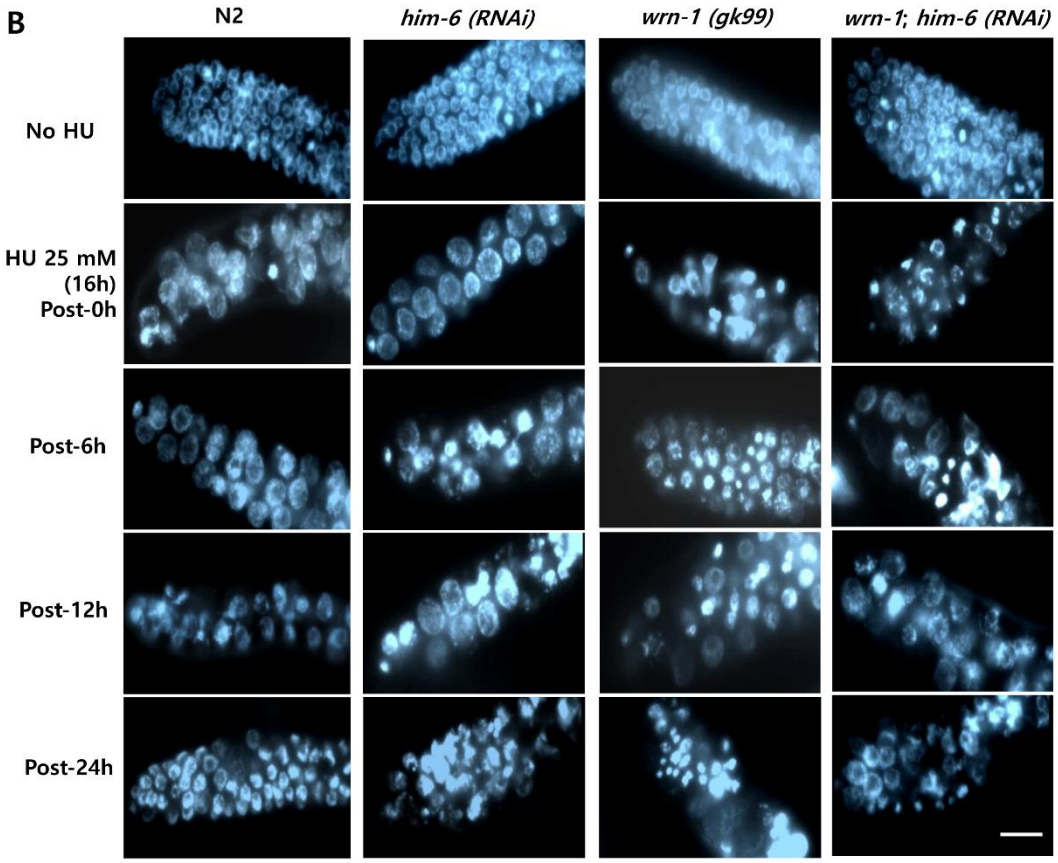


Figure 5. Responses to stalled replication forks

L4 worms were treated with 25 mM HU for 16 h and the transferred to NGM plates allowing recovery. Dissected gonads from the worms at the indicated times were stained with DAPI. Three gonads were observered and experiment was repeated once. Scale bar 5 μm

	Range of aberrant chromosomes	average
N2	**6**	**6**
N2; *mre-11 (RNAi)*	**7-12**	**8.8 ± 0.5**
N2; *him-6 (RNAi)*	**7-9**	**8.0 ± 0.7**
wrn-1	**7-9**	**7.5 ± 0.9**
wrn-1; mre-11 (RNAi)	**7-12**	**9.5 ± 0.3**
wrn-1; him-6 (RNAi)	**7-11**	**8.1 ± 0.3**

Table 1. The average number of chromosomes per oocytes.

DISCUSSION

Although single-gene loss-of-function analyses can identify components of particular processes, important molecules are missed owing to the robustness of biological systems. One approach is to use double mutants, so here, RNAi of *mre-11* and *him-6* genes with a functionally related mutant, *wrn-1*, was employed. Depletion of either *mre-11* or *him-6* in *wrn-1(gk99)* revealed high embryonic lethality and increased chromosome aberrations compared to the respective single gene mutants. This indicates that *wrn-1* functions together with *mre-11* or *him-6* in a shared process, meiotic recombination, through a functional relationship.

Although the biochemical activities of WRN-1 and mutant phenotypes implicate that WRN-1 functions in DNA repair and recombination (Ryu and Koo 2016), the roles of WRN-1 in normal processes are not well studied, regarding interactions with other genes. We have shown that combined loss of *mre-11* and *wrn-1* markedly increased embryonic lethality. Since *wrn-1* mutant animals show normal embryonic survival and *mre-11* single-gene depletions result in 74% embryonic lethality, it is likely that WRN-1 may act as MRE-11, but MRE-11 may play a major role enough to substitute embryonic survival in the absence of *wrn-1* function. On the other hand, WRN-1 would supplement the absence of *mre-11* function, displaying some embryonic survival.

If eggs comprise of dead aneuploid embryos, consequently most of them are inviable. Aneuploid can be detected by cytological analyses of oocyte nuclei in late meiotic prophase I (diakinesis) where paired homologous chromosomes (bivalents) held together by chiasmata are observed (Lui and Colaiacovo 2013). Chromosomal aberrations observed in the majority of *mre-11(RNAi)* oocytes is similarly detected in *mre-11* mutants (Chin and Villeneuve 2001). In addition, a defect in crossover formation was observed in *mre-11* mutants, resulting in the absence of chiasmata (Chin and Villeneuve 2001). Crossover is formed by a meiotic homologous recombination process, which is initiated by double-strand breaks (DSBs) followed by resection of DSBs and a subsequent strand invasion (Schvarzstein, Wignall et al. 2010). Genetic studies of *mre-11* mutants and an analogy to the roles of yeast Mre11p have implicated that MRE-11 nuclease activity may function in DSB end resection in *C. elegans*. However, the biochemical activity of *C. elegans* MRE-11 has not been reported.

A chromosome aberration of *wrn-1(gk99);mre-11(RNAi)* is higher than that of the respective single mutants. What might be the function of WRN-1 and MRE-11 during meiotic recombination? Since it has excluded the possibility that *C. elegans mre-11* functions solely in initiation of meiotic recombination (Hayashi, Chin et al. 2007). In supporting this, a study of yeast Mre11/Exo1 double mutants has documented that DNA end resection can still occur in this mutant (Moreau, Morgan et al. 2001), which is suggestive of further redundancy in this pathway. Thus, we propose a possibility that WRN-1 would also initiate DSB resection with a nuclease in the absence of MRE-11 function, although WRN-1 might not be fully responsible for DSB resection. When two proteins are absent, the initial resection of DSB would be greatly defected, thus showing a high chromosome aberration. Although Mre11, Rad50, Exo1, Dna2, and RecQ helicases are highly conserved in *C. elegans*, little is known about end resection in this model organism shown to cooperate with DNA-2 in end resection (Ryu and Koo 2017). Thus, it will be interesting to test whether DNA-2 and WRN-1 are responsible for the residual DNA end resection activity observed in the absence of MRE-11.

A high incidence of male (or “Him” phenotype), one type of meiotic mutants, possess a single X chromosome and arise among the self-progeny of XX hermaphrodites as a consequence of chromosome missegregation (Hodgkin, Horvitz et al. 1979). *him-6* embryos also suffer autosomal missegregation and consequently are mainly dead aneuploid embryos, with a few euploid adult survivors. Since univalent formation at diakinesis can be caused by a failure of homologous recognition and/or synapsis, or to the inability to undergo crossing-over or to transform to crossovers into the chiasmata, *him-6* may function in one of these processes. It has been reported that *him-6* mutants exhibit severe defects in chiasmata formation and that HIM-6 could act after the initiation of recombination and might be necessary for a late step of recombination (Wicky, Alpi et al. 2004). Our study shows that combined loss of *him-6* and *wrn-1* increased chromosome aberrations at diakinesis. Although we know very little about HIM-6 and WRN-1 functions during *C. elegans* meiosis, *him-6* and *wrn-1* may be involved in one of these processes. A recent literature proposes a unified model of a critical meiotic role of the RecQ helicase BLM and its orthologs, in which BLM uses its unwinding activity to increase the probability for a DSB to be processed by the

appropriate pathway (Hatkevich and Sekelsky 2017). Thus, crossovers are properly placed throughout the genome, promoting proper chromosomal disjunction at the end of meiosis. Furthemore, WRN and WRN-1 also are able to resolve D-loop, which is a recombination intermediate formed by DNA strand invasion (Hyun, Bohr et al. 2008). Taken together, we suggest that WRN-1 may also have overlapping function relationship with HIM-6 BLM in this process. Conceivably, in a HIM-6-impaired condition, WRN-1 may partially substitute for BLM. Therefore, in the absence of *him-6* and *wrn-1* functions the recombination pathways are compromised, resulting in a high chromosome aberration.

As unhatching of laid eggs is related to lethality, it would be expected that unhatched eggs show embryonic arrest. Laying eggs normally takes places when embryos are in the gastrula stage (Denich, Schierenberg et al. 1984). Indeed, unhatched eggs from *wrn-1(gk99);him-6(RNAi)* or *wrn-1(gk99);mre-11(RNAi)* were arrested as embryos and found in the gastrula stage. This indicates that embryos were not developed to later stages. The gastrula stage arrest is likely caused by either the disruption of morphogenetic processes taking place. In particular, the embryo arrest of *him-6(RNAi)* was shifted from the 3-fold stage to the gastrula stage when *wrn-1* gene was defected, indicating that the observed embryonic arrest may be the result of the combined loss of *wrn-1* and *him-6*. However the embryonic arrest is also likely caused by the consequence of an earlier embryonic defect. Because worms carrying large autosomal deficiencies are unhealthy and exhibit numerous morphological defects, chromosome aberrations, having an abnormal number of chromosomes in oocytes cause developmental abnormality in *C. elegans* (Sigurdson, Herman et al. 1986).

Many of the factors shown to be involved in meiotic HR are also contributing to mitotic HR. Accordingly, several mutants that exhibit defects in HR during the meiotic stages of the germline also display increased genomic instability in mitotic cells (Lemmens and Tijsterman 2011).

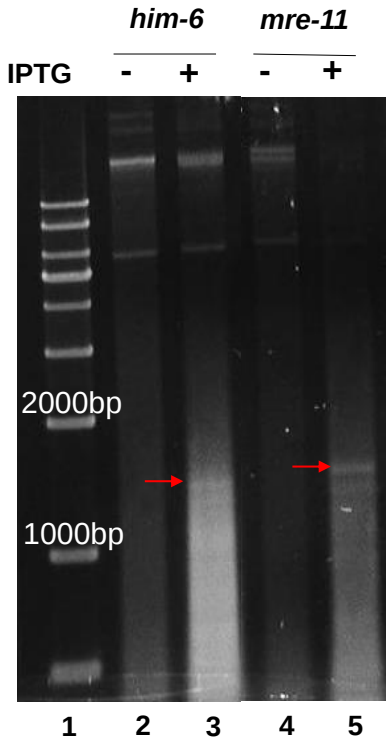
As an example, previously discussed *him-6* and *xpf-1* mutants show elevated levels of RAD-51 foci also in the mitotic zone of the adult germline, suggesting that these HR factors act both on programmed meiotic DSBs and on spontaneous DSBs that arise in mitotic cells (Wicky, Alpi et al. 2004, Saito, Youds et al. 2009). However, our results showed that single mutant or double mutants of *mre-11* and *him-6* in N2 and w*rn-1 (gk99)* had the similar morphological pre-mitotic germ cell nuclei, the shape and the number of nuclei were normal as N2 wild-type (supplemental figure 3). This suggests that mitosis in germline is not be affected to morphology but could be affected at molecular level by losing of *mre-11* or *him-6.*

From here, we examined the consequences of perturbing DNA replication in mitotic germ cells by treating worms with the ribonucleotide reductase inhibitor hydroxyurea (HU). During and after DNA replication, sister chromatids are held in close proximity to each other by cohesins, providing a convenient template for homology-based DSB repair (Nasmyth and Haering 2009). Accordingly, HR is mainly active in S/G2 (Beucher, Birraux et al. 2009). When a replication fork is stalled at a DNA lesion on the leading strand, an HJ can be formed through regression of the nascent leading and lagging strands. This type of HJ can be resolved by repair or bypassed (Michel, Grompone et al. 2004). A recent in vivo study showed that human RecQ helicases, WRN and BLM, function in the reactivation of forks after treatment with HU (Sidorova, Kehrli et al. 2013). In addition, BLM localized to repair centers at collapsed replication forks in response to HU (Sengupta, Linke et al. 2003). Our result suggests HIM-6 may operate in repair and recovery from replication fork collapse and MRE-11 responds differently to DNA damage than either wild-type or checkpoint-defective mutants to HU.

Overall, our data show that loss *of mre-11* or *him-6* results in the deficiency in maintain chromosomal integration without DNA damage, which retards embryonic and post-embryonic development.

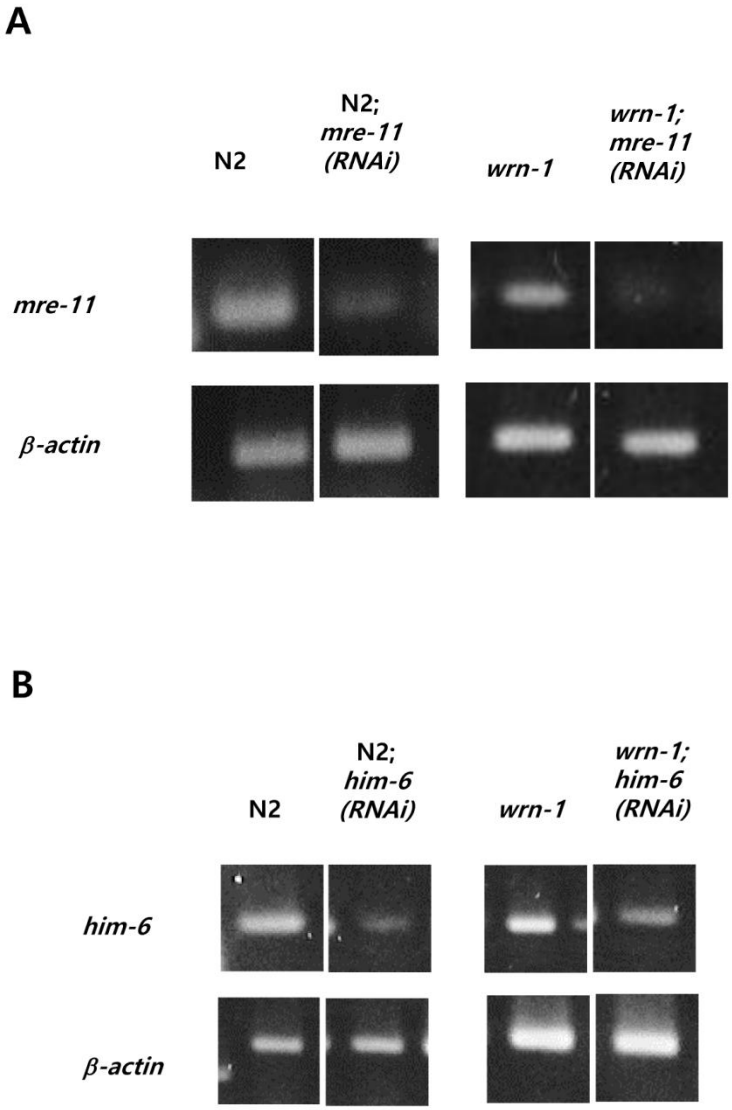
Supplemental information

The presence of dsRNA in *E. coli* HT115 using in RNAi was checked by RNA extraction method. After induction by IPTG, RNA was extracted and running on 1% agarose gel. The result showed that dsRNA of *him-6* and *mre-11* was expressed by IPTG (lane 3 and lane 5, respectively) compare with lane 2 and lane 4 were absence of dsRNA since without IPTG adding. (Supplemental figure 1). The efficiency of RNAi was determined by reverse transcription of mRNAs followed by quantitative PCR amplification. Total RNA was isolated from F1 adult worms after performing feeding RNAi. Reverse transcription after random priming and PCR using gene-specific *mre-11* and *him-6* primers were followed. *β-actin* was used as a loading control. The amplified cDNA fragments were separated on 1% agarose gels after 30 PCR cycles, the bands of *mre-11(RNAi), him-6(RNAi), wrn-1;mre-11(RNAi) and wrn-1;him-6(RNAi)* were thinner than bands of N2 wild-type and *wrn-1(gk99).* This indicated RNAi was efficient for knock-downing gene (Supplemental figure 2A, B).



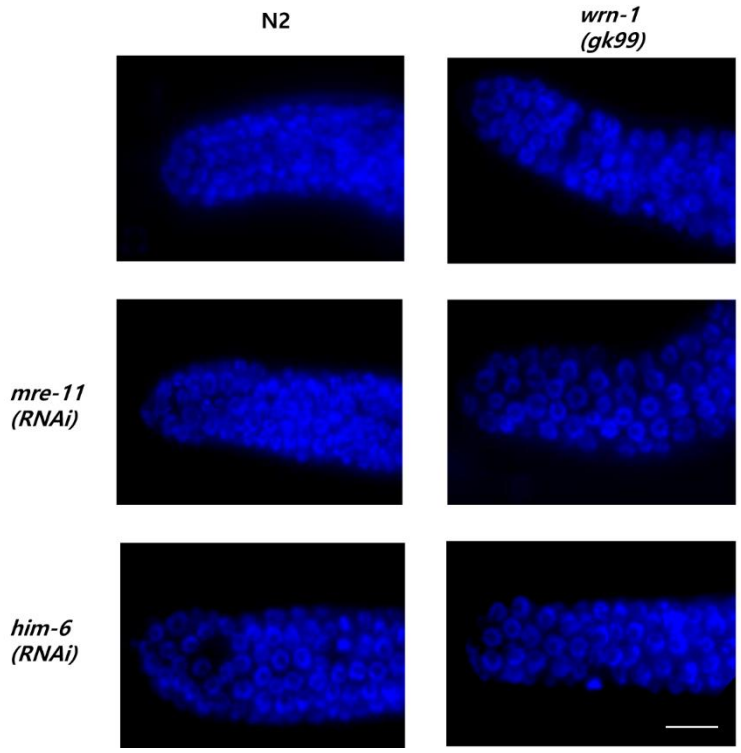
Supplemental Figure 1. Detection of double-stranded RNA induced from *mre-11* and *him-6* genes in *E. coli*

dsRNA of *him-6* (lane 3), *mre-11* (lane 6) from HT115 *E. coli* expression by 1 mM of IPTG. Lane 2 and 5 were from HT115 *E. coli* expression without adding IPTG. Lane 4 and 7 were dsRNA of *him-6* and *mre-11*, respectively as positive control. Lane 1 was DNA ladder 1 kb.



Supplemental Figure 2. Knockdown of target mRNAs

Total RNA extraction from F1 progeny of worms knock-downing *mre-11* and *him-6* in 1 μg of mRNA was subjected to cDNA synthesis. RT-PCR was performed on representative cDNAs of (A) *mre-11* (214 bp), (B) *him-6* (301 bp) and *β-actin* as a loading control (470 bp) following electrophoresis on a 1% agarose gel and visualized by staining with GelRed (Biotium).



Supplemental Figure 3. Morphology of pre-meiotic germline cell nuclei of N2 and *wrn-1 (gk99)* when losing *mre-11* or *him-6*

Images are DAPI-stained nuclei in F1 adult gonads. Twenty worms were dissected, stained and took under fluorescent microscope, and each experiment was repeated three times. Scale bar 5 μm.

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