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Nucleotide Excision Repair, XPA-1, and Translesion Synthesis Complex, POLZ-1 and REV-1, are Critical for Interstrand Crosslink Repair in *C. elegans* Germ Cells

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Abstract

Interstrand crosslinks (ICLs) are adducts of covalently linked nucleotides in opposing DNA strands that obstruct replication and prime cells for malignant transformation or premature cell death. ICLs may be caused by alkylating agents or ultraviolet (UV) irradiation. These toxic lesions are removed by diverse repair mechanisms such as Fanconi anemia (FA) pathway, nucleotide excision repair (NER), translesion synthesis (TLS), and homologous recombination (HR). In mammals, xeroderma pigmentosum group F (XP-F) protein participates in both FA pathway and NER, while DNA polymerase ζ (POLZ-1) and REV-1 mediate TLS. Nevertheless, little is known regarding the genetic determinants of these pathways in ICL repair and damage tolerance in germ cells. In this study, we examined the sensitivity of *C. elegans* germ cells to ICLs generated by trimethylpsoralen/ultraviolet A (TMP/UV-A) combination, and embryonic mortality was employed as a surrogate for DNA damage in germ cells. Our results show that XPA-1, POLZ-1, and REV-1 were more critical than FA pathway mediators in preserving genomic stability in *C. elegans* germ cells. Notably, mutant worms lacking both XPA-1 and POLZ-1 (or REV-1) were more sensitive to ICLs compared to either single mutant alone. Moreover, knockdown of XPA-1 and REV-1 leads to retarded disappearance of RPA-1 and RAD-51 foci upon ICL damage. Since DNA repair mechanisms are broadly conserved, our findings may have ramifications for prospective therapeutic interventions in humans.

Graphical Abstract

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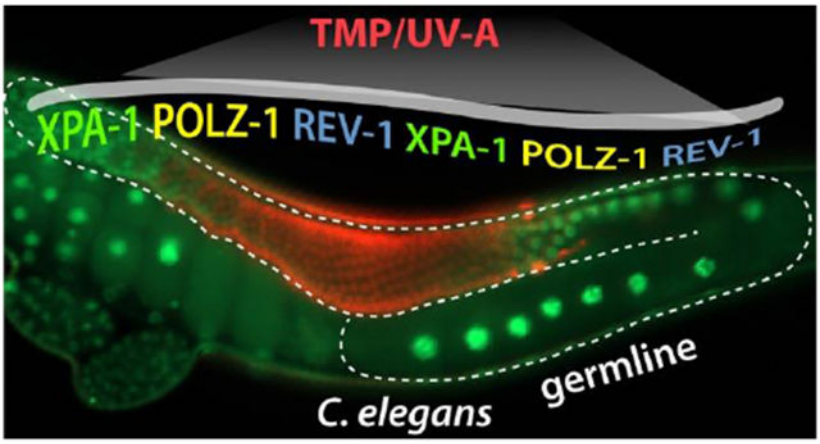
Declaration of Competing Interest

The authors declare no competing interests.

Supporting Information

UV-A dose-dependent effect on *C. elegans* germ cells (Figure S1), TMP dose-dependent effect on *C. elegans* germ cells (Figure S2), Sensitivity of XP mutants to ICLs (Figure S3), Strain information (Table S1), and One-way ANOVA analysis (Table S2).



Keywords

Interstrand DNA Crosslinks (ICLs); Nucleotide Excision Repair (NER); Translesion Synthesis (TLS); Fanconi Anemia Pathway; *Caenorhabditis elegans*

Introduction

Interstrand DNA crosslinks (ICLs) covalently link nucleotides in antiparallel DNA strands, impeding DNA replication and cell survival. ICLs are formed by ultraviolet (UV) radiation (induces DNA strand breaks) and DNA crosslinking agents including cisplatin, mitomycin C, and trimethylpsoralen (TMP) [1, 2]. No or very few DNA ICLs were observed without UV treatment. In particular, TMP induces severe ICLs upon UV-A activation, which facilitates studying molecular mechanisms specifically related to ICL repair *in vivo* and *in vitro* [3]. Although the Fanconi anemia (FA) pathway plays a major role in ICL repair in mammals [2, 4, 5], other pathways also respond to ICL at different phases of the cell cycle.

In replication-independent (G0/G1) phase (Fig. 1A), proteins of nucleotide excision repair (NER) recognize ICLs and recruit incision factors, XPA, RPA, and TFIIH, to the damaged lesion as to allow XPF-ERCC1 to make an incision at one side of the lesion, while XPG cleaves the other side [6]. Translesion synthesis (TLS) recruits polymerases, including POL κ, POL ζ, and REV-1, to the incision site by ubiquitinated proliferating cell nuclear antigen (PCNA) to commence strand extension [1]. POL ζ and REV-1 are also activated independently of PCNA ubiquitination upon UV radiation in POL δ mutants [7].

In replication-dependent (late S) phase (Fig. 1B), when replication forks are stalled by ICLs, the FANCM-FAAP24-MHF complex binds the damaged site, and recruits the FA complex FANCA/B/C/E/F/G, FAAP20, and FANCL (a ubiquitin ligase). The FANCI/FANCD2 complex is then mono-ubiquitinated by FANCL, which activates structure-specific endonucleases XPF/FANCQ-ERCC1, SLX/FANCP-SLX1, MUS81-EME1, and FAN1 [1]. The incision made produces double-strand breaks (DSBs) repaired by homologous recombination (HR). In particular, TLS by REV-1 and POL ζ proceeds during ICL repair, at the step between DSB formation and HR. Mono-ubiquitinated FANCI/FANCD2 complex promotes both incision and TLS before HR [8]. Ubiquitinated FANCI/FANCD2 complex is

finally deubiquitinated by ubiquitin specific peptidase 1-USP1-associated factor 1 (USP1-UAF1). Collectively, the NER+TLS pathway functions in G0/G1 phase, whereas the FA+HR pathway predominates in late S/G2 phase.

In ICL repair, TLS polymerases insert nucleotides after incisions are made [2]. Mammalian cells deficient in POL κ, POL ζ, REV-1, or POL ν are hypersensitive to ICLs, but those deficient in Pol η are not. Also, when unhooking of ICLs generates DSBs at late S/G2 phase, TLS is activated to bypass the unhooked lesion. In replication-independent ICL repair, TLS polymerases are essential to bypass the unhooked ICL and fully extend DNA strands [9]. POL ζ and REV-1 insert nucleotides opposite to the unhooked ICL and bypass the lesion in replication-dependent and –independent ICL repair. REV-1 is involved in bypass by inserting a nucleotide to the opposite DNA strand, and POL κ performs strand extension [10].

In mammals, the FA pathway is the main pathway of ICL repair. There are 21 FA genes in mammals [11], some of which are conserved in *C. elegans* [12], while NER and TLS genes are conserved from *C. elegans* to mammals [13-15]. In this report, we investigate the function of TLS polymerases (POLZ-1 and REV-1) and their genetic relationship with XPA-1 in the germline of the nematode *C. elegans*. This worm has a short life cycle and its germline has also been widely used as a model system to study cellular processes *in vivo.* The *C. elegans* germline is organized in a simple linear fashion that progresses from germline stem cells at one end to maturing gametes at the other (Fig. 2A). The *C. elegans* germ cells are sensitive to diverse DNA damage that is often associated with infertility or embryonic mortality. Thus, it has been recognized as a suitable model to investigate DNA repair pathways *in vivo* [3, 4, 16-19]. Our genetic and cellular studies demonstrate that XPA-1 and TLS are critical for *C. elegans* ICL repair, which drive HR progression beyond RPA-1 and RAD-51 recruitment. Since these regulators are broadly conserved, similar molecular mechanism may exist in humans.

Materials and Methods

Strains and maintenance

All *C. elegans* strains were maintained at 20°C as previously described unless otherwise noted [20]. Wild type (N2) and *xpa-1(ok698)* mutants were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). The *fcd-2(tm1298)*, *xpc-1(tm3886)*, *xpf-1(tm2842)*, *polz-1(tm8927)*, and *rev-1(tm8701)* mutants were obtained from National BioResource Project (NBRP, Japan). See Tables S1 and S2 for strain information and motif analysis of POLZ-1(tm8927) and REV-1(tm8701). The *fcd-2(tm1298), xpf-1(tm2842), xpa-1(ok698), xpc-1(tm3886), polz-1(tm8927)*, and *rev-1(tm8701)* mutants were outcrossed with N2 at least three times to eliminate background mutations. All mutations were confirmed by worm PCR (polymerase chain reaction). *polz-1(tm8927); rev-1(tm8701)* double mutant was generated from *polz-1(tm8927)* single and *rev-1(tm8701)* single mutants using a standard genetic method. Genotypes were validated by PCR.

RNA interference (RNAi)

RNAi was conducted by feeding worms with *E. coli* HT115 (DE3) expressing the double-stranded RNAs of *xpa-1* from the *C. elegans* RNAi V1.1 feeding library (Open Biosystems). RNAi bacteria were cultured in Lysogeny Broth (LB) containing ampicillin (50 μg/ml) for 16 h at 37°C and were seeded onto nematode growth medium (NGM) plates containing ampicillin (50 μg/ml) and IPTG (1 mM). RNAi was started in synchronized L1 larvae at 20°C.

ICL induction

Animals were synchronized as per [21] and grown to L4 stage (48 h past L1 stage).

To induce ICLs, L4 stage worms were treated with 0.8, 1.6, 4, and 20 μM 4,5',8-Trimethylpsoralen (TMP) (Acros, NJ, USA) for 40 min, and exposed to UV-A light (100 J/m^2) by Sylvania UV lamp. Generally, worms are exposed to TMP for 1 hour as per [3]. However, to examine the effect of ICLs on *C. elegans* germ cells, TMP exposure time was slightly reduced to 40 min as per [22].

Embryonic survival

To examine the sensitivity of germ cells to ICLs, embryonic survival was measured. Briefly, L4-larval worms (P0) exposed to ICL-inducing agents were allowed to lay embryos on NGM for 24 h at 20°C. P0 worms were removed, their embryos were maintained at 20°C, and survival rates were determined 24 h later.

$$\text{Embryonic survival rate}(\%) = [\text{No. of hatched worms}/(\text{No. of dead embryos} + \text{No. of hatched worms})] \times 100$$

Immunostaining

Germline immunostaining was performed as previously described [19]. Briefly, after ICL induction, gonads were incised on a glass bath with 1x PBST (0.1% Tween20 in 1x PBS). Gonads were fixed in 10% formalin for 15 min at 25°C, post-fixed in cold 100% methanol for 12 h at −20°C, washed in PBST, treated with blocking solution (goat serum: 1x PBST = 1:1) for 30 min at 25°C, and incubated with primary antibodies overnight at 4°C. Gonads were washed three times in 1x PBST, treated with a FITC-conjugated goat anti-rat secondary antibody (Molecular probes, 1:1000 dilution) for 1 h at 25°C, washed again in 1x PBST, stained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) for 15 min, and washed for the last time in 1x PBST. The foci of RPA-1 or RAD-51 were counted in 10 mitotic germ cells close to the distal end of each gonad under a fluorescence microscope (DMR HC, Leica). This was repeated for 10 gonad arms in each strain. Biological experiments were performed at least three times.

Statistics

Data are presented as means ± standard error of the mean (SEM) of at least three independent experiments. One-way ANOVA was used to analyze differences among the

experimental groups while comparisons between two groups were accomplished by Student's *t*-test. A cutoff for statistical significance was set at a *P* value of <0.05.

Results

XPA-1 and XPF-1 are required for ICL repair in C. elegans germline

To examine whether NER is required for ICL repair in *C. elegans* germline, the survival of progeny embryos after L4 stage worms treated with TMP and UV-A (TMP/UV-A) was measured (Fig. 2A). Optimal treatment conditions for TMP/UV-A treatment were determined as per [3, 22] (Fig. S1 and S2). To examine the effect of ICLs on *C. elegans* germ cells, TMP exposure time was slightly reduced to 40 min instead of 60 min as per [3]. Two null mutant alleles *fcd-2(tm1298)* [23] and *xpf-1(tm2842)* [24] were used as positive controls for ICL hypersensitivity. The *fcd-2* gene, an ortholog of human FANCD2 (FA complementation group D2), participates in FA pathway. The *xpf-1* gene, an ortholog of human ERCC4 (ERCC excision repair 4), functions at the initial stage of FA pathway and in NER. We found that *xpa-1(ok698)* null mutants were more sensitive than *fcd-2(tm1298)* mutants at 20 μM TMP/UV-A (Fig. 2B; Table S2 for ANOVA analysis). This indicates XPA-1 and XPF-1 are required for ICL repair in *C. elegans* germline. Similarly, Wilson *et al.* previously reported that XPA-1 and XPF-1 are also required for ICL repair during *C. elegans* development and aging [3].

TLS polymerases, POLZ-1 and REV-1, are also required for ICL repair in C. elegans germline

XPA recognizes ICLs, and RPA, TFIIH, XPG, and XPF-ERCC1 make incisions at both sides of the lesion. PCNA and TLS polymerases (e.g., POL κ, POL ζ, and REV-1) are then recruited to the damaged sequence [6] (Fig. 1A and 1B). To test whether TLS polymerases are required for ICL repair in *C. elegans* germline, we scored embryonic survival of TLS polymerase-deficient mutants, *polz-1(tm8927)* and *rev-1(tm8701)*, after ICL induction. Notably, both *polz-1(tm8927)* and *rev-1(tm8701)* mutants were more sensitive to ICL than *fcd-2(tm1298)* mutants at low-dose 1.6 μM TMP/UV-A (Fig. 2C). Particularly, the survival of *polz-1(tm8927)* mutants was non-significantly different to that of *xpa-1(ok98)* mutants at the same dose (Fig. 2C). The *rev-1(tm8701)* mutants, were more sensitive to ICL than *fcd-2(tm1298)* mutants, but were not as sensitive as *polz-1(tm8927)* mutants (Fig. 2C, Table S2 for ANOVA analysis). Collectively, these results indicate that an ICL repair pathway involving XPA-1 and POLZ-1 may play a more prominent role than the FA pathway and REV-1 in *C. elegans* germline.

Double POLZ-1 (or REV-1) and XPA-1 deficiency synergistically increase sensitivity to ICLs compared with single deficiencies

To determine the genetic relationship between XPA-1 and TLS polymerases (POLZ-1 and REV-1), wild type (N2), *polz-1(tm8927)*, and *rev-1(tm8701)* mutants were fed *E. coli* expressing double-stranded RNA (dsRNA) for *xpa-1.* The *xpa-1(RNAi)* worms were not as sensitive to ICLs as *xpa-1(ok698)* mutants, which indicates that the knockdown of *xpa-1* was not complete. Nevertheless, *polz-1(tm8927); xpa-1(RNAi)* mutants were more sensitive to ICLs than single mutants at 1.6 μM but not at 0.8 μM TMP/UV-A (Fig. 2D, Table S2 for

ANOVA analysis). Also, *rev-1(tm8701)*; *xpa-1(RNAi)* mutants were more sensitive to ICLs at 0.8 and 1.6 μM TMP/UV-A than those with either mutation (Fig. 2D). Altogether, these data suggest that POLZ-1 and REV-1 may work in parallel (Fig. 1A and 1B) and that double deficiency of POLZ-1 (or REV-1) and XPA-1 confers hypersensitivity to ICLs compared to either deficiency.

Loss of REV-1 delays the disappearance of RPA-1 and RAD-51 foci

We next investigated the number of RPA-1 and RAD-51 foci in the mitotic germ cells of *C. elegans* upon treatment with TMP/UV-A [25]. RPA-1 and RAD-51 bind single-stranded DNA (ssDNA) [26], and are thus used as surrogates to measure the efficiency of DNA repair. We measured the formation of RPA-1 and RAD-51 foci in response to ICLs in wild type (N2), *xpa-1(RNAi)*, *rev-1(tm8701)*, and *rev-1(tm8701)*; *xpa-1(RNAi)* mutants (Fig. 3A-3C). The formation of RPA-1 foci was increased in *rev-1(tm8701)* and *rev-1(tm8701)*; *xpa-1(RNAi)* mutants, but not in *xpa-1(RNAi)* worms. In wild type (N2) and *xpa-1(RNAi)* mutants, the number of RPA-1 foci reached a peak at 6 h after ICL induction and disappeared almost completely at 18 h (Fig. 3A and 3C). In *rev-1(tm8701)* mutants, the number of RPA-1 foci reached a peak at 6 h and almost disappeared at 24 h (Fig. 3B and 3C). Remarkably, in *rev-1(tm8701)*; *xpa-1(RNAi)* mutants, RPA-1 foci remained at 24 h (Fig. 3B and 3C, Table S2 for ANOVA analysis). Furthermore, there were no significant differences among strains in the number of RAD-51 foci at 12 h after ICL induction (Fig. 4A-4C). However, at 24 h, RAD-51 foci disappeared in wild type (N2) and *xpa-1(RNAi)* mutants (Fig. 4A and 4C). In contrast, in *rev-1(tm8701)* and *rev-1(tm8701); xpa-1(RNAi)* mutants, the numbers of RAD-51 foci at 24 h were non-significantly different from those observed at 12 h (Fig. 4B and 4C, Table S2 for ANOVA analysis). These results indicate, in germlines lacking REV-1, HR did not progress further after loading RPA-1 and RAD-51 on ssDNA.

POLZ-1 and REV-1 influence another ICL-responsive pathway

POLZ-1 works in tandem with REV-1 during ICL repair at both replication-independent (G0/G1) and replication-dependent (late S/G2) phases [9, 27, 28]. Figures 2-4 show that POLZ-1 and REV-1 act in parallel, not serially, in the NER+TLS pathway. To test this, ICL sensitivity of *polz-1(tm8927); rev-1(tm8701*) double mutants was determined. Significantly enhanced sensitivity to ICLs was exhibited by *polz-1(tm8927); rev-1(tm8701)* double mutants compared to *xpa-1(ok698)*, *polz-1(tm8927)*, or *rev-1(tm8701)* single mutants at 0.8 and 1.6 μM TMP/UV-A (Fig. 5, Table S2 for ANOVA analysis). This synergistic increase of sensitivity to ICLs implies that POLZ-1 and REV-1 may function redundantly in the same pathway or work in the undefined parallel pathways to repair ICLs in *C. elegans* germlines (see Discussion for details).

Discussion

In mammals, FA pathway is more prominent during ICL repair than NER [29]. However, Wilson *et al*. recently reported that NER components (e.g., XPA-1 and XPF-1) are more critical for ICL repair than FA pathway during development and aging in *C. elegans* [3]. Notably, increasing evidence indicates that NER plays an important role in ICL repair in mammalian cells [30-36]. Our studies also demonstrate that germlines lacking in XPA-1,

XPC-1, and XPG-1 display increased sensitivity to ICLs compared to those deficient in *fcd-2* (Fig. 2A and Fig. S3). XPA-1 is a damage recognition protein in NER whose mutation induces arrested development and reduced lifespan following TMP/UV-A exposure [3]. Similarly, dysfunctional growth and reproduction, DNA lesions, and germ cell apoptosis were more pronounced in XPA1-deficient mutants compared to wild type (N2) worms treated with aflatoxin B1 [37]. In human keratinocyte HaCaT cells exposed to arsenic, downregulation of XPA exacerbated DNA damage, an effect reversed by inhibition of histone deacetylase [38].

We have also demonstrated that POLZ-1 and REV-1 protect against embryonic mortality caused by germline ICL damage (Fig. 2B-2D and 5). The REV-1-POLZ complex, called the mutasome, inserts nucleotides and initiates replication opposite damaged lesions [39]. We have demonstrated that *polz-1(tm8927)* mutation confers hypersensitivity to ICLs more significantly than *rev-1(tm8701)* mutation (Fig. 2C). Moreover, *polz-1(tm8927); xpa-1(RNAi)* and *rev-1(tm8701); xpa-1(RNAi)* double deficiencies synergistically sensitize worms to ICLs compared to either deficiency (Fig. 2D). In *Xenopus* eggs, the mutasome extends the leading strand beyond the damage [40]; a step significantly obstructed by *polz* depletion [41]. Notably, blocking the catalytic activity of REV-1 does not influence survival following DNA damage in mammalian cells [42]. Collectively, these observations suggest POLZ-1 is indispensable to TLS, likely by acting as a strand extender beyond damaged termini.

It has been reported that *rev1* knockout mice display stunted growth and reduced lifespan [43]. Furthermore, proliferation of fibroblasts and hematopoietic stem cells obtained from these mice was blunted [44, 45]. In particular, combined deletion of *rev1* and *xpc*, leads to anemia and death in those mice [45]. Accordingly, cells with either deficiency display hypersensitivity to ICL-causing compounds [46]. This is in congruence with earlier studies describing the participation of REV1 in repair of alcohol-induced ICLs [47].

RPA-1 and RAD-51 coat ssDNA to form a complex involved in DNA repair [48, 49]. Our kinetic studies demonstrate that recruitment of RPA-1 is significantly amplified in *rev-1(tm8701)* and *rev-1(tm8701); xpa-1(RNAi)* mutants compared to wild type (N2) and *xpa-1(RNAi)* mutants (Fig. 3). Likewise, RAD-51 persisted for a significantly longer time in *rev-1(tm870)* and *rev-1(tm870); xpa-1(RNAi)* mutants unlike wild type (N2) or *xpa-1(RNAi)* worms (Fig. 4). This implies that TLS, through REV-1, is indispensable to ICL repair presumably by driving HR beyond RPA-1 and RAD-51 recruitment.

It has been demonstrated that binding of RPA-1 to damaged sites may be a prerequisite for subsequent recruitment of RAD-51 [49]. Unlike this sequential action of RPA-1 and RAD-51, our results in Figure 5 indicate that REV-1 and POLZ-1 function in tandem as *polz-1(tm8927); rev-1(RNAi)* mutants were more sensitive to ICLs than wild type (N2) or *xpa-1(RNAi)* worms. It has been suggested that HR is the main ICL repair pathway in which RAD-51 plays a central role. Congruently, cells with mutated *Rad51C* are sensitive to mitomycin C [50], and display aberrant centrosome numbers and mitotic spindles [51]. Also, in human and yeast cells, RPA-1 appears at the site of damage within 5-10 min of

microirradiation [52], and persists for 6 h [49]. Similarly, RAD-51 is recruited within the same timeframe in irradiated fibroblasts but exhibits faster kinetics than RPA-1 [53].

C. elegans germlines have germ cell population with different cell cycle phases. Distal mitotic region possesses germline stem cells (GSCs) and mitotic germ cells. Once mitotic germ cells enter meiotic cell cycle, they differentiate into either sperm or oocytes in the proximal region. Previous studies by Kimble's and Schedl's groups demonstrated that, at any given time, 50-60% of mitotic germ cells exist at the S phase [54, 55] (Fig. 6A). This finding speculates that 50-60% of ICL-induced cells are repaired by replication-dependent mechanism (i.e., FA) while the remaining cells are not (e.g., NER and TLS) (Fig. 6B). As depicted in Figure 1, damaged cells with ICLs employ different repair mediators depending on cell cycle phase. Thus, germ cells lacking either FA repair pathway (e.g., *fcd-2(tm1298)*) or NER pathway (e.g., *xpa-1(ok698)*) are partially repaired in a cell cycle-dependent manner. However, both pathways require the activities of TLS polymerase complex including POLZ-1 and REV-1 to remove ICLs, which is reflected in our findings that germ cells lacking *polz-1* and/or *rev-1* are more sensitive to ICLs than those lacking either *fcd-2* or *xpa-1* (Fig. 5). It is important to mention that *fcd-2* is dispatched to replication foci under stress [56], following incisions to unhook the ICL by endonucleases, which then progresses to HR in order to repair DSBs [57]. Accordingly, it is reasonable to assume that the lack of *fcd-2* involvement in the genomic maintenance following ICLs suggests that TMP/UV-A-induced ICLs occur outside the S phase. Alternatively, it may take place during replication but that would necessitate for repair proteins to be recruited by upstream, damage-sensing proteins unrelated to FA.

In mammals, REV1 and DNA Polymerase ζ (REV3 and REV7) play important roles in TLS and repair of DSBs [28]. TLS polymerase complex including REV1 and POLZ-1 may function in the same step. However, double mutation is more sensitive to ICLs than single mutation could be explained by three possibilities. First, *polz-1(tm8927)* and *rev-1(tm8701)* alleles may be loss-of-function, but not null, mutants. Second, their function may be redundant with each other. Third, both proteins may have shared and discrete functions in ICL repair pathways. Our recent studies also showed that subunits of the DNA polymerase α-primase complex promote Notch-mediated proliferation with shared and discrete functions in *C. elegans* germline [58]. Distinguishing between these possibilities is beyond the scope of this work but will be an important challenge for the future.

In conclusion, this study presents *in vivo* evidence in support of the major role played by NER, as opposed to FA pathway, in ICL repair in *C. elegans*, which may be reciprocated in higher animals. We also report that TLS polymerases, POLZ-1 and REV-1, are essential to ICL repair, and that lack of either protein in addition to XPA-1, synergistically exacerbates ICL-induced mortality and impedes HR repair. Unlike normal cells or post-mitotic cells, cancer cells are continuously dividing without differentiation. Therefore, our findings may provide insights into cell cycle-targeted chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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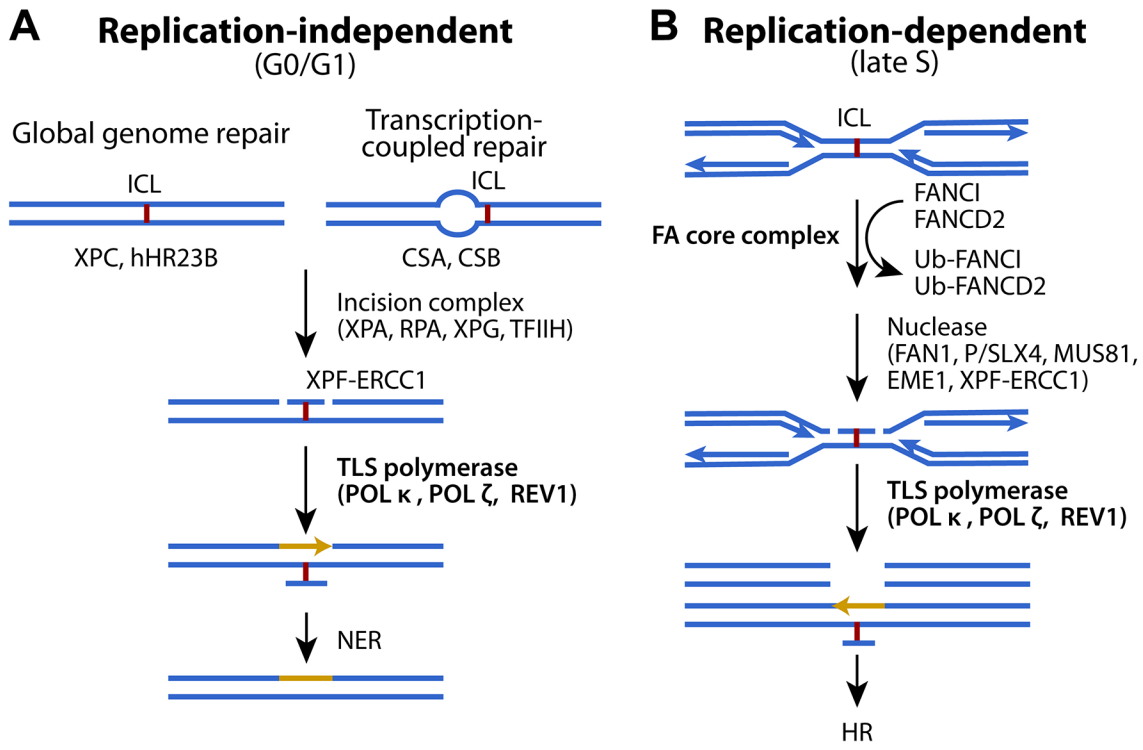

Figure 1. ICL repair pathway at different cell cycle phases.

(A) Replication-independent and (B) replication-dependent ICL repair mechanism. See text for details. Adapted with permission from ref. 1. Copyright 2016 BioMed Central (BMC).

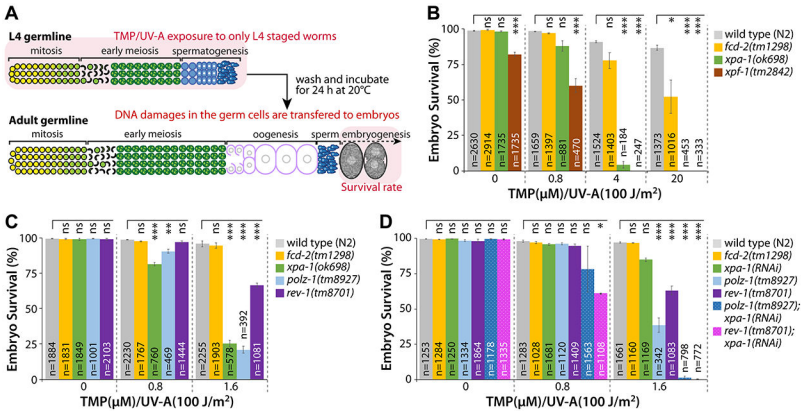

Figure 2. XPA-1, POLZ-1, and REV-1 are required for ICL repair.

(A) Schematic of ICL experiment. TMP/UV-A exposed to only L4 staged whole worms. DNA damages in the germ cells were determined by scoring the survival rate of fertilized embryos. See "Materials and Methods" for details. (B and C) *xpa-1(ok698)*, *polz-1(tm8927)*, or *rev-1(tm8701)* single mutants are more sensitive to ICLs than *fcd-2(tm1298)* mutants. L4 staged worms were treated with TMP/UV-A, and their F1 embryos were collected between 24 and 48 h post treatment. Latched embryos were counted 24 h later. (D) Double deficiencies of *polz-1(tm8927)* or *rev-1(tm8701)* together with *xpa-1(ok698)* significantly increase sensitivity to ICLs. Wild type (N2), *polz-1(tm8927)*, and *rev-1(tm8701)* mutants were fed *E. coli* expressing double-stranded RNA for *xpa-1* from the L1 stage. L4 staged worms were treated with TMP/UV-A and their F1 embryos' survival was scored. *P* values: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, no statistical significance. See Table S2 for One-way ANOVA analysis.

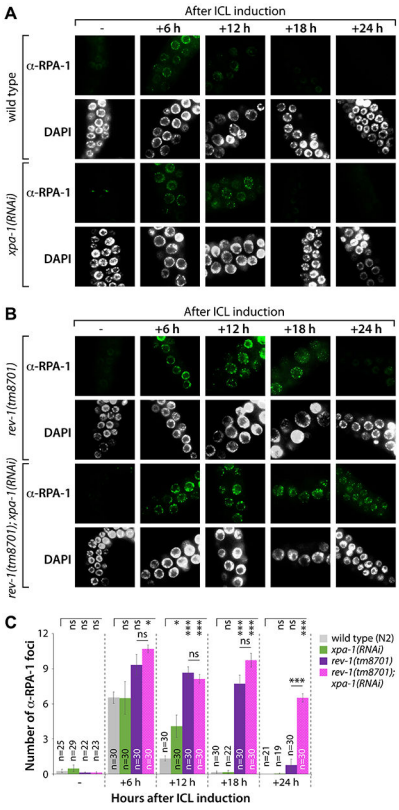

Figure 3. Formation and disappearance of RPA-1 foci after ICL induction are influenced by *rev-1* mutation.

(A and B) L4 stage worms of wild type (N2), *xpa-1(RNAi)*, *rev-1(tm8701)*, and *rev-1(tm8701); xpa-1(RNAi)* were treated with TMP (200 μM) for 40 min and exposed to UV-A light (100 J/m^2). Gonads were dissected, fixed, and immunostained using RPA-1 antibody at 0, 6, 12, 18 and 24 h after ICL induction. Scale bar, 10 μm. (C) The number of RPA-1 foci per nuclear focal plane was counted for over 100 mitotic germ cells in each stain. *P* values: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, no statistical significance. See Table S2 for One-way ANOVA analysis.

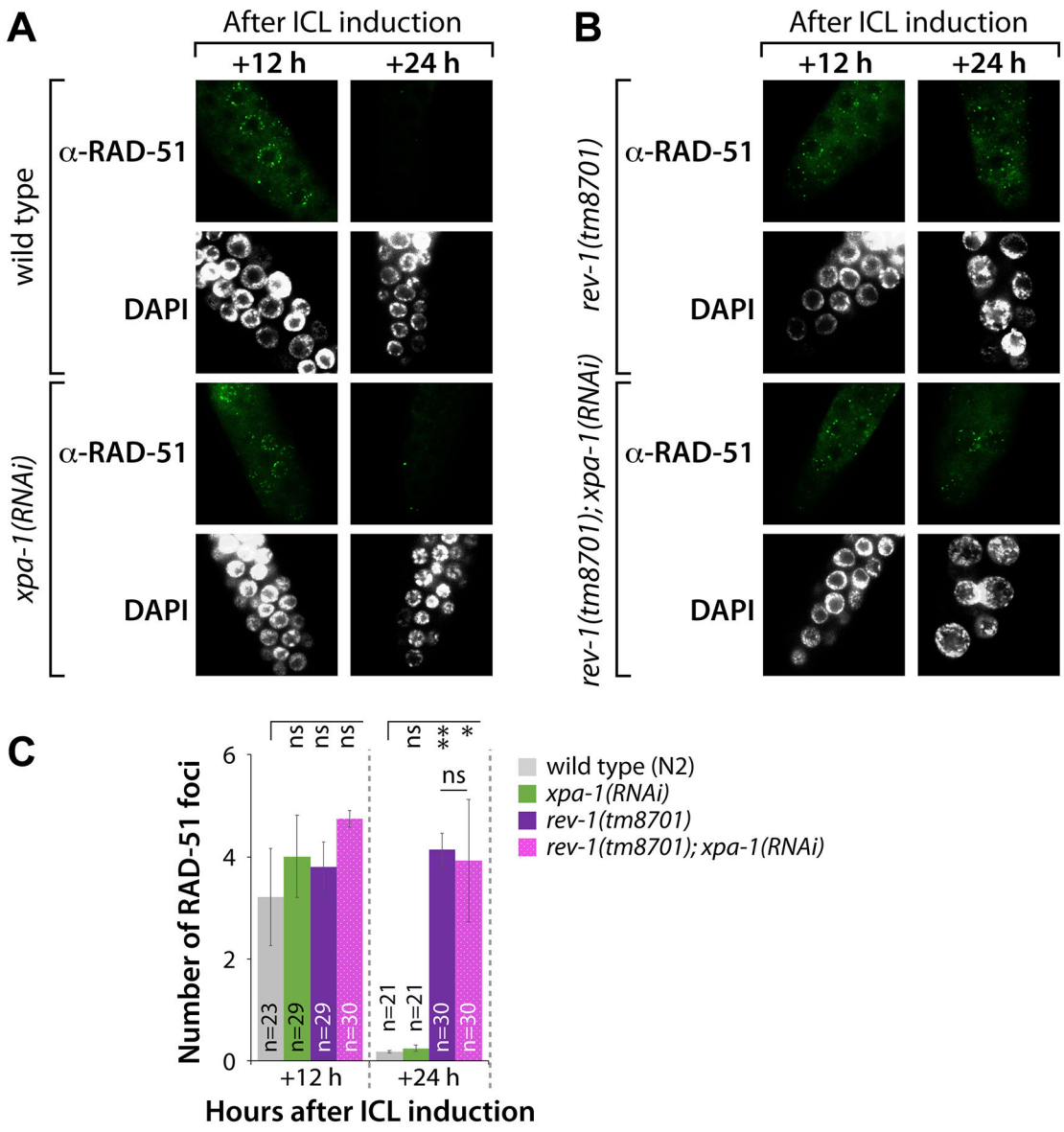

Figure 4. Disappearance but not formation of RAD-51 foci is delayed by *rev-1* mutation after ICL induction in mitotically proliferating germ cells.

(A and B) L4 stage worms of wild type (N2) and *xpa-1(RNAi)*, *rev-1(tm8701)* and *rev-1(tm8701); xpa-1(RNAi)* were treated with TMP (200 μM) for 40 min and exposed to UV-A light (100 J/m^2). Gonads were dissected, fixed, and immunostained at 12 and 24 h using RAD-51 antibody. Scale bar, 10 μm. (C) The number of RAD-51 foci per nuclear focal plane was scored for over 100 mitotic germ cells in each stain. *P* values: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, no statistical significance. See Table S2 for One-way ANOVA analysis.

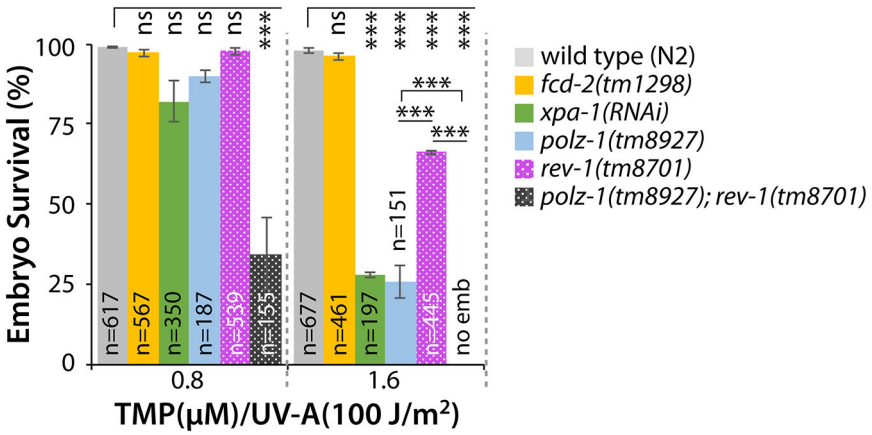

Figure 5. *polz-1* and *rev-1* double mutants are more hypersensitive to ICLs than either single mutants.

L4 stage worms were treated with TMP (0.8 and 1.6 μM) and exposed to UV-A (100 J/m^2) to score embryonic mortality. *P* values: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, no statistical significance. See Table S2 for One-way ANOVA analysis.

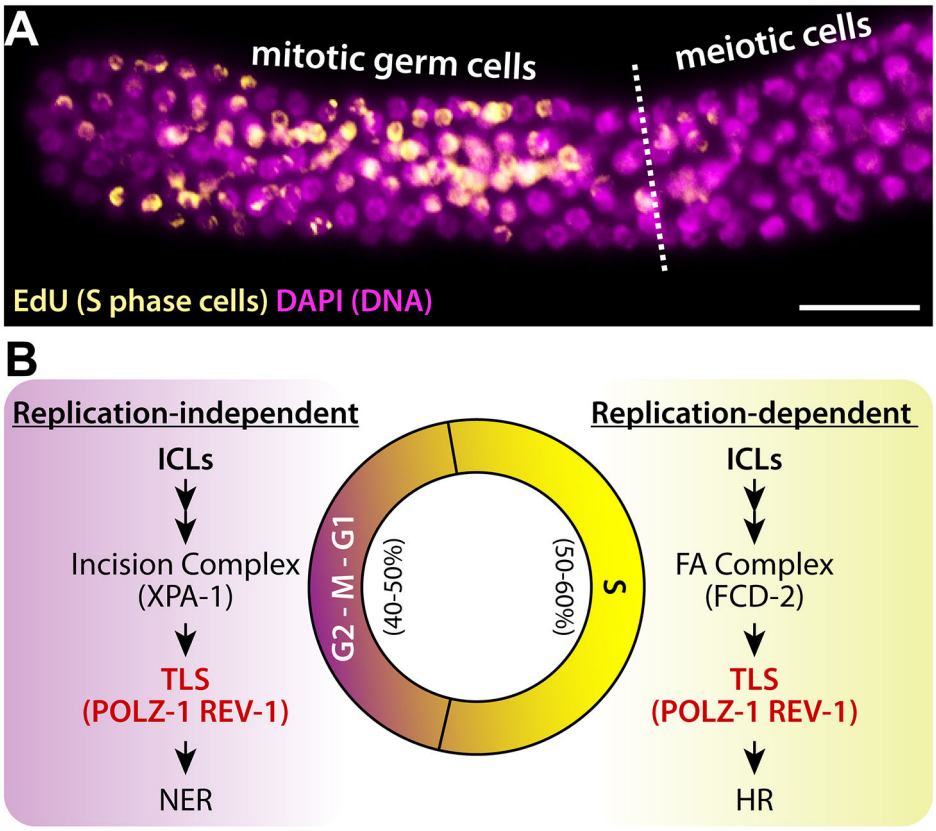

Figure 6. A proposed model for ICL repair in *C. elegans* germline.

(A) EdU stained adult *C. elegans* germline. Scale bar: 20 μm (B) Cell cycle-dependent ICL repair mechanism in the *C. elegans* germline.