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WRNIP1 functions upstream of DNA polymerase η in the UV-induced DNA damage response

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ABSTRACT

WRNIP1 (WRN-interacting protein 1) was first identified as a factor that interacts with WRN, the protein that is defective in Werner syndrome (WS). WRNIP1 associates with DNA polymerase η (Polη), but the biological significance of this interaction remains unknown. In this study, we analyzed the functional interaction between WRNIP1 and Polη by generating knockouts of both genes in DT40 chicken cells. Disruption of *WRNIP1* in Polη-disrupted (*POLH*$^{-/-}$) cells suppressed the phenotypes associated with the loss of Polη: sensitivity to ultraviolet light (UV), delayed repair of cyclobutane pyrimidine dimers (CPD), elevated frequency of mutation, elevated levels of UV-induced sister chromatid exchange (SCE), and reduced rate of fork progression after UV irradiation. These results suggest that WRNIP1 functions upstream of Polη in the response to UV irradiation.

1. Introduction

Werner syndrome (WS) is a rare autosomal recessive disorder characterized by accelerated aging and genomic instability. WS is caused by mutation in the *WRN* gene, which encodes a member of the RecQ family of DNA helicases [1]. In the course of our studies of WRN function, we identified a novel WRN-interacting protein, originally named WHIP (Werner helicase-interacting protein) and later renamed WRNIP1 (WRN-interacting protein 1) [2]. WRNIP1 belongs to the AAA + ATPase family of proteins, and is conserved from *Escherichia coli* to humans. WRNIP1 exhibits amino-acid sequence homology with the replication factor C (RFC) family of clamp-loader proteins and possesses an ATPase domain containing Walker A and B motifs in the middle region of the molecule. In addition, WRNIP1 contains a ubiquitin-binding zinc-finger (UBZ) domain and two leucine-zipper motifs in its N-terminal and C-terminal regions, respectively.

The budding yeast ortholog of WRNIP1, called Mgs1 (maintenance of genome stability 1), interacts with proliferating cell nuclear antigen (PCNA) and Polδ [3,4]. Overexpression of Mgs1 is lethal in mutants defective in proteins related to DNA replication [5]. Loss-of-function *MGS1* mutations exhibit synthetic-lethal and synthetic-sick interactions with mutations in *RAD6* and *RAD18*, respectively [6]. The products of both *RAD6* and *RAD18* are involved in ubiquitylation of PCNA [7]. This Rad6/Rad18-dependent ubiquitylation of PCNA mediates post-replication repair, which is a mechanism for bypassing lesions during DNA replication [8,9]. Post-replication repair is mediated by translesion synthesis (TLS) or template switching, pathways involving mono- and poly-ubiquitylation of PCNA, respectively [10,11]. Mgs1 is targeted to sites of replication stress by ubiquitylated PCNA [12]. WRNIP1 interacts with Rad18 and interferes with its binding to DNA [13]. In addition, WRNIP1 binds ubiquitin and polyubiquitin with its UBZ domain [14]. In light of these observations, it is likely that WRNIP1 functions in an early step of TLS and/or template switching.

In TLS, replication is taken over by a pathway-specific polymerase such as DNA polymerase η (Polη), which can elongate nascent DNA despite the presence of lesions on the template [15,16,9]. Thus, Polη can efficiently and accurately bypass UV-induced cyclobutane pyrimidine dimers (CPDs). *POLH*, the gene that encodes Polη, is mutated in the variant form of xeroderma pigmentosum (XPV), an autosomal recessive disorder characterized by early onset of skin cancer [17]. UV-induced ubiquitylation of PCNA promotes polymerase switching from the replicative polymerases, DNA polymerase δ (Polδ) and DNA polymerase ε (Polε), to TLS-specific polymerases such as Polη, in order to complete the replication process [18]. WRNIP1 interacts physically with Polη [19]. In addition, WRNIP1 interacts with Polδ and stimulates its DNA-synthesizing activity *in vitro*, probably by promoting recycling of

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Polδ [20]. Because WRNIP1 interacts with both of these polymerases, the circumstantial evidence described above indicates that WRNIP1 acts in the TLS pathway and facilitates the switch from Polδ to Polη. However, the precise role of WRNIP1 in the regulation of TLS in response to DNA damage remains unknown because no previous study has demonstrated a functional interaction between WRNIP1 and Polη.

To elucidate the functional relationship between Polη and WRNIP1, we performed genetic analysis. Disruption of *WRNIP1* resulted in suppression of the UV sensitivity of *POLH*$^{-/-}$ cells, suggesting that WRNIP1 functions upstream of Polη in the TLS pathway. To understand the role of WRNIP1 in the TLS pathway, as well as the mechanism underlying suppression of UV sensitivity of *POLH*$^{-/-}$ cells by disruption of *WRNIP1*, we investigated the rate of disappearance of CPDs, frequencies of mutation and SCE, and progression of replication forks after UV irradiation in these knockouts.

2. Materials and methods

2.1. Immunoprecipitation

293E cells were transfected with constructs encoding Myc-tagged human WRNIP1 and HA-tagged human Polη, using Lipofectamine 2000 Reagent (Invitrogen). Immunoprecipitation from cell lysates using anti-HA agarose beads (SIGMA) was described previously [13]. Proteins were separated by SDS–PAGE and detected by western blotting using anti-HA or anti-Myc antibody (Sigma).

2.2. Cell lines and cell culture

To generate *WRNIP1*-deficient DT40 cells, three *WRNIP1* disruption constructs were generated from polymerase chain reaction (PCR)-amplified genomic DNAs containing histidinol (his), blasticidin (bsr), or Ecogpt selection-marker cassettes. DT40 cells were successively transfected with his-*WRNIP1*, bsr-*WRNIP1*, and Ecogpt-*WRNIP1*. To generate *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells, the three *WRNIP1* gene-disruption constructs (his-*WRNIP1*, Ecogpt-*WRNIP1*, and bleo-*WRNIP1*) were introduced sequentially into *POLH*$^{-/-}$ cells. Chicken *WRNIP1* (c*WRNIP1*) cDNA was prepared by RT-PCR, and an HA tag was added to the N-terminus by PCR. HA-*WRNIP1* was inserted into expression vector pcDNA 3.1. Cells expressing *WRNIP1* were obtained by transfection of HA-*WRNIP1* into *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells. Cell growth was analyzed as described previously [21].

2.3. Assay for sensitivity to UV

UV sensitivity was evaluated using a Cell Counting Kit-8 (Dojindo). After being washed with PBS, cells (1×10^4) were irradiated with UV, and then further cultured for 48 h. Viability was assessed by the ability of cells to convert WST-8 into formazan, which was quantitated by spectrophotometry. Viability was expressed as a percentage relative to unirradiated cells (defined as 100%).

2.4. Detection of CPD by ELISA

Cells were irradiated with UV, and then harvested immediately or at various times after UV irradiation (to allow for repair). Genomic DNA was purified using the Easy DNA kit (Life Technologies). DNA concentrations were calculated based on the absorbance at 260 nm. CPDs were detected by ELISA using a monoclonal antibody, as described previously [22].

2.5. Assay for mutation frequency

Cells were irradiated with 6 J/m^2 UV and incubated for 2 days. Cells (1.0×10^7) that survived after UV exposure were suspended in 100 ml of RPMI-1640 medium containing 6-thioguanine (1.5 μg/ml), and 200 μl of each cell suspension was dispensed into five 96-well plates. Colony-forming efficiency at the time of selection was determined by dispensing 100 cells into two 96-well plates in medium without 6-thioguanine. Colonies were counted after 8 days. Damage-induced mutation frequencies were calculated as follows: (number of resistant colonies)/[(number of cells plated for selection) × (colony-forming efficiency at time of selection)].

2.6. Detection of SCE

Detection of SCE was performed as described previously [21]. For UV-induced SCE, cells were suspended in PBS and irradiated with 1 J/m^2 UV, followed by BrdU labeling.

2.7. DNA fiber assay

The DNA fiber assay was carried out as described previously [28]. Cells were pulse-labeled with CldU for 10 min, and then irradiated with UV (8 J/m^2). Fifteen minutes after irradiation of UV, cells were pulse-labeled with IdU for 10 min.

3. Results and discussion

3.1. WRNIP1 is implicated in the UV-induced TLS pathway upstream of Polη

A previous mass-spectrometric analysis suggested that WRNIP1 interacts with Polη [19]. To test this idea, we investigated whether WRNIP1 physically interacts with Polη in cells. WRNIP1 and Polη co-precipitated in pull-down experiments, suggesting that the two proteins do in fact interact, although the amount of WRNIP1 co-immunoprecipitated with Polη was low (Fig. 1A).

To further investigate the functional interaction between WRNIP1 and Polη, we generated *WRNIP1*$^{-/-/-}$ and *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ derivatives of the chicken cell line DT40 (note that *WRNIP1* resides on *Gallus gallus* chromosome 2, which is trisomic in DT40). A previous study by our group showed that *WRNIP1*$^{-/-/-}$ DT40 cells expressed a low level of WRNIP1 containing a 52 amino-acid deletion [21]. Therefore, we designed new gene-targeting constructs to delete exon 1 of *WRNIP1*, and then generated *WRNIP1*$^{-/-/-}$ and *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells by sequential transfection of the new constructs into wild-type and previously generated *POLH*$^{-/-}$ cells [23], respectively. Disruption of *WRNIP1* in both *WRNIP1*$^{-/-/-}$ and *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells was confirmed by RT-PCR (Fig. 1B). *WRNIP1*$^{-/-/-}$ cells and *POLH*$^{-/-}$ cells grew at almost the same rate as wild-type cells, whereas *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells grew slightly more slowly than either single knockout (Fig. 1C).

As reported previously, *POLH*$^{-/-}$ cells were highly sensitive to UV, consistent with the phenotype of XP-V cells [23]. On the other hand, *WRNIP1*$^{-/-/-}$ cells exhibited UV sensitivity similar to that of wild-type cells, suggesting that WRNIP1 is not essential for the TLS pathway itself. Remarkably, disruption of *WRNIP1* suppressed UV sensitivity of *POLH*$^{-/-}$ cells: the *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells were less sensitive to UV than *POLH*$^{-/-}$ cells (Fig. 1D). To further confirm this result, we introduced an expression vector carrying chicken *WRNIP1* cDNA into *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells. The presence of wild-type WRNIP1 protein restored the UV sensitivity of *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells to the level of *POLH*$^{-/-}$ cells (Fig. 1D).

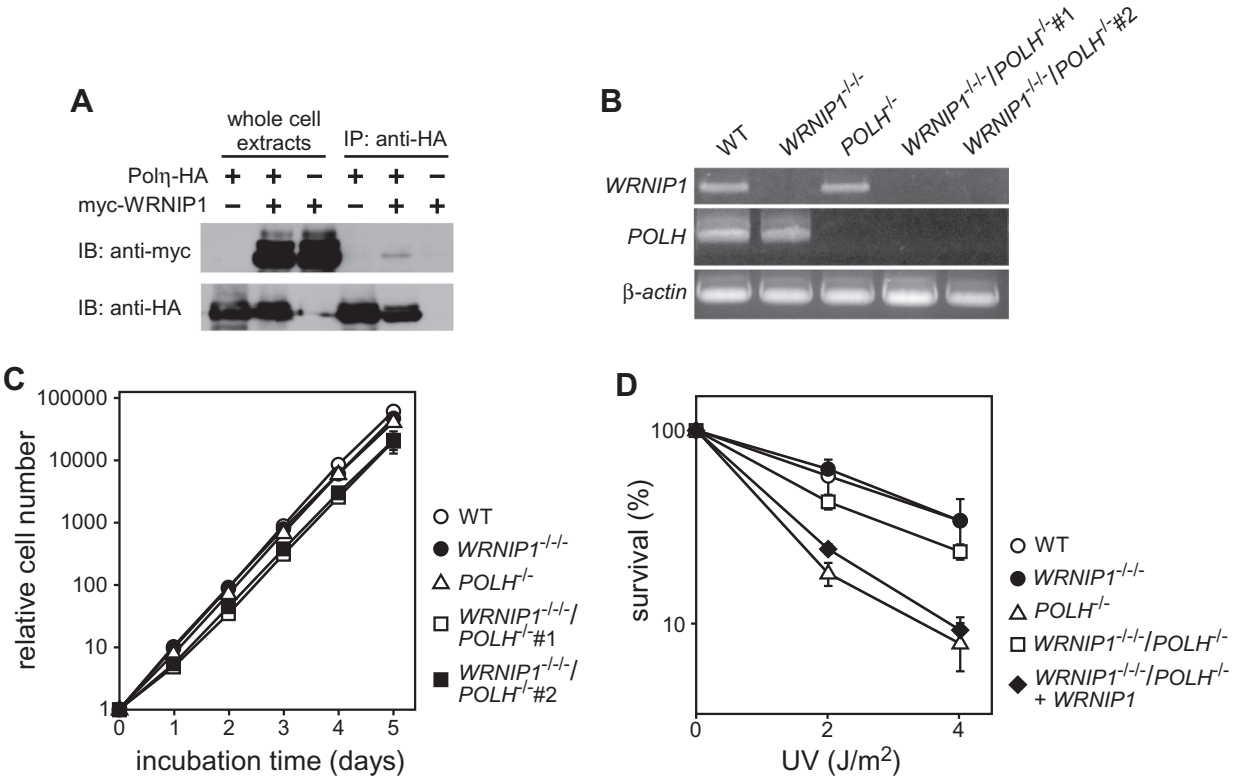

Fig. 1. (A) Co-immunoprecipitation of WRNIP1 and Polη. 293E cells were transfected with c-myc-*WRNIP1* and HA-*POLH*. Immunoprecipitation was performed using HA-beads, and precipitated proteins were analyzed by western blotting. (B) Confirmation of *WRNIP1* and *POLH* gene disruption by RT-PCR. β-actin mRNA was amplified as a control. (C) Growth curve. Numbers of viable cells were counted and expressed as relative values. (D) UV sensitivity of mutant cells. Wild-type and various mutant cells were irradiated with UV; 2 days after UV irradiation, viability was measured and expressed as a percentage relative to unirradiated cells (defined as 100%).

These results suggest that WRNIP1 functions upstream of Polη in the TLS pathway, and that in the absence of WRNIP1, DNA damage caused by UV is bypassed or dealt with by a Polη-independent pathway.

3.2. How are UV-induced lesions resolved in the absence of WRNIP1 and Polη?

Progressing replication forks can deal with DNA lesions and avoid collapse via several pathways, including the TLS pathway and recombination-mediated pathways that use the sister chromatid as a template, including homologous recombination (HR) and template switching. The suppression of UV sensitivity by *WRNIP1* disruption in $POLH^{-/-}$ cells suggests that in the absence of WRNIP1, cells may deal with DNA lesions by one of the aforementioned pathways that do not use Polη.

We first analyzed the disappearance of CPDs from UV-irradiated cells to determine whether the suppression of UV sensitivity in the $WRNIP1^{-/-/-}/POLH^{-/-}$ cells is associated with alterations in repair of UV-induced DNA lesions. CPDs are the most prominent UV-generated DNA lesions; Polη can efficiently and accurately synthesize DNA on template containing CPDs, especially thymine dimers, which are later removed by nucleotide excision repair. We used ELISA to monitor removal of CPDs after UV irradiation. In wild-type cells, about 50% of the CPDs were removed within 24 h, whereas almost no removal of CPDs was observed in $POLH^{-/-}$ cells at the same time point. In $WRNIP1^{-/-/-}/POLH^{-/-}$ cells, the extent of removal of CPD was similar to that in $WRNIP1^{-/-/-}$ cells, i.e., close to the level in wild-type cells. These results suggest that disruption of *WRNIP1* activates a pathway for repairing CPDs that does not require Polη.

One possible mechanism for dealing with CPD in the absence of Polη is to use TLS DNA polymerases other than Polη, which are mutagenic on templates containing CPDs [24]. To determine whether these TLS polymerases are utilized in the $WRNIP1^{-/-/-}/POLH^{-/-}$ cells, we assayed mutation frequency in *HGPRT*, which encodes hypoxanthine–guanine phosphoribosyl transferase, by measuring the appearance of colonies in the presence of 6-thioguanine (6-TG). Spontaneous mutation frequencies of wild-type, $WRNIP1^{-/-/-}$, $POLH^{-/-}$, and $WRNIP1^{-/-/-}/POLH^{-/-}$ cells were almost the same, but the frequency of UV-induced mutations was about 10-fold higher in $POLH^{-/-}$ cells than in wild-type and $WRNIP1^{-/-/-}$ cells (Fig. 2B). However, $WRNIP1^{-/-/-}/POLH^{-/-}$ cells had a mutation frequency as low as that of wild-type cells, indicating that mutagenic TLS polymerases are not involved in bypassing CPDs in $WRNIP1^{-/-/-}/POLH^{-/-}$ cells.

It is conceivable that recombination with the sister chromatid is involved in bypassing CPDs in an error-free manner in $WRNIP1^{-/-/-}/POLH^{-/-}$ cells. SCE is a very sensitive index of HR accompanying crossover [25]. Therefore, to determine whether the suppression of UV sensitivity of $POLH^{-/-}$ cells by disruption of *WRNIP1* is due to channeling of DNA lesions to HR, we measured the frequency of SCE in the gene-disrupted cells. The level of spontaneous SCE was slightly elevated in $POLH^{-/-}$, $WRNIP1^{-/-/-}$, and $WRNIP1^{-/-/-}/POLH^{-/-}$ cells relative to wild-type cells (Fig. 2C). After UV irradiation, however, $POLH^{-/-}$ cells exhibited significantly more SCE than wild-type cells [26]. In $WRNIP1^{-/-/-}/POLH^{-/-}$ cells, the level of SCE was higher than that of wild-type cells, but lower than that of $POLH^{-/-}$ cells, suggesting that the reduction in UV sensitivity of $POLH^{-/-}$ cells by disruption of *WRNIP1* is not due to an increased contribution of HR to repair UV-induced DNA lesions. However, because the assay detects only HR that accompany crossovers

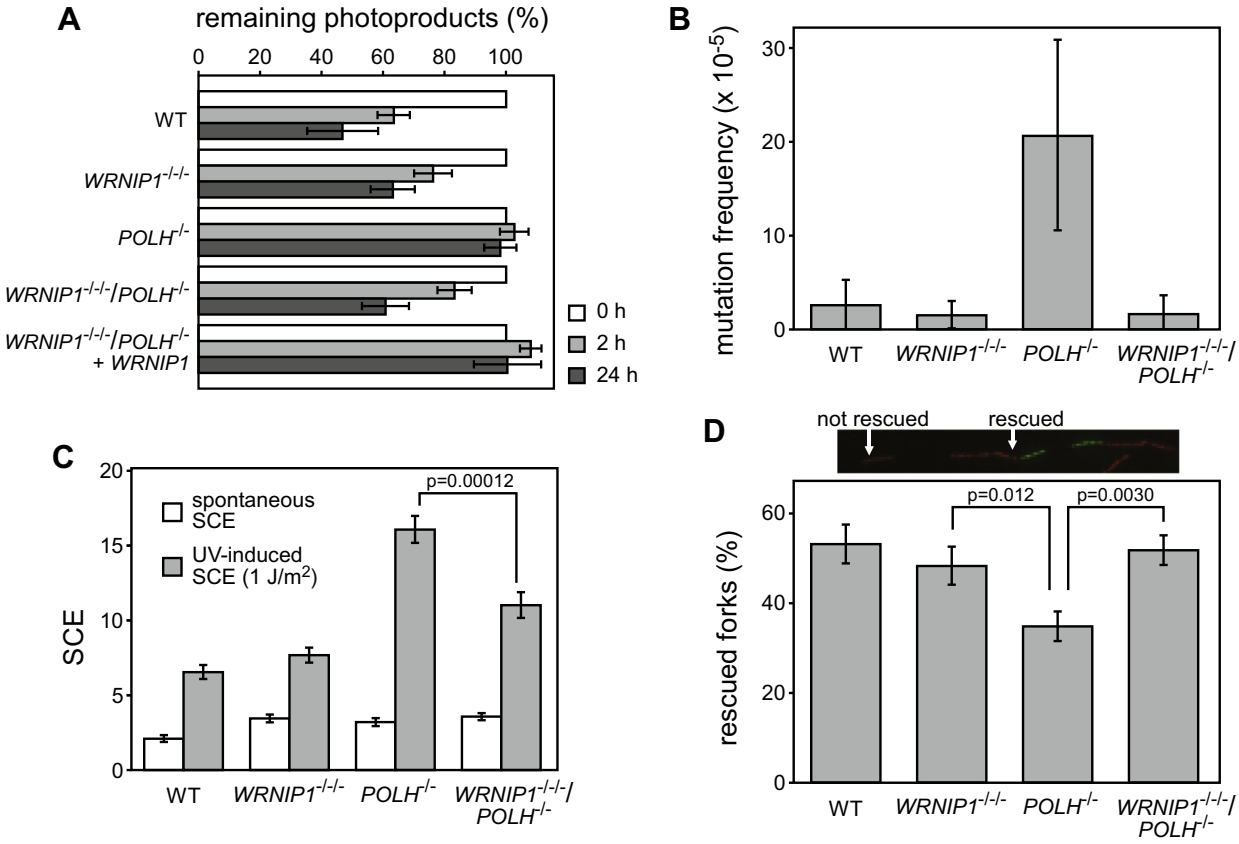

Fig. 2. (A) Removal of UV-induced CPDs. Cells were irradiated with 2 J/m^2 UV and incubated for the indicated time periods to allow repair. Cells were harvested, and CPD levels in their DNA were determined by ELISA. (B) Mutation frequency. Cells were irradiated with 6 J/m^2 UV and incubated for 2 days. Mutation frequency was measured by monitoring the appearance of 6-thioguanine–resistant cells. Data are representative of four independent experiments. Error bars indicate S.D. (C) Histograms of SCE. Bars represent mean scores from 50 metaphases of cells irradiated by UV (black) or unirradiated (white). Error bars indicate S.D. from three independent experiments. (D) Restarting of stalled forks. Top panel: image of DNA fibers showing a red track only, corresponding to an arrested, not rescued fork (NR), and a red–green color track corresponding to a rescued fork (R). Percentage of fork rescue was calculated as [R/(NR + R) × 100]. Error bars indicate S.D. from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between sister chromatids, we cannot exclude the possibility that *WRNIP1* disruption activates HR without crossover. It is also possible that depletion of WRNIP1 in *POLH*$^{-/-}$ cells promotes template switching or synthesis-dependent strand annealing, resulting in the bypass of DNA lesions.

Replication-fork progression on UV-damaged DNA is delayed or blocked in the absence of Polη [27,28]. Thus, we investigated whether suppression of the UV sensitivity of *POLH*$^{-/-}$ cells by depletion of WRNIP1 is somehow related to recovery from the defect in replication-fork progression. To this end, we analyzed replication-fork progression on UV-damaged DNA using the DNA fiber assay. Cells were pulse-labeled with the thymidine analog CldU (red), and then irradiated with UV (8 J/m^2). After incubation, the cells were pulse-labeled with the thymidine analog IdU (green). Arrest of replication-fork progression due to UV irradiation was indicated by reduced levels (or absence) of IdU in CldU-labeled fibers (Fig. 2D. Upper panel). *WRNIP1* disruption restored replication-fork progression in *POLH*$^{-/-}$ cells to the level in *WRNIP1*$^{-/-/-}$ cells (Fig. 2D. Lower panel). This result suggests that WRNIP1 may deploy Polη to bypass DNA lesions induced by UV. Thus, in the absence of WRNIP1, another polymerase that is not mutagenic takes the place of Polη in synthesizing DNA on a UV-damaged template.

In this study, we compared UV sensitivity, elimination of CPDs, frequency of mutations and SCE, and progression of replication forks between *POLH*$^{-/-}$ and *WRNIP1*$^{-/-/-}$/*POLH*$^{-/-}$ cells. The results showed that all phenotypes observed in *POLH*$^{-/-}$ cells were suppressed by disruption of *WRNIP1*, indicating that WRNIP1 functions upstream of Polη in the TLS pathway, and that in the absence of WRNIP1, DNA damage caused by UV was bypassed by a Polη-independent pathway. Recently, a new polymerase accompanying primase activity, PrimPol, was discovered in eukaryotic cells [28,29]. PrimPol can incorporate nucleotides opposite a T–T 6–4 photoproduct, but not a T–T CPD; however, PrimPol can elongate DNA starting from a dA opposite a T–T CPD. The PrimPol-mediated pathway for dealing with UV-induced lesions is unlikely to be epistatic to the Polη-dependent pathway. Under certain circumstances, Polδ incorporates two dA residues opposite a T–T CPD *in vitro* [30,31]. The budding yeast ortholog of WRNIP1, Mgs1, interacts with Polδ [3] and PCNA [4], and inhibits the interaction between Polδ and PCNA [32]. Furthermore, WRNIP1 interacts with Polδ and stimulates its DNA-synthesizing activity by increasing recycling of Polδ [20]. In addition, WRNIP1 forms foci after UV irradiation in a UBZ domain-dependent manner [33].

Based on all of these observations, we propose a working model in which Polδ and PrimPol are involved in dealing with UV damage in the absence of WRNIP1 and Polη. When a replication fork encounters UV lesions such as CPD, WRNIP1 is recruited to the site containing the UV lesions, where it removes Polδ to promote recruitment of Polη to the damaged site (Fig. 3). Then, in the absence of WRNIP1, Polδ will be retained in the replication machinery and continue DNA synthesis in a template containing a CPD; subsequently, PrimPol engages in DNA replication at the damage site, resulting in non-mutagenic translesion DNA synthesis. This scenario explains the suppression of *POLH*$^{-/-}$ phenotypes by depletion of WRNIP1, i.e., the suppression of UV sensitivity,

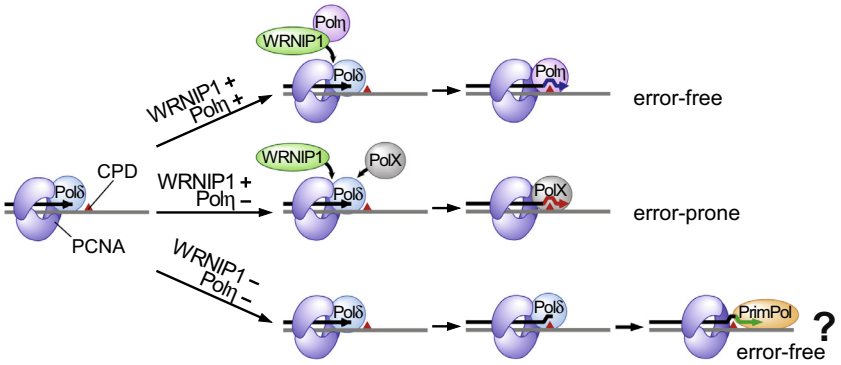

Fig. 3. Working model for the mechanism for dealing with CPDs in the absence of WRNIP1 and Polη.

reduction in the frequencies of mutation and SCE formation, and restoration of replication-fork progression. Further studies of the functions of WRNIP1 should provide insights into a novel error-free damage-tolerance mechanism.

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