

## The role of WRNIP1 in genome maintenance

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### ABSTRACT

WRNIP1 interacts with WRN helicase, which is defective in the premature aging disease Werner syndrome. WRNIP1 belongs to the AAA+ ATPase family and is conserved from *Escherichia coli* to human. The protein contains an ubiquitin-binding zinc finger (UBZ) domain at the N terminus and an ATPase domain in the middle region. In addition to WRN, WRNIP1 interacts with proteins involved in multiple cellular pathways, including RAD18, monoubiquitylated PCNA, DNA polymerase  $\delta$ , RAD51, and ATMIN. Mgs1, the yeast homolog of WRNIP1, may act downstream of ubiquitylation of PCNA to mobilize DNA polymerase  $\delta$ . By contrast, the functions of WRNIP1 in higher eukaryotic cells remain obscure, although data regarding the roles of WRNIP1 in DNA transactions have emerged recently. Here, we first describe the functions of Mgs1 in DNA transaction. We then describe various features of WRNIP1 and discuss its possible roles based on recent studies of the function of WRNIP1.

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### Introduction

Genome stability is essential for life. However, living cells are constantly exposed to environmental and endogenous DNA-damaging agents that cause the formation of DNA lesions, which block replication. In addition to DNA adducts, other factors such as topological stress, aberrant DNA structure, and depletion of nucleotide pools can induce replication block. Multiple mechanisms have evolved in prokaryotic and eukaryotic cells to remove DNA lesions, allowing cells to cope with replication block.

DNA helicases, enzymes that unwind structured DNA, play essential roles in preserving genome integrity. In particular, the helicases implicated in the DNA damage response play prominent roles in DNA damage sensing, fork maintenance, replication restart, and DNA repair. The RecQ family consists of highly conserved helicases with diverse roles in multiple DNA metabolic processes. One member of the RecQ family, WRN, is mutated in patients with Werner syndrome (WS), a genetic disease characterized by premature aging associated with genome instability and an elevated risk of cancer.<sup>1</sup>

WRNIP1 was originally identified as Werner helicase-interacting protein (WHIP) in a yeast 2-hybrid screen<sup>2</sup> and subsequently renamed Werner helicase-interacting protein 1 according to HUGO nomenclature conventions. WRNIP1 belongs to the AAA+ ATPase family and is conserved from *Escherichia coli* to human. The homology of WRNIP1 to members of the replication factor C family and its ability to stimulate the DNA-synthesis activity of DNA polymerase  $\delta$ <sup>3</sup> suggest a possible role for WRNIP1 in DNA replication and/or replication-related DNA

transactions. The *Saccharomyces cerevisiae* homolog of WRNIP1, Mgs1 (maintenance of genome stability 1), is an evolutionarily conserved AAA+ ATPase that is crucial for maintenance of genomic stability.<sup>4</sup>

In this review, we will provide an overview of the properties and functions of Mgs1, and then focus on WRNIP1. Specifically, we will address the protein's structure and interacting partners, and recent advances in understanding the roles of WRNIP1 in DNA replication and maintenance of stalled replication forks.

### Function of Mgs1

*MGS1* was identified by computational research for *S. cerevisiae* proteins homologous to RuvB.<sup>4</sup> Accordingly, Mgs1 is similar to *E. coli* RuvB and the eukaryotic clamp loader protein RFC, and all 3 of these proteins share the Walker A/B and sensor I/II motifs characteristic of the AAA+ ATPase family. Mgs1 has DNA-dependent ATPase and single-strand DNA annealing activities. Deletion of *MGS1* results in an elevated rate of mitotic recombination, and this phenotype is synergistic with mutation in *SGS1*, which encodes a RecQ family helicase considered to be the yeast homolog of WRN: the *mgs1 sgs1* double mutant exhibits more severe growth defects and genome instability than either single-gene mutant.<sup>4,5</sup>

Mutation in *MGS1* is synthetically lethal with mutation in *RAD6*, a gene involved in DNA damage tolerance, suggesting that Mgs1 participates in handling stalled replication forks.<sup>6</sup> The Rad6-dependent DNA damage tolerance pathway allows

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stalled replication forks to bypass the causative damage, via either error-prone translesion DNA synthesis (TLS) or an error-free damage avoidance mechanism that may involve template switching to the undamaged sister chromatid. The Rad6–Rad18 complex, which conjugates ubiquitin to PCNA, participates in both branches of the DNA damage tolerance pathway.<sup>7</sup> In the damage avoidance mechanism, the Mms2–Ubc13–Rad5 complex ubiquitylates monoubiquitylated PCNA to generate polyubiquitylated PCNA.<sup>8</sup>

Mgs1 physically associates with PCNA and is thought to suppress the Rad6-mediated DNA damage tolerance pathway in the absence of exogenous DNA damage.<sup>9</sup> Like WRNIP1, Mgs1 has a ubiquitin-binding zinc finger (UBZ) domain (Fig. 1) and preferentially interacts with ubiquitylated PCNA, especially polyubiquitylated PCNA, both *in vitro* and *in vivo*.<sup>10</sup> Overexpression of Mgs1 sensitizes cells to DNA-damaging agents such as MMS and HU, and also suppresses damage-induced mutagenesis.<sup>4,11</sup> The increase in DNA damage sensitivity upon overexpression of Mgs1 is dependent on the UBZ domain and the presence of Rad18,<sup>10</sup> implying that excess Mgs1 cripples the post-replication repair pathway by interacting with ubiquitylated PCNA. Overexpression of Mgs1 exacerbates the temperature sensitivity and DNA damage sensitivity of DNA polymerase  $\delta$  mutants, whereas deletion of *MGS1* reduces temperature and damage sensitivity.<sup>6,11,12</sup> Mgs1 inhibits the interaction of Pol32, a subunit of DNA polymerase  $\delta$  with ubiquitylated PCNA<sup>10</sup>; thus Mgs1 acts downstream of PCNA ubiquitylation to mobilize DNA polymerase  $\delta$ . Because Pol32 is also a subunit of the error-prone DNA polymerase  $\zeta$ ,<sup>13</sup> competition between Mgs1 and Pol32 for interaction with ubiquitylated PCNA could explain the suppression of damage-induced mutagenesis by Mgs1 overexpression.

The synthetic lethality of the *mgs1* and *rad6* mutations can be rescued by expression of Mgs1 lacking the UBZ domain,<sup>10</sup> suggesting that Mgs1 functions in other pathways in which ubiquitylation of PCNA is not required. In the context of DNA replication, Mgs1 may contribute to processing of Okazaki fragments by stimulating Fen1, an endonuclease required for the removal of the flap ends of these fragments.<sup>14</sup>

PCNA is modified with SUMO as well as ubiquitin; sumoylated PCNA then recruits Srs2 DNA helicase, which displaces Rad51 from the single-stranded DNA–Rad51 complex. Synthetic lethality seems to be rescued by the Rad51-dependent homologous recombination pathway since mutation of *SRS2* or genes involved in sumoylation of PCNA suppresses synthetic lethality (Fig. 2A).<sup>9</sup> Thus, when the Rad6-dependent DNA damage tolerance and HR pathways are blocked, an alternative pathway involving Mgs1 becomes indispensable for life, even in the absence of exogenous DNA damage.<sup>15</sup> Physical association of Mgs1 with non-ubiquitylated PCNA, along with the genetic and physical interaction of Mgs1 with DNA polymerase  $\delta$ , suggests that the role of Mgs1 in the alternative pathway is related to the regulation of DNA polymerase  $\delta$ . This issue should be addressed in a future study.

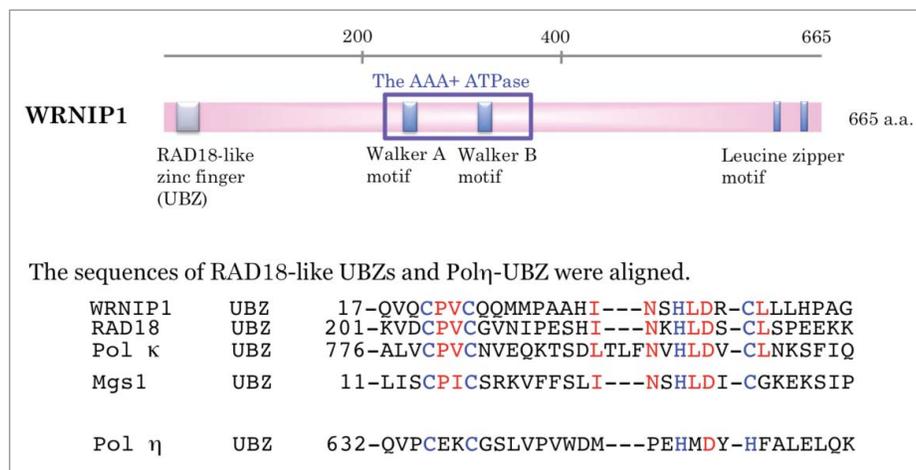
The observations described above suggest that Mgs1 acts to mobilize DNA polymerase  $\delta$  at the replication fork by directly interfering with the PCNA–DNA polymerase  $\delta$  interaction. Mgs1 may facilitate release of the polymerase by blocking the interaction of the enzyme with ubiquitylated PCNA during the polymerase switch associated with TLS and the events preceding strand exchange in preparation for template switching (Fig. 2A).

### The *WRNIP1* gene and structure of the *WRNIP1* protein

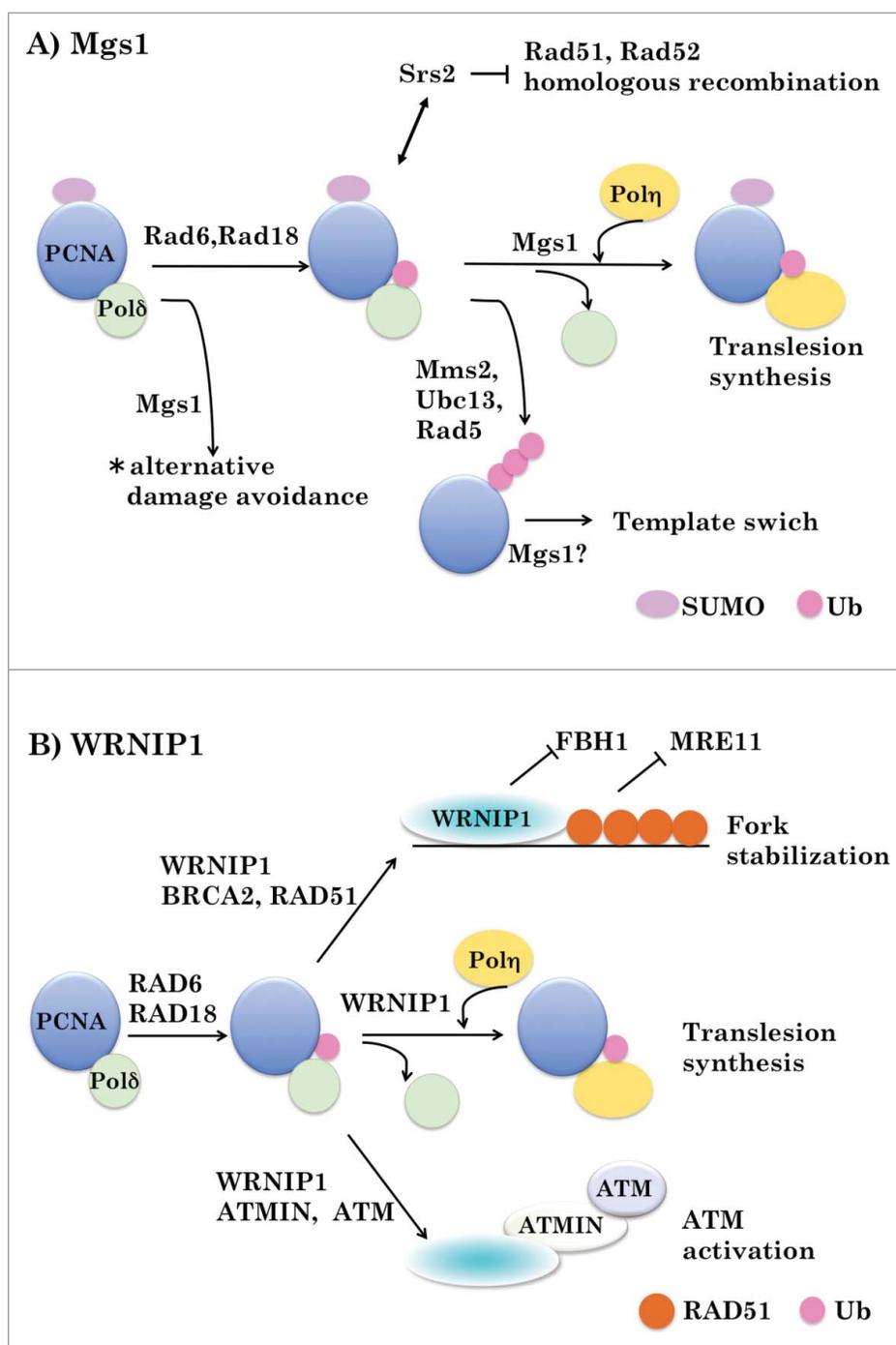
The human *WRNIP1* gene, which maps to chromosome 6 p25.2, encodes a protein similar to the replication factor C (RFC) family of clamp loader proteins; however, *WRNIP1* does not form a stable complex with RFC proteins.<sup>3</sup> Annotated domains in *WRNIP1* include a Rad18-like zinc finger domain at the N terminus, an ATPase domain in the middle region, and 2 leucine zipper motifs in the C terminus (Fig. 1). Gel-filtration chromatography and glycerol gradient sedimentation revealed that *WRNIP1* exists in a homo-oligomeric complex, most likely an octamer.<sup>3</sup>

### The UBZ domain of *WRNIP1*

The UBZ domain, identified in members of the Y-family of translesion DNA polymerases (polymerase  $\eta$  and  $\kappa$ ), is required



**Figure 1.** Schematic showing the functional domains of *WRNIP1*. The sequence of the Rad18-like ubiquitin-binding zinc finger (UBZ) within *WRNIP1* is aligned with those of RAD18, Pol $\kappa$ , and Mgs1, and that of pol $\eta$  UBZ. Zinc ion-coordinating residues are denoted by blue letters. RAD18-like CCHC zinc fingers are denoted by red letters.



**Figure 2.** Possible roles of Mgs1 and WRNIP1 in DNA transaction. A. Role of Mgs1. PCNA plays the important role in regulating the DNA damage tolerance pathway via its modification with ubiquitin and SUMO in yeast. SUMOylated PCNA interacts with Srs2 helicase, which prevents homologous recombination by disrupting DNA-Rad51 filaments. The 2 types of ubiquitylated PCNA (monoubiquitylated PCNA and polyubiquitylated PCNA) channels the lesion bypass to translesion synthesis and template switch, respectively. Mgs1 induces polymerase switch from Pol $\delta$  to Pol $\eta$  during TLS. Mgs1 may also act in the template switch. \*This pathway is essential when the RAD6 pathway and homologous recombination are disabled. B. Role of WRNIP1. WRNIP1 stabilizes RAD51 nucleofilaments formed by BRCA2, by counteracting FBH1 and protects replication forks from nucleolytic degradation by MRE11. WRNIP1 induces polymerase switch from Pol $\delta$  to Pol $\eta$  during TLS. WRNIP1 links ubiquitylated PCNA with ATMIN/ATM to activate ATM signaling in response to replication stress.

for post-replicative DNA repair. UBZ domains, which chelate zinc ions via cysteine or histidine residues, are classified into 2 families: CCHH-type zinc fingers (found in polymerase  $\eta$ ) and CCHC-type zinc fingers (found in DNA polymerase  $\kappa$ , WRNIP1, and RAD18) (Fig. 1).<sup>16</sup> In addition, UBZ domains can be categorized into at least 2 types, DNA polymerase  $\eta$  and WRNIP1, based on their ubiquitin-binding surfaces.<sup>17</sup>

WRNIP1 can interact with monoubiquitin, as well as polyubiquitin chains linked via lysine 48 or 63.<sup>16,18</sup> Moreover, WRNIP1 itself is modified by ubiquitylation and sumoylation, and the UBZ domain of WRNIP1 is involved in the ubiquitylation reaction.<sup>18</sup> Polyubiquitin chains conjugated to WRNIP1 are linked through lysines 11, 48, or 63.<sup>19</sup> The physiologic importance of these modifications remains unclear.

The localization of WRNIP1 to nuclear foci upon exposure to DNA-damaging agents such as UVC light is dependent on the UBZ domain. In addition, WRNIP1 accumulates very rapidly at laser-irradiated sites, and this repositioning also requires the UBZ domain.<sup>16,20</sup> Mutation of the conserved aspartate in the UBZ domain to alanine (D37A) abolishes WRNIP1 binding to both mono- and polyubiquitin. In addition, WRNIP1 D37A cannot form nuclear foci after UVC irradiation nor accumulate at laser-irradiated sites. Because ubiquitylated PCNA is bound by WRNIP1,<sup>21</sup> it may be one of the targets of the WRNIP1 UBZ domain upon UVC or laser light irradiation.

### The ATPase domain of WRNIP1

The ATPase domain in the middle region of WRNIP1 contains the Walker A/B and sensor I/II motifs characteristic of AAA+ ATPase family proteins. WRNIP1 has DNA-dependent ATPase activity, preferring template-primer mimic DNA such as poly(dA)/oligo(dT), and binds DNA in an ATP-dependent manner.<sup>3,22</sup> In addition, the interaction between WRNIP1 and WRN requires ATP, and mutation of the Walker A motif of WRNIP1 abolishes this interaction.<sup>23</sup> However, the ATPase domain of WRNIP1 is dispensable for accumulation of WRNIP1 at sites of DNA damage.<sup>20</sup>

As in the WRNIP1-WRN interaction, p97 (also known as valosin-containing protein, VCP), another AAA+ ATPase, interacts with WRN in an ATP-dependent manner.<sup>24</sup> p97/VCP is a ubiquitin-dependent protein segregase that separates ubiquitylated proteins from tightly bound partner proteins, enabling their selective degradation. For example, p97/VCP facilitates degradation of the largest subunit of RNA polymerase II, Rpb1, at sites of stalled transcription.<sup>25</sup>

Following DNA damage by UV light, p97/VCP is recruited to blocked replication forks by the adaptor protein DVC1 (also known as Spartan), where it facilitates the switch from replicative to TLS polymerase, as well as the removal of TLS polymerase.<sup>26,27</sup> This function is similar to the activities of Mgs1, discussed in the previous section, and WRNIP1, discussed in a later section.

### The leucine zipper domain of WRNIP1

WRNIP1 contains 2 leucine zipper motifs in its C-terminal region (leucine zipper domain), which is required for oligomerization of the protein.<sup>16</sup> The oligomerization of WRNIP1 is important for its localization to nuclear foci following UV irradiation. Both the N-terminal region containing the UBZ domain and the C-terminal region containing the leucine zipper domain, but not the ATPase domain in the middle region, are required for accumulation of WRNIP1 at laser-irradiated sites.<sup>20</sup> These observations indicate that oligomerization of WRNIP1, mediated by the leucine zipper domain, is important for proper subnuclear localization of WRNIP1.

### WRNIP1-interacting proteins

WRNIP1 interacts with multiple proteins involved in various cellular pathways; these include FGFR1OP (fibroblast growth factor receptor 1 oncogene partner),<sup>28</sup> Nup107,<sup>29</sup> p97/VCP,<sup>30</sup>

RAD18,<sup>22</sup> ubiquitylated PCNA,<sup>21</sup> DNA polymerase  $\delta$ ,<sup>3</sup> DNA polymerase  $\eta$ ,<sup>31,32</sup> RAD51, BRCA2,<sup>33</sup> ATMIN,<sup>21</sup> and WRN.<sup>2</sup> In this section, we focus on the WRNIP1-interacting proteins involved in DNA replication and/or repair: WRN, RAD18, DNA polymerase  $\delta$ , and DNA polymerase  $\eta$ .

### WRN

WRN is mutated in patients with WS, a rare autosomal recessive disorder characterized by premature aging and early onset of age-related diseases, including arteriosclerosis, melituria, cataracts, and malignant neoplasms. Somatic cells derived from WS patients have reduced lifespans *in vitro*<sup>34</sup> and elevated rates of chromosomal translocations, rearrangements, and deletions.<sup>35</sup> WRN, a member of the RecQ DNA helicase family, has 3'-5' DNA helicase and 3'-5' exonuclease activities, and is involved in many DNA transactions such as DNA recombination, repair, transcription, and telomere maintenance.

A yeast 2-hybrid screen revealed that WRN interacts with WRNIP1, and that this interaction is mediated by the N-terminal region of WRN.<sup>2</sup> Subsequently, we learned that the WRN-WRNIP1 interaction is dependent on ATP.<sup>23</sup>

The functional relationship between WRNIP1 and WRN was investigated by generating *WRNIP1/WRN* double-knockout (*wrnip1/wrn*) cells in the chicken DT40 cell line. In contrast to yeast *mgs1/sgs1* mutants, *wrnip1/wrn* cells grow at a rate similar to that of wild-type cells; however, the frequency of spontaneously occurring sister chromatid exchange (SCE) is slightly higher in *wrnip1/wrn* cells than in the wild-type or either single knockout.<sup>23</sup> In addition, *wrnip1/wrn* cells are more sensitive to camptothecin than either single mutant, suggesting that WRNIP1 acts independently of WRN in dealing with some types of DNA lesions.

### RAD18

RAD18, which has single-stranded DNA-binding activity, is a ubiquitin ligase that participates in monoubiquitylation with the aid of the ubiquitin-conjugating enzyme RAD6. WRNIP1 and RAD18 interact both *in vitro* and in living cells.<sup>22</sup> In contrast to the WRNIP1-WRN interaction, the WRNIP1-RAD18 interaction is not dependent on ATP. WRNIP1 binds DNA in an ATP-dependent manner, and the affinity of binding is increased by RAD18. By contrast, WRNIP1 inhibits DNA binding by RAD18, suggesting that WRNIP1 preferentially recognizes the RAD18-DNA complex via an interaction with RAD18 and binds the DNA, thereby displacing RAD18.<sup>22</sup>

The yeast *mgs1* and *rad18* mutations are synthetically lethal, whereas *WRNIP1/RAD18* double-knockout (*wrnip1/rad18*) DT40 cells are viable but grow slightly slower than wild-type cells. The frequency of spontaneously occurring SCE is higher in *wrnip1/rad18* cells than in either single mutant, suggesting that these 2 proteins function in different pathways in the absence of exogenous DNA damage.<sup>36</sup> Moreover, the high sensitivity of *rad18* cells to camptothecin is partially suppressed by disruption of *WRNIP1*, suggesting that WRNIP1 acts upstream of RAD18 in the process of dealing with DNA lesions induced by camptothecin.

### DNA polymerase $\delta$

DNA polymerase  $\delta$  a replicative polymerase involved in lagging-strand synthesis, was recently suggested to play a role in leading-strand synthesis as well.<sup>37</sup> DNA polymerase  $\delta$  in higher eukaryotic cells has 4 subunits, of which WRNIP1 interacts with 3: p125, p50, and p12.<sup>3</sup> On poly(dA)/oligo(dT) template, purified WRNIP1 stimulates the DNA-synthesis activity of DNA polymerase  $\delta$  by increasing initiation frequency, but the mechanism underlying this increase remains unclear. One possibility is that WRNIP1 strengthens DNA polymerase  $\delta$  binding to template primers. Alternatively, given that WRNIP1 (like Mgs1) acts as a mobilizer for DNA polymerase  $\delta$ , it may promote detachment of DNA polymerase  $\delta$  from the template after DNA synthesis, making DNA polymerase  $\delta$  molecules available for a new round of initiation.

### DNA polymerase $\eta$

DNA polymerase  $\eta$ , encoded by *POLH* (which is the gene responsible for the xeroderma pigmentosum variant), is a translesion DNA polymerase that can efficiently and accurately bypass UV-induced cyclobutane pyrimidine dimers (CPDs). The interaction between WRNIP1 and DNA polymerase  $\eta$  has been confirmed by proteomics<sup>31</sup> and co-immunoprecipitation analyses.<sup>32</sup>

*WRNIP1/POLH* double-knockout (*wrnip1/polh*) DT40 cells grow slightly slower than the wild type or either single knockout, suggesting that the encoded proteins play non-overlapping roles under non-stressed conditions. *wrnip1/polh* cells exhibit suppression of the phenotypes observed in *polh* cells upon UV irradiation, including UV sensitivity, delayed repair of CPDs, elevated rates of mutation and SCE, and reduced rate of replication fork progression.<sup>32</sup> Therefore, it is likely that WRNIP1 acts upstream of DNA polymerase  $\eta$  after UV irradiation.

### Functions of WRNIP1 in DNA transactions

WRNIP1 was first identified 16 y ago, but its functions in the cell remained obscure. Recently, however, data have accumulated regarding the roles of WRNIP1 in DNA transactions. In this section, we discuss the contributions of WRNIP1 to TLS after UV irradiation, as well as to maintenance of replication forks and checkpoint activation following DNA replication stress.

#### Translesion DNA synthesis (TLS)

TLS enables DNA synthesis on a damaged template via insertion of correct or incorrect bases into the daughter strand, allowing bypass of DNA lesions. This process requires a polymerase switch in which the standard replicative polymerase is replaced by specialized translesion polymerase. In UV-irradiated cells, the RAD6–RAD18 complex is recruited to the irradiated sites, where it monoubiquitylates PCNA. This modification of PCNA triggers the exchange of a replicative polymerase (e.g., DNA polymerase  $\delta$ ) for the translesion polymerase DNA polymerase  $\eta$ . WRNIP1 accumulates at UV-irradiated sites in a UBZ domain-dependent manner,<sup>16,20</sup> interacts with RAD18, and displaces it from the DNA. WRNIP1 also interacts directly with DNA polymerase  $\delta$  to

stimulate its DNA-synthesis activity by promoting recycling of DNA polymerase  $\delta$  on template/primer DNA.<sup>3</sup> Furthermore, WRNIP1 interacts with DNA polymerase  $\eta$ , and genetic data suggest that it functions upstream of DNA polymerase  $\eta$ .<sup>32</sup> Based on these observations, WRNIP1 may stimulate detachment of DNA polymerase  $\delta$  from ubiquitylated PCNA and recruit DNA polymerase  $\eta$  to sites of DNA damage during TLS (Fig. 2B). The high affinity of WRNIP1 for polyubiquitin and its interaction with RAD51 suggest that WRNIP1 itself is involved in the template switching mechanism. This issue should be addressed in a future study.

#### Stabilization of replication forks

DNA replication can be stalled for many reasons, including topological stress, DNA lesions, and depletion of nucleotide pools. Proper handling of stalled replication forks is essential for the maintenance of genome stability. BRCA2 is involved in stabilization of stalled replication forks, independent of its double-strand break repair function, by stabilizing RAD51 filaments and blocking degradation of replication forks by MRE11.<sup>38,39</sup>

WRNIP1 is recruited to stalled replication forks, where it interacts with BRCA2 and RAD51.<sup>33</sup> In WRNIP1-depleted cells, MRE11 generates more ssDNA than in control cells under both unperturbed and HU-treated conditions, and the amount of RAD51 on stalled replication forks is considerably lower. The aforementioned phenotypes and chromosome instability in WRNIP1-depleted cells are suppressed by depletion of FBH1, which can disrupt RAD51 filaments. Thus, WRNIP1 is directly involved in preventing MRE11-mediated degradation of stalled replication forks by stabilizing RAD51 on ssDNA, and this function is not dependent on the ATPase activity of WRNIP1. The ATPase activity is essential for recovery of perturbed replication forks, but the contribution of WRNIP1 to this process remains unclear.

#### Checkpoint activation

The ataxia telangiectasia mutated (ATM) kinase responds to the presence of DNA damage, especially damage induced by ionizing radiation (IR), by activating the cell cycle checkpoint and promoting DNA repair.<sup>40,41</sup> However, the molecular mechanism by which ATM is activated by replication stress remains obscure. The contribution of ATMIN (ATM interactor) to ATM activation is complementary to that of NBS1, i.e., it is dispensable for IR-induced ATM activation.<sup>42</sup> ATMIN is required for ATM-mediated signaling and recruitment of 53BP1 to DNA damage sites upon replication stress.<sup>43</sup> Moreover, WRNIP1 binds ATMIN and facilitates its ATM-activating function.<sup>21</sup> Upon induction of replication stress by aphidicolin, WRNIP1 interacts with monoubiquitylated PCNA and forms foci, dependent upon ubiquitylation of PCNA by RAD18. Depletion of RAD18, WRNIP1, or ATMIN prevents activation of ATM upon replication stress, but not after IR. Thus, WRNIP1 recognizes monoubiquitylated PCNA and connects with ATMIN/ATM to activate ATM signaling in response to replication stress.

## Conclusions and future perspectives

WRNIP1 and its yeast homolog, Mgs1, play important roles following ubiquitylation of PCNA, acting downstream of RAD18 to mobilize DNA polymerase  $\delta$ . Although the *mgs1* and *rad18* mutations are synthetically lethal, *wrnip1/rad18* double knockouts are viable and grow slightly slower than wild-type cells. Given that activation of the recombination pathway by inhibition of Srs2 function rescues the synthetic lethality of *mgs1/rad18* cells, it seems likely that this pathway is active in *wrnip1/rad18* cells. In fact, the frequency of spontaneous SCE is considerably higher in *wrnip1/rad18* cells than in wild-type cells. Future studies should seek to determine the UBZ domain-independent function of Mgs1 in the alternative pathway, which becomes essential when the RAD6 and HR pathways are blocked, as well as whether WRNIP1 has a similar function. In addition, it would be interesting to elucidate the coordination of the 2 events in which WRNIP1 participates, i.e., checkpoint activation involving ATM and stabilization of replication forks. Recently, we obtained results suggesting that WRNIP1 regulates the function of PrimPol, which is thought to function in TLS and restart DNA synthesis by synthesising primers (to be published elsewhere).

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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