INTRODUCTION

Werner syndrome (WS) is a rare autosomal recessive disease characterized by accelerated aging. WS patients exhibit several clinical features associated with human physiological aging, including diabetes mellitus, arteriosclerosis, and cancer. The WRN protein, encoded by the causative gene WRN, belongs to the RecQ family of DNA helicases.\(^1\) WRN participates in a variety of processes that maintain genome stability processes, including DNA replication, repair, recombination, and telomere maintenance.

Twenty years ago, we identified WRNIP1, the WRN interacting protein 1 (WRNIP1), as a member of the AAA+ ATPase family.\(^2\) WRNIP1 is a conserved protein that is evolutionarily conserved from \textit{Escherichia coli} to humans.\(^3\) The \textit{Escherichia coli} orthologue MgsA has DNA-dependent ATPase and ssDNA annealing activities.\(^4\) The Walker A motif of WRNIP1 AAA+ ATPase is required for the interaction between WRNIP1 and WRN.\(^5\)

In addition to the ATPase domain residing in the middle region of WRNIP1, human WRNIP1 has a ubiquitin-binding zinc-finger (UBZ) domain and two leucine zipper motifs in the N-terminal and C-terminal regions, respectively (Fig. 1A). The UBZ domain coordinates a zinc ion with cysteine or histidine residues, and interacts with ubiquitin. Indeed, the WRNIP1 UBZ domain binds ubiquitin in a similar manner to the UBZ domain of \textit{E. coli} ubiquitin ligase \textit{RAD18}\.\(^6\) Leucine zipper motifs were initially described as highly conserved motifs involved in interactions between transcription factors and DNA.\(^7\) WRNIP1 exists in a homo-oligomeric complex, most likely an octamer. The oligomerization of WRNIP1 requires the WRNIP1 C-terminal domain, including both leucine zipper motifs.\(^7\)

To date, several studies from our and other groups reported that WRNIP1 physically or genetically interacts with proteins involved in multiple cellular pathways (Fig. 1B). For instance, WRNIP1 interacts with the components of DNA replication machinery, such as proliferating cell nuclear antigen (PCNA) and replicative DNA polymerase \(\delta\) (Pol\(\delta\)). Moreover, WRNIP1 stimulates Pol\(\delta\) activity \textit{in vitro}.\(^8,9\) Mgs1 (maintenance of genome stability 1; the budding yeast orthologue of WRNIP1) also interacts with PCNA.\(^10\) Overexpression of Mgs1 is lethal in the cells that possess mutations in proteins related to DNA replication, including Pol\(\delta\).\(^11\) Mutation of \textit{MGS1} is synthetically lethal with the mutation of \textit{RAD6} and \textit{RAD18}\.\(^12\) Both of which are involved in translesion synthesis (TLS) through ubiquitylation of PCNA. TLS is a DNA damage tolerance mechanism that allows DNA synthesis across the damaged DNA. TLS uses specialized DNA polymerases, termed TLS polymerases, that bypass damaged DNA templates. Monoubiquitinated PCNA facilitates the substitution of the replicative DNA polymerase Pol\(\delta\) with TLS polymerases for the bypass of DNA lesions.\(^13\)

WRNIP1 interacts with RAD18, which plays key roles in mono-ubiquitylation of PCNA in response to DNA damage. WRNIP1 interacts with the binding of RAD18 to DNA.\(^14\) WRNIP1 binds to DNA polymerase \(\eta\) (Pol\(\eta\)), a specialized DNA polymerase in TLS.\(^15\) During DNA replication, UV-induced DNA lesions are bypassed mainly by Pol\(\eta\) rather than Pol\(\delta\); accordingly, Pol\(\eta\)-deficient cells show sensitivity to UV irradiation.

We previously demonstrated that deletion of \textit{WRNIP1} suppressed the UV sensitivity of Pol\(\eta\)-deficient cells,\(^16\) suggesting that a novel pathway began to function in the absence of both WRNIP1 and Pol\(\eta\) upon UV-induced DNA damage. We hypothesized that PrimPol, a TLS polymerase, was the protein involved in this function; hence, we investigated the relationship between WRNIP1 and PrimPol. Pull-down experiments showed that the two proteins interact, and that the amount

Regular Article

Functional Domain Mapping of Werner Interacting Protein 1 (WRNIP1)

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Werner helicase–interacting protein 1 (WRNIP1) belongs to the AAA+ ATPase family and is conserved from \textit{Escherichia coli} to human. In addition to an ATPase domain in the middle region of WRNIP1, WRNIP1 contains a ubiquitin-binding zinc-finger (UBZ) domain and two leucine zipper motifs in the N-terminal and C-terminal regions, respectively. Here, we report that the UBZ domain of WRNIP1 is responsible for the reduced levels of UV-induced proliferating cell nuclear antigen (PCNA) monoubiquitylation in \textit{POLH}-disrupted (polymerase \(\eta\) (Pol\(\eta\))-deficient) cells, and that the ATPase domain of WRNIP1 is involved in regulating the level of the PrimPol protein. The suppression of UV sensitivity of Pol\(\eta\)-deficient cells by deletion of WRNIP1 was abolished by expression of the mutant WRNIP1 lacking the UBZ domain or ATPase domain, but not by the mutant lacking the leucine zipper domain in \textit{WRNIP1/POLH} double-disrupted cells. The leucine zipper domain of WRNIP1 was required for its interaction with RAD18, a key factor in TLS (DNA translesion synthesis), and DNA polymerase \(\delta\) catalytic subunit, POLD1. On the basis of these findings, we discuss the possible role of WRNIP1 in TLS.

Key words Werner helicase–interacting protein 1 (WRNIP1); translesion synthesis (TLS); RAD18; polymerase \(\eta\) (Pol\(\eta\)); Pol\(\delta\); PrimPol
of WRNIP1 in cells negatively correlated with the amount of PrimPol.\textsuperscript{17}

Although information on the molecular structure of WRNIP1 and its physical and genetic interactors has accumulated, the relationships among WRNIP1, RAD18, PCNA ubiquitylation, Pol\textsubscript{\textgamma}, and PrimPol remain elusive (Fig. 1B). In this study, we constructed human or chicken WRNIP1 (cWRNIP1) mutants lacking the UBZ domain, the ATPase domain, or leucine zipper domain (Fig. 1A). Using these mutants, we determined which domains of WRNIP1 are related to its physical or genetic interactions with the interacting proteins. We will discuss the function of WRNIP1 based on the results of our functional domain mapping experiments.

**MATERIALS AND METHODS**

**Cell Culture and Reagents** Human 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum in an atmosphere containing 5% CO\textsubscript{2} at 37°C. \textit{WRNIP1\textsuperscript{−/−}/PolH\textsuperscript{−/−}} cells were generated from chicken DT40 cells as described previously.\textsuperscript{36} \textit{WRNIP1} is located on chromosome 2, which is trisomic in DT40 cells. Cells were cultured in RPMI 1640 supplemented with 100\textmu g/mL kanamycin, 10% fetal bovine serum, and 1% chicken serum at 39°C.

**Immunoprecipitation** Immunoprecipitation experiments were performed as previously described.\textsuperscript{14} For overexpression, 293 cells were transfected with constructs encoding FLAG-tagged human WRNIP1 using the Lipofectamine 3000 Reagent (ThermoFisher Scientific, Waltham, MA, U.S.A.). Cell lysates were prepared with lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail (ethylenediaminetetraacetic acid (EDTA)-free)) (Roche Diagnostics, Basel, Switzerland). The lysate was cleared by centrifugation and incubated with immunoglobulin G (IgG)-Sepharose (GE Healthcare UK Ltd., Bucks, U.K.) at 4°C for 30 min. After centrifugation, the supernatant was incubated with anti-FLAG agarose beads (Sigma-Aldrich, St. Louis, MO, U.S.A.) at 4°C for 1 h. The beads were washed five times with lysis buffer, resuspended in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol), and boiled for
3 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and detected by Western blotting using an anti-FLAG antibody (Sigma-Aldrich) or anti-c-myc antibody (Santa Cruz Biotechnology, Dallas, TX, U.S.A.).

**Protein Analysis**

For Western blotting, samples were lysed in Laemmli buffer and separated with SDS-PAGE. Primary antibodies used in this study were anti-FLAG (Sigma: anti-FLAG M2 monoclonal antibody), anti-myc (Sigma: anti-c-myc antibody, 9E10), anti-PrimPol (anti-CCDC111: Abcam ab185306, Abcam, Cambridge, U.K.), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam ab9485). Secondary antibodies were horseradish peroxidase (HRP)-linked anti-rabbit IgG and HRP-linked anti-mouse IgG (Cell Signaling, Danvers, MA, U.S.A.), and signals were visualized using ECL prime Western blotting Detection Reagent (Cytiva, Tokyo, Japan).

**Generation of WRNIP1−/−/POLH−/+ cWRNIP1 Cells**

To generate WRNIP1−/−/POLH−/+ cWRNIP1 mutant cells, an expression vector pcDNA 3.1 carrying chicken WRNIP1 mutants was prepared by PCR using appropriate primers from full-length hemagglutinin (HA)-WRNIP1 inserted into expression vector pcDNA 3.1.16,18) Cells expressing the WRNIP1 mutants were obtained by electroporation of HA-WRNIP1 mutant constructs into WRNIP1−/−/−/−/POLH−/−/− cells. Colonies were selected in 96-well plates in medium containing G418, and expression of mutant proteins was monitored as follows. Cells (5 × 10⁵) were suspended in SDS sample buffer. Samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, U.S.A.). HA-tagged WRNIP1 was detected using an anti-HA primary antibody (Roche Diagnostics). Bands were visualized using ECL Prime detection reagents (GE Healthcare U.K.).

**Assay for Sensitivity to UV**

After a phosphate buffered saline (PBS) wash, cells (1 × 10⁶) were irradiated with UVC and then cultured for an additional 48 h. UV sensitivity was evaluated using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). 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%).

**Construction of Plasmids**

Human WRNIP1 cDNA was described previously.8,14,18) To prepare deletion mutants of WRNIP1, mutant DNA fragments were amplified by PCR using the appropriate primers from the FLAG-tagged full-length WRNIP1 in the expression vector pIRES neo3 (TaKaRa Bio., Shiga, Japan).

**RESULTS**

**Effect of Mutated WRNIP1 Expression on UV Sensitivity of WRNIP1−/−/POLH−/+ DT40 Cells**

The UV sensitivity of chicken DT40 POLH−/− cells is suppressed by deletion of WRNIP1, implying that WRNIP1 functions upstream of Polη in the process of TLS on UV-induced DNA lesions.16) To determine which domain of chicken WRNIP1 (cWRNIP1) affects the UV sensitivity in WRNIP1−/−/−/−/POLH−/−/− cells, we assessed the sensitivity of WRNIP1−/−/−/−/POLH−/−/− cells to UV radiation by expressing various cWRNIP1 deletion mutants (Fig. 2A). Expression of wild-type cWRNIP1 (making the double mutant equivalent to POLH−/− cells), cWRNIP1 lacking the UBZ do-

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**Fig. 2. Effect of WRNIP1 Mutants Expressed in WRNIP1−/−/POLH−/+ DT40 Cells on Sensitivity to UV**

A. Western blot analysis of WRNIP1−/−/POLH−/+ cells expressing WT or deletion mutants of WRNIP1. Data are representative of three independent experiments. B. UV sensitivity of WRNIP1−/−/POLH−/+ cells expressing WT or deletion mutants of WRNIP1. Cells were exposed to UV and cultured for 48 h. Live cells were counted. Data points show the averages and standard deviation (S.D.) of five independent experiments.
main, or cWRNIP1 lacking the ATPase domain restored the UV sensitivity of the WRNIP1−/−/POLH−/− cells to the level in POLH−/− cells, whereas double mutant cells expressing cWRNIP1 lacking the leucine zipper domain had UV sensitivity similar to that of double mutant cells (Fig. 2B). These results suggest that the leucine zipper domain of cWRNIP1 affects the UV sensitivity of the POLH−/− cells. Because deletion of the leucine zipper of cWRNIP1 suppressed the UV sensitivity of POLH−/−, it is important to identify other phenomena related to the leucine zipper of WRNIP1.

The WRNIP1 Domain Affects the Level of PrimPol PrimPol is involved in error-free TLS.19) The suppression of the UV sensitivity of the POLH−/− cells by disruption of WRNIP1 could be due to up-regulation of some type of error-free TLS upon UV exposure.16) Because the level of PrimPol increased in WRNIP1−/− cells, we considered PrimPol as a candidate factor responsible for error-free TLS in WRNIP1−/−/POLH−/− cells. Moreover, exogenous or endogenous PrimPol decreased in WRNIP1-overexpressing 293 cells. Thus, it is conceivable that the increase in PrimPol results in error-free TLS upon UV exposure in WRNIP1−/−/POLH−/− cells.

Among the 293 cells expressing various WRNIP1 deletion mutants, those expressing the WRNIP1 mutant lacking the ATPase domain only exhibited the PrimPol band, suggesting

Fig. 3. The UBZ Domain of WRNIP1 Is Responsible for the Reduction of UV-Induced PCNA Monoubiquitylation, and the ATPase Domain Is Required for the Reduction in the Level of PrimPol

A. Western blot analysis of cell extracts from WRNIP1- and PrimPol-overexpressing cells. 293 cells were transfected with WRNIP1 c-myc/pIRES neo3, WRNIP1 mutant c-myc/pIRES neo3, or PrimPol FLAG/pIRES neo3. Cells overexpressing PrimPol FLAG/pIRES neo3 alone were used as controls. Cell extracts were collected after 48h. Data are representative of three independent experiments. B. (a) Analysis of PCNA monoubiquitylation using extracts of the indicated cells. Cells were irradiated with 0.8 J/m² UV and incubated for 4h. Ubiquitylation of PCNA was determined by immunoblotting chromatin-enriched fractions of UV-exposed cells with an anti-PCNA antibody. Bands corresponding to unmodified PCNA and monoubiquitylated PCNA (Ub-PCNA) were quantified by densitometry. (b) The graph shows quantification of the Western blots of four independent experiments. Data points show the mean and SD of the Ub-PCNA/PCNA ratio. C (a) Immunoblot analysis of DT40 WRNIP1−/−/POLH−/− cells containing the indicated plasmids. Ubiquitylation of PCNA was determined by immunoblotting chromatin-enriched fractions of UV-exposed cells with an anti-PCNA antibody. (b) The Ub-PCNA/PCNA ratio was analyzed as in B (b).
that the amount of PrimPol was downregulated by WRNIP1 through its ATPase domain (Fig. 3A). Thus, in opposition to our previous hypothesis, upregulation of PrimPol seems not to be the main factor involved in promoting error-free TLS upon UV exposure in $WRNIP1^{-/-}/POLH^{-/-}$ cells, as the suppression of UV sensitivity was observed only with the leucine zipper mutants (Fig. 2B).

**WRNIP1 Domain Affecting Ubiquitylation of PCNA**

PCNA is an essential factor in eukaryotic DNA replication, and ubiquitylation of this protein is a central event in the regulation of TLS. Pol $\eta$ bridges RAD18 and PCNA to efficiently promote monoubiquitylation of PCNA at stalled forks. Indeed, UV irradiation-induced monoubiquitylation of PCNA is reduced in $POLH^{-/-}$ cells.

We analyzed the ubiquitylation of PCNA by immunoblotting chromatin-enriched fractions of UV-exposed cells using an anti-PCNA antibody. As shown in Fig. 3B (a), UV-induced monoubiquitylation of PCNA in $POLH^{-/-}$ cells was partially rescued by deletion of WRNIP1. We wished to examine whether restoration of PCNA monoubiquitylation is associated with error-free TLS upon UV exposure in $WRNIP1^{-/-}/POLH^{-/-}$ cells. To this end, we sought to determine which domain of WRNIP1 is responsible for restoration of PCNA monoubiquitylation. The level of PCNA ubiquitylation in $WRNIP1^{-/-}/POLH^{-/-}$ cells expressing the WRNIP1 mutant lacking the ATPase domain or the leucine zipper domain was similar to that in cells expressing wild-type WRNIP1 (pseudo $POLH^{-/-}$ cells), whereas the level of PCNA monoubiquitylation in cells expressing the WRNIP1 mutant lacking the UBZ domain was higher than that in cells expressing wild-type WRNIP1 (Fig. 3C). These results suggest that the UBZ domain of WRNIP1 is related to the reduction in UV-induced PCNA monoubiquitylation.

In addition, although PCNA monoubiquitylation is a key step during TLS, the result showing that the expression of the WRNIP1 mutant lacking the leucine zipper domain did not increase PCNA monoubiquitylation excludes the possibility that the restoration of PCNA monoubiquitylation is associated with error-free TLS upon UV exposure in $WRNIP1^{-/-}/POLH^{-/-}$ cells.

**The WRNIP1 Domain Interacts with RAD18 or DNA Polymerase $\delta$**

UV-induced ubiquitylation of PCNA is catalyzed by Rad6 and Rad18. We previously reported that WRNIP1 interacted with RAD18 and interfered with the interaction between RAD18 and DNA in vitro. The interaction of WRNIP1 with RAD18 occurred in cells, as well. In addition, we found that WRNIP1 interacted with DNA polymerase $\delta$ (Pol$\delta$) via its subunits, including POLD1, in vitro. Because RAD18 and Pol$\delta$ are central players in DNA polymerase switching during TLS, we next sought to determine which domain of WRNIP1 is responsible for the interactions with RAD18 and Pol$\delta$ in the cells.

As shown in Fig. 4A, we expressed myc-tagged WRNIP1 mutants in cells and performed immunoprecipitation experiments with an anti-myc antibody. WRNIP1, lacking the leucine zipper domain, did not co-precipitate with RAD18 (Fig. 4A) or POLD1 (Fig. 4B). By contrast, WRNIP1 mutants lacking the UBZ domain or the ATPase domain were co-precipitated with these proteins. Thus, WRNIP1 forms a complex with these proteins via its leucine zipper domain.

Results obtained by functional domain mapping suggest that the disappearance of the interactions between WRNIP1 and RAD18 or Pol$\delta$ in $WRNIP1^{-/-}/POLH^{-/-}$ cells is due to the absence of the leucine zipper domain.

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*Fig. 4. The Leucine Zipper Domain of WRNIP1 Is Required for Binding of RAD18 or Pol$\delta$ to WRNIP1*

A. Physical interaction of WRNIP1 mutants with RAD18. Western blotting was performed on anti c-myc immunoprecipitates of 293 cells transfected with plasmids encoding the indicated proteins. 293 cells transfected with plasmids encoding the indicated proteins were subjected to c-myc immunoprecipitation (IP) followed by immunoblotting with c-myc and RAD18 antibodies. Data are representative of three independent experiments. B. Physical interactions of WRNIP1 mutants and POLD1. Western blot analysis of anti–c-myc immunoprecipitates of 293 cells transfected with plasmids encoding the indicated proteins. 293 cells were transfected with plasmids encoding the indicated proteins and subjected to c-myc IP, followed by immunoblotting with antibodies against c-myc and POLD1. Data are representative of three independent experiments.
family protein, VCP/p97, extracts ubiquitylated proteins from the structural transformation of complexes using the energy erones involved in diverse cellular functions characterized by proteins belonging to this family function as molecular chap-

**POLH** induced PCNA monoubiquitylation in (1) the UBZ domain is responsible for the reduction in UV-
domain are summarized in Fig. 5. Briefly, we found that: (2) the ATPase domain is responsible for down-regulation of PrimPol (Fig. 3A); and (3) the leucine zipper domain is responsible for the interactions of WRNIP1 with RAD18 and Pol₁ (Fig. 4). In addition, error-free TLS upon UV exposure in WRNIP₁⁻/⁻/POLH⁻/⁻ cells is activated by loss of the function performed by the leucine zipper domain (Fig. 2). Based on these results, we speculate that the function of each domain is as follows.

Previously, we reported that the recruitment of WRNIP1 to sites of DNA damage was dependent on the UBZ domain. WRNIP1 binds ubiquitin and polyubiquitin via the UBZ do-

**WRNIP1** and RAD18 or WRNIP1 and POLD1 is closely re-

**DISCUSSION**

Eukaryotic WRNIP1 contains three distinct domains, UBZ, ATPase, and leucine zipper, in the N-terminal, middle, and C-terminal regions, respectively. The results of functional domain mapping experiments using deletion mutants of each domain are summarized in Fig. 5. Briefly, we found that: (1) the UBZ domain is responsible for the reduction in UV-induced PCNA monoubiquitylation in POLH⁺/⁺ cells (Fig. 3C); (2) the ATPase domain is responsible for down-regulation of PrimPol (Fig. 3A); and (3) the leucine zipper domain is responsible for the interactions of WRNIP1 with RAD18 and Pol₁ (Fig. 4). In addition, error-free TLS upon UV exposure in WRNIP₁⁻/⁻/POLH⁻/⁻ cells is activated by loss of the function performed by the leucine zipper domain (Fig. 2). Based on these results, we speculate that the function of each domain is as follows.

Previously, we reported that the recruitment of WRNIP1 to sites of DNA damage was dependent on the UBZ domain. WRNIP1 binds ubiquitin and polyubiquitin via the UBZ domain. In addition, a previous study reported that WRNIP1 binds to ubiquitylated-PCNA. It seems likely that WRNIP1 might be able to recruit deubiquitinating enzymes to ubiquitylated-PCNA to which WRNIP1 binds. Thus, the UBZ domain of WRNIP1 might interact with ubiquitylated-PCNA at the stalled replication sites and indirectly eliminate ubiquitin from modified PCNA in POLH⁺/⁺ cells.

WRNIP1 belongs to the AAA+ ATPase family, and some proteins belonging to this family function as molecular chaperones involved in diverse cellular functions characterized by the structural transformation of complexes using the energy gained from hydrolysis of ATP to mediate the binding and dissociation of complex components. One AAA+ ATPase family protein, VCP/p97, extracts ubiquitylated proteins from membranes or cellular structures, or segregates them from binding proteins, thus facilitating the degradation of target proteins in the proteasome. A previous study by our group suggested that WRNIP1 is involved in the proteasomal degra-
dation of PrimPol. Thus, it is possible that WRNIP1 plays a role similar to that of VCP/p97, dependent upon the ATPase domain.

WRNIP1 requires the leucine zipper domain to form multimeric complexes, and multimerization is necessary in order for WRNIP1 to form a structure suitable for binding to RAD18 and Pol₁. Because deletion of the leucine zipper domain suppressed the UV sensitivity of POLH⁻/⁻ cells, the leucine zipper domain must be involved in suppression of the error-free TLS observed in WRNIP₁⁻/⁻/POLH⁻/⁻ cells. In regard to the error-free TLS that occurs upon UV irradiation in WRNIP₁⁻/⁻/POLH⁻/⁻ cells, we propose a working model in which Pol₁ performs TLS and PrimPol elongates DNA synthesized by Pol₁. In fact, Pol₁ itself has TLS activity *in vitro* when its exonuclease domain is mutated. One of the possible roles of WRNIP1 in TLS is to replace Pol₁ with Pol₁. Given that the leucine zipper domain is required for the interaction of WRNIP1 and Pol₁, deletion of WRNIP1 or the leucine zipper domain stabilizes Pol₁ at sites of damage. This situation partly supports our working model. However, the mechanism underlying activation of error-free TLS upon UV exposure in WRNIP₁⁻/⁻/POLH⁻/⁻ cells remains elusive, and further experiments are required to clarify this issue.

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**Conflict of Interest**

The authors declare no conflict of interest.

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