Proliferating Cell Nuclear Antigen (PCNA)-binding Protein C1orf124 Is a Regulator of Translesion Synthesis*\textsuperscript{[s]}

Received for publication, July 11, 2012, and in revised form, August 7, 2012. Published, JBC Papers in Press, August 17, 2012, DOI 10.1074/jbc.M112.400135

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**Background:** Translesion synthesis involves proliferating cell nuclear antigen (PCNA) monoubiquitination and polymerase switching.

**Results:** C1orf124 is required for cell survival following UV damage. It binds to monoubiquitinated PCNA and participates in polymerase switching.

**Conclusion:** C1orf124 serves as a central platform that facilitates translesion synthesis.

DNA damage-induced proliferating cell nuclear antigen (PCNA) ubiquitination serves as the key event mediating post-replication repair. Post-replication repair involves either translesion synthesis (TLS) or damage avoidance via template switching. In this study, we have identified and characterized C1orf124 as a regulator of TLS. C1orf124 co-localizes and interacts with unmodified and mono-ubiquitinated PCNA at UV light-induced damage sites, which require the PIP box and UBZ domain of C1orf124. C1orf124 also binds to the AAA-ATPase valosin-containing protein via its SHP domain, and cellular resistance to UV radiation mediated by C1orf124 requires its interactions with valosin-containing protein and PCNA. Interestingly, C1orf124 binds to replicative DNA polymerase POLD3 and PDD1 under normal conditions but preferentially associates with TLS polymerase η (POLH) upon UV damage. Depletion of C1orf124 compromises PCNA monoubiquitination, RAD18 chromatin association, and RAD18 localization to UV damage sites. Thus, C1orf124 acts at multiple steps in TLS, stabilizes RAD18 and ubiquitinated PCNA at damage sites, and facilitates the switch from replicative to TLS polymerase to bypass DNA lesion.

During DNA replication, replication forks may stall when they encounter secondary DNA structures, repetitive sequences, certain protein-DNA complexes, or lesions generated by DNA-damaging agents. Especially in response to UV light-induced DNA lesions, replicative DNA polymerases stall because they are unable to accommodate altered DNA bases in their active sites. Although stalled replication forks are normally stabilized following the activation of DNA damage checkpoints, they may also collapse and thus result in double-strand break formation, gross chromosomal rearrangements, and genomic instability (1). DNA damage tolerance pathways, also known as post-replication repair (PRR)\textsuperscript{2} pathways, function in preventing replication fork collapse in response to DNA damage by allowing stalled replication forks to progress through lesions (1–3). Earlier studies in both yeast and mammalian cells suggest two major pathways for PRR: translesion synthesis (TLS) and damage avoidance by template switching. During TLS, the stalled replicative polymerase is replaced by TLS polymerases, which are a class of specialized polymerases with low processivity that can replicate over distortions in DNA and directly bypass lesions (4, 5). Depending on the TLS polymerase that is recruited, UV light-induced cyclobutane pyrimidine dimers can be bypassed either in a relatively error-free mode (for example, when using DNA polymerase (pol) η) or by an error-prone mechanism using pol ζ and Rev1 (4, 5). The mechanism of lesion bypass by damage avoidance is unclear but is thought to involve template switching with the undamaged sister chromatid and/or the use of homologous recombination (6, 7). Thus, both of these direct (TLS) and indirect (template switching) bypass pathways allow for resumption of DNA replication and leave lesions for repair at a later time point.

A critical step in the regulation of PRR is the post-translational modification of proliferating cell nuclear antigen (PCNA), the replicative sliding clamp that plays an essential role in DNA replication. Following DNA damage and/or replication stress, PCNA is either mono- or polyubiquitinated on Lys-164 (3, 8–10). Studies suggest that monoubiquitination of PCNA promotes direct lesion bypass by recruiting TLS polymerases to stalled replication forks (5, 11, 12), whereas polyubiquitination of PCNA promotes damage avoidance through a process that is still unclear (8). In yeast, ubiquitination of PCNA is mediated by the Rad6 epistasis group and two RING domain-containing E3 ligases, Rad18 and Rad5. Rad18 mediates the monoubiquitination of PCNA, whereas Rad5 facilitates the further addition of Lys-63-linked polyubiquitin chains (5, 11, 12). In humans, monoubiquitination of Lys-164 is the major-modification of PCNA, facilitating the recruitment of TLS polymerases.

\*This work was supported, in whole or in part, by National Institutes of Health Grants CA089239, CA092312, and CA100109 (to J. C.).
\[s\]This article contains supplemental Fig. 1.
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\[2\]The abbreviations used are: PRR, post-replication repair; pol, polymerase; TLS, translesion synthesis; PCNA, proliferating cell nuclear antigen; HU, hydroxyurea; Ub-PCNA, monoubiquitinated PCNA; TAP, tandem affinity purification; MMC, mitomycin C; VCP, valosin-containing protein.
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C1orf124 was detected at much lower levels (14). Monoubiquitination of PCNA is the key event regulating PRR; however, it is insufficient, by itself, to account for the specificity of PRR pathway choice, as several TLS polymerases have ubiquitin-interacting motifs (5). Furthermore, the precise molecular mechanism and regulatory events underlying the switch from replicative to translesion polymerase in response to DNA damage is largely unknown.

In this study, we have identified a previously uncharacterized protein (C1orf124) as a regulator of TLS. C1orf124 is a multi-domain protein and contains an SprT-like domain at its N terminus, an SHP box and a PIP box in the middle region, and a UBZ (ubiquitin-binding zinc finger) domain at the C terminus. We show that C1orf124 localizes to sites of UV light-induced DNA damage in the cells and is required for cell survival following UV radiation. On the basis of the results presented below, we propose that C1orf124 is a key mediator protein involved in TLS, which plays an important role in the switch from replicative DNA polymerase to TLS polymerase for efficient lesion bypass upon UV damage.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-C1orf124 antibodies were raised by immunizing rabbits with GST-C1orf124 fusion proteins containing residues 1–250 and 1–489 of human C1orf124 protein. Antibodies were affinity-purified using an AminoLink Plus immobilization and purification kit (Pierce). Anti-β-actin and anti-FLAG antibodies were obtained from Sigma. Anti-RPA2 antibody was from Abcam. Anti-PCNA antibody (PC10) was obtained from Sigma.

GST Pulldown Assays and Immunoprecipitations—293T cells were transfected with constructs encoding Myc-tagged PCNA and incubated for 24 h. Cells were lysed with high-salt buffer (50 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM EDTA, 0.6% Triton X-100, 8% glycerol, 1 mM DTT, 1 mM PMSE, and 1 mM NaF). The supernatant was clarified and then incubated with GST-C1orf124, GST-C1orf124APIP, or GST protein pre-bound to glutathione-Sepharose beads for 1 h at 4 °C. After three washes with HEPES/Triton buffer, the beads were resuspended in 1 × SDS sample buffer and analyzed by Western blotting using anti-Myc antibody. For co-immunoprecipitation experiments following UV radiation, cells were treated with 100 J/m² UV-C light and allowed to recover for 4 h. Cells were then collected, lysed in 600 mM NaCl/HEPES/Triton buffer, diluted to 150 mM NaCl, sonicated, and clarified by centrifugation before performing co-immunoprecipitation experiments.

Cell Culture, Transfection, siRNAs, and shRNAs—HeLa and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Non-silencing control shRNA and shRNA target sets were purchased from Sigma. The C1orf124 targeting sequences are as follows: 1, 5′-CTATGTCACAGGCTACTAACTCTCGAGTTAGTAGCTGTTTGCATAG-3′; and 2, 5′-GTACAA-CCACAGCTGAATTCTCGAGAAATTCTCGACTTGGTGTAC-3′. The shRNA-resistant wild-type and mutant C1orf124 constructs were generated by changing nucleotides in the shRNA1 targeting region (5′-GCACTCTGGC-ACACTGATCCTAGCGACGATCGCTATGAGCATTA-3′). The shRNAs were packaged into lentiviruses by cotransfection with packaging plasmids pMD2G and pSPAX2 into 293T cells. 48 h later, the supernatant was collected for infection of HeLa cells. Infection was repeated twice with an interval of 24 h to achieve maximal infection efficiency. Infected cells were selected with medium containing puromycin (2 µg/ml).

Recombinant Proteins—GST proteins were expressed in Escherichia coli BL21(DE3) cells and purified as follows. Cells were pelleted and lysed in NETN buffer A (150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0) and 0.5% Nonidet P-40) supplemented with 1 mM PMSF, 1 mM DTT, and 50 µg/ml lysozyme. Cells were sonicated and clarified by centrifugation at 12,000 rpm for 20 min at 4 °C. After clarification, the supernatant was incubated with glutathione-Sepharose beads (Sigma) for 2 h at 4 °C. After three washes with NETN buffer A, beads coated with the indicated proteins were used for pulldown experiments.

Tandem Affinity Purification (TAP)—TAP was performed as described previously (20). Briefly, 293T cells were transfected with plasmids encoding SFB (S-protein, FLAG, and streptavidin-binding peptide)-tagged constructs. Cell lines stably expressing tagged proteins were selected, and the expression of exogenous proteins was confirmed by immunoblotting and immunostaining. For affinity purification, a total of 20 10-cm dishes of 293T cells stably expressing SFB-tagged protein were collected for infection of HeLa cells. Infection was repeated twice with an interval of 24 h to achieve maximal infection efficiency. Infected cells were selected with medium containing puromycin (2 µg/ml).

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C1orf124 is a DNA damage protein that functions in response to replication stress. SLX4, also known as FANCP, is a Fanconi anemia protein that functions in the repair of interstrand DNA cross-links generated by agents such as MMC, cisplatin, and platinum-based drugs. The precise molecular function of SLX4 in MMC-induced DNA damage repair is unclear. To obtain a better understanding of how SLX4 is recruited to DNA damage sites and the molecular function of SLX4 in DNA cross-link repair, we performed TAP using cell lysates prepared from 293T cells stably expressing full-length C1orf124 and the C1orf124 mutant co-localized with γH2AX. These data suggest that C1orf124 localizes to DNA damage sites via its association with Ub-PCNA.

C1orf124 Participates in the PRR Pathway—Because both the PIP box motif and the UBZ domain of C1orf124 are important for its interaction with PCNA and its localization to DNA damage sites, we suspected that C1orf124 might function downstream of PCNA ubiquitination. However, we found that...
C1orf124 knockdown cells also showed reduced UV light-induced PCNA monoubiquitination (Fig. 3A). This reduction in PCNA ubiquitination was rescued by reconstitution of C1orf124 knockdown cells with full-length C1orf124, but not with the C1orf124ΔIP or C1orf124ΔUBZ mutant (Fig. 3A). These results indicate that although binding to PCNA is required for the recruitment of C1orf124 following UV radiation, C1orf124 is also required for maintaining the level of ubiquitinated PCNA at DNA damage sites.

The E3 ubiquitin ligase RAD18 is exclusively required for PCNA monoubiquitination, which is believed to be critical for the switch from normal replicative DNA polymerase to Y-family polymerase following DNA damage and therefore allows lesion bypass (9, 10, 13). Consistent with a key role of RAD18 in TLS, RAD18 depletion causes hypersensitivity to DNA-damaging agents (27–29). The reduction in PCNA ubiquitination observed in C1orf124 knockdown cells suggests that C1orf124 may also regulate RAD18 function. RAD18 is a putative
C1orf124-associated protein identified by our mass spectrometry analysis (Fig. 2A). We confirmed this association by co-immunoprecipitation experiments (Fig. 3B). Interestingly, we found that the localization of RAD18 to UV light-induced damage foci was diminished in C1orf124 knockdown cells (Fig. 3C). Moreover, the chromatin association of RAD18 following UV radiation was also reduced in C1orf124 knockdown cells (Fig. 3D). Cells with co-depletion of RAD18 and C1orf124 exhibited similar UV sensitivity as RAD18−/− cells or C1orf124 knockdown cells (Fig. 3E), indicating that C1orf124 and RAD18 function in the same PRR pathway.

C1orf124 Regulates TLS—DNA damage inhibits replication fork progression by blocking replicative DNA polymerases. To overcome this blockade, cells recruit specialized TLS polymerases, which can insert nucleotides opposite the damaged bases. In particular, TLS by DNA pol η (also called POLH/RAD30/XPV) is the major pathway for bypassing UV photoproducts (30). Recruitment of pol η and other TLS polymerases to stalled replication forks is mediated by monoubiquitination of PCNA (13). Y-family polymerases possess UBZ motifs, and the direct binding of TLS polymerases to Ub-PCNA facilitates their recruitment to stalled replication forks (11). However, there are also other mechanisms that contribute to TLS polymerase recruitment. For example, RAD18 has been shown to associate directly with pol η and to guide the polymerase to sites of DNA damage (16).

When we compared the results of TAP of C1orf124-containing protein complexes obtained from untreated cells (Fig. 2A) with those from UV light-irradiated cells (Fig. 4A), we found that we obtained more peptides derived from POLD3 (pol delta 3/p66) and PDIP1 (POLD3-interacting protein 1/POLDIP1/KCTD13) in the untreated sample than in the UV

**FIGURE 2. C1orf124 interacts and co-localizes with PCNA at UV light-induced damage sites.** A, TAP was performed using 293T cells stably expressing SFB-tagged C1orf124. The results from mass spectrometry analysis are shown. B, sequence alignment of the PIP box motif of C1orf124 with conserved PIP box motifs of other PCNA-interacting proteins, namely FEN1, p21, and XPG. The consensus PIP box sequence (Q\(\psi(x)\psi(\theta,\theta)\), where \(\psi = L/V/I/M\) and \(\theta = Y/F\)) is indicated. C, C1orf124 interacts with PCNA via the PIP box motif. 293T cells were transfected with plasmid encoding Myc-tagged PCNA. Cell lysates were incubated with GST, GST-C1orf124/PIP, or GST-C1orf124, and immunoblotting was performed using the indicated antibodies. WB, Western blot. D, PCNA interacts with C1orf124 in the absence and presence of UV damage. 293T cells were cotransfected with plasmids encoding SFB-tagged PCNA and Myc-tagged C1orf124. 24 h later, cells were left untreated or treated with 100 J/m² UV-C light and collected 4 h later. Coprecipitation was carried out using S-protein beads, and immunoblotting was performed using the indicated antibodies. E, the UBZ domain of C1orf124 is required to bind to ubiquitinated PCNA. 293T cells were transfected with plasmids encoding SFB-tagged C1orf124 and C1orf124ΔUBZ. 24 h later, cells were treated with 100 J/m² UV-C light and collected 4 h later. Coprecipitation was carried out using S-protein beads, and immunoblotting was performed using the indicated antibodies. F and G, the PIP box motif and UBZ domain mediate the recruitment of C1orf124 to DNA damage sites. HeLa cells were transfected with constructs encoding SFB-tagged full-length (FL) C1orf124, C1orf124ΔSHP, C1orf124ΔPIP, or C1orf124ΔUBZ. Cells were treated with laser micro-irradiation (F) or 10 J/m² UV-C light (G) and incubated for 6 h prior to immunostaining with the indicated antibodies. RPA, replication protein A.
light-irradiated sample. Interestingly, POLH (pol η) was identified only as a C1orf124-associated protein in the UV light-treated sample (Figs. 2A and 4A), suggesting that C1orf124 may associate with different polymerases before and after DNA damage. Co-immunoprecipitation experiments showed that the binding of C1orf124 to POLD3 or PDIP1 was dramatically reduced following UV irradiation (Fig. 4B and C). On the other hand, the association of C1orf124 with translesion polymerase POLH was markedly increased in cells treated with UV radiation (Fig. 4D). These results suggest that C1orf124 may directly participate in the switching of replicative polymerase to translesion polymerase following UV damage and therefore mediate lesion bypass.

C1orf124 Coordinates with Valosin-containing Protein (VCP) in Mediating Cellular Resistance to UV Damage—Besides DNA replication and repair proteins, we also identified VCP and VCP-associated proteins (VCPIP and UBXD10) as C1orf124-binding proteins (Figs. 2A and 4A). VCP (also known as p97 and Cdc48) belongs to the hexameric AAA-ATPase family and functions in diverse cellular activities that include ubiquitin-dependent endoplasmic reticulum-associated protein degradation (31, 32), autophagy (32), endosomal sorting (33), and protein degradation at the outer mitochondrial membrane (34). Recent studies imply that VCP acts by extracting protein complexes bound to chromatin rather than promoting protein degradation. For example, Aurora B has been shown to be extracted from mitotic chromosomes by VCP/p97 and its cofactors (35). Furthermore, VCP/p97 regulates DNA replication by mediating the removal of replication licensing factor Cdt1 that is bound to PCNA (36). VCP/p97 has also been shown to function in repair of ionizing radiation-induced double-strand breaks. VCP/p97 regulates DNA repair following UV light damage, and the expression of C1orf124 is required for UV light-induced PCNA ubiquitination (Fig. 3A).
protein substrates to allow for proper assembly of downstream signaling effectors, including RAD51, BRCA1, and 53BP1 (37). In addition, VCP/p97 also mediates the removal of the RNA polymerase II complex when it is stalled at UV light-induced DNA lesions (38). These studies indicate that VCP plays a role in the DNA damage response. Thus, it is possible that the association between C1orf124 and VCP may be important for C1orf124 function following UV irradiation.

We found that C1orf124 binds to VCP via the SHP box (Fig. 5A). Deletion of the PIP box or UBZ domain of C1orf124 did not influence its interaction with VCP (Fig. 5A). To assess the function of C1orf124-VCP interaction following UV irradiation, we generated constructs encoding shRNA-resistant SFB-tagged wild-type C1orf124, C1orf124/ΔH9004, C1orf124/ΔSHP, or C1orf124/ΔUBZ. Only the expression of shRNA-resistant wild-type C1orf124, but not any of these C1orf124 deletion mutants, could rescue the UV hypersensitivity in C1orf124-depleted cells (Fig. 5B). These data indicate that the binding of C1orf124 to VCP and to ubiquitinated PCNA is required for its in vivo function mediating cell survival following UV damage.

**DISCUSSION**

In this study, we have identified and characterized the PCNA-binding protein C1orf124. Cells depleted of C1orf124 showed marked increases in cellular sensitivity to UV and HU treatment (Fig. 1, F and G), suggesting a function of C1orf124 in the DNA damage response. C1orf124 interacted with unmodified and ubiquitinated PCNA (Fig. 2, C–E) and localized to UV light-induced DNA damage sites. Depletion of C1orf124 resulted in a marked decrease in PCNA monoubiquitination, which was accompanied by a reduction in RAD18 chromatin association and RAD18 localization to DNA damage sites (Fig. 3, A–C). These results suggest that C1orf124 is required to stabilize RAD18 and Ub-PCNA at the sites of DNA damage.

Our findings agree with the observations in a recent study characterizing the function of C1orf124 in the UV light-induced damage response (39). However, the precise mechanism by which C1orf124 confers cellular resistance to UV damage is still unclear. In this study, we have shown that C1orf124 binds VCP via the SHP box and that the binding of C1orf124 to VCP/p97 is crucial for cellular resistance to UV damage (Fig. 5, A and B). Studies have shown that VCP/p97 functions in regulating DNA metabolic processes by mediating proteasomal degradation or catalyzing the extraction of proteins or protein complexes from the chromatin (35, 38). On the basis of this information and the data presented in our study, we propose that C1orf124 may function to stabilize Ub-PCNA and RAD18 on DNA damage sites by preventing their removal or extraction from the chromatin by VCP/p97 during TLS (Fig. 5C). C1orf124 may carry out its regulatory function by sequestering the substrates (RAD18 and PCNA) away from the enzymatic action of VCP/p97 either by directly inhibiting VCP activity or by physically disrupting VCP-substrate interaction via steric hindrance. Thus, in C1orf124-depleted cells, VCP can gain access to its substrates and catalyzes the removal of RAD18 and Ub-PCNA from the chromatin (Fig. 5C, right panel) and thus results in the reduction of RAD18 and Ub-PCNA at damage sites. Our hypothesis is supported by the observation that
overexpression of a catalytically inactive dominant-negative mutant of VCP (E305Q/E578Q) restored RAD18 chromatin association in C1orf124 stable knockdown cells (supplemental Fig. 1, A and B). Furthermore, the reduction of PCNA ubiquitination observed in C1orf124 knockdown cells was also rescued by the expression of this catalytically inactive mutant of VCP (supplemental Fig. 1C).

Interestingly, we also found that C1orf124 preferentially bound to replicative polymerase POLD3 or TLS polymerase POLH before or after DNA damage (Fig. 4, B–D). Thus, we propose that C1orf124 may directly regulate the switch from replicative polymerase to translesion polymerase following DNA damage (Fig. 5C). It is likely that this function of C1orf124 may involve its regulated associations with these polymerases and potentially also the function of VCP/p97. Further studies are needed to elucidate precisely how this switch occurs at the molecular level.

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