Human HLTF functions as a ubiquitin ligase for proliferating cell nuclear antigen polyubiquitination

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Human helicase-like transcription factor (HLTF) is frequently inactivated in colorectal and gastric cancers. Here, we show that HLTF is a functional homologue of yeast Rad5 that promotes error-free replication through DNA lesions. HLTF and Rad5 share the same unique structural features, including a RING domain embedded within a SWI/SNF helicase domain and an HIRAN domain. We find that inactivation of HLTF renders human cells sensitive to UV and other DNA-damaging agents and that HLTF complements the UV sensitivity of a *rad5*Δ yeast strain. Also, similar to Rad5, HLTF physically interacts with the Rad6–Rad18 and Mms2–Ubc13 ubiquitin-conjugating enzyme complexes and promotes the Lys-63-linked polyubiquitination of proliferating cell nuclear antigen at its Lys-164 residue. A requirement of HLTF for error-free postreplication repair of damaged DNA is in keeping with its cancer-suppression role.

yeast Rad5 | damage bypass | K63 polyubiquitination | tumor suppressor

Lesions in DNA impose a block to synthesis by the replicative polymerases (Pols), and unless replication is rescued by the timely action of lesion bypass processes, stalled replication forks can collapse, leading to genomic instability. In eukaryotes the Rad6–Rad18 enzyme complex regulates lesion bypass processes that ensure the completion of replication. Rad6, a ubiquitin-conjugating enzyme, forms a tight complex with Rad18, a RING-finger type ubiquitin ligase that binds DNA (1, 2), and in cells treated with DNA-damaging agents, Rad6–Rad18 monoubiquitinates proliferating cell nuclear antigen (PCNA), a DNA Pol sliding clamp that is a key component of the replication machinery (3). Ubiquitination at the Lys-164 residue of PCNA and its subsequent polyubiquitination serves as a molecular switch between various DNA damage bypass processes (3–5).

In the yeast *Saccharomyces cerevisiae*, Rad6–Rad18 governs at least three alternative pathways for promoting replication through DNA lesions (6). Two pathways activated by PCNA monoubiquitination are carried out by specialized translesion synthesis (TLS) DNA Pols, such as Pol η and Pol ζ, which are able to copy DNA directly from the damaged template on an error-free or error-prone way (6). The third pathway called postreplication repair (PRR), however, is activated by PCNA polyubiquitination and operates by template switching using the information of the undamaged newly synthesized nascent strand on the sister duplex for DNA synthesis across damaged DNA (7–10). The PRR pathway depends on the Rad5, Mms2, and Ubc13 proteins and promotes error-free replication through DNA lesions. Recently, we have shown that yeast Rad5 has a DNA helicase activity that is specialized for replication fork regression, as Rad5 can concertedly unwind and anneal the nascent and the parental strands of the fork without exposing any single-stranded regions (7). This Rad5 activity would ensure damage bypass by promoting replication fork regression where the newly synthesized DNA strand of the sister duplex can be used as a template. In addition to its role as a DNA helicase, Rad5 has a C_3HC_4 RING domain that is indicative of a ubiquitin ligase function. In DNA-damaged yeast cells, Rad5 is required together with the Mms2–Ubc13 ubiquitin-conjugating complex for the polyubiquitination of PCNA at its Lys-164 residue via a Lys-63-linked ubiquitin chain (3). *In vivo*, Rad5 associates with the Mms2–Ubc13 complex via Ubc13, and it also interacts with the Rad18 subunit of the Rad6–Rad18 complex and, presumably through these interactions, coordinates the action of these two enzyme complexes in PCNA polyubiquitination (11). Importantly, the inactivation of the DNA helicase function or the ubiquitin ligase function of Rad5 causes the same high degree of PRR defect as is seen in the *rad5*Δ mutant, indicating that both these activities are indispensable for Rad5 function in PRR (8).

In DNA-damaged human cells also, PCNA is monoubiquitinated at its K164 residue by Rad6–Rad18 and then polyubiquitinated by Mms2–Ubc13 via a Lys-63-linked ubiquitin chain. Previously, we and others have shown that human SHPRH, which resembles Rad5 in having a SWI/SNF helicase domain and a C_3HC_4 RING motif, exhibits a ubiquitin ligase activity able to promote PCNA polyubiquitination in a Rad6–Rad18- and Mms2–Ubc13-dependent manner (12, 13).

In addition to SHPRH, humans have another SWI/SNF protein, helicase-like transcription factor (HLTF), which displays a much higher degree of sequence conservation to Rad5 than SHPRH. Like Rad5, HLTF contains a RING domain, the SWI/SNF helicase domains, and a HIRAN domain not present in SHPRH. Furthermore, SHPRH differs from both Rad5 and HLTF in possessing sequence motifs not present in either protein. Although a role for HLTF in transcriptional regulation is indicated from previous studies where it has been shown to activate transcription by binding to specific regions of promoters and enhancers, the high degree of structural similarity of HLTF to Rad5 raised the possibility that HLTF contributes to DNA repair in ways similar to Rad5.

Here, we show that, similar to Rad5, HLTF has a ubiquitin-ligase activity that promotes the polyubiquitination of PCNA in collaboration with Rad6–Rad18 and Mms2–Ubc13 both *in vitro* and *in vivo*. Also, we provide evidence that the knockdown of HLTF levels results in elevated sensitivity to UV in human cells and that HLTF can rescue the UV sensitivity of a *rad5*Δ yeast mutant strain. Based on these and other findings, we conclude there is a role for HLTF in error-free PRR of damaged DNA.

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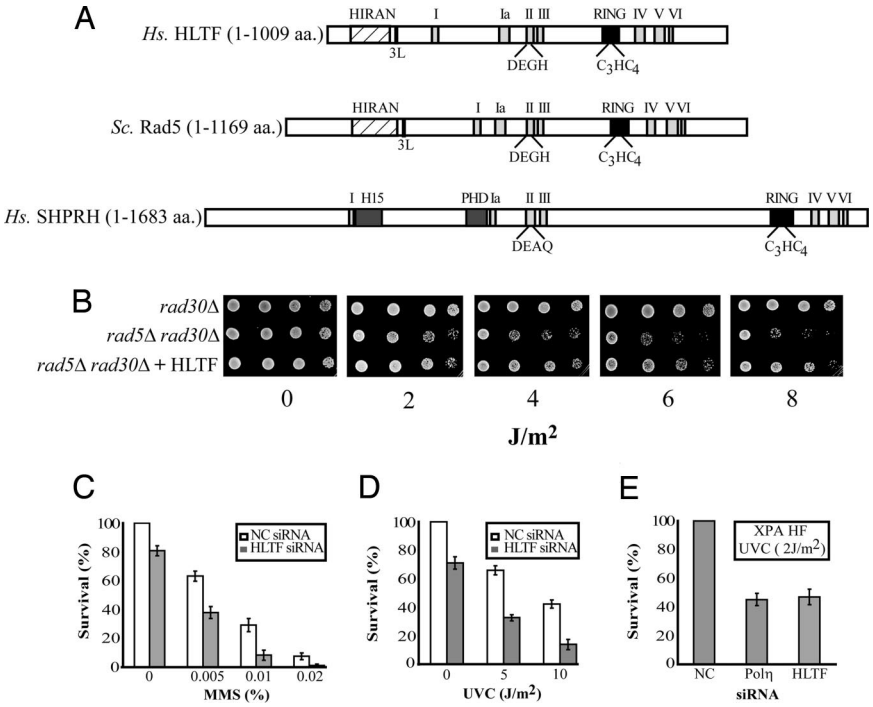

Fig. 1. HLTF complements the UV sensitivity of the yeast *rad5*Δ mutant and contributes to UV and MMS resistance of human cells. (*A*) Schematic representation of HLTF, SHPRH, and Rad5 proteins. All three proteins exhibit the conserved SNF2-type helicase domain shown by gray boxes numbered I–VI and a C3HC4-type RING-finger domain represented by a black box. The HIRAN motif of HLTF and Rad5 is shown by a striped box that is followed by a leucine heptad repeat motif labeled by a black line and 3L. In SHPRH, the H1,5 domain found in linker histone 1 and histone 5 families and a PHD domain are shown by dark gray boxes. (*B*) Complementation of the UV sensitivity of the *rad5*Δ yeast strain by HLTF. The indicated yeast strains, *rad30*Δ, *rad5*Δ*rad30*Δ, and *rad5*Δ*rad30*Δ in which human HLTF was expressed, were grown until midlog phase and spotted onto plates in 10× serial dilutions at equal cell concentrations. Cells were UV-irradiated with the indicated doses and incubated in the dark at 30°C. Cell growth was evaluated after 2 days. (*C*) Effect of siRNA inhibition of HLTF on MMS sensitivity of human cells. Normal human fibroblasts (HF) were transfected with HLTF-specific or negative control (NC) siRNAs followed by incubation in the presence of the indicated concentration of MMS for 3 h. After 2 days, cell survival was determined by MTT assay. Data shown as mean value and the SD presented as an error bar were calculated from three independent experiments. (*D*) Effect of siRNA inhibition of HLTF on UV survival of human cells. Normal HF cells were transfected with HLTF siRNA and irradiated with the indicated doses of UVC light, and cell survival was measured as described above. (*E*) Effect of HLTF knockdown on UV survival in XPA$^-$ cells. HLTF or Pol η expression was inhibited in XP-A-deficient human fibroblasts by siRNA and treated with 2 J/m^2 UV light before cell survival was examined. The first bar represents a control sample transfected with nonspecific siRNA.

Results

HLTF Is Able to Complement the UV Sensitivity of a Yeast *rad5*Δ Mutation and Contributes to UV and Methyl Methanesulfonate (MMS) Resistance of Human Cells. Human HLTF shows 43% similarity to *Saccharomyces cerevisiae* Rad5 protein at the amino acid level [supporting information (SI) Fig. 5]. Both proteins contain the seven motifs found in helicases and the SNF2 family of ATP-hydrolyzing proteins, a RING domain characteristic of ubiquitin ligases, and they share the unusual domain structure in which the RING domain is inserted between helicase domains III and IV (Fig. 1*A*). Although SHPRH also shows similarity to Rad5 (Fig. 1*A*), it has two additional protein domains: a histone H1 and H5 linker sequence and a PHD domain that are not present in Rad5 or HLTF (Fig. 1*A* and SI Fig. 5). Moreover, only HLTF and Rad5 have the HIRAN domain in the N terminus (Fig. 1*A* and SI Fig. 5) that has been proposed to function as a DNA-binding domain for recognizing damaged DNA or a stalled replication fork (14), followed by a leucine heptad repeat motif. Thus, throughout the entire sequence, HLTF shows a much higher degree of homology to yeast Rad5 than does SHPRH.

Prompted by the high degree of sequence conservation, we tested whether HLTF could complement the UV sensitivity of yeast strains devoid of Rad5 function. Because we were not able to observe a considerable increase in UV resistance when we expressed HLTF in a *rad5*Δ yeast strain, to increase the sensitivity of the assay further, we examined the complementation of the UV sensitivity of the *rad5*Δ*rad30*Δ strain by HLTF. Because of the simultaneous inactivation of two lesion bypass pathways, the *rad5*Δ*rad30*Δ strain exhibits a much higher level of UV sensitivity than the *rad5*Δ strain. Interestingly, we found that HLTF greatly increases the UV resistance of *rad5*Δ*rad30*Δ mutant cells (Fig. 1*B*). The observed increase in UV resistance was the result of HLTF complementing the Rad5 function and not the Pol η function because the expression of HLTF in a *rad30*Δ strain did not show any evidence of complementation. Furthermore, we did not detect any complementation of the UV sensitivity of the *mms2*Δ*rad30*Δ strain, in which similar to the *rad5*Δ*rad30*Δ strain, both Rad5–Mms2-dependent PRR and Pol η-dependent TLS have been inactivated (data not shown). From these observations, we conclude that HLTF is able to substitute for the DNA repair function of Rad5 in yeast cells.

The ability of HLTF to complement the *rad5*Δ mutation points to a high degree of conservation of the Rad5-dependent error-free lesion bypass pathway from yeast to humans. Inactivation of such a pathway would be expected to render human cells sensitive to DNA-damaging agents. To examine this possibility, we knocked down the expression of HLTF by siRNA in human fibroblasts by >80% (data not shown) and examined the UV and MMS sensitivity of the cells. As shown in Fig. 1 *C* and *D*, the HLTF knockdown has some inhibitory effect on cell survival even in the absence of any DNA-damaging treatment. However, exposure of human cells to UV or MMS caused a much further reduction in the survival of cells deficient in HLTF function (Fig. 1 *C* and *D*). A Rad5-like role of HLTF in promoting replication

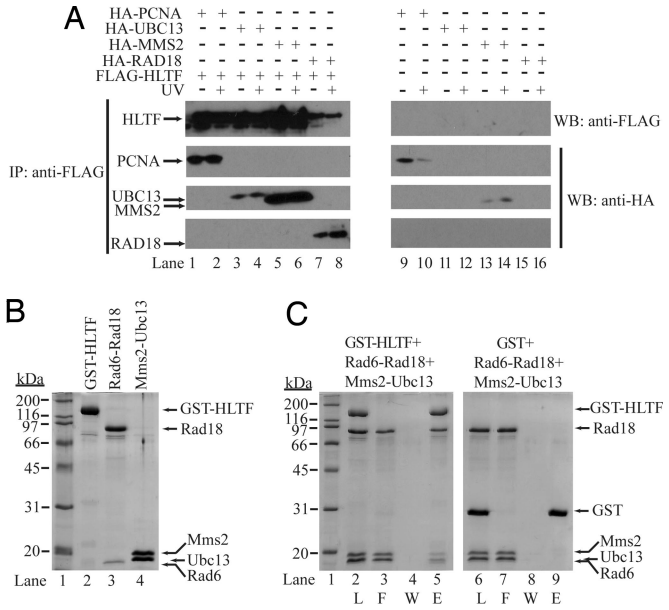

Fig. 2. Complex formation of HLTF with Rad6–Rad18 and Mms2–Ubc13. (*A*) Immunoprecipitation of human HLTF in complex with PCNA, Ubc13, Mms2, or Rad18. HEK293FT cells were transiently transfected with plasmids expressing HA–PCNA, HA–Ubc13, HA–Mms2, or HA–Rad18 alone or together with FLAG-HLTF as indicated. Thirty-six hours after transfection, half of the cells were treated with 40 J/m^2 UV, and 6 h later total cellular lysates were prepared. Immunoprecipitations (IP) were carried out with anti-FLAG antibodies, and subsequently the precipitates were analyzed by Western blots (WB) using anti-FLAG and anti-HA antibodies as indicated (lanes 1–16). (*B*) Purity of the proteins. One microgram of each protein was analyzed on 12% denaturing SDS-polyacrylamide gel. Lane 1, molecular mass standards; lane 2, GST–HLTF; lane 3, Rad6–Rad18 complex; lane 4, Mms2–Ubc13 complex. (*C*) GST pull-down of purified HLTF with Rad6–Rad18 and Mms2–Ubc13. GST–HLTF (10 μg) immobilized on glutathione-Sepharose beads was incubated with Rad6–Rad18 (7.5 μg) and Mms2–Ubc13 (6 μg). After washing, bound proteins were eluted by glutathione. Aliquots of each sample, taken before addition to the beads (L), from the flow-through fraction (F), from the last wash (W), and from the eluted proteins (E), were analyzed on a 12% SDS-polyacrylamide gel (lanes 2–5). The results for the control experiment using GST instead of GST–HLTF are shown in lanes 6–9.

through DNA lesions would suggest that the UV sensitivity of XPA-deficient cells, defective in nucleotide excision repair, will increase upon the inactivation of HLTF. As shown in Fig. 1*E*, the knockdown of HTLF in XPA$^-$ cells results in increased UV sensitivity, with ≈40% of cells surviving at 2 J/m^2, an effect similar to that seen for Pol η knockdown. We conclude from these observations that HLTF contributes to the resistance of human cells to DNA-damaging agents.

HLTF Associates with PCNA, Rad18, and Mms2-Ubc13. To establish that HLTF functions in human cells in a Rad5-like manner, we next examined whether it could associate *in vivo* with Rad6–Rad18, Mms2–Ubc13, and PCNA. We examined these interactions in human embryonic kidney 293FT cells, and to facilitate the detection of the interactions, plasmids expressing HA-tagged Rad18, Mms2, Ubc13, or PCNA were cotransfected into cells together with FLAG-tagged HLTF. The expression of these proteins was confirmed in total cell lysates by Western blot using anti-FLAG and anti-HA antibodies (data not shown). Next, we immunoprecipitated FLAG-HLTF and its associated proteins from cell lysates by using anti-FLAG beads, and the precipitated proteins were analyzed for the presence of HA-tagged proteins with anti-HA antibody. As shown in Fig. 2*A* (lanes 1, 3, 5, and 7), we detected association of HLTF with PCNA, Ubc13, Mms2, and Rad18. The specificity of these interactions was verified in control experiments using empty FLAG construct (Fig. 2*A*, lanes 9–16). In parallel experiments, we found that UV irradiation of cells followed by incubation for 6 h before immunoprecipitation did not affect any of these interactions (Fig. 2*A*, compare lanes 1, 3, 5, and 7 with 2, 4, 6, and 8, respectively). From these experiments we conclude that, similar to yeast Rad5, human HLTF associates with Rad18, Mms2, Ubc13, and PCNA *in vivo* and that this association occurs in the absence of UV irradiation.

To provide further evidence for the complex formation of HLTF with Rad6–Rad18 and Mms2–Ubc13, we purified these proteins and tested them for direct physical interaction. To facilitate the purification of human HLTF protein, we fused the *HLTF* cDNA downstream of the *GST* gene and expressed it in a protease-deficient *S. cerevisiae* strain. Purification of the GST-HLTF was carried out on glutathione beads, from which the GST–HLTF was eluted with glutathione that was subsequently dialyzed out. GST–Mms2, GST–Ubc13, and GST–Rad18, coexpressed with Rad6, were purified similarly but their GST tag was removed by Pre-Scission protease. As shown in Fig. 2*B*, GST–HLTF and the Rad6–Rad18 and Mms2–Ubc13 complexes were purified to near homogeneity. In GST pull-down assays purified Rad6–Rad18 and Mms2–Ubc13 proteins were added to GST–HLTF immobilized on gluhathione-Sepharose affinity beads, and after incubation bound proteins were released from the beads by incubating them with glutathione. As shown in Fig. 2*C*, lane 5, Rad6–Rad18 and Mms2–Ubc13 eluted together with GST—HLTF, indicating that these proteins formed a complex together. The specificity of the complex was verified in control experiments, where using GST instead of GST-HLTF in a similar assay did not result in any complex formation (Fig. 2*C*, lanes 6–9). In conclusion, purified HLTF, Rad6–Rad18, and Mms2–Ubc13 can interact directly and form a stable multisubunit protein complex.

HLTF Stimulates the Polyubiquitination of PCNA *In Vivo*. In yeast, upon DNA damage Rad6–Rad18 monoubiquitinates PCNA, and the monoubiquitinated form of PCNA is further polyubiquitinated by the combined action of Rad5 and Mms2–Ubc13. Similar to Rad5, complex formation of HLTF with the Rad6–Rad18 and Mms2–Ubc13 enzymes indicated that they might act together as a multisubunit ubiquitin-conjugating complex for PCNA ubiquitination. To explore this possibility, first we examined whether HLTF could promote the polyubiquitination of PCNA in human cells. We expressed GFP–HLTF, DsRed–Rad18, or a combination of these two proteins in HEK293FT cells and examined total cell lysates for PCNA ubiquitination by Western blot using anti PCNA antibody (Fig. 3*A Upper*). Further, we also precipitated PCNA from the lysates with anti-PCNA antibody and visualized ubiquitinated-PCNA forms by antiubiquitin antibody (Fig. 3*A Lower*). Because UV irradiation is known to enhance PCNA ubiquitination, in parallel, we also compared the samples from UV-irradiated and nonirradiated cells. In the absence of HLTF, we detected a very weak signal for monoubiquitinated PCNA, which was only slightly enhanced upon UV irradiation and Rad18 overexpression (Fig. 3*A*, compare lanes 3 and 4). Importantly, overexpression of HLTF resulted in PCNA polyubiquitination, which was enhanced by UV irradiation and overexpressing Rad18 (Fig. 3*A*, lanes 5–8). We note that in these experiments HLTF enhanced not only the polyubiquitination but also the monoubiquitination of PCNA (Fig. 3*A Upper*, compare lanes 5–8), which may be caused by the higher degree of activity of Rad6–Rad18 in complex with HLTF or a competition for monoubiquitinated PCNA between HLTF and a deubiquitinating enzyme, such as USP1. We have noticed that the anti-PCNA antibody has considerably weaker affinity to monoubiquitinated and polyubiquitinated PCNA than to PCNA.

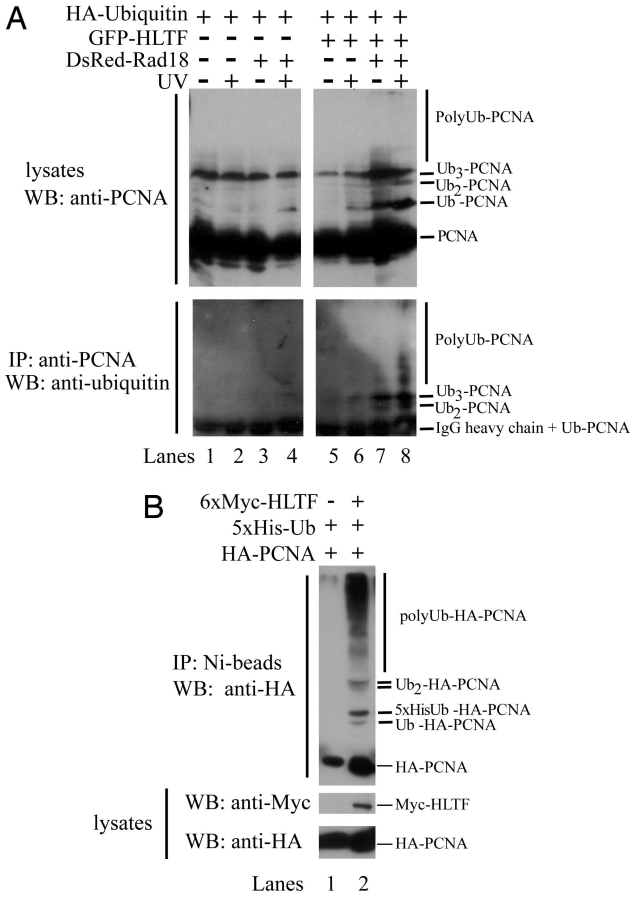

Fig. 3. HLTF promotes PCNA polyubiquitination in human cells. (*A*) PCNA polyubiquitination is stimulated by HLTF. HEK293FT cells were transfected with various combinations of plasmids expressing HA–ubiquitin, GFP–HLTF, and DsRed–Rad18 and UV-irradiated at 40 J/m^2. (*Upper*) After 48 h, cells were lysed and analyzed with anti-PCNA antibody. (*Lower*) From the lysates PCNA was immunoprecipitated (IP) with anti-PCNA antibody and PCNA ubiquitination was confirmed with anti-polyubiquitin (FK2) antibody. Because the IgG heavy chain migrates to the same position as HA–Ub–PCNA and is recognized by the secondary antibody, the differences in the levels of monoubiquitinated PCNA in different lanes are difficult to visualize. (*B*) Stimulation of polyubiquitination of PCNA by HLTF. HEK293FT cells were transfected with plasmids expressing HA–PCNA, 5His-ubiquitin, and with or without MYC–HLTF, as indicated. Forty-eight hours after transfection, cells were lysed and ubiquitinated proteins were immobilized on Ni-beads. Among the bound proteins ubiquitinated forms of PCNA were visualized by Western blot (WB) using anti-HA antibody.

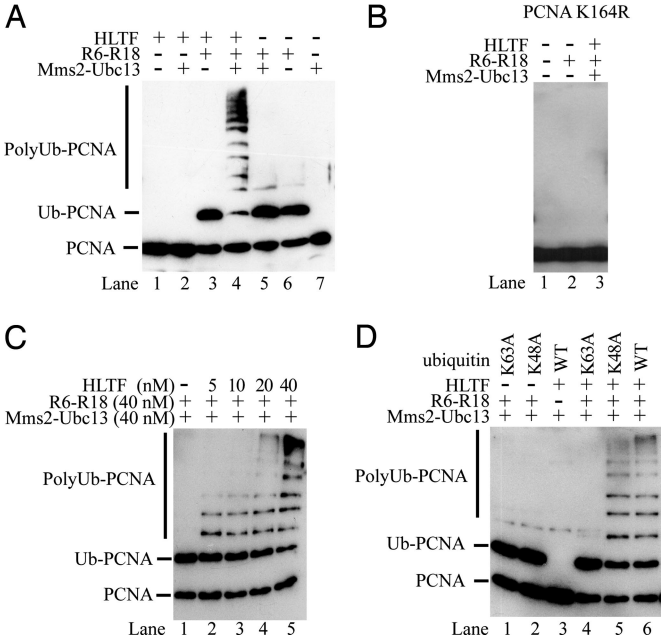

Fig. 4. HLTF is a ubiquitin ligase for Mms2–Ubc13-dependent K63-linked polyubiquitination of PCNA. (*A*) HLTF is required for PCNA polyubiquitination. PCNA (50 nM) was incubated in the absence or presence of various combinations of Rad6–Rad18 (100 nM), Mms2–Ubc13 (100 nM), and HLTF (100 nM) as indicated. Ubiquitination (Ub) of PCNA was followed by Western blot using anti-PCNA antibody. (*B*) Polyubiquitination occurs at the K164 residue of PCNA. K164R mutant PCNA (50 nM) was incubated with Rad6–Rad18 (100 nM) or with Rad6–Rad18 (100 nM), Mms2–Ubc13 (100 nM), and HLTF (100 nM) as indicated. (*C*) Concentration dependence of the ubiquitin ligase activity of HLTF. Polyubiquitination of PCNA (50 nM) was carried out at constant Rad6–Rad18 and Mms2–Ubc13 concentration (40 nM) but at increasing levels of HLTF (5–40 nM). (*D*) HLTF promotes K63-linked polyubiquitination of PCNA. PCNA ubiquitination reactions were carried out with K63A or K48A mutant ubiquitins or wild-type ubiquitin in the presence of various combinations of Rad6–Rad18, Mms2–Ubc13, and HLTF as indicated.

To overcome this limitation and further confirm the effect of HLTF on PCNA polyubiquitination, we also examined overexpressed HA–PCNA for ubiquitination in the presence of overexpressed 5His-ubiquitin. In this experimental system, we first enriched His-ubiquitinated proteins on nickel beads followed by detection of ubiquitinated HA–PCNA by HA antibody. Importantly, overexpression of HLTF markedly stimulated PCNA polyubiquitination (Fig. 3*B*, compare lanes 1 and 2). We note that under the nondenaturing conditions of the precipitation on nickel beads, we detected both ubiquitinated and unmodified PCNA monomers, which indicates that a PCNA trimer can have unmodified and ubiquitinated subunits within the same trimeric molecule. In summary, we found that HLTF greatly enhances polyubiquitination of endogenous and overexpressed PCNA *in vivo*.

HLTF Functions as a Ubiquitin Ligase for K63-Linked Polyubiquitination of PCNA. Next, we investigated whether HLTF can catalyze the polyubiquitination of PCNA *in vitro* by using highly purified proteins. We have already shown that for PCNA monoubiquitination to occur by Rad6–Rad18, PCNA first has to be loaded onto DNA by replication factor C (RFC) (13, 15). Therefore, first we preincubated PCNA with RFC and DNA followed by the addition of ubiquitin, Uba1, and various combinations of HLTF, Rad6–Rad18, and Mms2–Ubc13 (Fig. 4*A*). As expected, Rad6–Rad18 monoubiquitinated PCNA and the addition of Mms2-Ubc13 to this reaction did not have any effect on PCNA ubiquitination (Fig. 4*A*, compare lanes 5 and 6). Importantly, however, the addition of HLTF to the Rad6–Rad18 and Mms2–Ubc13-containing reaction resulted in the polyubiquitination of PCNA, where almost all of the monoubiquitinated form of PCNA was converted to polyubiquitinated species (Fig. 4*A*, lane 4). In contrast, in the absence of Mms2–Ubc13, HLTF alone or together with Rad6–Rad18 did not stimulate PCNA polyubiquitination (Fig. 4*A*, lanes 1 and 3, respectively), and neither did HLTF together with Mms2–Ubc13 in the absence of Rad6–Rad18 (Fig. 4*A*, lane 2). These results show that HLTF has a ubiquitin ligase activity for Rad6–Rad18- and Mms2–Ubc13-dependent polyubiquitination of PCNA. Ubiquitination occurred on the K164 residue of PCNA, as the K164R PCNA mutant was defective in both monoubiquitination and polyubiquitination (Fig. 4*B*). Increasing the concentration of HLTF up to the concentration of Rad6–Rad18 and Mms2–Ubc13 resulted in increased efficiency of PCNA polyubiquitination (Fig. 4*C*), which supports the notion that HLTF acts in a stoichiometric complex with Rad6–Rad18 and Mms2–Ubc13 protein complexes.

To determine the linkage specificity of the polyubiquitination, we used mutant ubiquitins, in which the K48 or the K63 residue

was mutated to alanine. Although both mutant ubiquitins were proficient in PCNA monoubiquitination, the K63A ubiquitin did not support PCNA polyubiquitination, whereas the K48A mutant behaved like wild-type ubiquitin (Fig. 4*D*), indicating that HLTF-catalyzed PCNA polyubiquitination is linked through the Lys-63 residue of ubiquitin. These observations provide strong biochemical evidence for a ubiquitin-ligase activity in HLTF that functions together with the Rad6–Rad18 and Mms2–Ubc13 enzyme complexes and stimulates the K63-linked polyubiquitination of PCNA at its K164 residue.

Discussion

In this study we provide evidence that human HLTF is a functional homologue of yeast Rad5. We show that similar to yeast Rad5, HLTF forms a stable complex with Rad6–Rad18 and Mms2–Ubc13, and we provide evidence for the interaction of HLTF with PCNA. Further, we show that HLTF promotes PCNA polyubiquitination *in vivo* and that purified HLTF functions as a ubiquitin ligase in promoting Mms2–Ubc13-dependent K63-linked polyubiquitination of PCNA at its K164 residue. Additionally, we present evidence that expression of HLTF can complement the UV sensitivity of a yeast *rad5*Δ strain, and as would be expected for a human Rad5 homologue, inhibiting the expression of HLTF renders human cells sensitive to UV light and MMS.

Yeast genetic studies have been instrumental for understanding the Rad6–Rad18-dependent PRR pathway, in which Rad5 plays a prominent role in promoting error-free replication of UV-damaged DNA (8, 9). Our recent finding that Rad5 has a replication fork-specific helicase activity has provided insight into the mechanism of this PRR pathway. On model replication fork substrates, Rad5 can concertedly unwind the nascent strands from their respective templates and then anneal them with one another and reanneal the parental strands (7). By this mechanism Rad5 can support a copy-choice type of synthesis, in which the information to fill in the gap opposite the DNA lesion is obtained by copying the newly synthesized strand of the sister duplex. In addition to the fork reversal activity, however, Rad5 has a ubiquitin ligase activity that acts in the polyubiquitination of PCNA (3). Yeast genetic data have shown that not only the *rad5* ATPase mutant but also the *rad5* RING mutant exhibit the same high degree of PRR defect as the *rad5*Δ mutant or the K164R mutation of PCNA, indicating that polyubiquitination of PCNA by the Rad5 ubiquitin ligase and the Rad5 helicase function are both necessary for PRR (5, 8, 9).

DNA damage bypass mechanisms are well conserved among eukaryotes from yeasts to humans. The human homologues of most of the yeast genes belonging to the Rad6–Rad18 group have been identified. However, in humans, additional layers of complexity have been added because humans have two homologues of yeast Rad6 and a family of Pol η homologues that include, in addition to Pol η, Pol ι, and Pol κ (6). Interestingly, yeast Rad5 has also two human orthologues, the *HLTF* and *SHPRH* genes. Although both have a RING-domain inserted between the seven SNF2-type helicase motifs, structurally HLTF resembles yeast Rad5 more closely than SHPRH, as only HLTF and Rad5 possess a recently identified HIRAN domain that has been predicted to have a role in recruiting repair activities to damaged DNA (14). On the other hand, SHPRH has an H1 and H5 linker sequence and a PHD domain not present in either Rad5 or HLTF. Because SHPRH can also promote the polyubiquitination of PCNA (12, 13), it will be important to examine the overlaps and differences between the functions of HLTF and SHPRH, suggested by their domain structures.

A number of previous studies have indicated a role for HLTF as a transcription factor. For example, HLTF activates the PA 1-1 promoter via specific binding to a promoter element (16), and in other studies, HLTF has been shown to bind to the simian virus 40 (SV40) enhancer, the myosin light chain locus enhancer, and HIV long terminal repeats (17–20). Interestingly, humans have at least two different forms of HLTF protein that differ in their translation initiation site, and it is the shorter form that lacks the ≈120 N-terminal residues that exhibit the sequence-specific DNA binding activity and is transcriptionally active (16). Because the sequence-specific DNA binding region in HLTF has been mapped to lie between residues ≈125 and 220, it would seem that the HIRAN domain common to both HLTF and Rad5 is not involved in the binding of the gene regulatory regions. It would be of considerable interest to identify the role of the HIRAN domain in the HLTF and Rad5 proteins and determine whether this sequence contributes to the DNA repair role of these proteins.

In yeast cells, Rad5 functions in an error-free PRR pathway through which it would mediate replication through a large variety of DNA lesions by template switching. In this role Rad5 functions as a ubiquitin ligase for promoting PCNA polyubiquitination by the combined action of the Rad6–Rad18 and Mms2–Ubc13 enzymes and as a DNA helicase for carrying out replication fork regression. As would be expected from an error-free role in PRR, mutational inactivation of the *RAD5*, *MMS2*, and *UBC13* genes confers enhanced mutagenesis in yeast cells treated with DNA-damaging agents (6). In humans, the various elements of this PRR pathway have been conserved, the inactivation of *MMS2* leads to an elevated mutation frequency, and PCNA polyubiquitination provides protection against mutations (21, 22). A role for HLTF in promoting Rad6–Rad18- and Mms2-Ubc13-dependent PCNA polyubiquitination implicates its involvement in promoting error-free PRR through DNA lesions in a manner similar to that of Rad5 in yeast cells.

A cancer suppressor role for HLTF is indicated from the observations that loss of HLTF expression accompanied by HLTF promoter methylation occurs in as many as 50% of colon and gastric cancers (23–25). Taken together, our observations that human HLTF functions together with Rad6–Rad18 and Mms2-Ubc13 in PCNA polyubiquitination, and that it can complement the UV sensitivity of *rad5*Δ yeast cells, strongly support a role for HLTF in promoting error-free DNA damage bypass. Hence, by preventing mutagenesis, HLTF DNA repair function would contribute to minimizing the incidence of carcinogenesis in humans.

Materials and Methods

Plasmids, Proteins, and Antibodies. The cDNA of HLTF in pZL2 plasmid was a gift from Alexandra Belayew (University of Leuven, Belgium) (16). The HLTF cDNA was cloned into N-terminal fusion with the GST gene in the yeast *GAL-PGK* expression vector pBJ842, resulting in plasmid pIL1000. Plasmids with CMV promoter-driven N-terminal fusions of GFP in pEGFPC3 (pIL1125), MYC in pCS2MT (pIL1370), and FLAG in pRK2F (pIL1490) with HLTF were constructed for expression in human cells. For expression in human cells, the cDNA of human Mms2 was cloned into pRK2F, resulting in pIL1757, and human ubiquitin was cloned into pRK2H and pRK5His, resulting in plasmids pIL1377 and pIL1376, respectively. cDNAs of human PCNA and human Rad18 were cloned into pRK2H and pDSREDC1, resulting in pIL1460 and pIL1263, respectively. Human Rad6–Rad18, Mms2–Ubc13, RFC, and ubiquitin-activating enzymes were purified as described (13). GST–HLTF was expressed in yeast and purified on glutathione-Sepharose beads. Bovine ubiquitin was purchased from Sigma. K63 and K48 yeast ubiquitins were gifts from Cecile Pickart (The Johns Hopkins University, Baltimore). The antibodies were obtained commercially: PCNA (PC10; Santa Crux), ubiquitin (FK2; Sigma), FLAG (M2; Sigma), HA (3F10; Roche), and MYC (9E10). Secondary antibodies were purchased from Pierce.

UV and MMS Survival. SV40-transformed normal (MRC5) and XPA-deficient (XP12BE) human fibroblasts obtained from Gerd Pfeifer (Beckman Research Institute of the City of Hope, Duarte, CA) were used for UV sensitivity assays. Two predesigned synthetic duplex siRNAs for human HLTF (ID codes 12487 and 12580) and for human Pol η (26) and a nonspecific negative control siRNA (NC siRNA no. 1) were purchased from Ambion. Duplex siRNAs (50 pmol) were transfected by using Lipofectamine 2000 (Invitrogen). Forty-eight hours after

transfection, RT-PCR was carried out to check the knockdown efficiency and cells were treated with UV or MMS with the indicated doses. After 48 h, UV and MMS survival was determined by the MTT assay (Promega) according to the manufacturer's instructions. For yeast complementation experiments, cells transformed with GST-HLTF expressing plasmid (pIL1000) or empty GST vector (pBJ842) were grown until midlog phase in selective medium, washed and spotted in 10× serial dilutions onto selective media, and irradiated with different UV doses. Plates were incubated in the dark at 30°C for 2 days before the experiments were evaluated.

Western Blots and Immunoprecipitation. To check *in vivo* interactions, HEK293FT cells were transfected with different combinations of expression plasmids as indicated in Fig. 3. Twenty-four hours later, half of the cultures were irradiated with 40 J/m^2 UV light, and after 6 h of additional incubation cells were lysed in buffer A (10 mM Tris·HCl, pH 7.6/1 mM EDTA/400 mM NaCl/15% glycerol/0.5% Nonidet P-40). Clarified lysates were diluted two times in buffer A without NaCl, and FLAG-tagged proteins and their complexes were immobilized on FLAG-beads for 2 h at 4°C. Bound proteins and the input lysates were analyzed by Western blot. For immunoprecipitation of PCNA, HEK293FT cells, transfected as indicated in Fig. 3, were UV-treated 24 h after transfection as noted above and lysed with 0.5% SDS at 100°C for 5 min. Sonicated and clarified lysates were diluted five times with buffer A containing 200 mM NaCl and 25 mM *N*-ethylmaleimide (NEM), and incubated with PCNA antibody overnight at 4°C. The next day, lysates were incubated for 2 h with protein-A agarose beads, and bound proteins and the input lysates were analyzed by Western blot. For enriching ubiquitinated PCNA, 48 h after transfection cells were lysed in 100 mM NaH_2PO_4 (pH 8.0), 10% glycerol, 100 mM NaCl, 0.2% Triton X-100, 1 mM PMSF, and 10 mM NEM, and lysates were incubated with NiNTA beads (Qiagen) for 1 h at 4°C. Bound proteins and the input lysates were analyzed.

GST Pull-Down Assay. Purified GST or GST-HLTF proteins (10 μg) were incubated with glutathione-Sepharose beads (Amersham Pharmacia) for 1 h at 4°C in buffer A (50 mM Tris·Cl, pH 7.5/250 mM NaCl/1 mM EDTA/1 mM DTT/10% glycerol). After washing beads were further incubated with Rad6–Rad18 (7.5 μg) and Mms2–Ubc13 (6 μg) overnight at 4°C in buffer B (same as buffer A but with 50 mM NaCl). Beads were washed five times with buffer B, and bound proteins were eluted in buffer B containing 20 mM reduced glutathione. Various fractions were analyzed by SDS/PAGE.

***In Vitro* Ubiquitination.** A standard *in vitro* ubiquitination reaction (10 μl) was carried out in P0 buffer (40 mM Tris·HCl, pH 7.5/8 mM $MgCl_2$/100 mg/ml BSA/10% glycerol/500 μM ATP) in the presence of 100 nM Uba1, 50 μM ubiquitin, 10 nM RFC, and 0.5 nM of a double-stranded pUC19 plasmid nicked by BstNBI enzyme (New England BioLabs) at 30°C for 60 min. As indicated in Fig. 4, ubiquitination reactions of PCNA (50 nM) were initiated by adding Rad6–Rad18 (40–100 nM), Mms2–Ubc13 (40–100 nM), and HLTF (5–100 nM) at various concentrations and in combinations. Samples containing unmodified and ubiquitinated PCNA were separated on 12% denaturing polyacrylamide gel and visualized by Western blot by using anti-PCNA antibody (Santa Cruz Biotechnology).

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