Sequential assembly of translesion DNA polymerases at UV-induced DNA damage sites

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ABSTRACT In response to DNA damage such as from UV irradiation, mammalian Y-family translesion synthesis (TLS) polymerases Polη and Rev1 colocalize with proliferating cell nuclear antigen at nuclear foci, presumably representing stalled replication sites. However, it is unclear whether the localization of one polymerase is dependent on another. Furthermore, there is no report on the in vivo characterization of the Rev3 catalytic subunit of the B-family TLS polymerase Polζ. Here we describe the detection of endogenous human Polη, Rev1, and Rev3 by immunocytochemistry using existing or newly created antibodies, as well as various means of inhibiting their expression, which allows us to examine the dynamics of endogenous TLS polymerases in response to UV irradiation. It is found that Rev1 and Polη are independently recruited to the nuclear foci, whereas the Rev3 nuclear focus formation requires Rev1 but not Polη. In contrast, neither Rev1 nor Polη recruitment requires Rev3. To further support these conclusions, we find that simultaneous suppression of Polη and Rev3 results in an additive cellular sensitivity to UV irradiation. These observations suggest a cooperative and sequential assembly of TLS polymerases in response to DNA damage. They also support and extend the current polymerase switch model.

INTRODUCTION

DNA damage tolerance (DDT) is defined as a strategy by which cells complete genome replication in the presence of DNA damage to avoid mitotic catastrophe. DDT can occur by at least two mechanisms; one is called damage avoidance, in which cells use a newly replicated sister chromatid as a template to synthesize across replication-blocking lesions, and another is called translesion synthesis (TLS), in which cells use a set of specialized, nonreplicative DNA polymerases to synthesize across the damaged template. The latter process can be either error free or error prone (Friedberg and Gerlach, 1999; Lehmann et al., 2007; Andersen et al., 2008).

In the budding yeast *Saccharomyces cerevisiae*, DDT is initiated by Rad6/Rad18-mediated monoubiquitination of proliferating cell nuclear antigen (PCNA) (Hoege et al., 2002), the replication processivity factor that functions as the primary scaffold for the replication machinery (Moldovan et al., 2007), whereas another E2-E3 complex, Mms2-Ubc13-Rad5, is required to polyubiquitinate PCNA via a noncanonical Lys63-linked chain (Hofmann and Pickart, 1999; Hoege et al., 2002). This poly-Ub chain differs from the conventional Lys48-linked chain in that it is not for target protein degradation but is believed to be involved in signaling (Pickart and Fushman, 2004). Genetic data indicate that PCNA monoubiquitination promotes TLS, whereas subsequent polyubiquitination promotes error-free DDT (Hoege et al., 2002; Stelter and Ulrich, 2003).

Mammalian homologues of yeast genes involved in monoubiquitination and polyubiquitination of PCNA have been identified, indicating the existence of both DDT mechanisms in human cells. It turns out that human cells contain four Y-family polymerases, three of which (Polη, Polι, and Polκ) contain one or two Ub-binding domains (UBM or UBZ) (Bienko et al., 2005), a PCNA-interacting peptide (PIP), and a Rev1-binding domain. Rev1, another member of the Y family of polymerases, possesses two UBM Ub-binding motifs (Guo et al.,

2006b), a C-terminal domain required to interact with all three other Y-family polymerases (Murakumo et al., 2001; Guo et al., 2003; Ohashi et al., 2004), and a unique N-terminal BRAC1 C-terminal domain required to interact with PCNA (Guo et al., 2006a). Observations that both the Ub-interacting domain and the PCNA-binding domain are required for the Y-family polymerase function (Bienko et al., 2005) support the notion that monoubiquitinated PCNA has enhanced affinity for Y-family polymerases and recruits them to the damage site, although whether the Polη Ub-binding domain plays a crucial role in TLS is still subject to debate (Acharya et al., 2007).

Y-family polymerases exhibit high substrate flexibility, low fidelity, and lack of proofreading ability (Prakash et al., 2005; Lehmann et al., 2007; McCulloch and Kunkel, 2008). Unlike the high-fidelity replicative polymerases, Y-family polymerases are capable of accommodating DNA distortions in the active site and are thus capable of DNA synthesis across the damaged template (Ling et al., 2001; Yang and Woodgate, 2007). Of interest, although Y-family polymerases have relaxed active sites, they exhibit remarkable substrate or lesion specificity. For example, Polη from yeast to human exhibits very high affinity for UV-induced cis-syn thymine dimers and remarkable accuracy and efficiency when bypassing this lesion (Johnson et al., 1999, 2000b; Washington et al., 2000; Biertumpfel et al., 2010; Silverstein et al., 2010). Hence it is thought that Y-family polymerases have evolved to handle specific replication-blocking lesions and so are referred to as specialized DNA polymerases (Ling et al., 2003). Rev1 differs from other Y-family polymerases in that its catalytic activity does not play a pivotal role for TLS; instead, it may serve as a scaffold (Guo et al., 2003). In addition to binding other Y-family polymerases, the C-terminal polymerase-interaction domain of Rev1 also binds to the Rev7 subunit of Polζ (Guo et al., 2003). Polζ is composed of the Rev3 catalytic subunit and the Rev7 regulatory subunit and is the only B-family DNA polymerase involved in TLS (Nelson et al., 1996b). In budding yeast, deletion of REV1, REV3, or REV7 results in greatly decreased spontaneous and DNA damage-induced mutations (Lawrence, 2004), indicating that Rev1 and Polζ activities account for the majority of mutagenesis. Similarly, suppression of human REV1 (Gibbs et al., 2000) or REV3 (Gibbs et al., 1998) also results in phenotypes reminiscent of a lack of TLS in yeast. Furthermore, the altered somatic hypermutation of immunoglobulin genes in Rev1-null mice is consistent with Rev1 functioning as a deoxycytidine transferase across abasic sites (Jansen et al., 2006). These observations suggest that, as in budding yeast, mammalian Rev1 and Polζ play critical roles in TLS. However, how they play such central roles is unclear, largely because of a lack of reagents to characterize mammalian Rev3 (Gan et al., 2008).

One well-characterized function of yeast Polζ is its ability to elongate from insertions made by Y-family polymerases (Johnson et al., 2000a), which forms the basis for the two-step TLS model (Prakash and Prakash, 2002). It is thought that although Y-family polymerases can insert limited bases to a damage site, either correctly or incorrectly, they cannot necessarily elongate from the distorted primer. In this case, Polζ may act sequentially to elongate downstream of the DNA distortion, albeit with a reduced fidelity. Similar elongation steps by Polζ have also been described following base insertion across from an abasic site initiated by Rev1 (Nelson et al., 1996a) or from a misincorporation initiated by the replicative polymerase Polδ (Haracska et al., 2001b). The two-polymerase model is further supported by a recent study in cultured mammalian cells (Shachar et al., 2009). Given its pivotal roles in the repression of chromosome abnormalities (Okada et al., 2005; Wittschieben et al., 2006), it is critical to characterize cellular functions of Rev3 in DNA damage response in mammalian cells. In this article, we describe the generation and characterization of antibodies specific for human Rev3 and the investigation of conditions required for the formation of Rev3 nuclear foci. We also examined UV-induced colocalization of Rev1, Rev3, and Polη with PCNA. Our observations for the first time illustrate the assembly and nuclear dynamics of endogenous TLS polymerases and provide supporting evidence for the polymerase switch model (Friedberg et al., 2005).

RESULTS

Characterization of an anti-Rev3 polyclonal serum

Based on cDNA analysis (Gibbs et al., 1998), hREV3 is expected to encode a 3130–amino acid protein with an estimated molecular mass of 353 kDa, which is consistent with its detected transcript size (Xiao et al., 1998). To investigate endogenous Rev3 protein dynamics we produced a mouse polyclonal antiserum directed against a recombinant C-terminal portion of Rev3. Western blotting analysis of human colorectal carcinoma HCT116 cells demonstrated a single major immunoreactive band that would agree in size with the predicted Rev3 protein (Figure 1A). Immunocytochemistry revealed that the polyclonal antiserum is localized in both cytoplasm and the nucleus, with a possible concentration in the nucleus in a subset of HCT116 cells (Figure 1B). The cytoplasmic localization of the polyclonal antiserum was unlikely to be due to nonspecific reactions
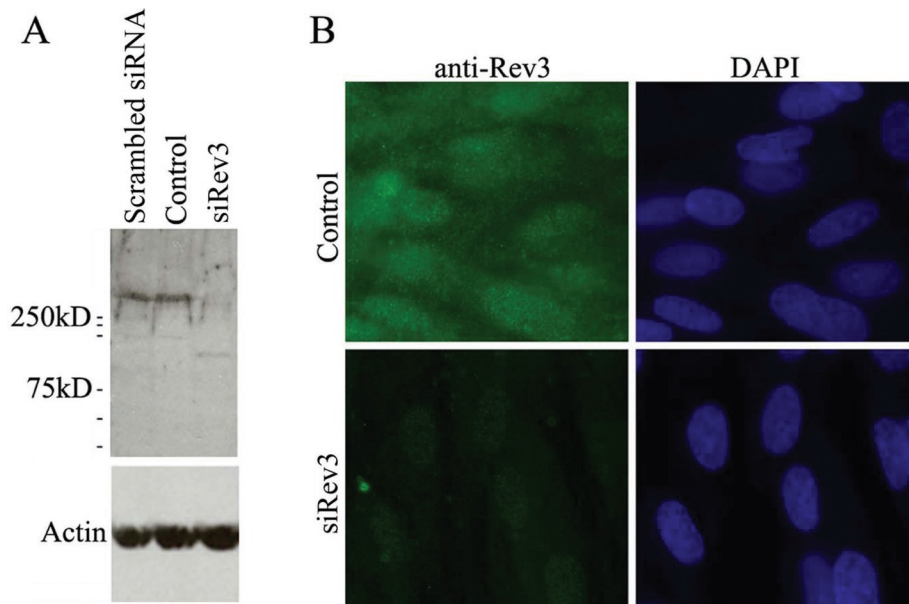
FIGURE 1: Characterization of the anti-Rev3 antibody using human colorectal carcinoma HCT116 cells. (A) Western blot analysis of an HCT116 cell lysate detecting Rev3 immunoreactivity as a single major band in excess of 250 kDa that was reduced >90% by Rev3-specific siRNA (siRev3) but not affected by nonspecific siRNA. (B) ICC using the anti-Rev3 antibody on HCT116 cells (top left) and on HCT116 cells pretreated with Rev3-specific siRNA (siRev3; bottom left). DAPI staining was used to show nuclei.

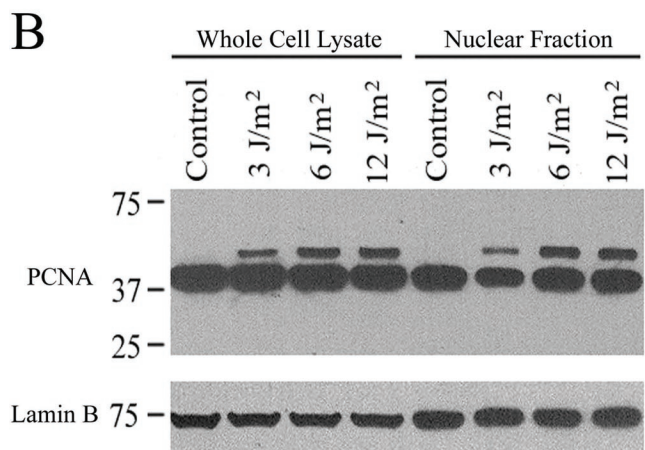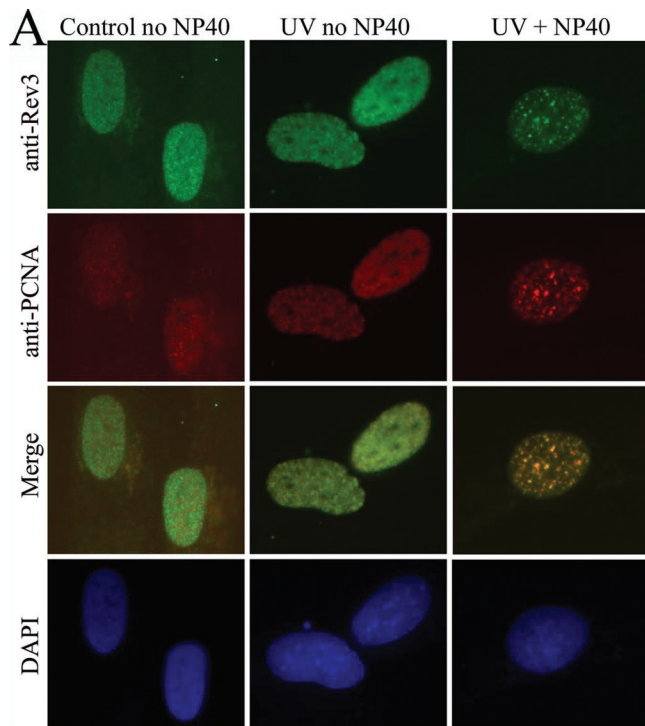**FIGURE 2:** PCNA nuclear focus formation and monoubiquitination in response to UV damage. (A) UV-induced colocalization of PCNA with Rev3 in the low-passage human fibroblast cell line GM08402 as revealed by ICC. Although Rev3 was found to be variably distributed in cytoplasm and the nucleus (left), a UV treatment (12 J/m² for 4 h, middle) caused a nearly complete translocation of Rev3 to the nucleus, regardless of the cell cycle stage. The staining patterns of Rev3 and PCNA in untreated S-phase (PCNA-positive) cells do not appear to overlap (left). However, after UV treatment, the discrete distribution of Rev3 appears to match that of PCNA (middle). This nuclear focus colocalization is further revealed following NP40 preextraction before fixation (0.4% on ice for 40 min, right). (B) NF1604 whole-cell lysates and nuclear fractions were loaded based on equal culture volume and analyzed by Western blotting to reveal the induction of a slow-migrating anti-PCNA immunoreactive band following UV exposure, indicative of monoubiquintinated PCNA. Cells were incubated for 12 h following UV irradiation at the indicated doses. Anti–lamin B immunoreactivity was used as a loading control in a parallel Western blot.

since it disappeared after preabsorption with purified antigen (unpublished data). To establish a means of ablating endogenous Rev3, we transfected HCT116 cells with a mixture of anti-Rev3 interfer-

ence RNA (siRev3) and found that the immunoreactivity was effectively reduced by up to 90%, whereas a mixture of nonspecific interference RNA did not affect the Rev3 level (Figure 1, A and B). Furthermore, qRT-PCR analysis also confirmed target-specific suppression of the *REV3* transcript that was consistent with the anti-Rev3 immunocytochemistry (ICC) results (Supplemental Figure S1). Hence we were able to conclude that the polyclonal antiserum prepared for this study contains antibodies specific for Rev3 and that the siRev3 used in this experiment is capable of suppressing endogenous Rev3 expression. Here we will refer to the positive detection using this antiserum as Rev3.

UV-induced Rev3 nuclear foci and their colocalization with PCNA

With the available antibody against Rev3, we wanted to characterize the cellular distribution of Rev3 and its response to DNA-damaging agents. The immortalized human lung fibroblast cell line NF1604 was initially used to determine experimental conditions. It was found that although as low as 6 J/m² UV was able to cause nuclear focus formation after a 4-h incubation, 12 J/m² induced discrete and bright foci (Supplemental Figure S2A). A time-course study indicated that at the UV dose of 12 J/m², it took 3–6 h to result in NP40-resistant nuclear focus formation (Supplemental Figure S2B) in the vast majority of cells. Furthermore, it was determined that ice-cold 0.4% NP40 treatment for 40 min before fixation effectively removed the majority of the soluble protein from the cells and resulted in an NP40-insoluble fraction that represents nuclear foci with chromatin structures (Supplemental Figure S2C). Unless specified, subsequent studies generally followed the aforementioned conditions (12 J/m² of UV irradiation, followed by 4 h of incubation and 40 min of NP40 preextraction), which consistently resulted in >75% cells positive for Rev3 nuclear foci in different cell line backgrounds.

To avoid the discrepancy and ambiguity associated with different cell lines with respect to Rev3 localization, we used the cultured low-passage normal human fibroblast cell line GM08402. Without DNA damage treatment, Rev3 was found in both the nucleus and cytoplasm but appeared to be enriched in the nucleus in S-phase (PCNA positive) cells (Figure 2A, left) similar to the findings in HCT116 cells (Figure 1B). Four hours after exposure to 12 J/m² of UV irradiation, discrete PCNA and Rev3 immunopositive dots were observed in S-phase cells, and the majority of them appeared to overlap (Figure 2, middle). Indeed, after NP40 preextraction, the remaining PCNA nuclear foci colocalize with Rev3 foci (Figure 2A, right). In contrast, no NP40-resistant Rev3 nuclear foci were observed in unirradiated cells, regardless of their cell cycle stage, although PCNA foci were visible in S-phase cells (unpublished data). On the basis of the foregoing observation and previous reports (Kannouche *et al.*, 2001), we suspect that the UV-induced, NP40-resistant PCNA nuclear foci represent stalled replication sites and further speculate that Rev3 also plays a role at these sites. To reinforce this argument, we analyzed PCNA ubiquitination in response to different doses of UV irradiation. As seen in Figure 2B, covalent PCNA modification, presumably monoubiquitination (Kannouche *et al.*, 2004; Bienko *et al.*, 2005), in the nucleus peaked after 6–12 J/m² UV treatment, which is in good agreement with that of NP40-resistent PCNA and Rev3 nuclear focus formation. In addition, cell survival after UV irradiation for each cell line used in this study was determined. As seen in Figure S3, 12 J/m² of irradiation did not cause significant cell death after 12 h of incubation for most cell lines, except for the XPV-derived cell line GM03617, which displayed ~70% viability.

Repeated attempts to coimmunoprecipitate Rev3 with PCNA were unsuccessful. This was probably due to the low abundance of

endogenous Rev3 or the unsuitability of the anti-Rev3 antibody for coimmunoprecipitation. To further address whether nuclear foci containing both Rev3 and PCNA represent stalled replication forks, we examined the colocalization of PCNA with Polη and Rev1. It has been previously established that following low-dose UV treatment, Polη accumulates at replication foci stalled at DNA damage (Kannouche *et al.*, 2001), that Rev1 colocalizes with Polη to the same replication foci (Tissier *et al.*, 2004), and that both colocalize with PCNA. Indeed, under our experimental conditions, both Polη (Supplemental Figure S4A) and Rev1 (Supplemental Figure S4B) nuclear foci colocalize with PCNA in a manner similar to that of Rev3 (Supplemental Figure S4C). These observations collectively indicate that upon DNA damage, PCNA, Polη, Rev1, and Rev3 all accumulate at stalled replication forks as revealed by NP-40 insoluble nuclear foci.

UV-induced Rev3 nuclear focus formation is dependent on Rev1 but independent of Polη

The C-terminal 100–amino acid region of Rev1 has been reported to physically interact with a number of Y-family polymerases as well as Rev7 (Murakumo *et al.*, 2001; Guo *et al.*, 2003; Ohashi *et al.*, 2004), a presumed regulatory subunit of Polζ that binds to Rev3 in an in vitro assay (Murakumo *et al.*, 2000). This observation predicts that Rev3 is colocalized with Rev1; however, such a physical interaction has not been reported in vivo, and it is unclear whether the interaction is dependent on DNA damage. We found that without DNA damage treatment, both Rev1 (Supplemental Figure S5A, first column) and Rev3 (Figure S5A, second column) are distributed in the cytoplasm and the nucleus, but their colocalization is not obvious (Figure S5A, third column). After UV irradiation, the Rev3 nuclear foci colocalize with Rev1 foci, and these nuclear structures are resistant to NP40 preextraction (Figure 3A, left).

To determine whether Rev1 is required for Rev3 localization to the damage site, we used NF1604 and its derivative Rz20, which stably expresses an *hREV1*-specific ribozyme that results in the suppression of Rev1 mRNA (Clark *et al.*, 2003). Under the condition that the cellular Rev1 mRNA level is reduced by up to 90% (Supplemental Figure S5B) and the Rev1 protein is barely detectable (Figure S5A, first column) in Rz20, total cellular Rev3 levels do not appear to be affected regardless of UV irradiation (Figure S5A, second column, and Supplemental Figure S5C). However, UV-induced Rev3 nuclear focus formation is severely compromised (Figure 3A, right) in essentially all Rev1-negative cells examined (Figure 3C), whereas the PCNA nuclear focus formation appears to be unaltered (Supplemental Figure S6, top two rows). Hence Rev1 appears to play a pivotal role in recruiting Polζ to the damage site.

Polη is recruited to the damage site after UV irradiation (Kannouche *et al.*, 2001). It has been reported through in vitro translesion DNA synthesis analysis that Polζ may act in concert with a Y-family polymerase to insert bases opposite the damaged template base(s) and then extended from them (Johnson *et al.*, 2000a). These observations predict that Polζ is colocalized with Polη at UV-induced damage sites. Indeed, we found that, as expected, normal human fibroblasts exhibited UV-inducible Polη foci that colocalized with Rev3 foci and were persistent after NP40 extraction (Figure 3B, left). To determine whether Polη is also required for Rev3 nuclear focus formation, we used an XPV cell line (GM03617) derived from a xeroderma pigmentosum variant patient that contains a four–base pair deletion in *XPV*, resulting in a truncation at amino acid 42 (Masutani *et al.*, 1999) and the loss of Polη functional domains, including the polymerase, Rev1-binding, PIP, and UBZ domains. ICC using an anti-Polη anti-

body revealed that the endogenous Polη was indeed undetectable in this XPV cell line; however, UV treatment was still able to induce NP40-resistant Rev3 focus formation at a level indistinguishable from that of matched normal human fibroblasts (Figure 3B, right, and Figure 3D). Furthermore, UV-induced Rev3 nuclear foci still colocalize with PCNA (Supplemental Figure S6, bottom two rows). These results demonstrate that although Rev3 colocalizes with Polη following UV treatment, its recruitment to the damage site is independent of Polη.

Polη and Rev1 are independently recruited to the stalled replication fork

The differential requirement of Rev1 and Polη for UV-induced Rev3 nuclear focus formation raised an interesting question of interdependence between Polη and Rev1. Surprisingly, despite numerous reports from different laboratories on the nuclear dynamics of Rev1 and Polη in response to DNA damage, very little attention has been paid to the in vivo interdependence of the two proteins. One report (Tissier *et al.*, 2004) examined the subcellular localization of various YFP-hRev1 derivatives in wild-type and XPV cells, and the authors concluded that Rev1 nuclear localization occurs independent of the presence of Polη. However, this study relied on experimentally transfected peYFP-REV1 cell lines, and the authors observed that artificial overexpression of YFP-hRev1 was sufficient to induce nuclear foci in up to 40% of cells in the absence of DNA damage (Tissier *et al.*, 2004), making its physiological relevance questionable. In contrast, spontaneous nuclear foci were not detected for endogenous Rev1 in our experiment (unpublished data). On the other hand, a recent report (Akagi *et al.*, 2009) demonstrated that UV-induced Rev1 nuclear focus formation is dependent on its physical interaction with Polη. In this study, we examined UV-induced colocalization of Polη and Rev1 in the nuclear foci and their interdependence. As shown in Figure 4, Polη and Rev1 indeed colocalized to the nuclear foci regardless of the cell lines examined. However, suppression of Rev1 did not affect Polη nuclear focus formation (Figure 4, A and C), nor did inactivation of Polη affect Rev1 (Figure 4, B and D). These observations allow us to conclude that Polη and Rev1 are independently recruited to the DNA damage sites upon UV-induced DNA damage, although one cannot formally rule out a possibility that the residual levels of Rev1 in Rz20 cells recruit Polη to nuclear foci after DNA damage.

Rev3 is not required for UV-induced nuclear focus formation of Rev1 or Polη

After knowing how Rev3 is recruited to the UV-induced damage sites, it would be of great interest to learn whether Rev3 is also required for the recruitment and/or retention of other TLS polymerases. To this end, we examined the Rev1 and Polη nuclear focus formation in NF1604 cells and when the expression of Rev3 is suppressed by interference RNA against Rev3 (siRev3). We found that suppression of Rev3 did not affect total cellular levels of Rev1 or Polη regardless of UV treatment (unpublished data). More important, UV-induced, NP40-resistant Polη (Figure 5A) or Rev1 (Figure 5B) focus formation did not appear to be affected by ablation of Rev3 (Figure 5, C and D). These observations allow us to conclude that Rev3 probably acts downstream of Rev1 in TLS.

Contribution of Rev3 and Polη to the protection of cells against killing by UV irradiation

If Rev3 acts downstream of Rev1 but independent of Polη, one would predict that Rev3 and Polη provide an additive effect toward
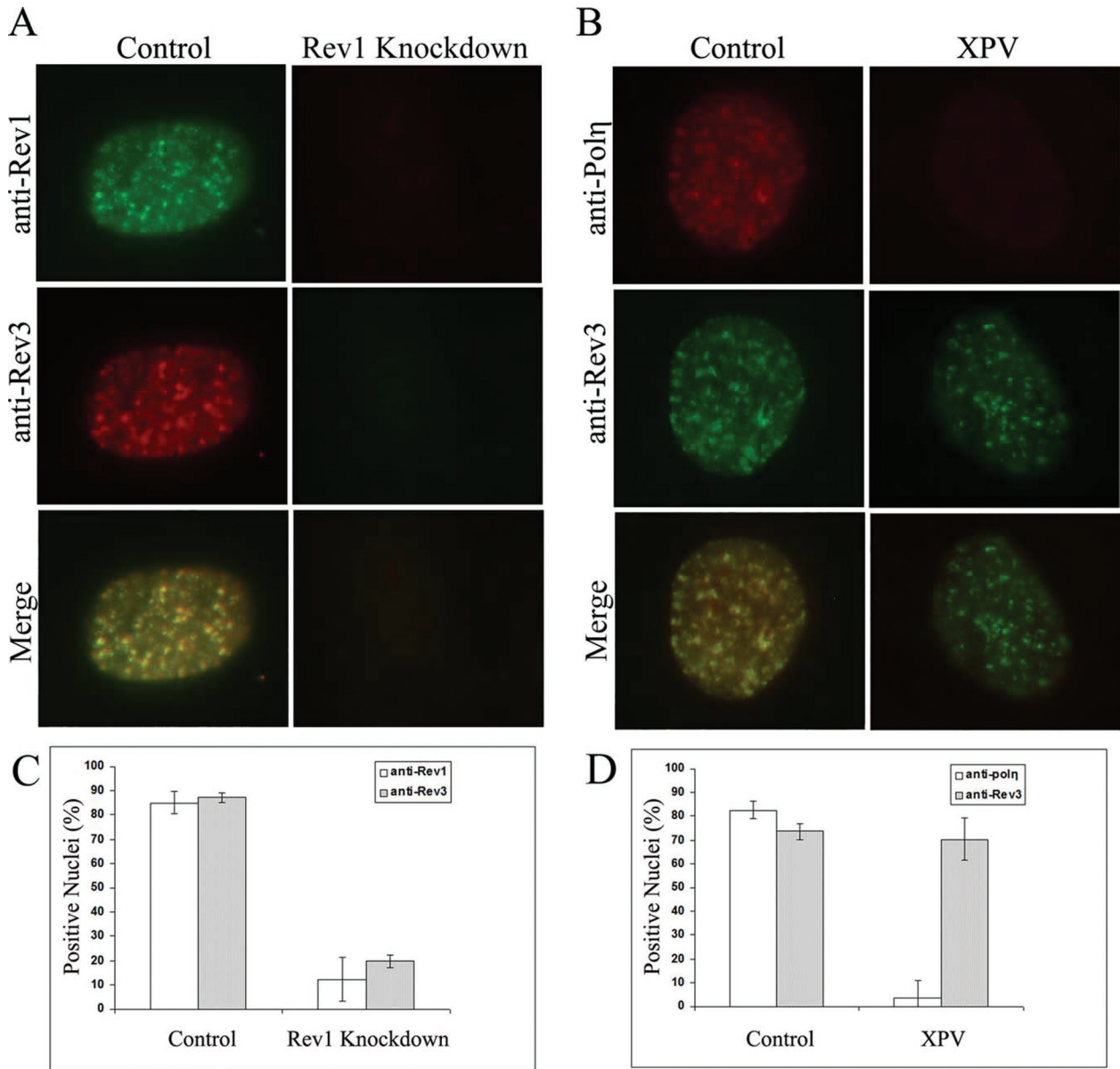
FIGURE 3: Dependence of Rev3 nuclear focus formation on other TLS polymerases following UV irradiation. (A) Rev3 nuclear focus formation is dependent on Rev1. In control immortalized fibroblasts (NF1604), UV-induced, NP40-resistant Rev3 nuclear foci colocalized with Rev1 foci (left). However, in genetically matched Rz20 cells in which Rev1 is depleted by a *REV1*-specific ribozyme, the Rev3 foci were not observed (right). (B) Rev3 nuclear focus formation is independent of Polη. In control normal fibroblasts (GM08402), UV-induced, NP40-resistant Rev3 nuclear foci colocalized with Polη foci (control, left). The Rev3 nuclear focus formation was not altered in an XPV cell line (GM03617) lacking endogenous Polη (right). (C, D) Statistical analyses of experimental results as shown in A and B, respectively.

protection of cells against UV-induced DNA damage. To test this hypothesis, we compared the normal human fibroblast cell line GM08402 with the XPV cell line in the presence or absence of Rev3 suppression for their viability following UV irradiation. As seen in Figure 6, 24 h after 6 J/m² of UV irradiation, Polη-null or Rev3-depleted cells displayed moderate UV sensitivity. In sharp contrast, Rev3-depleted XPV cells displayed remarkable sensitivity to UV damage (Figure 6A). As a control, XPV cells treated with a scrambled interference RNA did not display decreased cell viability upon UV treatment (Figure 6B). Hence we conclude that suppression of Rev3 and Polη has an additive effect with respect to UV-induced killing. This observation agrees with a recent report (Ziv *et al.*, 2009).

DISCUSSION

DNA damage-induced nuclear focus formation has been used as an important tool to characterize a number of proteins involved in DNA damage response. Lehmann and his colleagues used this method to elegantly demonstrate that following irradiation of cells with UV, Polη accumulates at replication foci stalled at the damage site (Kannouche *et al.*, 2001). Subsequently, it was shown that Rev1 colocalizes with Polη (Tissier *et al.*, 2004). One caveat for both of these studies is that the authors used transfected cells carrying the genes of interest to facilitate functional domain analysis. Here we report the generation of Rev3-specific antibody and characterization of three TLS polymerases, Polη, Rev1, and Rev3, in their native form. We found that in UV-irradiated cells, detergent-resistant
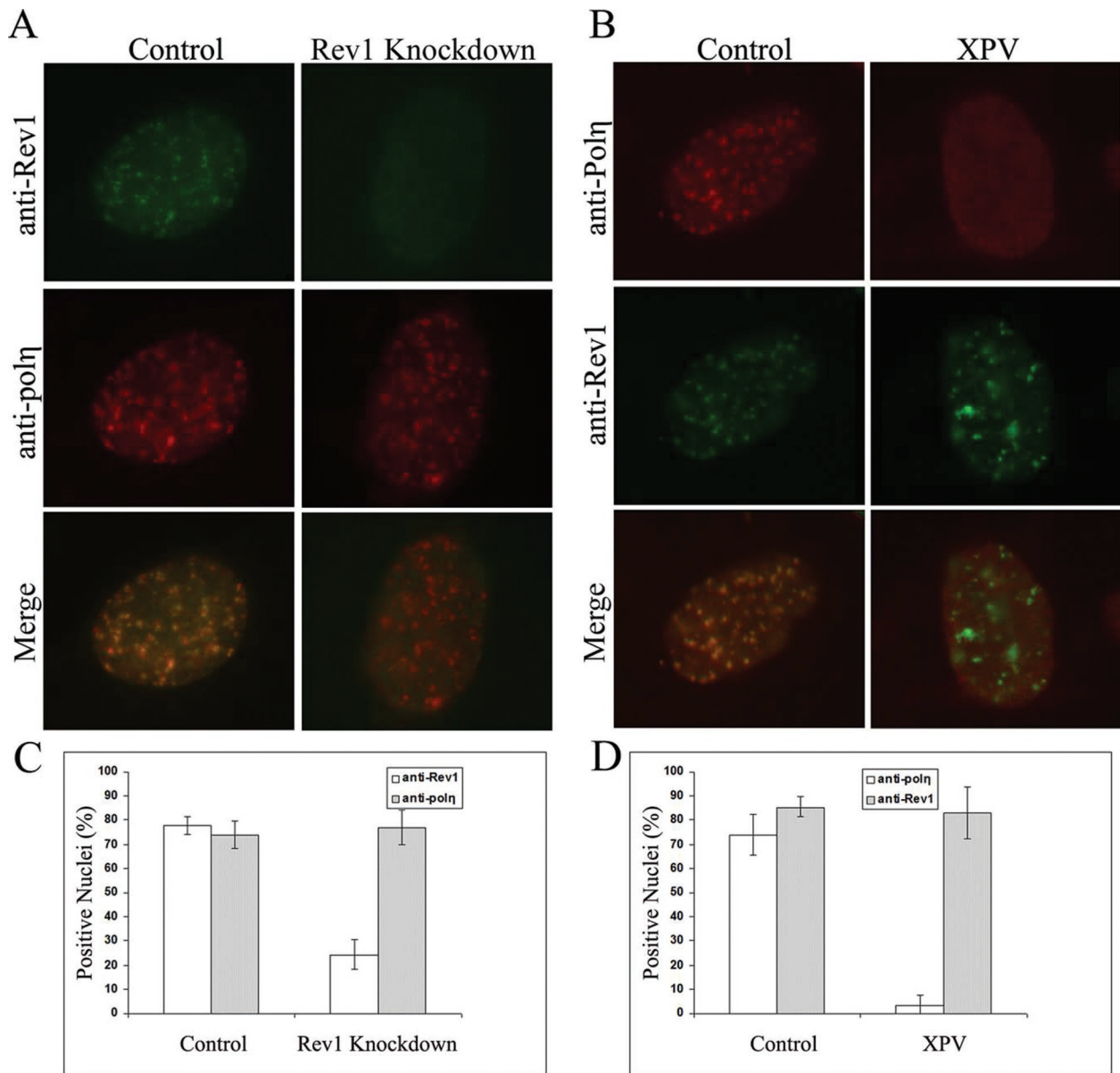
FIGURE 4: UV-induced nuclear foci of Rev1 and Polη are colocalized but independent of one another. (A) UV-inducible, NP40-resistant nuclear focus formation of Polη in the presence (NF1604, left) or absence (Rz20, right) of Rev1. Polη focus distribution and intensity remain unaltered regardless of the status of Rev1. (B) Rev1 nuclear foci are detected after UV treatment and NP40 preextraction in both normal human fibroblast cells (GM08402, left) and the corresponding XPV cells (GM03617, right). Note that in both types of cells, UV-induced, NP40-resistant nuclear foci of Rev1 and Polη colocalize (bottom left). (C, D) Statistical analyses of experimental results as shown in A and B, respectively.

(presumably chromatin-containing) nuclear foci of all three TLS polymerases colocalize with each other as well as with PCNA, which provides strong evidence that the TLS complex is assembled at UV-induced lesions. Furthermore, the formation of nuclear foci and their persistence appear to be correlated with PCNA monoubiquitination (unpublished data), suggesting that PCNA molecules found in these foci are predominantly ubiquitinated. A couple of discrepancies are noted between this and previous reports (Kannouche et al., 2001; Tissier et al., 2004). First, unlike previous reports, we did not observe detergent-resistant nuclear foci of any of the aforementioned three TLS polymerases in the absence of UV treatment, which is probably due to the low level of endogenous TLS polymerases detected in this study in contrast to the trans-

fected cell lines. Second, there appear to be fewer and apparently sizable heterogeneous detergent-resistant nuclear foci in our study compared with the previous reports. Although the difference may be attributed to the different UV doses or detailed detergent preextraction procedures, we believe that the transfected cell lines used in other studies may be primarily responsible for the discrepancy, since under our experimental conditions the pGFP-Polη transfectants also display nuclear focus images similar to those previously reported (P. L. Andersen and W. Xiao, unpublished data). Furthermore, UV-induced Polη and Rev1 nuclear foci have been thought to represent stalled replication forks. However, given recent reports indicating that *RAD18*-mediated DNA-damage tolerance can function in G2 (Karras and Jentsch, 2010) and be
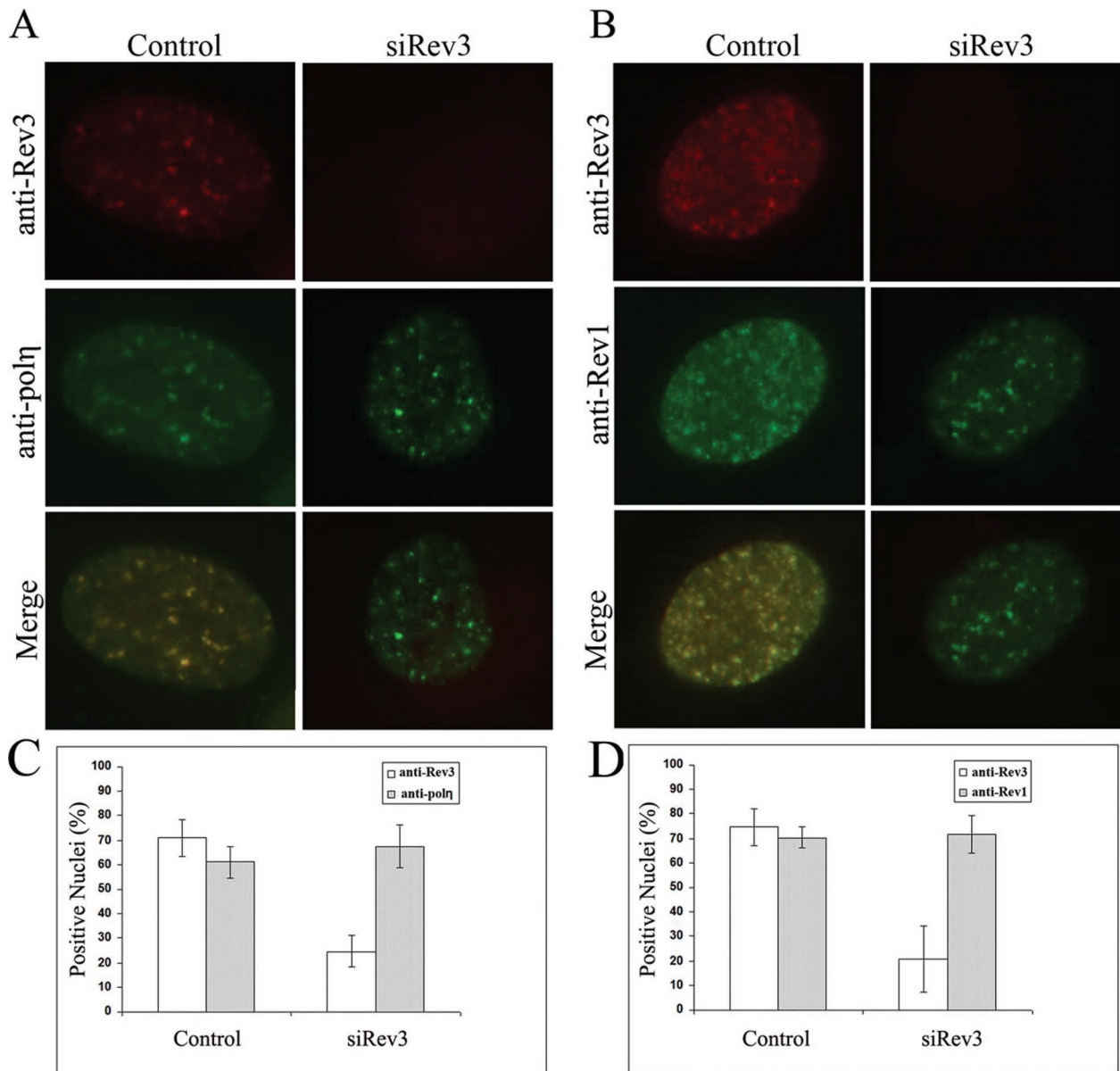
FIGURE 5: UV-induced nuclear focus formation of Polη and Rev1 does not depend on Rev3 in normal fibroblasts (GM08402). (A) Detection of UV-induced, NP40-resistant Polη nuclear foci in the presence (left) and absence (right) of Rev3. (B) Detection of UV-induced, NP40-resistant Rev1 nuclear foci in the presence (left) and absence (right) of Rev3. Note that UV-induced, NP40-resistant nuclear foci of Polη and Rev1 colocalize with Rev3 (bottom left). (C, D) Statistical analyses of experimental results as shown in A and B, respectively.

separated from genome replication (Daigaku *et al.*, 2010) in yeast, it remains highly possible that the TLS foci revealed in this study also represent postreplicative single-strand gaps.

The current protein interaction data from the literature could be compatible with several possibilities on how the TLS polymerases are assembled. To determine the actual order of such an assembly, we used different methods to reduce or eliminate one TLS polymerase and examined the UV-induced nuclear focus formation of the two remaining polymerases. As summarized in Table 1, we found that Polη and Rev1 are independently recruited to the stalled replication site, that Rev3 recruitment requires Rev1 but not Polη, and that suppression of Rev3 does not affect the assembly of either Polη or Rev1. Our observation that Rev1 is recruited to UV-induced damage sites independent of Polη agrees with a report from Tissier *et al.* (2004) but differs from the work of Akagi *et al.* (2009). We argue that if UV-induced Rev1 nuclear focus formation were dependent on

Polη, then the Polη mutation would be epistatic to *rev1* and *rev3*, which is inconsistent with the additive effect of Polη and Rev3 inactivation to UV damage as observed in this and a previous report (Ziv *et al.*, 2009). This order of assembly as revealed in this study could be consistent with observations that both the PCNA interaction domain and the Ub-binding domain of Polη (Kannouche *et al.*, 2004;

Foci	Require		
	Polη	**Rev1**	**Rev3**
Polη		No	No
Rev1	No		No
Rev3	No	Yes	

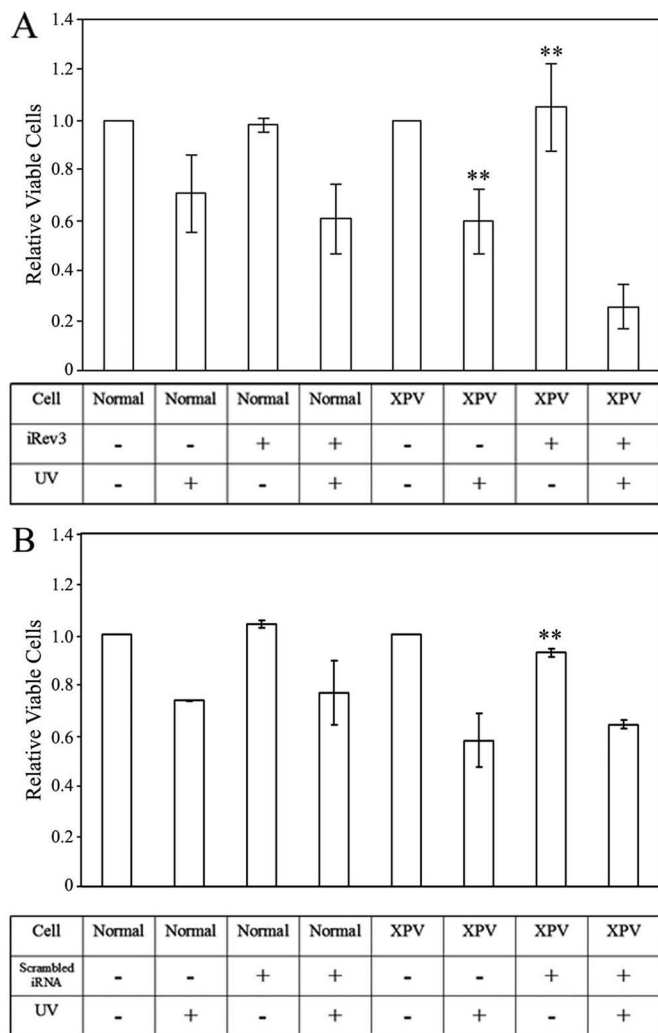**TABLE 1:** Summary of the nuclear focus formation data.

FIGURE 6: Cell survival following Rev3 ablation and/or UV treatment in normal and Polη-deficient XPV cells. Cells deemed viable by the presence of intact nuclei and nucleoli were counted in cultures of normal fibroblasts (GM08402) and Polη-deficient XPV (GM03617) fibroblasts 24 h after 6 J/m^2 of UV exposure. (A) Cells with or without prior siRev3 treatment. Each bar represents an average of four independent experiments normalized to the untreated control or untreated XPV cells. (B) Cells with or without prior treatment by a scrambled siRNA control. Each bar represents an average of two independent experiments. Error bars represent SD. **Statistically significant difference ($p < 0.01$) from the (iRNA + UV)-treated XVP cells (far right bar) as determined by a two-tailed Student's t test.

Bienko *et al.*, 2005) and Rev1 (Guo *et al.*, 2006a, 2006b) are required for the nuclear focus formation and in vivo functions, as well as for their in vitro TLS activities (Haracska *et al.*, 2001a; Garg and Burgers, 2005) but cannot explain the significance of the direct Polη–Rev1 interaction (Guo *et al.*, 2003). Our result does not favor a notion that the Polη–Rev1 interaction stabilizes the complex at the stalled replication site, since depleting either component does not appear to affect the number or intensity of the other nuclear foci. It is highly possible though that Rev1 may assist Polη for translesion synthesis, or alternatively that once both Polη and Rev1 are independently recruited to the damage site by ubiquitinated PCNA, their physical interaction provides a functional bridge for polymerase switching (Figure 7). Indeed, we have shown in this report that Rev3 is recruited by Rev1 to the DNA damage site independent of Polη. This observation invokes two competitive scenarios. First, it supports the
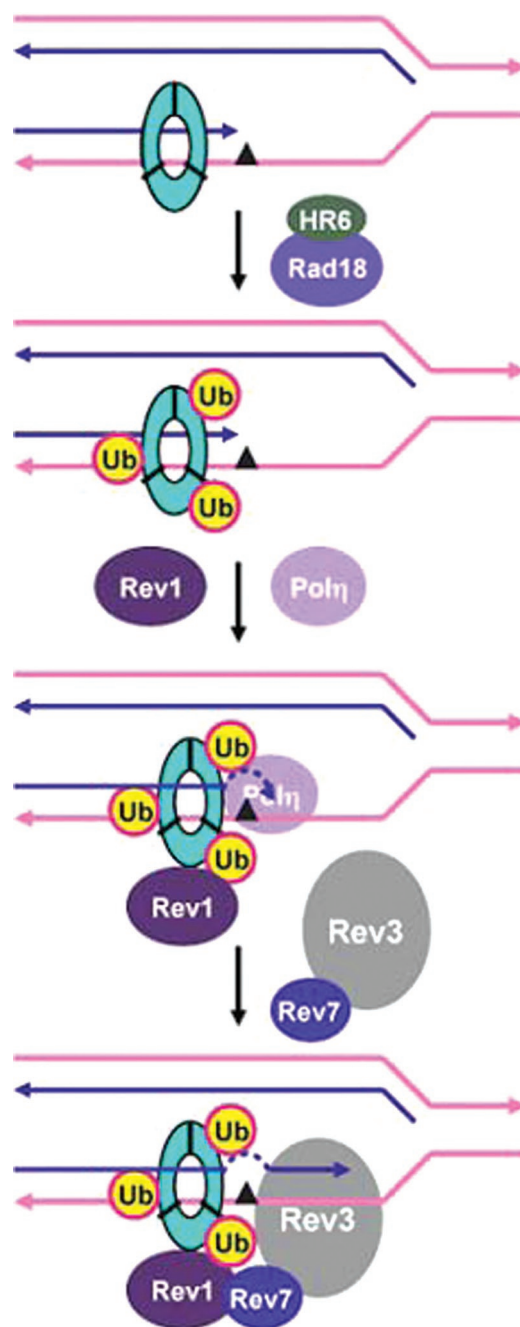

FIGURE 7: A proposed model depicting the ordered assembly and switch of mammalian TLS polymerases in response to UV irradiation. On UV irradiation, the replication machinery is stalled at the damaged template and invokes PCNA monoubiquitination by the HR6A/HR6B–Rad18 complex. Monoubiquitinated PCNA independently recruits both Rev1 and Polη to the damage site, and Polη is able to insert dAs across the thymine dimer with relatively high fidelity with or without assistance from Rev1. Meanwhile, Rev1 is able to recruit Polζ to the damage site probably through its interaction with the Rev7 subunit, and the Rev1–Polη interaction brings Polζ into proximity to replace Polη for primer extension. It is noted that for a different type of DNA damage, another preferred TLS polymerase may replace Polη for a similar reaction. This model also predicts that in the absence of Polη, Rev1 (or another TLS polymerase) may serve to perform translesion insertion, followed by Polζ extension. All three subunits of PCNA are monoubiquitinated in the diagram for convenience, and we understand that whether this is required for TLS is subject to debate.

notion that Rev1 serves as a trading place for the polymerase switch (Friedberg *et al.*, 2005), in this case between Polη and Polζ, for translesion insertion and extension, respectively. Alternatively, if the lesion is not a UV-induced thymine dimer, other TLS polymerases may be preferentially used over Polη at the insertion step. For example, it has been speculated that Polκ is specialized for bypassing lesions induced by chemicals like benzo[a]pyrene (Ogi *et al.*, 2002), which induces Polκ nuclear foci, but whether it induces Polη foci is still subject to debate (Bi *et al.*, 2005; Ogi *et al.*, 2005). Second, when the preferred TLS polymerase is not available or the lesion cannot be preferentially recognized by a specialized TLS polymerase, Rev1 may insert deoxycytidine opposite the damaged template or recruit an alternative TLS polymerase, followed by Polζ extension, which is expected to be highly mutagenic. The latter scenario may account for the observed XPV phenotypes with enhanced mutagenesis and predisposition to cancer and is supported through characterization of UV-induced mutagenesis in XPV cells (Ziv *et al.*, 2009). Indeed, yeast Rev1 and Polζ are responsible for most spontaneous and damage-induced mutagenesis (Lawrence, 2004), and in vitro studies indicate that yeast PCNA (Garg *et al.*, 2005) or Rev1 (Acharya *et al.*, 2006) is able to enhance TLS by Polζ. Furthermore, experimental suppression of mammalian Rev3 results in a decrease in mutagenesis induced by either benzo[a]pyrene (Li *et al.*, 2002) or UV (Diaz *et al.*, 2003), suggesting that Rev3 is responsible for bypassing a broad range of lesions. The notion of involvement of Polζ in both error-free and error-prone TLS is consistent with the polymerase switching model, which has been demonstrated recently in cultured mammalian cells (Shachar *et al.*, 2009).

It is unclear how Rev3 is recruited to the damage site by Rev1. A likely candidate is Rev7, since it binds to the C-terminal domain of Rev1 (Guo *et al.*, 2003) and also forms a stable complex with Rev3 (Murakumo *et al.*, 2000). Nonetheless, we note that in addition to the reported Rev1–Rev7 interaction (Acharya *et al.*, 2005), yRev3 can also directly interact with Rev1, and this interaction appears to be essential for Rev1 function (Acharya *et al.*, 2006). Unfortunately, due to the extremely large size of hRev3, its functional domains remain to be further characterized.

MATERIALS AND METHODS
Cell lines and UV treatment
XPV cells (GM03617, also called XP30RO) and their matched control of apparently normal human fibroblasts (GM08402) were obtained from the Coriell Institute for Medical Research (Camden, NJ). The creation of cells harboring the *REV1* ribozyme construct (Rz20 cells) and matched control fibroblasts (NF1604) has been previously described (Clark *et al.*, 2003). HCT116 cells originally derived from a human colorectal carcinoma were from S. Carlsen (University of Saskatchewan, Saskatoon, Canada). All cultures were grown in DMEM (Sigma D-7777) plus 15 mM HEPES and 10% horse serum (16050-122; Life Technologies, Carlsbad, CA) with reduced sodium bicarbonate to 1.8 g/l and grown in a 5% CO_2 humidified atmosphere. Ribozyme-containing cells were maintained in 100 μM Geneticin (11811-031; Life Technologies), which was routinely removed 2–3 d before each experiment.

For UV treatment, 80–90% of the culture medium was removed, and cultured cells in a dish were exposed to 254-nm UV irradiation at given doses. The culture medium was immediately replaced, and cells were returned for incubation.

Production of anti-Rev3 antibody
The C-terminal 0.9-kb coding region of *hREV3* (Xiao *et al.*, 1998) was cloned into the *Eco*R1–*Xho*I sites of pET30a (Novagen; EMD

Biosciences, San Diego, CA) to form pET-hREV3C, which was transformed into the *Escherichia coli* strain BL21(DE3)-RIPL (Stratagene, Santa Clara, CA) to produce a His6-hRev3C fusion protein. After IPTG induction, the fusion protein was found to be mainly insoluble. Crude cell extract was centrifuged repeatedly to remove soluble proteins, and the resulting pellet was resuspended, separated on an SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The nitrocellulose band was collected, crushed in liquid nitrogen, and used as an immunogen in CD1 mice. Serum was collected as a source of mouse polyclonal antiserum 30–40 d after initial immunization.

Immunocytochemistry
Cultured cells were routinely seeded onto polylysine-coated cover slips and fixed before confluency in 4% formaldehyde for 30 min. When the preextraction procedure was performed, the cells were first rinsed with ice-cold phosphate-buffered saline (PBS) and exposed to 0.4% NP40 (also called Igepal CA-630; Sigma I-3021) in PBS for 40 min or as specified on ice before fixation. Fixed cells were rinsed four times over 30 min with PBS plus 0.25 g/l of Tween-20 (PBST) before incubation with the primary antibody. The primary antibodies used in this study include rabbit anti-PCNA (sc-7907, 1:100, 1 h; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Polη (ab17725, 1:200, overnight; Abcam, Cambridge, MA), and goat anti-Rev1 (sc-13827, 1:50, overnight; Santa Cruz Biotechnology). Following washing with PBST, secondary antibodies were added. The secondary antibodies used in this study were all Molecular Probes purchased from Invitrogen (Carlsbad, CA) and include Alexa 488 goat anti-mouse (A11001, 1:3000), Alexa 546 goat anti-rabbit (A11035, 1:2000), Alexa 488 donkey anti-goat (A11055, 1:3000), Alexa 546 goat anti-mouse (A11030, 1:2000), Alexa 546 donkey anti-mouse (A10036, 1:2000), and Alexa 488 donkey anti-rabbit (A21206, 1:3000). When performing anti-Rev1 ICC, cells were blocked with 50% goat serum in PBST after application of the anti-goat secondary antibody and before application of the second primary antibodies in order to minimize cross-reactivity between antibodies. Secondary antibodies were applied for 30 min and mixed with 1.5 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) in PBST for 30 min before washing with PBST four times over 30 min. Microscopy was performed with an inverted Olympus (Center Valley, PA) IX70 microscope equipped with a 60× oil immersion lens. Images were acquired using the Image Pro-Plus, version 4.1, software (Media Cybernetics, Silver Spring, MD), and panels were compiled using Photoshop, version 9 (Adobe, San Jose, CA).

Samples for comparison in each panel were always included in the same experiment and treated identically, and at least three independent experiments were performed for each data set. Within each experiment, images containing at least 500 cells for each treatment were captured and analyzed, and those with representative cell(s) were presented. When necessary, images in each panel were processed identically in Photoshop.

Western blotting
Samples were collected and prepared on ice with sonication in a lysis solution containing 0.5% SDS and 0.5% sodium deoxycholate with a protease inhibitor cocktail (Sigma P-8340, 1:100) and 10 mM *N*-ethylmalimide (NEM). Samples for anti-Rev3 immunoreactivity were resolved on a 4% SDS–PAGE gel run at 30 V at 4°C for 20 h. Gels were blotted using the wet transfer system and blocked in 5% Carnation instant skim milk in PBST. Blots were probed with the anti-Rev3 polyclonal antibody (produced locally, 1:2000 overnight). Anti-actin blots used 12% gels and the mouse anti-actin antibody

(Sigma A-5316, 1:10,000, 2 h). To prepare nuclear extracts, cultures were first rinsed with ice-cold PBS three times and then exposed to 0.4% NP40 with 10 mM NEM for 30 min. Nuclei were collected by centrifugation at 2000 × g for 2 min, and the nuclear pellet was sonicated in the lysis solution. PCNA was resolved on a 12% SDS–PAGE gel using standard techniques. Antibodies used include mouse anti-PCNA (1:5000, overnight, clone Ab-1; Calbiochem, La Jolla, CA) and mouse anti–lamin B (1:5000, overnight; Oncogene Science, Cambridge, MA).

Immunoreactivity was detected using horseradish peroxidase–conjugated goat anti-mouse antibody (12-349, 1:10,000, 45 min; Upstate; Millipore, Billerica, MA) and developed using Western Lightning Plus (NEL104; PerkinElmer, Waltham, MA). For quantitative analysis of band intensity, the Western blot was scanned and analyzed using a BioRad (Hercules, CA) ChemiDoc XRS system.

Suppression of target genes by siRNA

Synthetic siRNAs including siRev3 (sc-37791) and scrambled siRNA (sc-37007) were purchased from Santa Cruz Biotechnology and applied to cultured cells as suggested by the supplier. The efficacy of target gene suppression was monitored by either Western blotting or ICC as well as qRT-PCR and typically achieved ~90% suppression. Cultures not exhibiting expected target protein suppression were excluded from further experiments.

Real-time RT-PCR (qRT-PCR)

Total RNA was isolated from three independent populations (1 × 10^6 cells each) of subconfluent NF1604 or Rz20 cells (Clark *et al.*, 2003), using an RNEasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was analyzed and quantified using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and reverse transcribed using reverse transcriptase (Promega, Madison, WI), oligo(dT) primers, and DNase I by standard protocols. The effect of the ribozyme on cellular levels of *REV1* mRNA was then determined by qRT-PCR, using TaqMan technology and a gene-specific probe. Relative amounts of target RNA for triplicate sample runs were quantitated with the instrument software and normalized to the control *GAPDH* values. Changes in values were calculated using the comparative C_T method (Livak and Schmittgen, 2001).

Cell survival assay

Cultures were treated with siRNA and 2 d later passaged to produce replicate plates. At 24 h after passaging, cells were irradiated with 254-nm UV in a UV cross-linker and incubated for an additional 24 h before being fixed with 4% formaldehyde. Fixed cultures were stained with DAPI, and 10 random fields of view (ranging from 70 to 250 cells per view) from each culture dish were photographed. Cells with round and intact nuclei were counted as viable cells.

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