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Vertebrate DNA damage tolerance requires the C-terminus but not BRCT or transferase domains of REV1

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ABSTRACT

REV1 is central to the DNA damage response of eukaryotes through an as yet poorly understood role in translesion synthesis. REV1 is a member of the Y-type DNA polymerase family and is capable of *in vitro* deoxycytidyl transferase activity opposite a range of damaged bases. However, non-catalytic roles for REV1 have been suggested by the *Saccharomyces cerevisiae rev1-1* mutant, which carries a point mutation in the N-terminal BRCT domain, and the recently demonstrated ability of the mammalian protein to interact with each of the other translesion polymerases via its extreme C-terminus. Here, we show that a region adjacent to this polymerase interacting domain mediates an interaction with PCNA. These C-terminal domains of REV1 are necessary, although not sufficient, for effective tolerance of DNA damage in the avian cell line DT40, while the BRCT domain and transferase activity are not directly required. Together these data provide strong support for REV1 playing an important non-catalytic role in coordinating translesion synthesis. Further, unlike in budding yeast, *rad18* is not epistatic to *rev1* for DNA damage tolerance suggesting that REV1 and RAD18 play largely independent roles in the control of vertebrate translesion synthesis.

INTRODUCTION

The ability to bypass DNA damage encountered during replication is critical to the survival of a cell. Failure to do so results in incomplete replication and cell death or the passage of an aberrant genome to the cell's progeny. Replication of a damaged template is facilitated by two pathways, homologous recombination and translesion synthesis. The former is generally accurate and makes use of an alternative undamaged template to allow the replicative polymerases to bypass the lesion. The latter employs direct bypass of a lesion by one or more of a number of specialized translesion polymerases (1). Because DNA lesions are often non- or misinstructional, and because these enzymes generally have a higher misincorporation rate than the replicative polymerases, this strategy will frequently result in mutation. Despite the obvious risks of mutation in a multicellular organism, it has recently become clear that not only do vertebrate genomes encode translesion polymerases, but that higher eukaryotes rely heavily on these enzymes for their ability to deal with DNA damage (2).

Saccharomyces cerevisiae has two major translesion polymerases: RAD30 (DNA polymerase η) and DNA polymerase ζ (comprising a catalytic subunit, REV3, and REV7). In addition, REV1, a member of the Y family of DNA polymerases, plays an important, but ill-defined, role in translesion synthesis. *REV1* was first identified in a screen for genes required for UV-induced mutagenesis (3). It possesses deoxycytidyl transferase *in vitro* (4) and, there is good evidence from diverse experiments in yeast that this activity contributes to abasic site bypass *in vivo* (5–8), although one report has claimed that the transferase activity is dispensable for mutagenesis (9). A second, not directly catalytic role for REV1 in DNA damage bypass has been inferred from an analysis of the *rev1-1* mutant. This mutant carries a point mutation in the N-terminal BRCT domain of the protein (10) and, while it retains much of its catalytic activity, it is deficient in damaged-induced mutagenesis (6). BRCT domains are found in a number of proteins involved in DNA repair (11) and have been implicated in mediating interactions with phosphoproteins (12,13). REV1 homologues are also found in higher eukaryotes where they too play a key role in mutagenesis and the DNA damage response (14–16) and, biochemically, human REV1 has many of the same properties as its yeast counterpart (17,18).

Clearly, the unrestrained activity of the translesion polymerases would be hazardous and there has been increasing

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interest in how these enzymes are controlled and recruited to sites of DNA damage. Recently, post-translational modifications of POL30, the *S.cerevisiae* homologue of the sliding clamp PCNA (proliferating cell nuclear antigen), have been shown to play a major role (19–21). In response to DNA damage, POL30 is monoubiquitinated at Lys-164 by the RAD6/RAD18 heterodimer. In *S.cerevisiae* RAD6/RAD18 are epistatic to both REV1/POLζ and POLη leading to the suggestion that this monoubiquitinated form of PCNA controls the use of these enzymes. RAD18 has also been shown to mediate the DNA damage-induced monoubiquitination of PCNA in human cells and this modification can recruit DNA polymerase η (22,23).

However, there are a larger number of specialized translesion polymerases in vertebrates than in yeast and so the regulation of translesion synthesis is likely to be more complex. It seems unlikely that RAD18 plays the same dominant controlling role as it does in yeast: to date at least one translesion polymerase, POLκ, appears not to be under the control of RAD18 (24). Recently, the observation that mouse and human REV1 are able to interact with each of the other translesion polymerases has also hinted at potential differences in the control of vertebrate translesion synthesis (25–28) since the region of REV1 responsible, the extreme C-terminus, reportedly bears no homology to the yeast protein (25). Although this observation has led to the suggestion that vertebrate REV1 may also play a critical role in choreography of translesion synthesis (26), albeit by a perhaps different mechanism to yeast REV1, the functional significance of the three main domains of vertebrate REV1 (N-terminal BRCT domain, transferase domain and C-terminus) in DNA damage tolerance has not been determined.

Here, we present evidence that the C-terminus of REV1 plays an essential role in the control of translesion synthesis, we suggest through coordinating the interaction of the specialized translesion polymerases with PCNA. Further, we find that *rad18* is not epistatic to *rev1* in DT40 suggesting that these genes play substantially, but perhaps not completely, independent roles in the control of this complex process in vertebrates.

MATERIALS AND METHODS

Mammalian-two-hybrid assays

pM and pVP16 plasmids (both from Clontech) were used to create fusion proteins with the GAL4 DNA binding domain and GAL4 activation domain (linked to the HSV transactivator, VP16) respectively. Pairs of potential interactors were transfected into 2.5×10^6 293T cells together with an internal control plasmid [pRL-CMV (Promega), encoding *Renilla* luciferase] and a GAL4 promoter-driven *Firefly* luciferase reporter plasmid. The cells were transfected using SuperFect (Qiagen) following manufacturer's procedures and harvested after 24 h. Luciferase activity was determined using an Orion Luminometer (Berthold Technologies). The *Renilla* luciferase readout was used as internal control to normalize all transfections within an experiment and interactions were calculated as fold induction in *Firefly* luciferase expression compared to negative controls.

DNA constructions and site-directed mutagenesis

The chicken β-actin expression construct, pXPSN was created by modifying the multiple cloning site of the pExpress plasmid (29) to give the following sites: HindIII–SalI–NheI–NotI. The human REV1 open reading frame, and mutants thereof, were cloned as SalI–NotI fragments into pXPSN cut HindIII–NotI in a three-way ligation with eYFP (Clontech) as a HindIII–SalI fragment. This creates an N-terminal YFP fusion (eYFP is referred to in this paper as simply YFP). The β-actin promoter-YFP-REV1 cassettes were subsequently cloned into pLoxBsr (29). *rev1*(1–826) was generated by PCR using REV1FS (5′-GC**GTCGAC**CATGAGGCGAGGTGGATGGAGGAAGCGAGC) with R1827RN (5′-TTTTCCTTTT**GCGGCCGC**TTAAGTTGGAACGAACTGATTCACG). *rev1*(333–1251) and *rev1*(923–1251) were generated using REV1RN (5′-TTTTCCTTTT**GCGGCCGC**TTATGTAACTTTTAATGTGCTTCC) with R1333F (5′-GC**GTCGAC**CATGGCAGCACCTTCAGTGCCATCC) and R1923F (5′-GC**GTCGAC**CATGTCGAGACTTAACCTGAGTATAGAGGTCCCG), respectively. Site-directed mutants were generated using Quik-Change (Stratagene) following manufacturer's protocol using R1mutB (5′-GATGTTGCATGGA**C**GTCAATACCATG) for the G76R BRCT mutant and R1mutC (5′-GAAGCTGTCAGTTGTG**C**TG**C**AGCGCTGGTAGACATT) for the D570A/E571A catalytic mutant. Mutations were confirmed by DNA sequencing.

DT40 culture and transfection

DT40 and mutants were cultured as described previously (15). pLoxBsr-YFP-hREV1 constructs were transfected by electroporation at 250 V and 950 μF into *rev1* DT40. Transfected clones were selected using 20 μg/ml Blasticidin (Invitrogen). Surviving clones were analysed by cytometry using a FACSCalibur flow cytometer to detect YFP expression. For generating the *rad18* and *rad18*/*rev1* mutants, a chicken *rad18* targeting construct (30) was modified to contain selectable markers for blasticidin and histidinol. Transfection was carried out at 550 V, 25 μF with selection at 24 h. Clones were screened for targeted integration by Southern blotting.

Mutagen sensitivity assays

Cells were exposed to 254 nm UV light delivered by a bench top lamp (UVP Inc.) whose output was equilibrated for 10 min and measured using a UV radiometer (UVP Inc.). They were then plated on DMEM containing 1% 4000 cP methylcellulose as previously described (15). For cisplatin and hydrogen peroxide sensitivity measurements, cells were exposed to freshly diluted cisplatin (Sigma) or H_2O_2 (BDH) for 1 h and then washed before being plated out. Experiments were repeated two to four times. For clarity only the positive error (SD) is shown.

Analysis of sister chromatid exchange

This was performed as described previously (15).

Confocal microscopy

Cells were allowed to adhere to poly-L-lysine coated slides and treated with Fix & Perm (Caltag) following manufacturer's instructions. Cells were mounted in VectaShield containing DAPI (Vector Laboratories Inc.) and were viewed using Nikon Eclipse TE300/Biorad Radiance Confocal Microscope.

Images were collected with LaserSharp2000 (Biorad) and processed using Adobe PhotoshopCS.

RESULTS

A region adjacent to the C-terminal translesion polymerase-interacting domain of hREV1 mediates an interaction with PCNA

During a yeast two-hybrid screen for proteins that interacted with human DNA polymerase η, we identified a C-terminal fragment of REV1 (C. Chu-Wai-Chow, Anna-Laura Ross and Julian E. Sale, unpublished observations). We confirmed this interaction in a mammalian two-hybrid system with full-length REV1 and showed dependence on the extreme C-terminus of REV1 (Figure 1A and B), as has been recently reported (26–28). A further survey of interactions between the human homologues of the yeast RAD6 epistasis group, again using a mammalian-two-hybrid system, revealed, among several known interactions, an interaction of human REV1 with PCNA (Figure 1A).

Although this interaction is only seen in one orientation (when PCNA is in pM, the GAL4 binding domain plasmid), it is clearly also mediated by the C-terminus of REV1 (Figure 1B). A finer dissection of the C-terminus suggests that the region responsible for this interaction lies adjacent to the polymerase interacting domain, likely between amino acids 923 and 1047 (Figure 1C). Although this region contains at least one sequence (QVDPEVF at 1015–1021) that remotely resembles a PCNA binding motif [QXXhXXa, where 'h' is a hydrophobic residue and 'a' is aromatic (31)], we could not detect an interaction between PCNA and REV1 in a yeast two-hybrid assay (data not shown) suggesting that it may be indirect.

Conservation of the C-terminus of REV1

While human and mouse REV1 have been shown to interact with each of the other TLS polymerases, to date no physical interaction between yeast REV1 and POLζ or RAD30 has been reported. Indeed, it has been suggested that a similar interaction between the C-terminus of yeast REV1 and the other yeast TLS polymerases is unlikely because of the lack of sequence homology between this region of the yeast and human proteins (25). We re-examined the homology in the C-terminus of REV1 from a range of eukaryotes. Using PSI-BLAST with the C-terminal 100 amino acids of human REV1, we were able to identify REV1 homologues down to *S.cerevisiae*, although notably plant sequences were absent (e.g. *Arabidopsis* and *Oryza*) (Figure 1D). All of the sequences identified using the human C-terminus, including that from *Gallus gallus*, also contain an N-terminal BRCT domain and central IMS/DinP nucleotidyl transferase/polymerase domain. An exception was a sequence from *Apis mellifera* (honeybee), which contained a transferase domain but no BRCT domain. As expected, conservation of the C-terminal region is high among vertebrates, 78% between pufferfish and human, and 96% between rat and human. Secondary structure prediction using 3D-PSSM (32) suggests this region in the human protein encodes a pair of coiled-coil domains with similarity to a number of domains known to mediate protein–protein interactions, including the bromodomain and spectrin repeat. Unexpectedly however, there is also significant conservation of this region between human and *Drosophila melanogaster* (19%), *Schizosaccharomyces pombe* (24%) and *Saccharomyces cerevisiae* (17%). Despite only 19% identity, the C-terminus of *Drosophila* REV1 has been used to purify the REV3 subunit of POLζ (33) suggesting that the function of this region is likely to be conserved at least between humans and flies, a point we consider further below.

Human REV1 complements the UV and cisplatin sensitivity of the DT40 *rev1* mutant

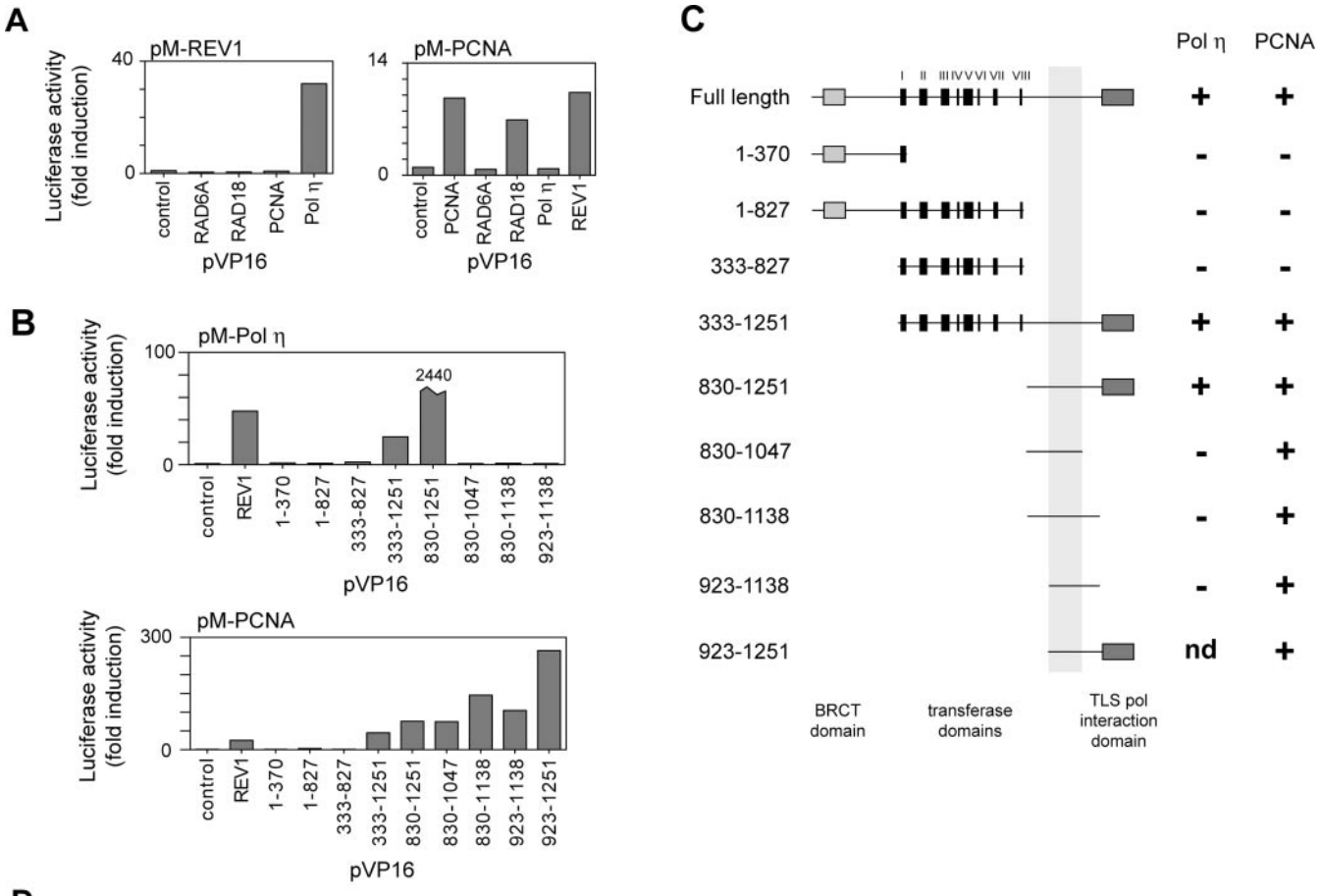
Vertebrate REV1 therefore appears to have three key regions: the N-terminal BRCT domain, central transferase domain and the TLS polymerase interaction region at the C-terminus. To clarify the functional importance of these three regions *in vivo*, we adopted a complementation strategy in a *rev1* mutant of the chicken cell line DT40 (15). As no good antibodies against REV1 have been reported to date, we constructed a fusion protein in which enhanced yellow fluorescent protein (eYFP, Clontech Laboratories) was fused to the N-terminus of human REV1. Attempts to induce stable expression of this construct using strong promoters, such as the cytomegalovirus IE promoter, were unsuccessful despite being able to demonstrate transient expression of the fusion protein from this construct in COS cells (data not shown). However, using a chicken β-actin promoter, we were able to reliably obtain stable YFP positive clones. Interestingly, the steady-state level of expression of the fusion protein in these clones was not high, producing between a half and one log shift in fluorescence (Figure 2A). Nevertheless, this level of expression was sufficient to restore the growth characteristics and hypersensitivity of *rev1* cells to UV light and cisplatin to wild-type levels (Figures 2B and 4D and data not shown), while YFP negative transfectants retained the *rev1* phenotype (Figure 2). Thus, human REV1 is able to complement the chicken *rev1* mutant.

The N-terminus is required for efficient nuclear localization of hREV1

Using the same technique, we created a panel of *rev1* DT40 expressing mutated/truncated human REV1 constructs. We assessed localization of the fusion proteins by examining YFP fluorescence using laser scanning confocal microscopy. The full-length REV1 and all mutants, with the exception of *rev1*(333–1251) and *rev1*(923–1251), were strongly localized to the nucleus (Figure 3). *rev1*(333–1251) is expressed both in the nucleus and cytoplasm despite the deleted N-terminal region containing no obvious nuclear localization signal. In none of the clones examined did we detect clear focus formation in either untreated or UV irradiated cells. We discuss both these observations below.

The N-terminus, including the BRCT domain, of hREV1 is dispensable for efficient DNA damage tolerance

The *rev1-1* mutant in yeast (3) carries a point mutation in the N-terminal BRCT domain resulting in a G193R amino acid substitution (10). This renders the cells sensitive to UV irradiation (34) and yet the mutant protein retains substantial deoxycytidyl transferase activity (6). This observation



D

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Hs 1152 ----------------VRPPAPNLAGAVEFN---------DVKTLLREWITTIS---DPMEEDILQVVKYCTDLIEE-------
Rn 1155 ----------------FRPAAPNLAGAVEFS---------DVKTLLKEWITTIS---DPMEEDILQVVKYCTDLIEE-------
Mm 1150 ----------------FRPAAPNLAGAVEFS---------DVKTLLKEWITTIS---DPMEEDILQVVRYCTDLIEE-------
Gg 1156 ----------------FRPQAPNLAGAVEFN---------DVKTLLKEWITTIS---DPMEEDILQVVKYCTDLIEE-------
Xl 1131 ----------------FKPKPPNLAGAIEFS---------DVKTLLREWITTIS---DPMEEDILQVVRYCTDLIDE-------
Tn 1214 ----------------LPRPTPTLAGACDLT---------DIRALLREWVTTIT---EPMEEDILQVVKYCTDLIED-------
Dm  878 -------VNPHILKLISHPVEMPELLMGD---------NYKDLLNDWVSREE---VPKPNDVDLILKQVSRMIKN-------
Gz 1024 ----------LPGGQLRIQFATLPRKISFASSGVTSTQEIKDMLDAWHSETRKN-GPHRGDIEVLEKFLERVVQEE------
Cg  768 ------------GKFSIFDPIKFQGQPKFK---------DICSLVKEWVHSTLKEAGPHDRDVQLFEKYSKKLCNTNR---A
Cn 1117 ----PPQQRQSVKIIRPPKPALMNSTELP---------DVLRTIELWIESREDS-PPADKDAIKVAAFLKKCLNDGEGRVG
Eg  698 ------SDHALKNLGILEPVTFQNLISQE---------KIKSLVREWISQTLRSDGPNHKDLDLFTNYLDRLISGGPRR-L
Mg 1072 ---AVRSEGAVDSQERLQFPKKPARASFTKQNLTSLPKIKDMLQLWHRDTVRT-GPHLRDTEVLETYLLKVIREE------
Um 1369 ------------NAEKSSRDIPTIRGLSHPR---------DVEILLSQWISAFARK-GPREGDVNRIATYLADVVRTASVTRV
Sp  835 ----------------KPHIVTFQHVQSLE---------DLRGLLTKWYSKASK--GPNIHDVNYFANYVCRVIREE------
Sc  856 ESLQNEKNHFMGQNSIFQPIKFQNLTRFK---------KICQLVKQWVAETLGDGGPHEKDVKLFVKYLIKLCDSNR---V

Hs 1201 KDLEKLDLVIKYMKRLMQQS--------------VESVWNMAFDFILDNVQVVLQQTYGSTLKVT---------- 1251
Rn 1204 KDLEKLDLVIKYMKRLMQQS--------------VESVWNMAFDFILDNVQVVLQQTYGSTLKVT---------- 1250
Mm 1199 KDLEKLDLVIKYMKRLMQQS--------------VESVWNMAFDFILDNVQVVLQQTYGSTLKVT---------- 1249
Gg 1205 KDLEKLDLVVKYMKRLMQSS-------------VESVWNMAFDFILDNVQVVLQQTYGSTLKVI---------- 1254
Xl 1180 KDLEKLDLVIKYMKRLMQQS--------------VESVWNMAFDFILDNIQVVLQQTYGSTLKVV---------- 1230
Tn 1263 KDLEKLDLIIKYMKRLMKQS--------------AESVWSMAFDFVLDNVQVVLQQTYGSTLKVS---------- 1313
Dm  934 DQLDHVCDVMKYWCRIINMK-----------RSSSCCWHVAYKHIEESIQNQMLTIEGYSLLFIEYIRCIKCS-  995
Gz 1089 RDLEKATKLVKWLDVLVEQD--------GRKGRGQETWRRSVEGIKVVVQQAVKQRGMAPLKF------------ 1143
Cg  826 HFLCRLAHIISMELDLMEDE---------SQNSEGFQEWESILLKRIIPLLNQNKHTFQTERKLDIEFDFT----- 887
Cn 1184 DGVEKVVEVLRWMRMIIKEKWAQDEGLNEGASEAGREWWRIWRQMRDEVNLICVKKFGAGLRI------------ 1247
Eg  763 PTLLSMARLISDQLRIHEAQ---------GDRPSFQEWEEYLVRVVLVRLNRCKQLYTQNVEILFDIYPQIDRP  828
Mg 1143 RNLEKAQVLVAWLDWLVEEEEEG-----DSSGKGKKQWQAAVERIKRVVQEALAERGLGPMDLG----------- 1201
Um 1430 EDTQKASSLLGFIQERLDEVQYK-----IDDGFASEEWETAKRKIRDAVQAKSREVFGGAELEE----------- 1489
Sp  885 KNLGKAQMMLKWLYQLNRKE-----------CNKPWEKAIDKIIETVQGECLQRNIPPLMIF------------ 936
Sc  925 HLVLHLSNLISRELNLCAFL---------NQDHSGFQTWERILLNDIIPLLNRNKHTYQTVRKLDMDFEV----- 986
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Figure 1. (**A**) Mammalian two-hybrid interactions of REV1 and PCNA. Luciferase activity is expressed as a multiple of the negative control. The bait, in pM is indicated at the top of each graph and the prey, in pVP16, under each column. (**B**) The C-terminus of REV1 is responsible for both the interaction with POLη and PCNA. Representative mammalian-two-hybrid luciferase assays for the interaction of POLη and PCNA with full-length (FL) REV1 and truncation mutants thereof. (**C**) Summary of mammalian two-hybrid readouts between REV1 mutants (in pM) and POLη or PCNA (in pVP16). The grey box indicates the region shared by all REV1 truncations that give a positive interaction with PCNA. (**D**) ClustalW (http://www.ebi.ac.uk/clustalw/) alignments of C-terminus of REV1 homologues identified during a PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search with the C-terminal 100 amino acids of human REV1. All these proteins additionally possess N-terminal BRCT domains and central transferase domains. The shades of grey indicate the degree of conservation derived from the BLOSUM62 score calculated in Jalview (http://www.jalview.org/) using the ClustalW alignment. The amino acid positons are indicated at the beginning of each line. The number of the final amino acid of each sequence is given at the end of the second line. Species abbreviations: Hs, *Homo sapiens*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Gg, *Gallus gallus* (chicken); Xl, *Xenopus laevis*; Tn, *Tetraodon nigroviridans* (pufferfish); Dm, *Drosophila melanogaster*; Gz, *Gibberella zeae*; Cg, *Candida glabrata*; Cn, *Cryptococcus neoformans*; Eg, *Eremothecium gossypii*; Mg, *Magnaporthe grisea*; Um, *Ustilago maydis*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*.

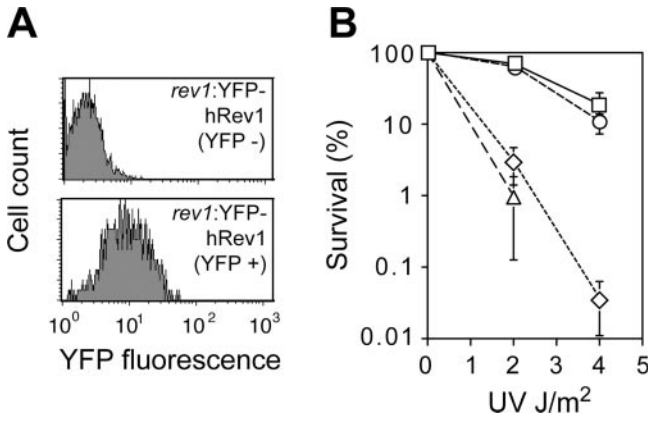

Figure 2. Complementation of *rev1* DT40 with human REV1. (**A**) Typical expression of eYFP-tagged human REV1 in *rev1* DT40 compared with a non-expressing control from the same transfection. (**B**) UV sensitivity of the two clones shown in Figure 2A compared with wild-type DT40 and the *rev1* mutant. Key: squares, WT; diamonds, *rev1*; triangles, *rev1:YFPhREV1* YFP negative clone; circles, *rev1:YFPhREV1* YFP positive clone.

thus appears to dissociate the deoxycytidyl transferase function of REV1 from a second role in translesion synthesis. The corresponding residue in human REV1 is G76 (35) and we therefore constructed a YFP-hREV1 fusion carrying a G76R substitution. Surprisingly, the expression of this construct was able to complement the UV and cisplatin sensitivity of *rev1* DT40 (Figure 4A).

We therefore asked whether the whole N-terminus, encompassing the BRCT domain, was dispensable (Figure 4B). A construct lacking the first 332 amino acids was also able to restore UV and cisplatin sensitivity of *rev1* DT40 to wild-type levels. However, expression of this construct is seen in both the nucleus and cytoplasm. Such delocalization was also noted by Tissier *et al*. (27) during transient expression of a more extensive N-terminal deletion of the first 729 amino acids. While in this latter system expression was predominantly nuclear, in our stably transfected lines *rev1*(333–1251) expression is predominantly cytoplasmic. Further, unlike full-length REV1, it was possible to obtain clones of *rev1*(333–1251) with much higher levels of expression (data not shown). This may reflect the poor nuclear retention of the mutant protein, allowing higher levels of expression without toxicity.

The catalytic activity of hREV1 does not play a major role in tolerance of damage induced by UV light, cisplatin or hydrogen peroxide

The transferase activity of REV1 depends on key motifs in polymerase domains III and IV. We mutated the critical DE motif in domain IV by changing D570 and E571 to alanine. This mutation has previously been shown to abolish the transferase activity of the protein (36). Expression of catalytically inactive REV1 in *rev1* DT40 resulted in complementation of the cellular hypersensitivity to UV light (Figure 4C). However, since neither T–T dimers nor 6–4 photoproducts are substrates for dCMP transfer by REV1 *in vitro*, this result does not exclude a role for the dCMP transferase activity in the bypass of other lesions. Hydrogen peroxide causes a wide range of oxidative base lesions including abasic sites, which are known substrates for REV1 (4,17). We therefore additionally examined the response of the catalytic mutant to hydrogen peroxide. The D10 values for hydrogen peroxide (dose at which 10% of cells survive) of wild-type cells was 19.4 ± 3.0 μM, while for *rev1* cells it was 7.2 ± 3.7 μM. The value for the *rev1*(D570A/E571A) mutant was 25.6 ± 6.9 μM. Thus, *rev1*(D570A/E571A) is also able to complement the hypersensitivity of *rev1* cells to hydrogen peroxide. This suggests that dC transfer is not the principal function of REV1 for tolerance of DNA damage created by these mutagens.

It has emerged recently that the efficient repair of interstrand cross-links in vertebrates requires the translesion polymerases POLζ (37) and POLη (38) as well as REV1 (15). The precise role they play is unclear, but one attractive model is that they bypass the adducted mononucleotide remaining following the incision and degradation of one strand of the cross-linked DNA (39). Similar to the findings for UV and hydrogen peroxide, the catalytic activity of REV1 is dispensable for its role in processing cisplatin-induced damage (Figure 4C).

The C-terminus of REV1 is necessary but not sufficient for effective DNA damage tolerance

Further confirmation of the dispensability of the BRCT domain and transferase activity of REV1 comes from complementation with *rev1*(1–827). This mutant contains both BRCT and transferase domains and yet fails to restore the hypersensitivity of *rev1* DT40 to UV and cisplatin (Figure 4D), despite being correctly localized in the nucleus (Figure 3B). This result also demonstrates the critical importance of the C-terminus of the protein. We noted that *rev1* cells expressing *rev1*(1–827) appeared slightly more sensitive to UV light than *rev1* cells suggesting a possible dominant negative role for this construct. However, expression of *rev1*(1–827) in wild-type cells did not confer any significant additional sensitivity to UV (data not shown). We next asked whether the C-terminus alone, *rev1*(923–1251), was sufficient to restore the DNA damage sensitivity of the *rev1* mutant. It is not (Figure 4E). In addition, we tested *rev1*(1150–1251) and found that it also failed to complement the *rev1* defect (data not shown). However, expression of the *rev1*(923–1251) mutant results in a rather granular YFP signal throughout both the nucleus and cytoplasm suggesting that the protein may be aggregating.

REV1 acts largely independently of RAD18

Our data suggest that REV1 is playing a structural role in translesion synthesis probably through coordination of the other translesion polymerases at a lesion, including those not found in yeast, POLκ and POLι. In yeast, RAD18 plays a key role in the control of the TLS polymerases POLζ and RAD30 (POLη) and is epistatic to both (19,20,40). In vertebrates, RAD18 has been shown to be involved in the regulation of POLη recruitment, but does not appear to be epistatic to POLκ (22–24). These data, the importance of REV1 in vertebrate TLS and the apparent universality of its interaction with the TLS polymerases, led us to examine the genetic relationship between RAD18 and REV1 in DT40 by constructing a *rev1*/*rad18* double mutant.

The *rev1* mutant grows more slowly than wild-type cells (15) whereas the *rad18* mutant does not exhibit such defect

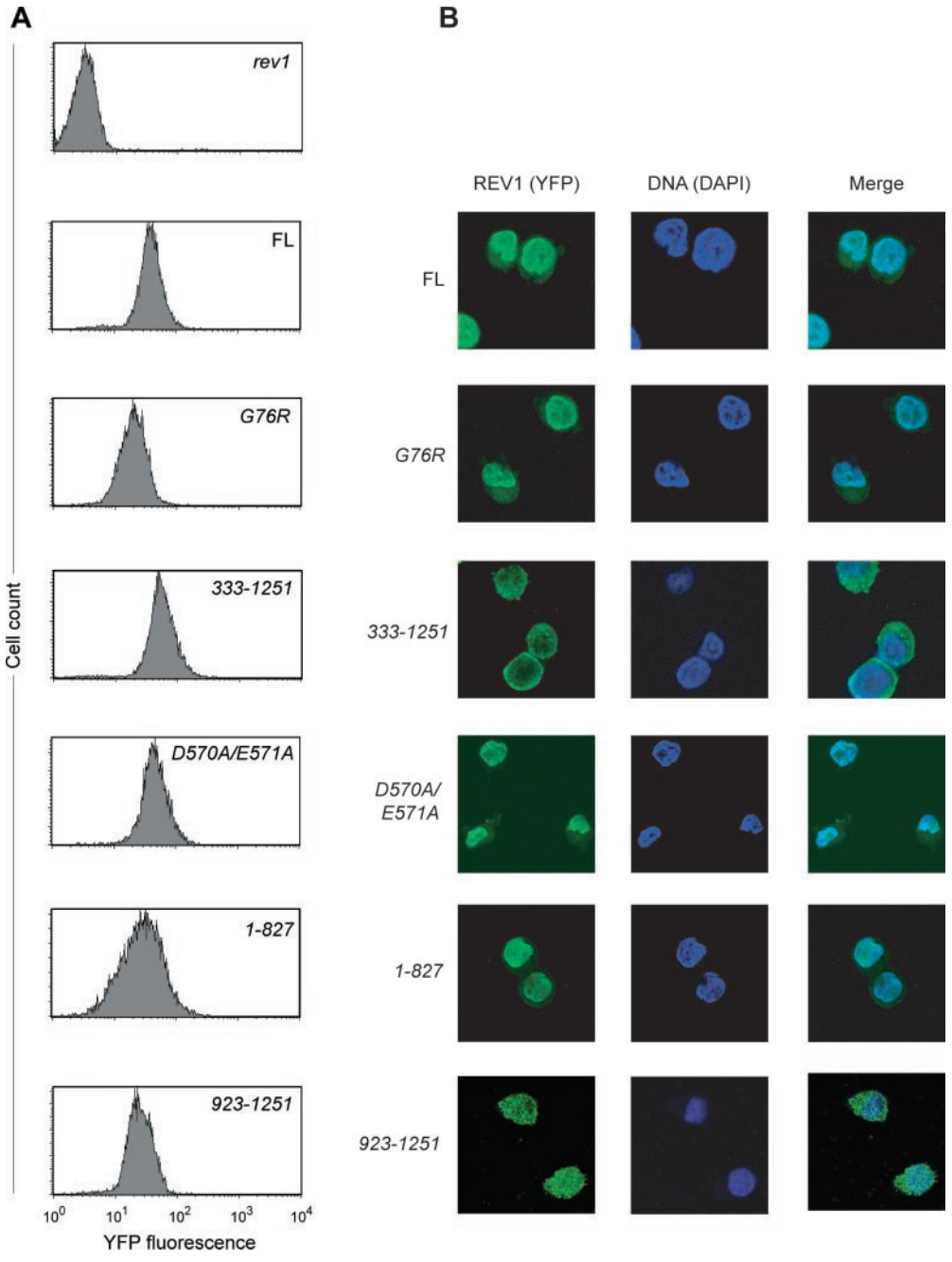

Figure 3. Expression and localization of REV1 and mutants in DT40 cells. (**A**) Expression levels by flow cytometry of eYFP fluorescence in *rev1* DT40 transfected with full-length and mutant YFP-hREV1 fusions. FL, Full-length hREV1. (**B**) Localization of REV1 and mutants by confocal microscopy. REV1 in green, DNA stained by DAPI in blue. Colocalization results in cyan.

(30). The *rev1*/*rad18* double mutant exhibits similar slow growth to the *rev1* single mutant (data not shown). A key feature of *rad18* mutants of both DT40 and mouse embryonic stem cells is the elevated levels of spontaneous sister chromatid exchange, which has been interpreted as channelling of lesions from post-replication repair into homologous recombination (30,41). *rev1* mutants do not show this elevation and, making the assumption that *rad18* would be epistatic to *rev1* in DT40, we previously suggested that this might mean that REV1 is beyond a point of commitment in lesion bypass that cannot be 'rescued' by homologous recombination (15). The observation (Figure 5A) that the *rev1*/*rad18* double mutant displays elevated SCE similar to the *rad18* single mutant is consistent with this. However, testing for sensitivity to UV light and cisplatin shows the *rev1*/*rad18* double mutant to be considerably more sensitive than either single mutant (Figure 5B). Further, and in contrast to yeast, we consistently observed the *rev1* mutant to be more sensitive than the *rad18* mutant to all agents tested. Taken together, these data are most readily explained by RAD18 and REV1 playing largely non-overlapping roles in DNA damage tolerance.

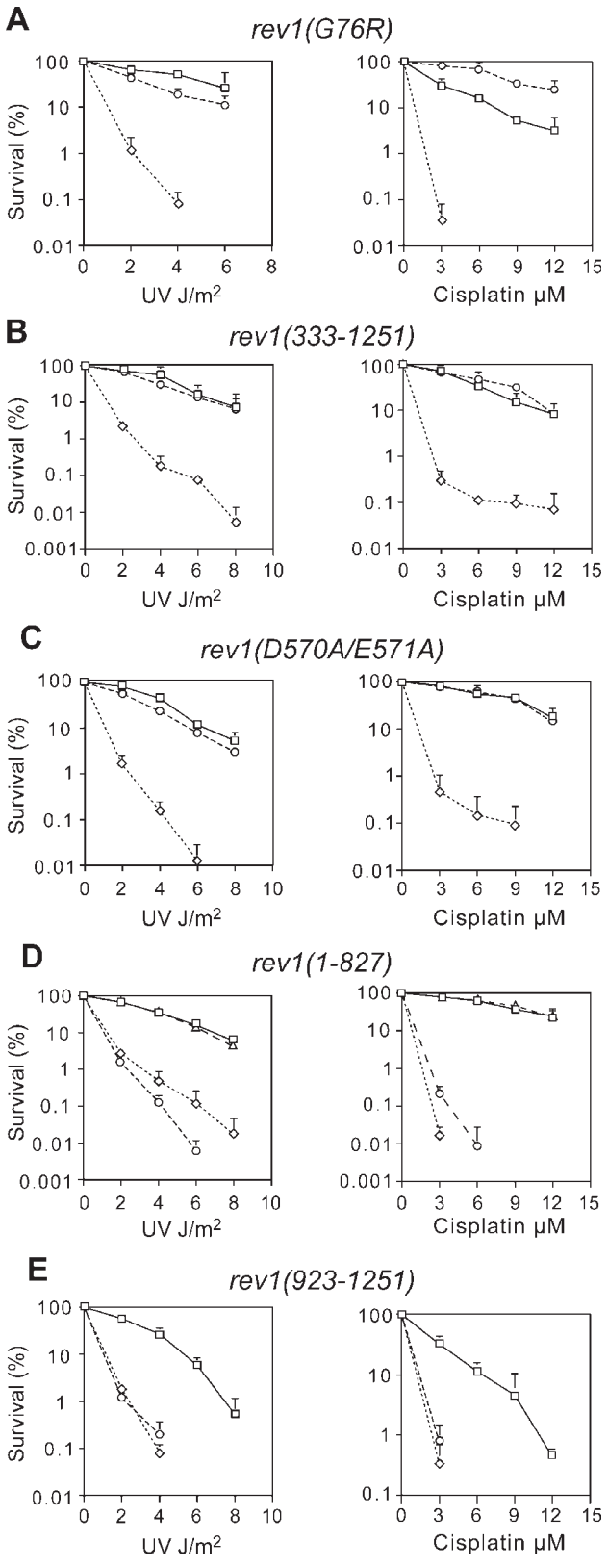

Figure 4. A 254 nm UV and cisplatin damage sensitivity of *rev1* mutants. (**A**) BRCT mutant, *rev1*(*G76R*) (**B**) N-terminal BRCT truncation mutant, *rev1*(333–1251). (**C**) Catalytic mutant *rev1*(*D570A/E571A*). (**D**) C-terminal truncation mutant, *rev1*(1–827). (**E**) C-terminus only mutant, *rev1*(923–1251). Key: squares, WT; diamonds, *rev1*; circles, *rev1* expressing the indicated YFP fusion; triangles, *rev1* expressing full-length YFP-hREV1.

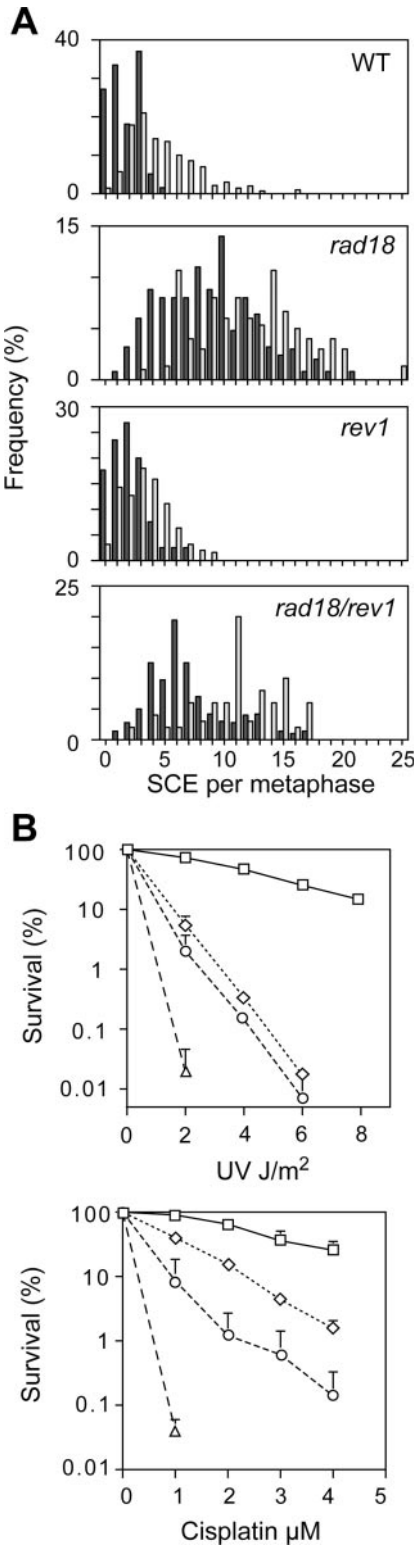

Figure 5. (**A**) Spontaneous and DNA damage-induced sister chromatid exchange (SCE) in *rev1*/*rad18* DT40. These histograms represent the percentage of metaphases (*y*-axis) containing a given number of SCE (*x*-axis). Untreated in dark grey, cells treated with NQO in light grey. Mean SCE: WT 1.4 ($n=254$) [+NQO 4.7 ($n=140$)]; *rad18* 8.5 ($n=125$) [+NQO 12.3 ($n=75$)]; *rev1* 2.0 ($n=119$) [+NQO 3.4 ($n=63$)]; *rev1*/*rad18* 7.1 ($n=72$) [+NQO 11.0 ($n=50$)]. (**B**) Epistasis analysis for survival following DNA damage in *rev1*/*rad18* DT40 following treatment with 254 nm UV and cisplatin. Key: squares, WT; diamonds, *rev1*; circles, *rad18*; triangles, *rev1*/*rad18*.

DISCUSSION

Expression and localization of Rev1

We believe that REV1 expression is likely to be tightly regulated at low levels in cells. Although human REV1 is able to complement the *rev1* defect in a chicken cell line, it does so only at modest expression levels. Attempts to obtain stably overexpressing lines using a strong (CMV-IE) promoter were unsuccessful whereas use of the chicken β-actin promoter appears to allow selection of clones with levels of expression closer to expected physiological levels. Like *REV3*, the human *REV1* locus contains an upstream ATG which will give rise to an out-of-frame transcript predicting that expression levels will be low (42) and to date there has been no successful detection of endogenous REV1 using immunofluorescence in vertebrate cells. In *S.cerevisiae*, it is estimated that there are only about 500 molecules of REV1 per cell (43).

The only mutant that could be readily driven to higher constitutive levels of expression was *rev1*(333–1251), which lacks the N-terminus including the BRCT domain. Expression of this mutant in our stably transfected cells is seen predominantly in the cytoplasm. We do not believe this result is inconsistent with that of Tissier *et al*. (27), although a direct comparison between the experimental systems employed is difficult as we believe that stable expression of REV1 selects against high nuclear levels of the protein. The N-terminus of REV1 does not contain any likely nuclear localization signal, so we think it is probable that efficient nuclear retention of REV1 requires the BRCT domain. However, *rev1*(333–1251) is still able to complement the DNA damage hypersensitivity of *rev1* DT40. This suggests that the N-terminus is not essential for the direct role played by REV1 in translesion synthesis. In the context of the endogenous *REV1* promoter, such a defect in the BRCT domain may manifest itself as a null or hypomorphic phenotype, not because this domain plays a direct role in the coordination of TLS, but because of insufficient nuclear levels of the C-terminus, which our data show to be essential. Indeed, murine ES cells carrying a REV1 BRCT domain deletion show a phenotype similar to *rev1* DT40 (16). That the equivalent mutation to yeast *rev1-1* in the human protein does not disrupt localization or function may be due to differences in the fine structure of the domain between yeast and vertebrates or a manifestation of a different mode of action of yeast REV1: it would be interesting to determine whether the *rev1-1* mutant in yeast is correctly localized.

Two recent reports (27,28) and our own unpublished observations have shown that a proportion of mammalian cells transiently overexpressing REV1 show spontaneous focus formation. Transient transfection, in COS or HeLa cells, of CMV-IE promoter-driven YFP-REV1 results in a spectrum of expression ranging from faint and diffuse, through foci of varying sizes to clearly unphysiological aggregates. In our stably transfected *rev1* DT40 cells, we do not observe such foci either spontaneously or following DNA damage: YFP fluorescence in the nucleus remains diffuse, and relatively faint. We do not believe that this reflects failure of recruitment of YFP-tagged REV1 to sites of stalled replication, since we see complementation of the DNA damage hypersensitivity of *rev1* DT40. Although we believe that the REV1 foci seen in transient overexpression experiments are probably tag sites of recruitment, we think it more likely that the actual physiological function of REV1 at sites of stalled replication does not require more than a few molecules.

The interactions of REV1 in higher and lower eukaryotes

Recent data (25–28), and that presented here, have shown that the extreme C-terminus of human and mouse REV1 is able to bind each of the other translesion polymerases. From the alignments presented in Figure 1 and the fact that the human gene is able to complement a chicken mutant, it seems likely that this will be a universal feature in vertebrates. Contrary to previous assertions (25), we do not agree that the C-terminus of REV1 of *S.cerevisiae* exhibits no significant homology with the vertebrate protein. The alignments of the extreme C-terminus of the human and yeast proteins reveal some strikingly conserved features despite a relatively low percentage overall identity. Highly relevant in this regard is the fact that a similar level of conservation is seen between human and *Drosophila* as is seen between human and *S.cerevisiae* REV1. Recently, biochemical experiments have shown that it is possible to purify *Drosophila* REV3 by affinity chromatography with the C-terminus of *Drosophila* REV1 (33). While it is unclear whether this is actually achieved via an intermediate REV7 interaction (25), it would seem likely that the human and fly REV1 are working in the same way. These observations beg a careful re-examination of the interactions and role of REV1 in yeast. Indeed, the observations of Larimer *et al*., some fifteen years ago, suggest that deletion of the C-terminal 128 amino acids of REV1 in *S.cerevisiae* also results in the null phenotype: UV-induced Lys^+ revertants from *lys1-1* in this mutant were comparable to the level seen in the *rev1-1* mutant, while a strain producing a *REV1* transcript truncated just 3′ of the stop codon was Rev^+ (10). However, there is no apparent conservation of this domain between vertebrates and plants, exemplified by *Arabidopsis* and *Oryza*, suggesting that the REV1-TLS polymerase interaction is not likely to be conserved in all eukaryotes.

A novel interaction identified in the current work is that between the C-terminus of REV1 and PCNA. Our data further suggests that the interactions of the TLS polymerases and PCNA involve adjacent regions of the C-terminus of REV1. Although the interaction of REV1 with PCNA may be indirect, transiently overexpresssed REV1 has been shown to colocalize with POLη and PCNA in 'replication factories' (27), with the localization of REV1 appearing to be independent of POLη. Thus, REV1 may be required to coordinate or stabilize the interaction of the incoming TLS polymerase with the PCNA clamp.

The roles of REV1 and RAD18 in the control of vertebrate translesion synthesis

REV1 is a member of the Y-type polymerase family and the human protein exhibits *in vitro* dCMP transferase activity over a wide range of lesions (17,18,44). However, although it is unable to bypass both UV-induced T–T cyclobutane dimers and 6–4 photoproducts, it is clearly required for normal tolerance of these lesions in DT40, suggesting that its role is

not a function of its catalytic activity. The data we present here unambiguously shows this to be the case, not only for UV-induced damage, but also for oxidative base damage induced by hydrogen peroxide and the complex damage introduced by cisplatin. The principal function of the protein appears to derive exclusively from the C-terminus further suggesting that its major role in vertebrates is non-catalytic. We suggest that REV1 is required for stabilization of the interaction between the incoming TLS polymerase and PCNA at sites of replication blockage. This stabilizing role may be enhanced by the as yet unidentified interactions of the N-terminal BRCT domain and possibly the DNA binding activity of the transferase domain. However, in the complementation system presented in this paper, any loss of function of these domains is overcome, possibly because there is still an element of REV1 overexpression.

In *S.cerevisiae* RAD6/RAD18 are epistatic to the translesion DNA polymerases. Recent work has shown that the RAD6/RAD18 heterodimer mediates the DNA damage-induced monoubiquitination of PCNA (POL30 in yeast) (19,21). In turn, this modification is required to recruit translesion synthesis by POLζ and RAD30. Human RAD18 has also been shown to be responsible for the monoubiquitination of PCNA and that monoubiquitinated PCNA specifically recruits POLη to sites of replication arrest (22,23). However, we show here that the function of REV1 in DNA damage tolerance in DT40 is significantly independent of RAD18. A similar observation has also been made for DNA polymerase κ (24). Together this suggests that the role RAD18 plays in the regulation of vertebrate translesion synthesis is not as central as its yeast counterpart. Indeed, it is possible that in vertebrates the RAD18-mediated monoubiquitination of PCNA is only required for the recruitment of POLη. However, while it is possible that in vertebrates there exist alternate REV1-dependent and RAD18-dependent modes of translesion synthesis, we favour the idea that both are required and that in the absence of either, the coordination of the polymerases becomes inefficient resulting in decreased DNA damage tolerance. The precise mechanism by which REV1 and RAD18 interact in translesion synthesis will be a fertile ground for further work.

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