

# Structure and Functional Analysis of the BRCT Domain of Translesion Synthesis DNA Polymerase Rev1

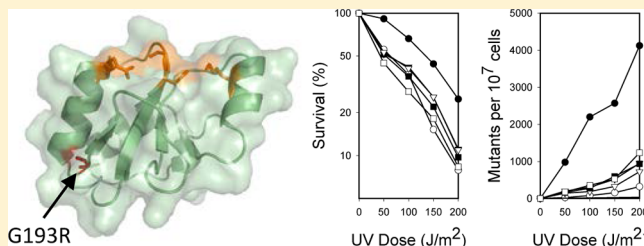
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## S Supporting Information

**ABSTRACT:** Translesion synthesis (TLS) is a pathway in which specialized, low-fidelity DNA polymerases are used to overcome replication blocks caused by DNA damage. The use of this pathway often results in somatic mutations that can drive carcinogenesis. Rev1 is a TLS polymerase found in all eukaryotes that plays a pivotal role in mediating DNA damage-induced mutagenesis. It possesses a BRCA1 C-terminal (BRCT) domain that is required for its function. The *rev1-1* allele encodes a mutant form of Rev1 with a G193R substitution in this domain, which reduces the level of DNA damage-induced mutagenesis. Despite its clear importance in mutagenic TLS, the role of the BRCT domain is unknown. Here, we report the X-ray crystal structure of the yeast Rev1 BRCT domain and show that substitutions in residues constituting its phosphate-binding pocket do not affect mutagenic TLS. This suggests that the role of the Rev1 BRCT domain is not to recognize phosphate groups on protein binding partners or on DNA. We also found that residue G193 is located in a conserved turn region of the BRCT domain, and our in vivo and in vitro studies suggest that the G193R substitution may disrupt Rev1 function by destabilizing the fold of the BRCT domain.



DNA damage presents a serious problem during DNA replication, because DNA synthesis by the high-fidelity, replicative DNA polymerases is blocked by DNA lesions. One way to overcome these blocks is translesion synthesis (TLS). In TLS, low-fidelity DNA polymerases, which have evolved to accommodate DNA lesions, are used to replicate through the damage.<sup>1–8</sup> Because of their low fidelity and lack of proofreading activity, TLS polymerases generate mutations. This is problematic, because an accumulation of somatic mutations drives carcinogenesis. It has previously been shown that the knockdown of TLS polymerases reduces the extent of carcinogen-induced tumor formation.<sup>9</sup> Moreover, TLS polymerases promote acquired chemoresistance, and inhibition of TLS polymerases enhances the efficacy of some chemotherapeutics.<sup>10,11</sup> Therefore, TLS polymerases are potential chemopreventative and chemotherapeutic drug targets.<sup>9–12</sup>

In eukaryotes, TLS polymerases Pol  $\zeta$  and Rev1 often function together and play critical roles in mediating mutagenic TLS.<sup>13–15</sup> For example, in the budding yeast *Saccharomyces cerevisiae*, these proteins are responsible for >90% of ultraviolet (UV) radiation-induced mutations, and in human cells, knockdown of either protein reduces the level of UV-induced mutagenesis nearly 75%.<sup>16–19</sup> The role of Pol  $\zeta$  in generating these mutations is well understood. Although Pol  $\zeta$  is not able to efficiently incorporate nucleotides directly opposite sites of DNA damage, it is able to extend from nucleotides incorporated either correctly or incorrectly opposite the damaged base by another TLS polymerase.<sup>1,15,20,21</sup> Amino acid substitutions that reduce the efficiency of nucleotide

incorporation by Pol  $\zeta$  in vitro reduce the level of DNA damage-induced mutagenesis in vivo.<sup>22</sup>

The role of the Rev1 protein in generating mutations, however, is less clear. Rev1 is able to efficiently incorporate nucleotides opposite several common types of DNA damage, including abasic sites, N<sup>2</sup>-adducted guanines, and N<sup>6</sup>-ethenoadenines.<sup>23–26</sup> In most cases, the catalytic activity of Rev1 is dispensable for mutagenesis. Thus, Rev1 plays a critical noncatalytic function in mutagenic TLS that likely involves mediating a key protein–protein interaction.<sup>27</sup> In addition to the polymerase domain, Rev1 also contains at least two other functional regions: a C-terminal domain and an N-terminal BRCT domain. The C-terminal domain of Rev1 interacts with other TLS DNA polymerases and is thought to promote mutagenic TLS by acting as a protein scaffold.<sup>28–33</sup> The BRCT domain of Rev1 also plays a critical role in mutagenic TLS. The *rev1-1* allele encodes a mutant form of Rev1 with a G193R substitution in the BRCT domain, which nearly eliminates UV-induced mutagenesis in yeast.<sup>34</sup> Targeted deletion of the Rev1 BRCT domain in mice also reduces the level of UV-induced mutagenesis and nearly eliminates mutagenesis at thymine dimers.<sup>35</sup> Despite its clear importance, the function of the Rev1 BRCT domain is unknown.

BRCT domains are typically found in proteins involved in the DNA damage response, and these domains have been

Received: November 21, 2012

Revised: December 11, 2012

Published: December 14, 2012

associated with several activities, including the binding of phosphorylated proteins, the binding of nonphosphorylated proteins, and the binding of DNA.<sup>36–38</sup> A previous study showed that the yeast Rev1 BRCT domain binds phosphorylated peptides with a higher affinity than nonphosphorylated peptides in vitro.<sup>39</sup> Another study demonstrated that the yeast and mouse Rev1 BRCT domains are capable of binding phosphate groups on DNA primer–template junctions in vitro.<sup>40</sup> It is unknown if either of these activities is required for its function in mutagenic TLS in cells, nor is it known if the G193R substitution disrupts either of these activities.

To gain insight into the role of the Rev1 BRCT domain in TLS, we determined its X-ray crystal structure and conducted structure–function analyses of key residues within the domain. We found that the yeast Rev1 BRCT domain has a canonical BRCT domain phosphate-binding pocket, but substitutions of conserved residues within this binding pocket did not lead to a reduction in the level of UV-induced mutagenesis in vivo. This suggests that recognition of phosphate groups on either proteins or DNA by the BRCT domain of Rev1 is not required for mutagenic TLS. By contrast, even conservative substitutions of Gly-193 resulted in substantial defects in UV-induced mutagenesis similar to those caused by the G193R substitution. Our analysis of the structure suggested that the substitution of residues at this position might destabilize the fold of the BRCT domain. Consistent with our structural analysis, we found that only the substitution of residues designed to disrupt the stability of the BRCT domain led to defects in induced mutagenesis. On the basis of these and other results, we conclude that that G193R substitution destabilizes the yeast Rev1 BRCT domain and that proper folding of the domain is necessary for its function in the TLS pathway.

## ■ EXPERIMENTAL PROCEDURES

**Protein Expression and Purification.** The yeast Rev1 G193R mutant protein (residues 1–746), which is lacking 239 amino acid residues from its C-terminus, but contains the N-terminal BRCT domain and the polymerase domain, was expressed and purified as described previously for the wild-type protein.<sup>41</sup> We used this truncated protein because it is overexpressed to a greater extent than the full-length protein and because this truncation does not affect catalytic activity.<sup>42</sup> Briefly, the mutant protein was expressed in yeast strain BJ5464 harboring the pKW385 plasmid that encoded the Rev1 mutant protein fused in frame with glutathione S-transferase (GST). The protein was purified by affinity chromatography using a glutathione column (GE Healthcare). The Rev1 mutant protein was eluted from the column by incubation with Prescission Protease (GE Healthcare) at 4 °C overnight. This resulted in the removal of the GST tag, which remained bound to the column. The concentration of the purified protein was determined by UV spectroscopy.

The yeast Rev1 BRCT domain (residues 159–250) was overexpressed and purified using the IMPACT kit (New England Biolabs). The coding sequence of the Rev1 BRCT domain was cloned into protein expression plasmid pTXB1 to yield pKW384, which expresses this domain as a fusion protein containing a C-terminal tag consisting of an intein and a chitin-binding domain. The fusion protein was produced in *Escherichia coli* Rosetta-2 (DE3) cells (Invitrogen) and was purified by affinity chromatography using a chitin column (New England Biolabs). The Rev1 BRCT domain was eluted from the chitin column by incubation in a buffer containing 50 mM

DTT at 25 °C overnight. This resulted in the removal of the intein and chitin-binding domain, which remained bound to the column. The Rev1 BRCT domain was further purified using size exclusion chromatography using a Superdex 75 column (GE Healthcare). The concentration of the purified protein was determined by UV spectroscopy.

**DNA and Nucleotide Substrates.** The synthetic oligonucleotides used as substrates in this study were a 24-mer primer strand with the sequence 5'-GCC TCG CAG CCG TCC AAC CAA CTC and a 45-mer template strand with the sequence 5'-GGA CGG CAT TGG ATC GAC CTG GAG TTG GTT GGA CGG CTG CGA GGC (Integrated DNA Technologies). Oligonucleotides were gel-purified, and their concentrations were determined using UV spectroscopy. The primer strand was <sup>32</sup>P-labeled on the 5' end using [ $\gamma$ -<sup>32</sup>P]ATP (Sigma) and polynucleotide kinase (New England Biolabs). The primer strand and template strand (1  $\mu$ M each) were annealed in 25 mM Tris-HCl (pH 7.5) and 100 mM NaCl by being heated to 90 °C for 2 min and then slowly cooled to room temperature over several hours. Labeled and annealed DNA substrates were stored at 4 °C for up to 1 week. Solutions of each dNTP (100 mM) were purchased from New England Biolabs and stored in 10  $\mu$ L aliquots at –80 °C.

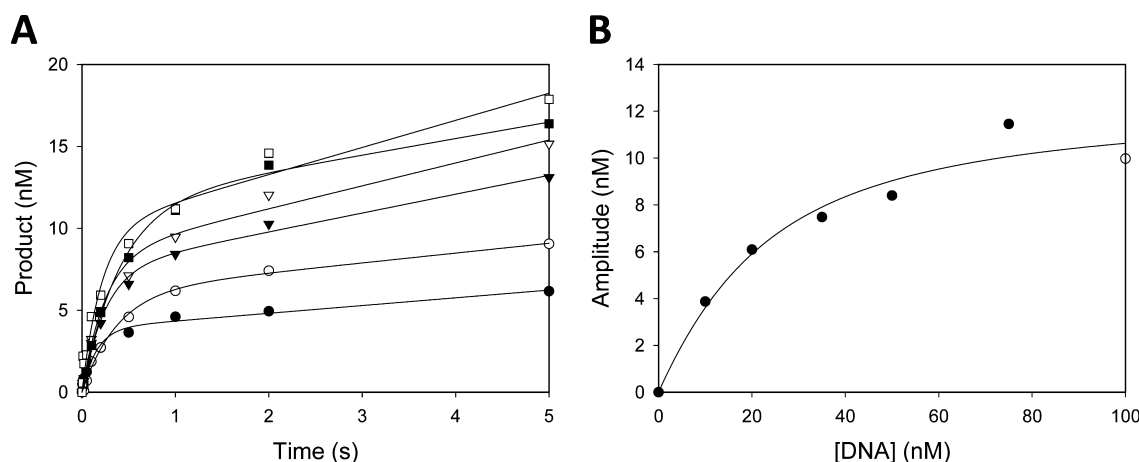
**Pre-Steady State Kinetics.** Analysis of the kinetics of incorporation of nucleotides by the Rev1 G193R mutant protein was conducted as described previously for the wild-type Rev1 protein.<sup>23</sup> Reactions were performed in a buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 5 mM DTT, and 10% glycerol at 30 °C using a rapid chemical quench flow apparatus (KinTek). The Rev1 G193R mutant protein (800 nM) was preincubated with various concentrations of <sup>32</sup>P-labeled DNA (10–100 nM), and reactions were initiated by the addition of 1 mM dCTP. Reactions were quenched after various time points ranging from 10 ms to 5 s and the products were resolved on a 15% polyacrylamide sequencing gel containing 8 M urea. The radiolabeled gel band intensities were quantified using a Phosphorimager (GE Healthcare). The concentrations of extended product (*P*) were graphed as a function of time (*t*). The amplitude of the burst phase (*A*), the observed first-order rate constants for nucleotide incorporation during the burst phase (*k*<sub>obs</sub>), and the observed rate for the steady state phase (*v*<sub>ss</sub>) were obtained from the best fit of the data to the burst equation:

$$P = A(1 - e^{-k_{\text{obs}}t}) + v_{\text{ss}}t$$

The amplitudes of the burst phases (*A*) were graphed as a function of DNA concentration ([DNA]). The apparent dissociation constant for the DNA (*K*<sub>d-DNA</sub>) and the active site concentration of Rev1 ([Rev1]) were obtained from the best fit of the data to the quadratic form of the binding equation:

$$A = 0.5(K_{\text{d-DNA}} + [\text{Rev1}] + [\text{DNA}]) - \sqrt{0.25(K_{\text{d-DNA}} + [\text{Rev1}] + [\text{DNA}])^2 - ([\text{Rev1}][\text{DNA}]}$$

**Crystallization and Structural Determination of the Rev1 BRCT Domain.** Crystallization of the Rev1 BRCT domain was performed using the hanging drop method with 200 nL drops that were set up with a mosquito crystallization robot (TTP Labtech). Optimally diffracting crystals were obtained by combining an equal volume of protein (8 mg/mL) with a reservoir solution containing 0.1 M HEPES (pH 7) and 20% PEG 2000 (w/v). Crystals formed within 24 h at 18



**Figure 1.** Pre-steady state kinetics of the Rev1 G193R mutant protein. (A) The Rev1 mutant protein (800 nM) was incubated with various concentrations of the DNA substrate [10 (●), 20 (○), 35 (▼), 50 (▽), 75 (■), and 100 nM (□)] and then mixed with dCTP (1 mM). The amount of nucleotide incorporation was plotted as a function of time, and the solid lines represent the best fits of the data to the burst equation. (B) The burst amplitudes were plotted as a function of DNA concentration, and the solid line represents the best fit of the data to the quadratic form of the binding equation. The  $K_{d-DNA}$  was equal to  $16 \pm 5$  nM, and the concentration of the active Rev1 mutant protein was equal to  $13 \pm 1$  nM.

°C. Protein crystals were flash-cooled in liquid nitrogen, and data were collected at 100 K on a Rigaku rotating anode X-ray source. The data were processed and scaled to a resolution of 1.96 Å using XDS and SCALA, respectively,<sup>43,44</sup> and the space group was determined to be  $P2_1$ . Molecular replacement was performed using the structure of the human Rev1 BRCT domain [Protein Data Bank (PDB) entry 2EBW] and PHASER.<sup>45</sup> Simulated annealing was conducted using PHENIX to remove any structural bias prior to any refinement.<sup>46</sup> Further refinement of the data was conducted using PHENIX, and model building was conducted using Coot.<sup>47</sup> The coordinates have been deposited in the PDB as entry 4ID3.

**Genetic Complementation Studies.** The full-length *REV1* gene with an additional 1000 bp of yeast genomic DNA containing the native *REV1* promoter was cloned into low-copy number *CEN/ARS LEU2* yeast shuttle vector pRS315 to yield pKW386. The QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) was used to introduce base substitutions into pKW386 to generate the following constructs that encode the Rev1 mutant proteins: pKW387 (N174A), pKW388 (R181A), pKW389 (K217A), pKW390 (R181A/K217A), pKW391 (L182A), pKW392 (E186A), pKW393 (L190A), pKW394 (K194A), pKW395 (F195A), pKW396 (G193V), pKW397 (G193D), pKW398 (G193R), and pKW399 (W231A).

To measure UV sensitivity, overnight cultures of the *rev1Δ* strain (Open Biosystems) harboring plasmids pKW386–pKW399 were washed and diluted in water and then plated on synthetic complete medium lacking leucine. The plated cells were exposed to various doses of UV radiation and incubated at 30 °C in the dark for 3–4 days. The percent of cell survival was obtained by comparing the number of colonies on the irradiated plates to the number of colonies on nonirradiated control plates. To measure UV-induced mutagenesis, cells were treated the same way except that they were placed on synthetic complete medium lacking leucine and arginine, but containing canavanine. The frequency of *CAN1<sup>S</sup>* to *can1<sup>r</sup>* forward mutations was obtained by comparing the number of colonies on irradiated plates lacking arginine and containing canavanine to those on irradiated plates containing arginine and lacking canavanine. All genetic complementation experiments were

performed at least four times to ensure reproducibility, and the data points shown are the averages of at least three independent experiments. The standard deviations for each of these data points are included in Tables S1 and S2 of the Supporting Information.

## RESULTS

**Impact of the Yeast Rev1 BRCT Domain on the Activity of the Polymerase Domain.** The BRCT domain of Rev1 plays an essential yet enigmatic role in mutagenic TLS. A single amino acid substitution within the Rev1 BRCT domain (G193R), which is encoded by the *rev1-1* allele, nearly eliminates UV-induced mutagenesis in yeast.<sup>34</sup> A previous study showed that the nucleotide incorporation activity of the full-length Rev1 G193R mutant protein is reduced by approximately 50% compared to that of the wild-type Rev1 protein.<sup>27</sup> This suggests that the BRCT domain may support the catalytic activity of the polymerase domain, perhaps through an intramolecular interaction. Thus, we have conducted pre-steady state kinetic analyses of the Rev1 G193R mutant protein to understand how disruptions in the BRCT domain impact the mechanism of nucleotide incorporation and DNA binding affinity.

In a previous study, we examined the kinetics of nucleotide incorporation for the wild-type Rev1 protein, and we found that the kinetics were biphasic.<sup>23</sup> This means that the nucleotide is incorporated faster in the first enzyme turnover than in subsequent turnovers. The Rev1 G193R mutant protein also displays biphasic (or burst) kinetics (Figure 1A). Because the amplitude of the burst phase is proportional to the amount of the active Rev1–DNA complex formed during the preincubation period, the apparent dissociation constant for the Rev1–DNA complex ( $K_{d-DNA}$ ) can be obtained by measuring the burst amplitudes at various DNA concentrations. Thus, we graphed the burst amplitude as a function of DNA concentration, and the best fit of the data yielded a  $K_{d-DNA}$  of  $16 \pm 5$  nM (Figure 1B). Thus, the affinity of the Rev1 G193R mutant protein for DNA does not differ significantly from that of the wild-type protein, which had a  $K_{d-DNA}$  of  $10 \pm 2$  nM.<sup>23</sup>

The observed rate of nucleotide incorporation in the first enzyme turnover ( $k_{obs}$ ) for the Rev1 G193R mutant protein



was  $10 \pm 4 \text{ s}^{-1}$ . This is very similar to the  $k_{\text{obs}}$  for the wild-type protein, which was  $16 \pm 1 \text{ s}^{-1}$ .<sup>23</sup> Thus, neither the DNA binding affinity nor the rate of the nucleotide incorporation step accounts for the lower catalytic activity observed with the Rev1 G193R mutant protein. Interestingly, the active site concentrations of the two proteins, which correspond to the fraction of Rev1 molecules capable of catalyzing nucleotide incorporation, were quite different. We previously showed that the wild-type Rev1 protein has an active site concentration that is approximately 7% of the total protein concentration, and we concluded that this is due to a high fraction of catalytically inactive Rev1 protein that does not bind the DNA substrate.<sup>23</sup> Here, we find that the Rev1 G193R mutant protein has an active site concentration that is approximately 1.6% of the total protein concentration (Figure 1B). This difference in active site concentrations does fully account for the previously reported differences in catalytic activity between the wild-type and mutant proteins. These results indicate that the G193R substitution does not affect DNA binding or nucleotide incorporation by the Rev1 polymerase domain but does appear to have a significant effect on the ability of the protein to maintain a stable, catalytically active conformation.

**Structure of the Yeast Rev1 BRCT Domain.** To gain insight into the function of the yeast Rev1 BRCT domain, we determined its X-ray crystal structure to a resolution of 1.96 Å (Table 1). There are two BRCT domain molecules per asymmetric unit. The buried solvent accessible surface area between these two molecules in the asymmetric unit and between these molecules and symmetry-related neighbors is less than 390 Å<sup>2</sup>, which suggests that the Rev1 BRCT domain is a monomer in the crystal. This is consistent with size exclusion chromatography results, which show that this domain is a

monomer in solution (data not shown). This domain (comprising residues 164–250) has the typical BRCT domain fold with a core of four parallel β-strands flanked by three α-helices (Figure 2). Overall, its structure is similar to the structures of other single-copy BRCT domains. Direct comparisons of the structure of the yeast Rev1 BRCT domain to the structures of other single-copy BRCT domains are shown in Figure S1 of the Supporting Information.

Consistent with previous work showing that the Rev1 BRCT domain preferentially interacts with phosphorylated peptides and the phosphate groups on DNA, we found that this domain has a conserved, canonical BRCT domain phosphate-binding pocket comprised of residues Asn-174, Gly-175, Arg-181, and Lys-217 (Figures 2B and 3A). The configuration of the phosphate-binding pocket is similar to that of the yeast RFC1 BRCT domain, which interacts with the phosphate groups on DNA.<sup>48,49</sup>

**Functional Significance of the Yeast Rev1 BRCT Domain Phosphate-Binding Pocket.** To assess the functional significance of the phosphate-binding pocket of the yeast Rev1 BRCT domain, we conducted genetic complementation studies. We examined whether full-length Rev1 mutant proteins with substitutions in the phosphate-binding pocket could complement the UV sensitivity of the *rev1Δ* strain. We tested four Rev1 mutant proteins: N174A, R181A, K217A, and the R181A/K217A double-mutant protein (Figure 2B). Residues corresponding to these are directly involved in phosphate binding in the BRCT domains of other proteins.<sup>38</sup> We found that all of the strains producing these Rev1 mutant proteins had nearly the same level of resistance to UV radiation as did the strain producing the wild-type protein (Figure 3B). All of these strains were more resistant than the negative control strain containing the empty vector, which did not produce Rev1.

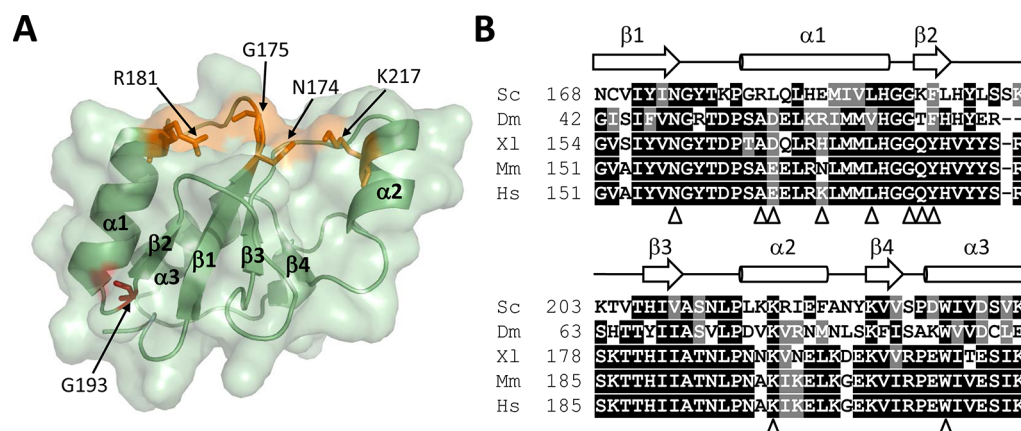
We next examined whether these Rev1 mutant proteins could complement the defect in UV-induced mutagenesis of the *rev1Δ* strain. All of the strains producing these Rev1 mutant proteins had nearly the same frequencies of *CAN1*<sup>S</sup> to *can1*<sup>r</sup> forward mutations as did the strain producing the wild-type protein (Figure 3C). By contrast, the frequency of UV-induced mutagenesis was reduced by nearly 95% in the empty vector control strain. Taken together, these data show that the phosphate-binding pocket of the Rev1 BRCT domain is dispensable for its function in TLS in vivo.

**The G193R Mutation Destabilizes the Yeast Rev1 BRCT Domain.** To better understand how the G193R substitution disrupts the function of the Rev1 BRCT domain, we introduced additional amino acid substitutions of and around Gly-193 and conducted genetic complementation studies. We reasoned that if Gly-193 is part of a protein–protein interaction surface, then amino acid substitutions near Gly-193, particularly along the solvent-exposed face of helix α1 and strand β2, should disrupt the function of the BRCT domain in a manner similar to that of the G193R substitution. The solvent-exposed residues on helix α1 have been shown to be involved in protein–protein interactions in the BRCT domains of several other proteins.<sup>50–52</sup> Thus, we examined five substitutions in this region of the protein: L182A, E186A, L190A, K194A, and F195A (Figures 2B and 4A). We found that all of the strains producing these Rev1 mutant proteins had sensitivity to UV radiation between that of the wild-type protein and that of the vector control (Figure 4B). All of these strains, however, had nearly the same frequencies of *CAN1*<sup>S</sup> to *can1*<sup>r</sup> forward mutations as did the strain producing the wild-

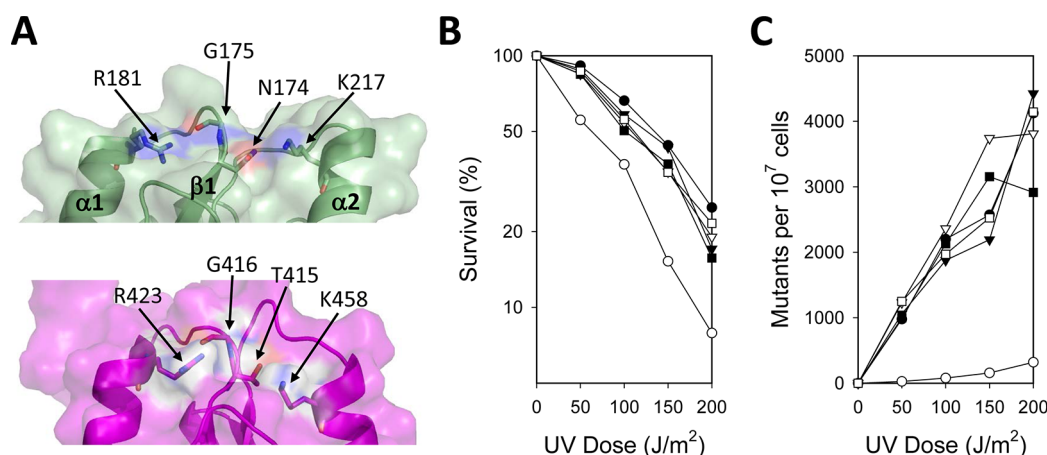
**Table 1. Data Collection and Refinement Statistics**

(A) Data Collection	
resolution (Å)	17.4–1.96
wavelength (Å)	1.54
space group	P2 <sub>1</sub>
cell dimensions	<i>a</i> = 32.0 Å, <i>b</i> = 82.5 Å, <i>c</i> = 32.7 Å, α = 90.0°, β = 91.5°, γ = 90.0°
completeness (%) <sup>a</sup>	94.1 (85.1)
redundancy <sup>a</sup>	2.8 (2.5)
⟨ <i>I</i> /σ( <i>I</i> )⟩ <sup>a</sup>	19.5 (4.7)
<i>R</i> <sub>merge</sub> (%) <sup>a</sup>	2.7 (25.5)
(B) Refinement	
resolution range (Å)	17.4–1.96
<i>R</i> (%)	19.08
<i>R</i> <sub>free</sub> (%)	23.32
rmsd for bonds (Å)	0.008
rmsd for angles (deg)	1.096
no. of protein atoms	1428 (186 amino acid residues)
no. of water molecules	120
average <i>B</i> factor (Å <sup>2</sup> )	
protein	35.7
solvent	39.4
Wilson <i>B</i> factor	30.4
Ramachandran analysis (%)	
most favored	97.65
allowed	2.35

<sup>a</sup>Values in parentheses are for the highest-resolution shell.



**Figure 2.** Structure of the yeast Rev1 BRCT domain. (A) The BRCT domain structure (green) is shown with the phosphate-binding pocket (orange) and G193 residue (red) highlighted. (B) Amino acid sequences of the Rev1 BRCT domain of *S. cerevisiae* (Sc), *Drosophila melanogaster* (Dm), *Xenopus laevis* (Xl), *Mus musculus* (Mm), and *Homo sapiens* (Hs) are shown. A black background indicates identical amino acid residues, and a gray background indicates similar amino acid residues. The secondary structural elements are indicated. Triangles denote the positions of amino acid substitutions used in the genetic complementation studies.



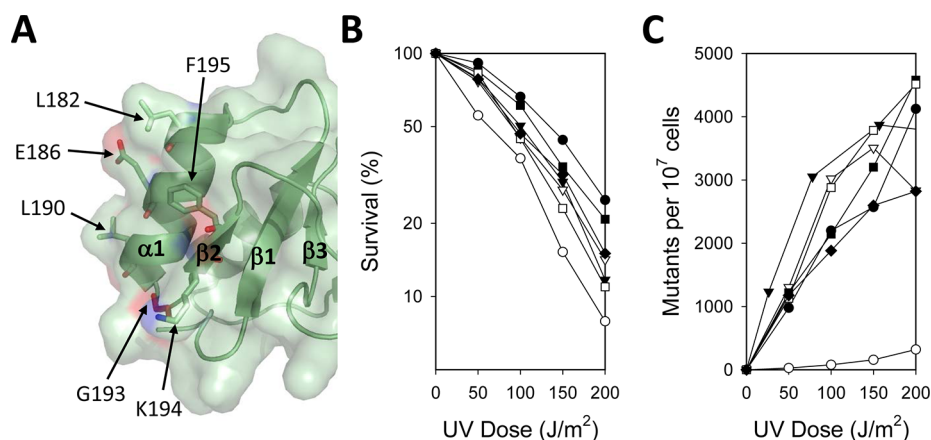
**Figure 3.** Genetic complementation studies of Rev1 mutant proteins with substitutions of residues in the phosphate-binding pocket. (A) The phosphate-binding pocket of the Rev1 BRCT domain (green) is shown in comparison to the RFC1 BRCT domain phosphate-binding pocket (PDB entry 2EBU, maroon). The locations of residues Asn-174, Gly-175, Arg-181, and Lys-217 in the Rev1 BRCT domain and Thr-415, Gly-416, Arg-423, and Lys-458 in the RFC1 BRCT domain are indicated. (B) UV sensitivity of *rev1Δ* yeast strains harboring plasmids producing either wild-type Rev1 (●), no Rev1 (○), the N174A mutant protein (▼), the R181A mutant protein (▽), the K217A mutant protein (■), or the R181A/K217A double-mutant protein (□). The percentage of cells surviving each UV dose is graphed. (C) UV-induced mutagenesis of these same yeast strains. The number of *CAN1*<sup>S</sup> to *can1*<sup>r</sup> forward mutations per 10<sup>7</sup> surviving cells for each UV dose is graphed.

type protein (Figure 4C). This suggests that these residues of the Rev1 BRCT domain are not necessary for its function in mutagenic TLS in vivo.

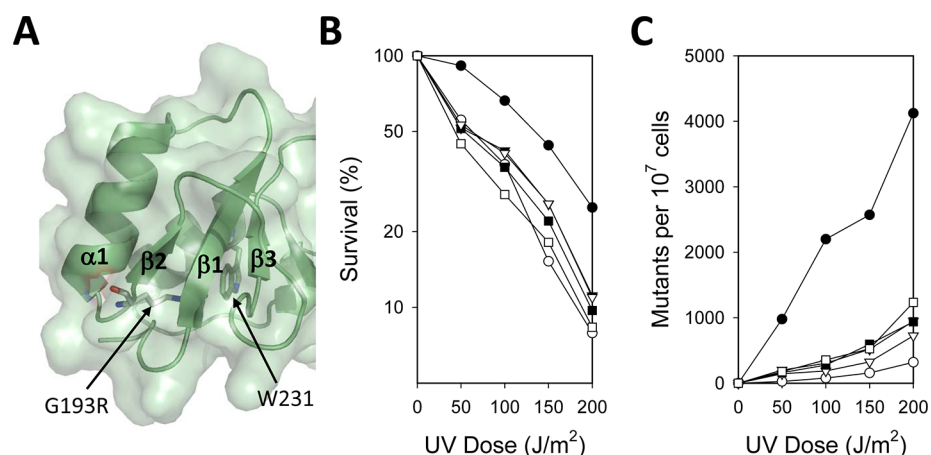
Analysis of the structure showed that, assuming no alterations to the position of the backbone, the substituted arginine side chain at position 193 would project directly into the hydrophobic core of the domain leading to serious steric clashes (Figure 5A). This raised the possibility that the G193R substitution destabilized the fold of the BRCT domain. To test this idea, we examined two additional substitutions of Gly-193: G193V and G193D. We also examined a substitution in a nearby conserved tryptophan residue (W231A); that substitution destabilizes the fold of BRCT domains in other systems.<sup>53,54</sup> Trp-231 is located on helix  $\alpha$ 3 and points into the hydrophobic core of the domain (Figure 5A). All of the strains producing these Rev1 mutant proteins had sensitivities to UV radiation similar to that of the strain producing the G193R mutant protein and the vector control strain (Figure 5B). Similarly, all of these strains had nearly the same defect in UV-

induced mutagenesis as the strain producing the G193R mutant protein and the vector control strain (Figure 5C). Thus, these substitutions prevent Rev1 from functioning in mutagenic TLS in vivo.

To test whether these amino acid substitutions destabilized the fold of the yeast Rev1 BRCT domain, we attempted to express and purify the G193R, G193V, G193D, and W231A mutant BRCT domains for in vitro studies. However, we were unable to do so. These mutant BRCT domains were overexpressed at greatly reduced levels as chitin-binding domain fusion proteins. These fusion proteins bound the chitin column, but we were unable to elute any significant amount of soluble mutant BRCT domains upon cleavage of the fusion protein bound to the column. The small amount of soluble BRCT domain that was released from the column was aggregated and eluted in the void volume during size exclusion chromatography. Taken together, these results strongly suggested that all of these substitutions destabilize the fold of the BRCT domain.



**Figure 4.** Genetic complementation studies of Rev1 mutant proteins with substitutions of residues near Gly-193. (A) Close-up view of the location of Gly-193. The locations of residues Leu-182, Glu-186, Leu-190, Gly-193, Lys-194, and Phe-195 are indicated. (B) UV sensitivity of *rev1Δ* yeast strains harboring plasmids producing either wild-type Rev1 (●), no Rev1 (○), the L182A mutant protein (▼), the E186A mutant protein (▽), the L190A mutant protein (■), the K194A mutant protein (□), or the F195A mutant protein (◆). The percentage of cells surviving each UV dose is graphed. (C) UV-induced mutagenesis of these same yeast strains. The number of *CAN1<sup>S</sup>* to *can1<sup>r</sup>* forward mutations per 10<sup>7</sup> surviving cells for each UV dose is graphed.



**Figure 5.** Genetic complementation studies of Rev1 mutant proteins with substitutions of Gly-193 or the W231A substitution. (A) Close-up view of the location of Gly-193. The location of Trp-231 is indicated. (B) UV sensitivity of *rev1Δ* yeast strains harboring plasmids producing either wild-type Rev1 (●), no Rev1 (○), the G193V mutant protein (▼), the G193D mutant protein (▽), the G193R mutant protein (■), or the W231A mutant protein (□). The percentage of cells surviving each UV dose is graphed. (C) UV-induced mutagenesis of these same yeast strains. The number of *CAN1<sup>S</sup>* to *can1<sup>r</sup>* forward mutations per 10<sup>7</sup> surviving cells for each UV dose is graphed.

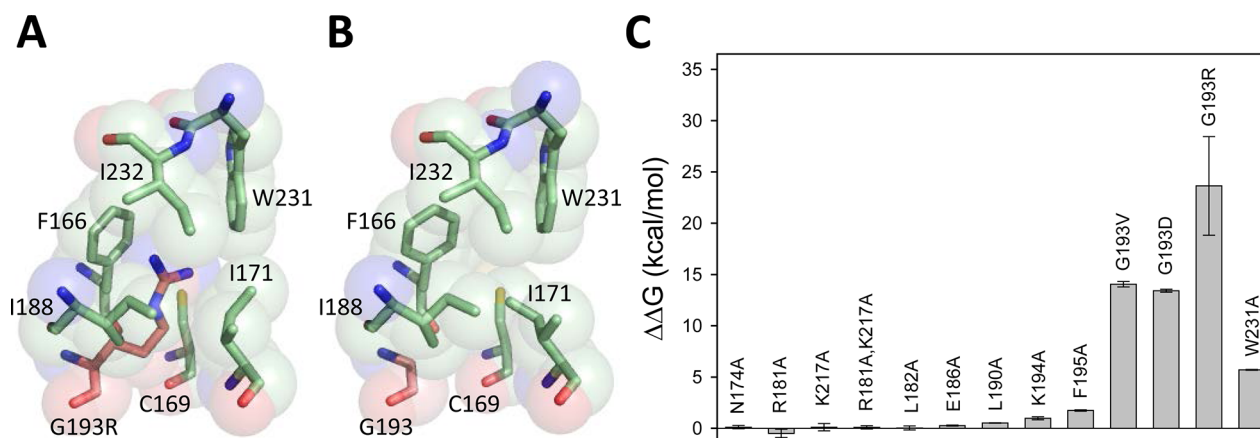
Because we were unable to express and purify these mutant BRCT domains, we conducted molecular modeling. To predict the changes in the stability of the BRCT domain resulting from each of these amino acid substitutions, we used the computer algorithm FoldX.<sup>55</sup> We calculated the  $\Delta\Delta G$  of unfolding of the mutant BRCT domain versus the wild-type BRCT domain for all of the mutant proteins examined in this study (Figure 6). As is commonly known, the absolute energies calculated by FoldX are not precise, but the relative energies are quite reliable. We found that the G193V, G193D, G193R, and W231A mutant Rev1 BRCT domains were all substantially less stable than the wild-type Rev1 BRCT domain. All of the other mutant Rev1 BRCT domains that we examined were nearly as stable as the wild-type domain. In fact, there is a direct correlation between the calculated stability of the Rev1 BRCT domain and the in vivo function of the mutant Rev1 proteins. Thus, these molecular modeling studies combined with the experimental work described above clearly show that the lack of function of

these mutant Rev1 proteins results from the destabilization of the Rev1 BRCT domain.

## DISCUSSION

The yeast Rev1 protein has three structural elements flanked by regions that are likely intrinsically disordered. These are an N-terminal region that contains a BRCT domain, the catalytic core that is comprised of a polymerase domain and a polymerase-associated domain (PAD), and the C-terminal region that contains two ubiquitin-binding motifs (UBMs) and a C-terminal domain (CTD). The X-ray crystal structure of the catalytic core has been previously determined, and this structure shows that this domain catalyzes nucleotide incorporation utilizing a remarkable protein template-directed mechanism of incoming nucleotide selection.<sup>42,56,57</sup> The NMR structure of a close homologue of the two UBM motifs has been determined, and its binding site on ubiquitin has been mapped.<sup>58</sup> These motifs are thought to mediate interactions of Rev1 and ubiquitin-modified PCNA. Recently, the X-ray crystal





**Figure 6.** Molecular modeling of the mutant Rev1 BRCT domains. (A) Model of hydrophobic core of the Rev1 G193R mutant BRCT domain generated by FoldX. The side chain of Arg-193 (red) projects into the core of the domain and contacts various hydrophobic residues (green). (B) Model of the hydrophobic core of the wild-type Rev1 BRCT domain generated by FoldX. (C) Plot of  $\Delta\Delta G$  of unfolding of the mutant Rev1 BRCT domains vs the wild-type Rev1 BRCT domain. These values represent the average and standard deviation of five independent calculations using FoldX.

structure of the Rev1 CTD bound to the Rev7 subunit of Pol  $\xi$  has been determined.<sup>32,33</sup> This domain is thought to allow Rev1 to function as a scaffold to bind other TLS polymerases.<sup>28,30,59</sup> The X-ray crystal structure of the Rev1 BRCT domain reported here now completes the set of structures for all the folded domains of the Rev1 protein. Although the Rev1 BRCT domain plays a central role in mutagenic TLS, its function is unknown.

The structure of the yeast Rev1 BRCT domain has guided our structure–function studies of this domain. This has allowed us to test several recent models regarding the role of the BRCT domain. First, it was previously shown that the yeast Rev1 BRCT domain binds phosphorylated peptides *in vitro* with higher affinity than it binds nonphosphorylated peptides.<sup>39</sup> This led to the model in which the Rev1 BRCT domain interacts with a phosphorylated binding partner. Our genetic complementation studies with yeast Rev1 mutant proteins with substitutions in the conserved phosphate-binding pocket show that any such interactions are not necessary for the *in vivo* function of Rev1. Thus, interactions with a phosphorylated binding partner are unlikely to be the principal function of this domain in mutagenic TLS. Second, previous studies showed that the yeast and mouse Rev1 BRCT domains bind DNA *in vitro* by interacting with recessed 5′-phosphates at primer–template junctions.<sup>40</sup> This led to the model in which Rev1 is recruited to stalled replication forks via the BRCT domain interacting with downstream 5′ primer–template junctions. Again, our genetic complementation results with yeast Rev1 mutant proteins with substitutions in the phosphate-binding pocket argue that this interaction is unlikely to be the principal function of this domain in TLS. Third, we considered additional models in which the BRCT domain supports the function of other domains in the Rev1 protein, such as the catalytic domain or the CTD, via intramolecular interactions. With respect to the catalytic domain, our pre-steady state kinetic studies of the Rev1 G193R mutant protein show that disrupting the BRCT domain does not affect DNA binding or nucleotide incorporation by Rev1. This suggests that the BRCT domain does not support the function of the catalytic domain, although it does have some effect on the ability of Rev1 to maintain a stable, catalytically active conformation. In any event, mutagenic TLS does not require the catalytic activity of

Rev1. Thus, supporting the function of the catalytic domain is not the principal function of the BRCT domain. With respect to the CTD, a previous study showed that the Rev1 CTD is sufficient for interacting with the Rev7 subunit of Pol  $\xi$ , another TLS polymerase.<sup>28</sup> This implies that supporting the function of the CTD is not the principal function of this domain.

The *rev1-1*-encoded G193R protein is defective in mutagenic TLS. Our structural and functional analyses of the BRCT domain strongly suggest that this substitution leads to a destabilization of the domain, rendering the Rev1 protein nonfunctional *in vivo*. If one were to fix the position of the backbone atoms around Gly-193, it would not be possible to substitute any other amino acid residue for this glycine, because the side chain would be protruding into the hydrophobic core of this domain making severe steric clashes (Figures 5A and 6A). Thus, in the case of the G193R, G193V, and G193D substitutions, the protein backbone in this region would have to reorient significantly, and we believe that this destabilizes the BRCT domain. Support for this notion comes from three observations. First, while it is possible to obtain full-length Rev1 with the G193R substitution, we were unable to obtain isolated Rev1 BRCT domains with the G193R, G193V, and G193D substitutions because of the lower levels of expression and problems with protein solubility and aggregation. These problems were not observed in the case of the wild-type Rev1 BRCT domain. Second, we made a Rev1 mutant protein with a W231A substitution, which we chose because it was expected to destabilize the BRCT domain. Substitutions of this conserved tryptophan destabilize the BRCT domains in other proteins.<sup>53,54</sup> This substitution significantly impaired the ability of Rev1 to support mutagenic TLS. Moreover, we were unable to isolate the Rev1 BRCT domain with the W231A substitution because of problems with protein solubility and aggregation. Third, molecular modeling showed that G193V, G193D, G193R, and W231A mutant Rev1 BRCT domains were substantially less stable than the wild-type Rev1 BRCT domain. Taken together, our results show that all of these amino acid substitutions destabilize the Rev1 BRCT domain and only these substitutions impaired the ability of Rev1 to support mutagenic TLS. Thus, we conclude that destabilization of the BRCT domain by these substitutions is what disrupts Rev1 function *in vivo*. It should be noted that in the case of the G193R

substitution, the expression levels of Rev1 mutant protein in cells are not affected.<sup>60</sup>

So why is the BRCT domain of Rev1 required for mutagenic TLS? It is likely that this domain interacts with another protein in a manner that is not dependent on this binding partner's phosphorylation state. The identity of this binding partner is unknown, but there are some interesting candidates suggested by the literature. For example, a previous study showed that when the BRCT domain of Dbf4 (Dumbbell forming 4), the regulatory subunit of the Cdc7 checkpoint kinase, was replaced by other BRCT domains, only the Rev1 BRCT domain was able to fully substitute with respect to function in vivo.<sup>61</sup> These data suggest that the Dbf4 BRCT domain and the Rev1 BRCT domain share a common binding partner. The binding of Dbf4 to Rad53 is well-characterized, suggesting Rad53 may be the critical interacting partner of the Rev1 BRCT domain.<sup>62</sup> Incidentally, this may explain why RAD53 influences the formation of nuclear foci of Rev1.<sup>63</sup> Another possible binding partner is PCNA, a key replication accessory factor. Using a pull-down assay, one group showed that the BRCT domain of both yeast and mouse Rev1 interacts with PCNA, and the G193R mutation in the yeast Rev1 BRCT domain blocks this interaction.<sup>64</sup> They also demonstrated that the extent of localization of Rev1 to DNA replication foci was reduced in cells expressing Rev1 mutant proteins with a BRCT domain deletion or the G193R mutation. However, these results remain controversial as other groups have been unable to detect direct interactions between the Rev1 BRCT domain and PCNA.<sup>40,65</sup> Moreover, PCNA stimulates the catalytic activity of the Rev1 G193R mutant protein, showing that this functional interaction remains intact even when the BRCT domain is disrupted.<sup>66</sup> In any event, more work will be required to identify the Rev1 BRCT domain binding partner.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

One figure showing the comparison of the yeast Rev1 BRCT domain to other BRCT domains (Figure S1), a table providing the means and standard deviations of the UV survival experiments (Table S1), and a table providing the means and standard deviations of the UV mutagenesis experiments (Table S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Funding

This work was supported by Grant GM081433 from the National Institute of General Medical Sciences to M.T.W.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank Christine Kondratik, Lynne Dieckman, Elizabeth Boehm, and Bret Freudenthal for valuable discussions.

## ■ ABBREVIATIONS

BRCT, BRCA1 C-terminal; CTD, C-terminal domain; dNTP, deoxynucleoside triphosphates; DTT, dithiothreitol; PAD, polymerase-associated domain; PCNA, proliferating cell nuclear antigen; Pol, polymerase; TLS, translesion synthesis; UBM, ubiquitin-binding motif; UV, ultraviolet.

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