Y-family DNA polymerases, such as polymerase $\eta$, polymerase $\iota$, and polymerase $\kappa$, catalyze the bypass of DNA damage during translesion synthesis. These enzymes are recruited to sites of DNA damage by interacting with the essential replication accessory protein proliferating cell nuclear antigen (PCNA) and the scaffold protein Rev1. In most Y-family polymerases, these interactions are mediated by one or more conserved PCNA-interacting protein (PIP) motifs that bind in a hydrophobic pocket on the front side of PCNA as well as by conserved Rev1-interacting region (RIR) motifs that bind in a hydrophobic pocket on the C-terminal domain of Rev1. Yeast polymerase $\eta$, a prototypical translesion synthesis polymerase, binds both PCNA and Rev1. It possesses a single PIP motif but not an RIR motif. Here we show that the PIP motif of yeast polymerase $\eta$ mediates its interactions both with PCNA and with Rev1. Moreover, the PIP motif of polymerase $\eta$ binds in the hydrophobic pocket on the Rev1 C-terminal domain. We also show that the RIR motif of human polymerase $\kappa$ and the PIP motif of yeast Msh6 bind both PCNA and Rev1. Overall, these findings demonstrate that PIP motifs and RIR motifs have overlapping specificities and can interact with both PCNA and Rev1 in structurally similar ways. These findings also suggest that PIP motifs are a more versatile protein interaction motif than previously believed.

Proliferating cell nuclear antigen (PCNA) is an essential protein that functions in DNA replication, repair, recombination, damage tolerance, and cell cycle control. It is a homotrimer that forms a ring-shaped clamp, which slides along DNA (1–3). Many proteins involved in DNA metabolism, the maintenance of genome stability, and cell cycle control possess PCNA-interacting protein (PIP) motifs that mediate their binding to PCNA (1, 4–6). These PIP motifs contain two adjacent aromatic residues, which bind in a hydrophobic pocket on the front face of the PCNA ring (2, 4), and the binding of these PIP motifs to PCNA represents a major point of regulation for these proteins.

Among the proteins that are regulated by PCNA are the Y-family DNA polymerases such as DNA polymerase $\eta$ (pol $\eta$), DNA polymerase $\iota$ (pol $\iota$), DNA polymerase $\kappa$ (pol $\kappa$), and Rev1 (7–12). These polymerases bypass DNA damage during translesion DNA synthesis in order to allow for continuous progression of the replication fork (13–16). Pol $\eta$, for example, catalyzes the bypass of UV-induced thymine-thymine dimers and 8-oxoguanine lesions (17, 18). Defects in pol $\eta$ cause the variant form of xeroderma pigmentosum, a genetic disorder characterized by sensitivity to sunlight and a high incidence of skin cancers (19, 20). Rev1, by contrast, bypasses damaged guanines and abasic sites (21–24).

In addition to its catalytic function, Rev1 plays an important non-catalytic role in translesion synthesis by binding to other polymerases, like pol $\eta$, pol $\kappa$, and pol $\iota$, and acting as a scaffold to recruit them to PCNA (25–29). Many Y-family polymerases, such as mammalian pol $\eta$, pol $\kappa$, and pol $\iota$, possess short, conserved Rev1-interacting region (RIR) motifs that contain two adjacent phenylalanine residues, which mediate their interaction with the C-terminal domain (CTD) of Rev1 (30–33). This interaction is essential for the function of pol $\kappa$ in mouse embryonic fibroblasts (30).

Although yeast pol $\eta$ and Rev1 are known to interact (29), there is no evidence that yeast pol $\eta$ contains an RIR motif or that this interaction involves the Rev1 CTD. Examining the yeast pol $\eta$ amino acid sequence revealed that the only candidate for an RIR motif was its PIP motif. We show here that the PIP motif of pol $\eta$ interacts with the Rev1 CTD. This was surprising because until now the sole purpose of PIP motifs has been thought to be binding PCNA. These results raised the intriguing possibility, which we also demonstrate here, that PIP and RIR motifs of other proteins interact with both PCNA and the CTD of Rev1. This shows that PIP motifs and RIR motifs have overlapping specificities and can interact with both PCNA and Rev1 in structurally similar ways. These findings also suggest that PIP motifs are a more versatile protein interaction motif than previously believed.
yeast strain BJ5464 harboring plasmids pKW546 and pKW560, respectively. These proteins both contained an Avi tag (GNLDIFEAQKIEWHE), which was inserted between the PreScission protease cleavage site (used to remove the GST tag) and the N terminus of pol η. Pol η was co-expressed with the Escherichia coli BirA biotin ligase from plasmid pKW547, which resulted in site-specific biotinylation of the Avi tag, when the cells were grown in the presence of 0.1 mM biotin. Otherwise, these proteins were expressed and purified as described previously (34). Wild-type yeast Rev1 and a mutant form with substitutions in the CTD (L889A/W893A/T897A/L898A/V910A) were expressed in yeast strain BJ5464 harboring plasmids pKW143 and pKW570, respectively, and purified as described previously (35).

The GST-Msh6 PIP motif fusion proteins were expressed in bacteria harboring plasmids pKW483. This fusion protein contained residues 21–40 of yeast Msh6 fused to the C terminus of GST using the Spel and HindIII sites in pET41a (Novagen). This protein was purified using glutathione-Sepharose (GE Healthcare) and eluted with 10 mM glutathione.

Wild-type yeast and human PCNA were overexpressed as N-terminally His6-tagged proteins in bacteria harboring plasmid pKW336 or pKW303, respectively. These proteins were purified as described previously (36). The isolated yeast Rev1 CTD (residues 875–985) was expressed in bacteria as a maltose-binding protein fusion. The codon-optimized gene encoding the yeast Rev1 CTD was inserted into pMAL-c5x vector (New England Biolabs) to form plasmid pKW540. Cells were lysed using an EmulsiFlex C3 homogenizer, and the lysate was spun at 12,000 rpm for 45 min. MBP-Rev1 CTD was purified using an amylose affinity column (New England Biolabs), followed by anion exchange (DEAE Sepharose Fast Flow, GE Healthcare) and size exclusion chromatography (HiLoad 16/600 Superdex 75, GE Healthcare). The plasmid for overexpressing the isolated human CTD was kindly provided by Dr. Dmitry Korzhnev (University of Connecticut).

**Protein Binding Assay**—The high-throughput protein-binding assay with an ELISA-based detection was carried out as described previously (37, 38). Each data point represents the average of three independent experiments. Parallel control reactions using bovine serum albumin (BSA) instead of Rev1 or PCNA were performed, and these absorbance values were subtracted from the absorbance of each sample.

**Single-molecule Imaging**—Purified yeast PCNA and Rev1 were dialyzed into buffer containing 50 mM potassium phosphate, pH 7.05, 100 mM sodium chloride, and 1 mM dithiothreitol. Protein solutions (5–15 μM) were then incubated with 1 mg of Cy3 maleimide (GE Healthcare) dissolved in 10 mM HEPES, pH 7.5, incubated at room temperature under fluorescent light with continuous rotation for 3–4 days. This reaction generates an oxido-redox mixture of Trolox (43).

**Analysis of Single-molecule Data**—The single-molecule fluorescence trajectories were extracted from recorded videos and visualized as described previously (40, 41). Trajectories were selected for further analysis if they showed a stable average signal intensity over time (44). After selection, trajectories were normalized, and binding events were identified using QuB (45). QuB output files were used for further analysis.

For each Cy3-PCNA concentration, 700–5,000 binding events were extracted from 150–275 trajectories and were analyzed collectively. The dwell times of these events were plotted in histograms with a bin size of 2.5 s and fit with single-exponential decay functions in Sigmaplot. For each Cy3-Rev1 concentration, 500–5,000 binding events were extracted from 200–350 trajectories and were analyzed collectively. The dwell times were plotted in histograms with a bin size of 5 s and fit with single-exponential decay functions in Sigmaplot, except in the case of 100 pm Rev1, which had fewer data points and was fit to a double-exponential decay function. The exponential decay functions from these dwell time histograms were then used to obtain the apparent dissociation rate constants $k_{off}$ (s$^{-1}$). The off times between subsequent binding events in a single trace were also plotted in histograms and fit with single-exponential decay functions in Sigmaplot. The apparent on rates from these histograms were then plotted versus either PCNA or Rev1 concentration to obtain the apparent association rate constants, $k_{on}$ (M$^{-1}$ s$^{-1}$). The dissociation constant, $K_D$, was then calculated as $k_{off}/k_{on}$.

**Isothermal Titration Calorimetry (ITC)**—The yeast pol η PIP peptide corresponding to residues 620–632 was purchased from GenScript. The isolated yeast Rev1 CTD or PCNA protein and the peptide were dialyzed overnight in 1 liter of 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, 1.8 mM potassium phosphate, at pH 7.4 at 4 °C. Final protein concentrations were adjusted to 8 μM for the Rev1 CTD.
or 30 µM for PCNA and 80 or 300 µM for the pol η PIP peptide. Protein samples were degassed, and ITC was performed at 25 °C using a MicroCal VP-ITC calorimeter. The Rev1 CTD or PCNA was in the sample cell, and the pol η PIP peptide was in the syringe. Twenty-one injections of the peptide were done at intervals of 180 s. Control experiments where the peptide was injected into buffer showed that the heats of dilution were constant across all injections. The heat of dilution was determined from the final 3–5 injections and was subtracted from the data, which were analyzed using a single-site binding model provided in the ITC analysis software package. To ensure reproducibility, three independent experiments from two different preparations of PCNA and two independent experiments from two different preparations of the isolated Rev1 CTD were carried out, and the $K_d$ stoichiometry, enthalpy change, and entropy change values were in close agreement.

Similar ITC experiments were carried out using human PCNA (60 µM in the sample cell) and the isolated human Rev1 CTD (60 µM in the sample cell) and peptides containing the PIP motif or the RIR motif from human DNA polymerases η, δ, and κ (600 µM in the injection syringe). The sequences of these peptides are given in Table 1.

Co-immunoprecipitation Experiments—Rev1 containing a N-terminal FLAG tag was expressed from its native promoter in the pRS314 yeast shuttle vector (ATCC). Pol η and Msh6 containing N-terminal His$_6$ tags were expressed from their native promoters in the pTB364 yeast shuttle vector. Cells expressing FLAG-Rev1 and His$_6$-pol η and cells expressing FLAG-Rev1 and His$_6$-Msh6 were grown to an OD$_{600}$ of 0.8–1.0 before being lysed in Y-PER (Thermo Scientific). A 1:50 dilution of rabbit α-FLAG antibody (Cell Signaling Technologies, catalog no. 23685, lot 6) diluted in Y-PER was used to immunoprecipitate the FLAG-Rev1, and a 1:25 dilution of rabbit α-His tag antibody (Cell Signaling Technologies, catalog no. 23655, lot 3) diluted in Y-PER was used to immunoprecipitate the His$_6$-pol η and the His$_6$-Msh6. Protein A beads (Sigma) were then used to recover the FLAG-Rev1-containing protein complexes. Equal amounts of protein were loaded in each lane of a 7.5% SDS-polyacrylamide gel before being transferred to a nitrocellulose membrane. FLAG-Rev1 was visualized first with Rabbit α-FLAG antibody at 1:1000 dilution in 5% BSA and 1× TBST followed by a 1:1,000 dilution of α-rabbit IgG-HRP (Cell Signaling Technologies, catalog no. 70745, lot 25) in 5% BSA and 1× TBST. His$_6$-pol η and His$_6$-Msh6 were visualized with HRP-α-His (Abcam, catalog no. GR163369-5, lot ab1187) at 1:1,000 in 5% BSA and 1× TBST. The mutant protein controls included the pol η PIP mutant (S621A/F627A/F628A), the Msh6 PIP mutant (F33A/F34A), and the Rev1 CTD mutant (G193R/L889A/W893A/T897A/L898A/V910A). To ensure reproducibility, three replicates of the Rev1-pol η co-immunoprecipitation were performed, and two replicates of the Rev1-Msh6 co-immunoprecipitation were performed.

Structural Modeling—Homology models were constructed using the SWISS-MODEL protein structure homology-modeling server (46–49) using the primary amino acid sequence of yeast Rev1 CTD spanning residues 859–985. Because several potential models are output by this web server, selection of the best output was based upon the sequence identity and homology context. The 2LSK.pdb-based homology model was selected based on the greatest sequence homology score (0.29). It was also selected due to this structure being a complex of the human Rev1 CTD and the RIR of human pol η (31), making it a suitable structure to approximate a similar interaction in the yeast system. The phenylalanine-phenylalanine dipetide was modeled into a putative binding pocket using VMD (Visual Molecular Dynamics) software.

**Results**

The interactions of many eukaryotic Y-family DNA polymerases with Rev1 are mediated by RIR motifs, which are short sequence elements that contain two adjacent phenylalanine residues. These RIR motifs bind in a hydrophobic pocket on the CTD of Rev1 (30–33). Yeast pol η and Rev1 are known to interact (29), but there is no evidence that pol η contains an RIR motif or that this interaction involves the Rev1 CTD. We examined the yeast pol η amino acid sequence and found that the only candidate for an RIR motif was the single PIP motif at its extreme C terminus (residues 621–628). We therefore tested the importance of the PIP of yeast pol η for binding PCNA and Rev1.

**The Pol η PIP Motif Binds PCNA and the Rev1 CTD**—We used a high throughput protein-binding assay to examine the binding of yeast pol η to PCNA and to Rev1. First, PCNA was immobilized in the wells of a 96-well plate (2 µg/well), and various amounts of wild type pol η (1–20 µg) were added. Unbound pol η was washed away, and bound pol η was observed via ELISA detection. Wild type pol η and PCNA interact strongly (Fig. 1A). Next, we carried out the experiment using a mutant form of pol η with three amino acid substitutions in the PIP motif (S621A, F627A, and F628A). The mutant form of pol η interacts with PCNA with much lower affinity (Fig. 1A). This was not surprising because the PIP motif has been shown previously to mediate the interaction of pol η with PCNA (7).

Using this same protein-binding assay, we showed that wild type pol η binds Rev1 (Fig. 1B). Using the same mutant pol η protein, we showed, quite unexpectedly, that the PIP motif of pol η is necessary for binding to Rev1 (Fig. 1B). This suggested that the PIP motif of pol η might also function as an RIR motif. If so, this would mean that the two conserved phenylalanine residues of the yeast pol η PIP motif are contacting the hydrophobic cleft on the CTD of yeast Rev1. To test this, we created a structural model of the yeast Rev1 CTD (Fig. 1C) and identified residues that could be involved in binding the pol η PIP motif. We then showed that substitutions in the CTD of yeast Rev1 (L889A, W893A, T897A, L898A, and V910A) blocked its interaction with pol η (Fig. 1B). This shows that the pol η PIP motif binds the CTD of yeast Rev1 at the same site where RIR motifs of other Y-family polymerases bind the Rev1 CTD.

To determine whether the pol η PIP motif binds the CTD of Rev1 in vivo, we carried out co-immunoprecipitation experiments from yeast cells (Fig. 1D). We observed the formation of a pol η-Rev1 complex that is disrupted by either substitutions in the PIP motif of pol η or substitutions in the CTD of Rev1.

Pol η Binding to PCNA and Rev1 Is Characterized by Similar Kinetics and Affinity—The binding kinetics of yeast pol η with PCNA and Rev1 was examined quantitatively using single mol-
The PIP Motif of DNA Polymerase $\eta$ Interacts with Rev1

**A** Yeast PCNA was immobilized, and various concentrations of either the wild type yeast pol $\eta$ protein (closed circles) or the PIP mutant (S621A/F627A/F628A) yeast pol $\eta$ protein (open circles) were added. After washing, retention of pol $\eta$ was determined by ELISA. B, wild type yeast Rev1 protein was immobilized, and various concentrations of either wild type pol $\eta$ (closed circles) or the PIP mutant pol $\eta$ (open circles) were added. The CTD mutant (L889A/W893A/T897A/L898A/V910A) Rev1 protein was immobilized, and various concentrations of wild type pol $\eta$ (closed triangles) were added. After washing, retention of pol $\eta$ was determined by ELISA. C, homology model of the yeast Rev1 CTD bound to the pol $\eta$ PIP motif shown as a schematic representation. The phenylalanine side chains of the PIP motif are shown in green, and the residues of the CTD involved in binding the PIP motif are shown in red. D, co-immunoprecipitation (IP) of yeast Rev1 (middle) and pol $\eta$ (bottom). Lanes 1–3 correspond to cells expressing wild type Rev1 and wild type pol $\eta$, the Rev1 CTD mutant and wild type pol $\eta$, and wild type Rev1 and the pol $\eta$ PIP mutant, respectively. A control demonstrating the production of wild type and mutant pol $\eta$ is shown (top).

In the case of PCNA, the $k_{off}$ was $1.0 \times 10^{8}$ M$^{-1}$ s$^{-1}$. This yielded a $K_d$ for the pol $\eta$-PCNA of 4.6 nm.

Next, pol $\eta$ was immobilized on the microscope slide, and various concentrations of Cy3-labeled Rev1 (100–700 pm) were added to the slide chamber (Fig. 3A). Increases of Cy3 fluorescence at particular positions on the slide represent the binding of PCNA to single pol $\eta$ molecules immobilized at those positions. Fluorescence trajectories were obtained that report the Cy3 fluorescence intensity at a specific position of an immobilized pol $\eta$ molecule as a function of time. A representative trajectory for PCNA binding is shown in Fig. 2B. Regions of increased fluorescence represent times when the immobilized pol $\eta$ is bound to PCNA; regions of decreased fluorescence represent times when the pol $\eta$ is free.

Analysis of the duration of the PCNA binding events (i.e. the dwell times) allows one to determine the $k_{off}$ for the pol $\eta$-PCNA complex. Histograms depicting the number of events as a function of their dwell time were fit to single exponential decay functions with rate constants equal to $k_{off}$ (Fig. 2C). The $k_{off}$ had an average value of 0.46 s$^{-1}$, and it did not vary with PCNA concentration. Similarly, analysis of the duration between PCNA binding events (i.e. the off times) allows one to obtain the $k_{on}$ for this complex. Histograms depicting the number of events as a function of their off time were fit to single exponential decay functions with apparent rates, $v_{on}$ (Fig. 2D). The $k_{on}$ was obtained from the best-fit line when the apparent $v_{on}$ was plotted as a function of PCNA concentration (Fig. 2E).

Control experiments were performed in which the mutant form of pol $\eta$ with substitutions in the PIP motif was immobilized on the microscope slide. In these experiments, we saw very few binding events with either PCNA or Rev1 showing that the binding affinity of the mutant protein for either PCNA or Rev1 was too weak to measure and further confirming that both interactions involve the pol $\eta$ PIP motif.

**Pol $\eta$ Binding to PCNA and Rev1 Is Largely Entropically Driven**—The binding of the yeast pol $\eta$ PIP motif with PCNA and the Rev1 CTD was examined quantitatively using ITC. First, PCNA (3 $\mu$M) was placed in the sample cell of the calorimeter, and the isolated pol $\eta$ PIP motif (residues 620–632) was injected. The $\Delta H$ was $-0.34$ kcal/mol, the $T\Delta S$ was 7.6 kcal/mol, and the $K_d$ for the complex of PCNA and the PIP
The PIP Motif of DNA Polymerase \( \eta \) Interacts with Rev1

The PIP Motif of DNA Polymerase \( \eta \) Interacts with Rev1

peptide was 1.6 \( \text{\textmu M} \) (Fig. 4A). Next, the isolated Rev1 CTD (residues 875–985; 2 \( \text{\textmu M} \)) was placed in the sample cell, and the pol \( \eta \) PIP motif was injected. The \( \Delta H \) was \( -1.6 \text{ kcal/mol} \), the \( T \Delta S \) was 8.4 kcal/mol, and the \( K_d \) for the complex of the Rev1 CTD and the PIP peptide was 48 nM (Fig. 4B). Thus, the isolated Rev1 CTD binds the pol \( \eta \) PIP peptide with 30-fold higher affinity than PCNA binds the pol \( \eta \) PIP peptide. These data show that in both of these cases, the binding is mostly driven by entropy changes, which is consistent with these interactions being largely hydrophobic in nature.

**PIP Motifs and RIR Motifs from Human Y-family Polymerases Have Different Specificities**—To determine whether the ability of PIP motifs to bind the CTD of Rev1 is evolutionarily conserved among Y-family polymerases, we carried out quantitative binding studies using PIP motifs of human pol \( \eta \), pol \( \kappa \), and pol \( \iota \) (Table 1). Using ITC, we show that the PIP motifs of human pol \( \eta \) and pol \( \iota \) bind PCNA with affinities in the low micromolar range. We do not detect binding for the pol \( \kappa \) PIP motif, which is consistent with previous reports (50). In these three cases, we do not detect binding with the CTD of human Rev1. It should be noted that this apparent lack of binding could be due to a low enthalpy change associated with the interaction rather than a genuine lack of binding.

We next examined whether the RIR motifs of human pol \( \eta \), pol \( \kappa \), and pol \( \iota \) bind to PCNA and the CTD of Rev1 using ITC (Table 1). Although we did not detect binding to PCNA for either the pol \( \eta \) RIR or the pol \( \iota \) RIR, we did find that the RIR motif of pol \( \kappa \) binds both PCNA and the Rev1 CTD with affinities in the low micromolar range. Thus, whereas some of the PIP and RIR motifs in the human Y-families polymerases are probably specific for binding either PCNA or Rev1, the RIR motif of human pol \( \kappa \) does have specificities for both PCNA and Rev1 similar to those that we observed with the PIP motif of yeast pol \( \eta \).

**The PIP Motif of Msh6 Binds Both PCNA and the Rev1 CTD**—The high affinity with which the yeast pol \( \eta \) PIP motif binds the yeast Rev1 CTD raised the intriguing possibility that PIP motifs from other proteins beyond Y-family polymerases might also bind the Rev1 CTD. To test this, we considered the mismatch repair protein Msh6. First, we fused the PIP motif of yeast Msh6 to GST and examined its ability to bind the isolated Rev1 CTD and PCNA using the high throughput protein-binding assay. This isolated PIP motif binds both PCNA and the Rev1 CTD (Fig. 5, A and B). Control reactions using GST with no PIP motif attached showed no binding to either PCNA or the Rev1 CTD. Next, we examined whether the Msh6 PIP motif binds the CTD of Rev1 in the context of full-length proteins in vivo. Using co-immunoprecipitation experiments, we observed the formation of an Msh6-Rev1 complex that is disrupted by either substitutions in the PIP motif of Msh6 or substitutions in the CTD of Rev1.
FIGURE 3. Kinetics of pol η binding to Rev1. A, single molecule TIRF microscopy was used to examine the kinetics of pol η binding to Rev1. Cy3-labeled Rev1 binds biotinylated pol η immobilized on the surface of the slide. B, representative fluorescence trajectory of Cy3-labeled Rev1 binding pol η obtained at 700 pM Rev1. C, histogram depicting the dwell time of Rev1 bound to pol η obtained at 700 pM Rev1. The solid line represents the best fit of the data to a single-exponential decay function with a $k_{off}$ of 0.19 ± 0.01 s$^{-1}$. D, histogram depicting the off time between Rev1 binding events obtained at 700 pM Rev1. The solid line represents the best fit of the data to a single-exponential decay function with a $v_{on}$ of 0.053 ± 0.008 s$^{-1}$. E, plot of the apparent $v_{on}$ values as a function of Rev1 concentration. The apparent on rate constant ($k_{on}$), $6.6 \times 10^7$ M$^{-1}$ s$^{-1}$, was determined from the slope of this line.

FIGURE 4. The pol η PIP motif binds PCNA and the Rev1 CTD with high affinity. A, isothermal titration calorimetry was used to measure the binding affinity of yeast PCNA and the isolated PIP motif of pol η (residues 620 – 632). From the best fit of the data, we obtained a $K_d$ of 6.4 ± 1.2 × 10$^5$ (corresponding to a $K_a$ of 1.6 μM), a Δ$H$ of −0.34 ± 0.01 kcal/mol, a Δ$S$ of 7.6 kcal/mol, and a stoichiometry of 0.59 ± 0.01. B, isothermal titration calorimetry was used to measure the binding affinity of the isolated yeast Rev1 CTD (residues 875–985) and the isolated PIP motif of pol η. From the best fit of the data, we obtained a $K_d$ of 2.1 × 10$^7$ ± 0.7 × 10$^7$ (corresponding to a $K_a$ of 48 nm), a Δ$H$ of −1.6 ± 0.1 kcal/mol, a Δ$S$ of 8.4 kcal/mol, and a stoichiometry of 0.95 ± 0.02.
Discussion

PCNA is a hub in the protein interaction network responsible for DNA metabolism, genome stability, and cell cycle control (1, 2, 5, 6). Many proteins in this network contain PIP motifs, which bind to PCNA. These motifs are eight amino acid residues in length and have conserved aromatic residues at positions 7 and 8. They generally have a conserved glutamate residue at position 1 and a conserved hydrophobic residue at position 4. Until now, the sole purpose of these motifs has been thought to be binding PCNA. The work presented here shows that some PIP motifs may also bind to the CTD of Rev1, demonstrating that they are a more versatile protein interaction motif than previously appreciated.

The regions on PCNA and on the CTD of Rev1 to which PIP motifs bind are structurally distinct but possess hydrophobic pockets that accommodate the two conserved, adjacent aromatic side chains (Fig. 6). In the case of PCNA, the PIP motif binds within a trench that stretches across the front surface of the ring. The two aromatic residues of the PIP motif are

| TABLE 1 | Affinities of PIP and RIR motifs from Y-family polymerases for PCNA and the Rev 1 CTD |
|---------|------------------------------------------------------------------|
| PIP-like motif | Sequence | $K_a$ (μM) | $K_d$ (μM) |
| Yeast Pol η PIP | 620 - SSKNILSFTRIKK$^a$ | 1.6 ± 0.3 | 0.958 ± 0.022 |
| Human PCNA | | | |
| Human Rev 1 CTD | | | |
| Human pol β PIP | 696 - PRPEGQMTLESSFFKPLTH$$^a$$ | 11 ± 2 | ND$^b$ |
| Human pol β RIR | 247 - KKTTSLESFFKQKAER | ND$^b$ | 11$^b$ |
| Human pol γ PIP | 444 - AKKGLDDYVL | 7.8 ± 0.8 | ND$^b$ |
| Human pol γ RIR | 364 - ASRGCLSSFSKQMDD | ND$^b$ | 69$^b$ |
| Human pol σ PIP | 862 - KITLHFFX$$^a$$ | ND$^b$ | ND$^b$ |
| Human pol σ RIR | 560 - ESMHKSFFDKRRSER | 6.6 ± 1.2 | 1.3 ± 0.6 |

$^a$ ND, binding was not detected because the change enthalpy was low.
$^b$ These $K_a$ values were obtained from surface plasmon resonance experiments reported in Ref. 30.
anchored in a hydrophobic pocket that is composed of residues from a β-sheet and the extended interdomain connecting loop of PCNA. In the case of the Rev1 CTD, the two aromatic residues of the PIP motif bind within a deep hydrophobic pocket that forms at the intersection of two α-helices.

Rev1, in addition to being a Y-family polymerase, plays an important non-catalytic role in translesion synthesis by acting as a scaffold to recruit other Y-family polymerases (25–29). Many of these other Y-family polymerases have short, conserved PIP-like motifs called RIR motifs, which bind to Rev1 (30–33). These motifs are six amino acid residues in length and have two conserved phenylalanine residues at positions 1 and 2. The only other sequence constraint is that the remaining four amino acid residues not be prolines.

Our results challenge the view that PIP motifs and RIR motifs are two strictly distinct classes of protein interaction motifs. We found that some PIP and RIR motifs have overlapping specificities and interact with both PCNA and Rev1. This is most notably the case for the PIP motif of yeast pol η, the RIR motif of human pol κ, and the PIP motif of yeast Msh6. These findings support the notion that PIP and RIR motifs are members of the same class of motif. Some of these motifs may bind PCNA tighter than Rev1, whereas others may bind Rev1 tighter than PCNA. Nevertheless, these motifs probably exist along a continuum of specificities and collectively should be referred to as PIP-like motifs.

Given the structural basis of these interactions, it is possible that proteins other than Y-family polymerases have PIP-like motifs that bind the CTD of Rev1. It has recently been shown that XRCC1, a scaffold protein involved in single strand break repair and base excision repair, possesses a PIP-like motif containing two phenylalanine residues that binds to the CTD of Rev1 (51). Moreover, it is possible that PIP-like motifs bind other proteins in addition to PCNA and Rev1 that possess similar hydrophobic pockets. For example, it has recently been shown that the RIR motif of human pol η binds the B subunit of the replicative DNA polymerase δ (pol δ) (52). Similarly, the PIP motif of mismatch repair protein Msh3 binds to a hydrophobic pocket on the C-terminal domain of the mismatch repair protein Mlh1 (53). Moreover, PIP-like motifs in the DNA glycosylase Ntg2 and the exonuclease Exo1 both bind to this same hydrophobic pocket on Mlh1 (54–56). Taken together, these findings suggest a very different view of the protein interaction network responsible for DNA metabolism, maintaining genome stability, and cell cycle control. In this new picture, PIP-like motifs mediate interactions with multiple binding partners, including PCNA, Rev1, pol δ, Mlh1, and perhaps other proteins.

In the case of yeast pol η, our data suggest some noteworthy differences in the way its single PIP motif is used to interact with PCNA and Rev1. The affinity of full-length pol η binding PCNA is measured by single-molecule TIRF microscopy is ∼350-fold greater than that of the isolated PIP peptide binding PCNA as measured by ITC. Moreover, amino acid substitutions in the PIP motif of full-length pol η greatly reduce but do not fully eliminate PCNA binding, as shown by the high throughput protein-binding assay. This implies that there are additional points of contact between pol η and PCNA that are outside of the PIP motif. The affinity of full-length pol η binding Rev1, by contrast, is only ∼10-fold greater than that of the isolated PIP peptide binding the isolated Rev1 CTD. In addition, amino acid substitutions in the PIP motif of full-length pol η completely eliminate Rev1 binding. This is consistent with a complex in which the interactions between these two proteins are almost entirely mediated by the PIP motif binding the CTD.

Both PCNA and Rev1 serve as hub proteins in the large macromolecular complexes that assemble at stalled replication forks to carry out translesion synthesis. Given 1) that the sole PIP motif of yeast pol η binds to both PCNA and to Rev1 and 2) that the structural bases of these interactions would make it nearly impossible for the PIP motif to bind both PCNA and Rev1 at the same time, there are only two possible architectures of the ternary complexes that forms between yeast pol η, PCNA, and Rev1: the PCNA tool belt architecture and the Rev1 bridge architecture. In the PCNA tool belt, pol η uses its PIP motif to directly bind one subunit of PCNA, whereas Rev1 is able to directly bind another subunit of PCNA. In the Rev1 bridge, pol η uses its PIP motif to directly bind the CTD of Rev1. In this model, pol η does not directly bind PCNA but is bridged to it by Rev1. The dual specificity of the yeast pol η PIP motif suggests that both types of higher order complexes should be formed at stalled replication forks.

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References
1. Moldovan, G. L., Pfander, B., and Jentsch, S. (2007) PCNA, the maestro of the replication fork. Cell 129, 665–679
2. Dieckmann, L. M., Freundenthal, B. D., and Washington, M. T. (2012) PCNA structure and function: insights from structures of PCNA complexes and post-translationally modified PCNA. Subcell. Biochem. 62, 281–299
3. Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. Cell 79, 1233–1243
4. Jönsson, Z. O., Hindges, R., and Hübischer, U. (1998) Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen. EMBO J. 17, 2412–2425
5. Warbrick, E. (2000) The puzzle of PCNA’s many partners. Bioessays 22, 997–1006
6. Maga, G., and Hübischer, U. (2003) Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J. Cell Sci. 116, 3051–3060
7. Haracska, L., Kondratick, C. M., Unk, I., Prakash, S., and Prakash, L. (2001) Interaction with PCNA is essential for yeast DNA polymerase η function. Mol. Cell 8, 407–415
8. Acharya, N., Yoon, J. H., Gali, H., Unk, I., Haracska, L., Johnson, R. E., Hurwitz, J., Prakash, L., and Prakash, S. (2008) Roles of PCNA-binding and
The PIP Motif of DNA Polymerase η Interacts with Rev1

ubiquitin-binding domains in human DNA polymerase η in translesion DNA synthesis. Proc. Natl. Acad. Sci. U.S.A. 105, 17724–17729

9. Guo, C., Sonoda, E., Tang, T. S., Parker, J. L., Bielen, A. B., Takekura, S., Ulrich, H. D., and Friedberg, E. C. (2006) Rev1 protein interacts with PCNA: significance of the Rev1 BRCT domain in vitro and in vivo. Mol. Cell 23, 265–271

10. Haracska, L., Johnson, R. E., Unk, I., Phillips, B., Hurwitz, J., Prakash, L., and Prakash, S. (2001) Physical and functional interactions of human DNA polymerase η with PCNA. Mol. Cell. Biol. 21, 7199–7206

11. Haracska, L., Johnson, R. E., Unk, I., Phillips, B. B., Hurwitz, J., Prakash, L., and Prakash, S. (2001) Targeting of human DNA polymerase iota to the replication machinery via interaction with PCNA. Proc. Natl. Acad. Sci. U.S.A. 98, 14256–14261

12. Haracska, L., Unk, I., Johnson, R. E., Phillips, B. B., Hurwitz, J., Prakash, L., and Prakash, S. (2002) Stimulation of DNA synthesis activity of human DNA polymerase η by PCNA. Mol. Cell. Biol. 22, 784–791

13. Ohmori, H., Friedberg, E. C., Fuchs, R. P., Goodman, M. F., Hanaoka, F., Hinkle, D., Kunkel, T. A., Lawrence, C. W., Livneh, Z., Nohmi, T., Prakash, L., Prakash, S., Todo, T., Walker, G. C., Wang, Z., and Woodgate, R. (2001) The Y-family of DNA polymerases. Mol. Cell 8, 7–8

14. Prakash, S., Johnson, R. E., and Prakash, L. (2005) Eukaryotic translesion DNA synthesis polymerases: specificity of structure and function. Annu. Rev. Biochem. 74, 317–353

15. Pryor, J. M., Dieckman, L. M., Boehm, E. M., and Washington, M. T. (2014) in Nucleic Acid Polymerases (Murakami, K. S., and Trakselis, M. A., eds) pp. 85–108, Springer, Berlin

16. Sale, J. E., Lehmann, A. R., and Woodgate, R. (2012) Y-family DNA polymerases and their role in tolerance of cellular DNA damage. Nat. Rev. Mol. Cell Biol. 13, 141–152

17. Johnson, R. E., Prakash, S., and Prakash, L. (1999) Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Polα. Science 283, 1001–1004

18. Haracska, L., Yu, S. L., Johnson, R. E., Prakash, L., and Prakash, S. (2000) Efficient and accurate replication in the presence of 7,8-dihydro-8-oxoguanine by DNA polymerase η. Nat. Genet. 25, 468–461

19. Johnson, R. E., Kondratick, C. M., Prakash, S., and Prakash, L. (1999) hRAD30 mutations in the variant form of xeroderma pigmentosum. Science 285, 263–265

20. Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K., and Hanaoka, F. (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase μ. Cell Biol. 170, 784–791

21. Nelson, J. R., Lawrence, C. W., and Hinkle, D. C. (1996) Deoxyctydylid transferase activity of yeast Rev1 protein. Nature 382, 729–731

22. Washington, M. T., Minko, I. G., Johnson, R. E., Haracska, L., Harris, T. M., Lloyd, R. S., Prakash, S., and Prakash, L. (2004) Efficient and error-free replication past a minor-groove N2-guanine adduct by the sequential template-specific DNA polymerase.

23. Washington, M. T., Prakash, L., and Prakash, S. (2001) Yeast DNA polymerase η utilizes an induced-fit mechanism of nucleotide incorporation. Cell 107, 917–927

24. Haracska, L., Unk, I., Johnson, R. E., Johannson, E., Burgers, P. M., Prakash, S., and Prakash, L. (2001) Roles of yeast DNA polymerases δ and ζ and of Rev1 in the bypass of abasic sites. Genes Dev. 15, 945–954

25. Freudenthal, B. D., Ramaswamy, S., Hingorani, M. M., and Washington, M. T. (2008) Structure of a mutant form of proliferating cell nuclear antigen that blocks translesion DNA synthesis. Biochemistry 47, 13354–13361

26. Dieckman, L. M., Boehm, E. M., Hingorani, M. M., and Washington, M. T. (2013) Distinct structural alterations in proliferating cell nuclear antigen block DNA mismatch repair. Biochemistry 52, 5611–5619

27. Biesiadecki, B. J., and Jun, J. P. (2011) A high-throughput solid-phase microplate protein-binding assay to investigate interactions between myofilament proteins. J. Biomed. Biotechnol. 2011, 421701

28. Ghomeini, M., and Spies, M. (2014) Direct correlation of DNA binding and single protein domain motion via dual illumination fluorescence microscopy. Nano Lett. 14, 5920–5925

29. Honda, M., Park, J., Pugh, R. A., Ha, T., and Spies, M. (2009) Single-molecule analysis reveals differential effect of ssDNA-binding proteins on DNA translocation by XPD helicase. Mol. Cell 35, 694–703

30. Masuda-Ozawa, T., Hoang, T., Seo, Y. S., Chen, L. F., and Spies, M. (2013) Single-molecule sorting reveals how ubiquitylation affects substrate recognition and activities of FBH1 helicase. Nucleic Acids Res. 41, 3576–3587

31. Haghighat Jahromi, A., Honda, M., Zimmerman, S. C., and Spies, M. (2013) Single-molecule study of the CUG repeat-MBNL1 interaction and its inhibition by small molecules. Nucleic Acids Res. 41, 6687–6697

32. Cordes, T., Vogelsang, J., and Tinnefeld, P. (2009) On the mechanism of Teloxin as antiblinking and antibleaching reagent. J. Am. Chem. Soc. 131, 5018–5019

33. Zhou, R., Kunzelmann, S., Webb, R. M., and Ha, T. (2011) Detecting intramolecular conformational dynamics of single molecules in short distance range with subnanometer sensitivity. Nano Lett. 11, 5482–5488

34. Milescu, L. S., Nicolai, C. L., Qin, F., and Sachs, F. (2002) New developments in the QUB software for single-channel data analysis. Biophys. J. 82, 267A–267A

35. Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Kapral, M., Bordoni, L., and Schwede, T. (2012) SWISS-MODEL: modelling protein tertiary and quaternary structure. Nucleic Acids Res. 40, W252–W258

36. Arnold, K., Bordoni, L., Kopp, J., and Schwede, T. (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201

37. Kiefer, F., Arnold, K., Künstl, M., Bordoni, L., and Schwede, T. (2009) The SWISS-MODEL Repository and associated resources. Nucleic Acids Res. 37, D387–D392

38. Gue, N., Peitsch, M. C., and Schwede, T. (2009) Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a
The PIP Motif of DNA Polymerase η Interacts with Rev1

50. Hishiki, A., Hashimoto, H., Hanafusa, T., Kamei, K., Ohashi, E., Shimizu, T., Ohmori, H., and Sato, M. (2009) Structural basis for novel interactions between human translesion synthesis polymerases and proliferating cell nuclear antigen. J. Biol. Chem. 284, 10552–10560

51. Gabel, S. A., DeRose, E. F., and London, R. E. (2013) XRCC1 interaction with the REV1 C-terminal domain suggests a role in post replication repair. DNA Repair 12, 1105–1113

52. Baldeck, N., Janel-Bintz, R., Wagner, J., Tissier, A., Fuchs, R. P., Burkovics, P., Haracska, L., Despras, E., Bichara, M., Chatton, B., and Cordonnier, A. M. (2015) FF483–484 motif of human Poleta mediates its interaction with the POLD2 subunit of Poldelta and contributes to DNA damage tolerance. Nucleic Acids Res. 43, 2116–2125

53. Iyer, R. R., Pluciennik, A., Genschel, J., Tsai, M. S., Beese, L. S., and Modrich, P. (2010) MutLα and proliferating cell nuclear antigen share binding sites on MutSβ. J. Biol. Chem. 285, 11730–11739

54. Gellon, L., Werner, M., and Boiteux, S. (2002) Ntg2p, a Saccharomyces cerevisiae DNA N-glycosylase/apurinic or apyrimidinic lyase involved in base excision repair of oxidative DNA damage, interacts with the DNA mismatch repair protein Mlh1p: identification of a Mlh1p binding motif. J. Biol. Chem. 277, 29963–29972

55. Dherin, C., Gueneau, E., Francin, M., Nunez, M., Miron, S., Liberti, S. E., Rasmussen, L. I., Zinn-Justin, S., Gilquin, B., Charbonnier, J. B., and Boiteux, S. (2009) Characterization of a highly conserved binding site of Mlh1 required for exonuclease I-dependent mismatch repair. Mol. Cell. Biol. 29, 907–918

56. Gueneau, E., Dherin, C., Legrand, P., Tellier-Lebegue, C., Gilquin, B., Bonnesoeur, P., Londino, F., Quemener, C., Le Du, M. H., Márquez, J. A., Moutiez, M., Gondry, M., Boiteux, S., and Charbonnier, J. B. (2013) Structure of the MutLα C-terminal domain reveals how Mlh1 contributes to Pms1 endonuclease site. Nat. Struct. Mol. Biol. 20, 461–468

57. Vijayakumar, S., Chapados, B. R., Schmidt, K. H., Kolodner, R. D., Tainer, J. A., and Tomkinson, A. E. (2007) The C-terminal domain of yeast PCNA is required for physical and functional interactions with Cdc9 DNA ligase. Nucleic Acids Res. 35, 1624–1637