Insights into the regulation of human Rev1 for translesion synthesis polymerases revealed by structural studies on its polymerase-interacting domain

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Supplementary Figure 1. Double titration experiments among the translesion synthesis polymerases \( \kappa, \eta \) and \( \iota \) for the hRev1-PID binding.

(A) Titration of Pol \( \kappa^{560-575} \) to the bound hRev1-PID saturated with Pol \( \eta^{524-539} \). (B) Titration of Pol \( \kappa^{560-575} \) to the bound hRev1-PID saturated with Pol \( \iota^{564-579} \). (C) Superimposition of hRev1-PID bound with Pol \( \kappa^{560-575} \), Pol \( \eta^{524-539} \) and Pol \( \iota^{564-579} \).

Supplementary Figure 2. Superimposition of hRev1-PIDs bound with translesion synthesis polymerases.

(A) Side view of bound hRev1-PID with polymerase \( \kappa \) (magenta) and polymerase \( \zeta \) (orange), respectively (B) Top view of the bound hRev1-PIDs with polymerase \( \kappa \) (magenta) and polymerase \( \zeta \) (orange), respectively

Supplementary Figure 3. Residues involved in the interaction between hRev1-PID (magenta ribbon) and Pol \( \kappa \) (green ribbon).

Salt bridge formed by hRev1-PID: E1174 (red stick) and Pol \( \kappa \): K571 (green stick); hydrophobic interaction formed by hRev1-PID: L1159, L1171, L1172, W1175 and V1190 (white stick) and Pol \( \kappa \): F567 and F568 (green stick).

Supplementary Figure 4. Sequence alignment for C-terminal translesion synthesis polymerase-interacting domain of Rev1 and Rev7 in vertebrates.

Residues labeled with asterisk are the key residues responsible for interaction with polymerase \( \kappa \) (green), Rev1 (magenta) and polymerase \( \zeta \) (blue), respectively.

Supplementary Figure 5. Fluorescence anisotropy on the interaction between Rev1, polymerase \( \kappa \) and polymerase \( \zeta \).

(A) Fluorescence anisotropy binding curve for the FITC-labeled Pol \( \kappa^{561-577} \) titrated with hRev1-PID. (B) Fluorescence anisotropy binding curve for the titration of Pol \( \zeta \).
into the FITC-labeled Pol κ 561-577, which saturated with hRev1-PID based on the condition obtained in (A).

**Supplementary Figure 6.** Circular dichroism spectrum for free synthesis peptide Pol kappa.

**Supplementary table legend**

**Supplementary Table 1.** Structure statistics for hRev1-PID in complex with Pol κ

**Supplementary Table 2.** Average values for T1, T2 and T1/T2 for free and bound form hRev1-PID with translesion synthesis polymerases κ, η and ι.

**Supplementary Table 3.** Structure statistics for hRev1-PID in complex with Pol ζ

**Supplementary Table 4.** Binding affinity between hRev1-PID and translesion synthesis polymerases.

**Supplementary methods**

**Protein expression and purification.** The gene encoding Rev1 1156-1251 and Pol κ 564-577 were sub-cloned into the pET-28a and pGEX4T-1 vectors, respectively. The plasmid was then transformed into E.coli BL21 (DE3) competent cell for expression. The competent cell cultured in Luria-Bertani (LB) at 37° was induced for protein expression with addition of 0.4mM IPTG when the cells reached an OD600 of 0.6-0.8. After the overnight post-culture at 16 ° and 4 hours post-culture at 37°, respectively for Rev1 and Pol κ, the cells were harvested and stored for the further protein purification. His-Rev11156-1251 was first purified using Ni-NTA column (Qiagen) and the eluted protein was collected for further TEV digestion. After the overnight TEV digestion at 4°C, the sample was dialyzed against Ni-NTA binding buffer and then reloaded onto
the Ni-NTA column. The unbound fractions were collected and loaded to the gel filtration chromatography (Superdex-75, Amersham Biosciences) for final purification and buffer exchange to 50 mM sodium phosphate (pH 6.8), 0.15M NaCl, 0.5mM EDTA and 1mM DTT. The GST-Pol κ was purified using GST column (Qiagen) and the purified GST-Pol κ was mixed with purified Rev11156-1251 and injected to the GPC column. The elution fraction, which contains GST-Pol κ/Rev11156-1251, was collected and then underwent the thrombin digestion to remove GST-tag. After addition of PMSF to terminate the digestion reaction, the sample was reloaded to the GPC column equilibrated with 50mM Bis-Tris pH 6.5, 100mM NaCl, 1mM DTT buffer for the final complex sample preparation. Pol ζ (Rev7/Rev3) was prepared following the previous reported expression and purification procedure(Hara et al, 2010).

**Peptides preparation.** Pol κ, η and ι peptides, used for the double titration experiment, share the same sequence as previously described(Ohashi et al, 2009). The peptides for fluorescence anisotropy experiments were labeled with FITC in the C-terminus of Pol κ: GSHKKSFFDKKRSERKW. The sequence for the FITC-Pol κ mutant is GSHKKSFFDKGRSERKW. All the peptides were chemically synthesized and purified by Peptron (Daejeon, S. Korea).

**Circular dichroism spectroscopy.** Circular dichroism (CD) spectroscopy was carried out at room temperature using a Jasco J-815 spectropolarimeter. Far-UV wavelengths were scanned from 260 nm to 190 nm at the speed of 100 nm/min. CD spectra were collected and averaged for 10 scans. Three samples were analyzed in 50mM NaPi, pH 6.8, 0.15M NaCl, 0.5mM EDTA and 1mM DTT buffer: synthetic Pol κ alone, Rev11156-1251 with and without synthetic Pol κ.

**Isothermal titration calorimetry (ITC).** Isothermal titration calorimetry experiments were conducted on a Microcal VP-ITC calorimeter (MicroCal, Northampton, MA, USA) at 25 ºC. The synthetic polypeptides Pol κ, η, ι and Pol ζ (Rev7/Rev3) were prepared in the buffer: 50mM NaPi, pH 6.8, 0.15M NaCl 0.5mM EDTA and 10mM Tris-HCl, pH7.5, 0.15M NaCl, 10mM β-mercaptoethanol, respectively. The reference power and
stirring speed were set to 10uAML/s and 300 rpm, respectively. Total 30 (60) times injection with 10 (5) ul injection and 20(10) second duration and 240 second space were used for ITC experiments. All the data were processed and fitted using Origin 7.0 software.

**Fluorescence anisotropy.** Fluorescence anisotropy experiments were conducted on a luminescence spectrometer 55 (Perkin Elmer Instrument) at 25 °C. All the samples: FITC labeled-Pol κ\(^{WT}\), FITC labeled-Pol κ\(^{K571G}\), Rev\(^{1156-1251}\) and Rev\(^{1156-1251\ E1174A}\) were dissolved in 50mM NaPi, pH 6.8, 0.15M NaCl 0.5mM EDTA. The excitation and emission were set to 495nm and 520 nm, respectively. The width of excitation and emission slits was 10nm and the integration times and duration time for titration was set to 5 second and 3 minutes, respectively.

**NMR spectroscopy.** Three dimensional HNCACB, CBCA(CO)NH, HN(CA)CO, HNCO, HBHA(CO)NH, HBHANH, HCCH-TOCSY, HN(CA)CO, (H)CCH-TOCSY, \(^{15}\)N-, \(^{13}\)C- simultaneous NOESY-HSQC, \(^{13}\)C- aromatic NOESY and two dimensional aromatic hbCBgcdHD, hbCBgdcdeHE spectra were measured on 800 and 900MHz Bruker NMR spectrometer from Korea Basic Science Institute (KBSI, S. Korea). The T1, T2 and htNOE experiments were carried out on a Varian Unity Inova spectrometer operating at the 600MHz Varian NMR at KAIST (Daejeon, S. Korea). \(^{15}\)N, \(^{13}\)C double labeled Rev\(^{1156-1251}\)/Pol κ was dissolved in the buffer containing 50mM Bis-Tris, pH 6.5, 0.1M NaCl, 0.5mM EDTA, 1mM DTT and 10%D\(_2\)O solvent for NMR measurements. All the spectra were processed using the nmrPipe program(Delaiglio et al, 1995) and analyzed with Sparky 3.1.\(^{16}\)

**Residual dipolar coupling (RDC) experiments.** Pf1-phage was used as alignment media for the RDC experiments. Despite the identical HSQC spectra with and without Pf1-phage, the decrease of peak intensity has been observed in the same NMR buffer used for the triple resonance experiments. An increasing salt concentration in the RDC medium recovered the peak intensity while remaining the peak position of HSQC spectrum unchanged. Therefore, the RDC NMR samples were prepared in
buffer (50mM Bis-Tris pH 6.5, 0.15M NaCl, 1mM DTT and 10% D₂O). The IPAP\textsuperscript{15}N-HSQC spectra were recorded for the isotropic and anisotropic (14mg/ml Pf1-phage; D₂O split, 12.9 Hz) condition respectively.

**NMR titration and relaxation experiments.** $^{15}$N-labeled hRev\textsubscript{1156-1251} at 150uM concentration was first titrated with synthetic peptide Pol η or ι to the saturation points. Then equivalent Pol κ was titrated to the Rev\textsubscript{1156-1251} saturated with Pol η or Pol ι. Similarly, equivalent Pol η was titrated to the Rev\textsubscript{1156-1251} saturated with Pol κ. The T1 and T2 relaxation experiments were measured for the Rev\textsubscript{1156-1251} saturated with polymerases κ, η and ι. $^{15}$N T1 and T2 relaxation was measured with delays of 50, 100, 150, 200, 300, 350, 400, 450, 500, 600, 700, 800 and 900ms for T1 and delays of 10, 30, 50, 70, 90, 110, 130, 150, 170, 190, 210, and 230 ms for T2. Peak intensities for the series T1 and T2 spectra were further fitted to a single exponential decay function, 

$I=I_0*\exp(-R^t)$, using Sparky program 3.1\textsuperscript{16}.

**NMR Structure determination.** The structure calculation and the automatic assignment of NOE cross-peaks were performed using the CYANA 3.0 program(Guntert et al, 1997) with additional NH-RDC restraints and dihedral angle restraints. The dihedral restraints were obtained from the program TALOS plus(Shen et al, 2009) using the assigned chemical shifts. The NOE-distance restraints from the CYANA were used for next structure calculation using the Xplor-NIH program(Schwieters et al, 2003), in which the same RDC and dihedral angle restraints were also implemented. The 100 accepted structures that were generated using the restrained simulated annealing protocol were further improved by the explicit water refinement (Linge et al, 2003; Schwieters et al, 2003). The ensemble of 15 structures were selected based on the criteria (1, total energy; 2, NOE violation; 3, RDC violations; 4, dihedral angle violation), and the statistics of the final structure are shown in Supplementary Table 2.

**Crystallization and data collection.** 9.6 mg/ml Rev\textsubscript{1156-1251} /Pol ζ (Rev7/Rev3) complex dissolved in the buffer (10mM Tris-HCl pH7.0, 0.15M NaCl, 10mM
β-mercaptoethanol) was used for the crystal screening and optimization. The Rev1\textsuperscript{1156-1251}/Pol ζ (Rev7/Rev3) complex was crystallized by the hanging drop vapour diffusion at 20 °C. The crystallization solution contains 0.1M HEPES-Na (pH 7.5), 0.1M NH\textsubscript{4}-citrate tribasic (pH 7.0), 19-21% PEG-3350, and 20% ethylene glycol was used for a cryo-protectant. Initial crystal screenings were performed using the Rigaku MicroMax\textsuperscript{TM}-007 HF (R-AXIS IV++) X-ray system in KBSI (S. Korea), and then the ultimate X-ray diffraction data were using the beamline-1A, KEK-PF (Japan).

**X-ray structure determination and refinement.** The diffraction data obtained were processed using HKL2000 (Otwinowski & Minor, 1997). The structure of Rev1\textsuperscript{1156-1251}/Pol ζ (Rev7/Rev3) complex was solved by the molecular replacement (MR) method. The MR was conducted using the Phaser (McCoy et al, 2007) program in the CCP4 package (Potterton et al, 2003) with the structure of apo Pol ζ (Rev7/Rev3) (Hara et al, 2010). The subsequent model building and structure refinement were performed using the Coot program (Emsley et al, 2010) and the CNSsolve-1.3 program (Brunger et al, 1998), respectively. The statistics of the x-ray structure calculation are shown in supplementary Table 3.

**Construction of Bait/Target expression vectors.** The wild type genes, PKC δ (BC043350) was obtained from Korean UniGene Information (KUGI, Korea). PKCδ-mRFP-REV1-PID expression vector was constructed using a combination of pPKCδ-mRFP-C3 (Lee et al, 2011), and PCR-amplified REV1-PID genes. To construct the eGFP-POLK (560-615) expression vector, the PCR-amplified POLK (560-615) gene was cloned into the plasmid peGFP-C3 (Clontech Inc.). To construct the TagBFP-REV7 expression vectors, the PCR-amplified REV7 genes was cloned into the plasmid pTagBFP-C3 (Evrogen, Moscow, Russia). To construct the C3N1-REV3 expression vectors, the PCR-amplified REV3 genes was cloned into the plasmid pC3N1 (GFP gene was removed from pEGFP-C3).

**Co-expression of the Bait/Target Protein in Cultured Cells.** HEK-293T cells were grown on round coverslips (Knittel glaser Inc.) in a well culture plate to 50–70%
confluence. Transient co-transfection of the desired bait/target protein pair was conducted using an ExGen 500 (Thermo Fisher Scientific Inc.) according to the manufacturer’s standard protocol.

**Confocal imaging.** For live cell imaging, transiently co-transfected cells on round coverslips in 12-well culture plate were treated with PMA (1 μM, Aldrich) for 5 min. and washed with DPBS, followed by fixing with 3.7% formaldehyde for 5 min. Images of the fixed cells were measured using a laser scanning confocal microscope (LSM 710, Carl Zeiss, Germany) with a C-Apochromat 40X/1.2 water immersion lens (405 nm diode laser with 410-475 nm detection range for TagBFP, 488 nm argon laser/505-550 nm detection range for eGFP, 561 nm solid state laser/586-662 nm detection range for mRFP).

**Supplementary References**


