Interactions of PIP and RIR Motifs in TLS Polymerases with PCNA and Rev1-CT

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UCONN HEALTH

Translesion Synthesis (TLS)

TLS is a mechanism of DNA damage tolerance utilized by cells to directly replicate across DNA lesions that alter DNA structure. TLS utilizes specialized DNA polymerases with spacious active sites which makes them capable of replicating past sites of damage otherwise untraversable by high-fidelity polymerases pol δ and ε. TLS is important for resolving stalled replication forks, which arise after pol δ and ε are uncoupled from DNA at sites of damage. However, this property also promotes resistance to genotoxic agents in cancer cells, limiting treatment efficacy. **Thus, understanding the protein-protein interactions that govern TLS is crucial for developing adjuvant drugs designed to inhibit TLS function in cancer cells during treatment.** In humans, this group of polymerases are comprised of the low-fidelity Y-family polymerases: **pol η, pol ι, pol κ and Rev1**, and the multi-subunit B-family polymerase: **pol ζ (Rev3/Rev7/PolD2/PolD3)**. Together these polymerases assemble onto PCNA forming the multi-protein **TLS complex**.[5,8,9]

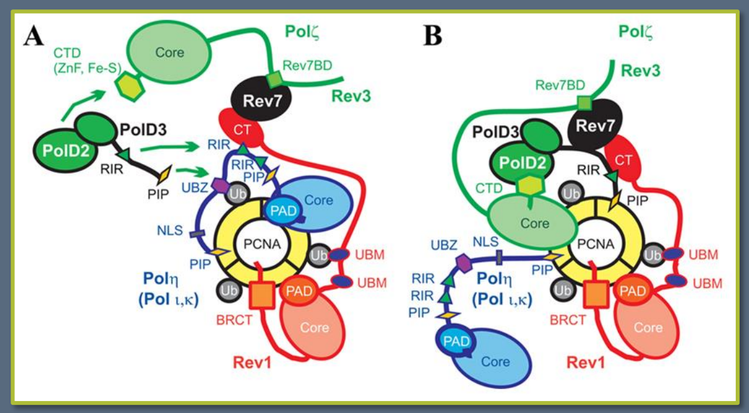

Figure 1. Model of the TLS Complex and Polymerase Switching [7]

Recruitment of TLS polymerases to sites of DNA damage occurs after monoubiquitination of the **proliferating cell nuclear antigen** (PCNA) by the E2/E3 ligases Rad6 and Rad18, promoting both the recruitment of TLS polymerases via ubiquitin-binding motifs (UBM/UBZ) leading to the formation of the **TLS complex.** TLS then proceeds through an **"insertion"** step (Figure 1A), where the appropriate polymerase (pol η, ι, κ or Rev1) is selected to incorporate nucleotides across a specific lesion. This is followed by an **"extension"** step (Figure 1B), as a polymerase (pol ζ) adds to the structurally aberrant DNA primer, past the lesion.[5,8,10]

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PIP-box and RIR Motifs

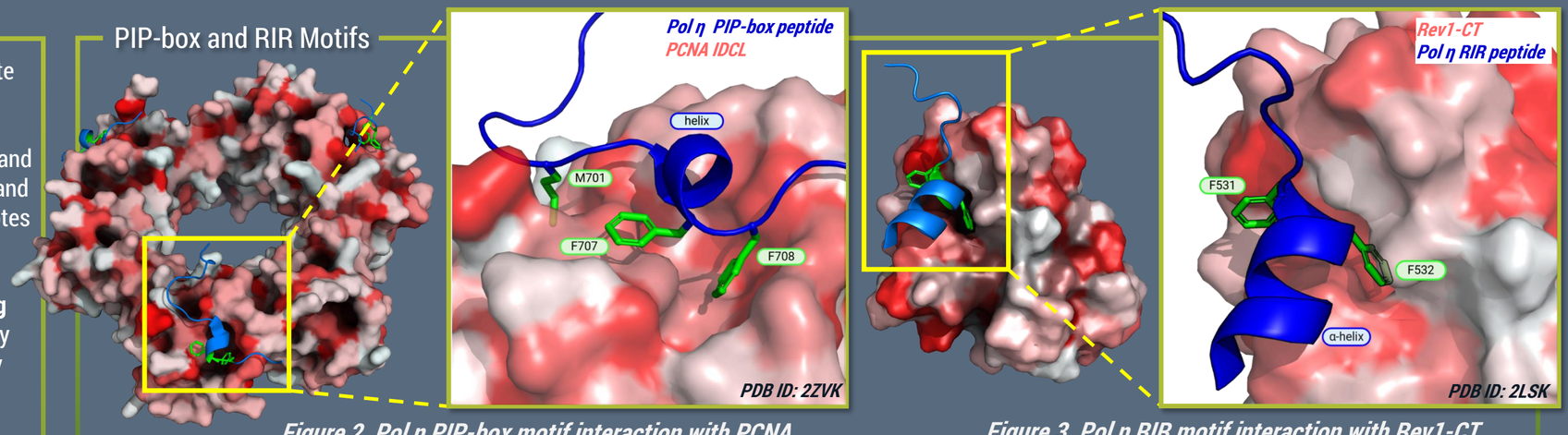

Figure 2. Pol η PIP-box motif interaction with PCNA

Figure 3. Pol η RIR motif interaction with Rev1-CT

Coordination of the TLS polymerases on PCNA is achieved through binding to a hydrophobic pocket in the **interdomain connector loop** (IDCL) of PCNA via a **PCNA-interacting peptide** or **"PIP" box motif** (Figure 2). In TLS, PIP box sequences follow the form: **(K/M/R)-x-x-[M/L/I]-x-x-[F/Y][F/Y/L]**, with the intermediate residues forming a helix that properly orients the two terminal residues (typically FF) for binding. Rev1 primarily functions as a regulatory scaffold for the other TLS polymerases which bind its C-terminal domain (Rev1-CT) via a **Rev1-interating region** or **"RIR" motif** (Figure 3). The RIR motif bears a strong resemblance to the PIP box, following the form: **n-FF-hhhh**, where n is an n-capping residue, followed by two phenylalanines that insert into a deep hydrophobic pocket, stabilized by 4 residues (h) forming an α-helix. **Together these motifs form relatively weak, competing protein-protein interactions, setting a basis by which the TLS complex can rearrange its configuration when selecting the correct polymerase during each step of TLS.**[2,3,4,5,6]

Hypothesis

Yeast Pol η, which lacks a RIR motif, was shown to bind to both PCNA and Rev1-CT using only its PIP-box motif with Kd's of 1.6 μM and 0.05 μM, respectively. A similar finding was shown for human pol κ RIR.[2] These results, together with compositional/structural similarities between PIP-box and RIR motifs have led to the question: **should they be redefined as a single class of motif or should they continue to be classified as functionally separate domains?** [2] To answer this question in the context of the TLS system, we performed nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC) titrations of PCNA and Rev1-CT with peptides comprising each of the PIP-box and RIR motifs in the TLS polymerases.

** Indicates sequences at the C-terminus; NBD = no binding detected*

			PCNA Titrations		Rev1-CT Titrations
			NMR (35°C)	ITC (25°C)	NMR (15°C)
Peptide (region)	Motif	Sequence	K_d	K_d	K_d
hPolι-(418-435)	PIP	TAK**K**GLI**D**Y**YL**MPSLSTT	46.94 ± 0.33 μM	12.05 ± 0.72 μM	NBD
hPolη-(698-713)	PIP2	PEG**M**QT**L**ES**FF**KPLTH*	80.10 ± 0.27 μM	36.4 ± 5.82 μM	45.01 ± 0.20 μM
hPolη-(434-451)	PIP1	PSS**S**TD**I**TS**FL**SSDPSSL	NBD	NBD	NBD
hPolκ-(859-870)	PIP	NNP**K**HT**L**DI**FF**K*	NBD	NBD	96.89 ± 0.86 μM
hPolη-(474-491)	RIR1	KKA**T**TS**L**ES**FF**QKAAERQ	NBD	127.40 ± 16.22 μM	6.28 ± 0.10 μM
hPolη-(522-539)	RIR2	TSQ**S**TGT**E**P**FF**KQKSLLL	NBD	NBD	10.38 ± 0.12 μM
hPolκ-(558-575)	RIR	PLE**M**SH**K**KS**FF**DKKRSER	NBD	NBD	9.55 ± 0.10 μM
hPolι-(537-554)	RIR	LHA**S**RG**V**LS**FF**SKKQMQD	NBD	NBD	11.36 ± 0.12 μM
hPolD3-(229-246)	RIR	PGK**G**NM**M**SN**FF**GKAAMNK	NBD	212.40 ± 36.95 μM	32.93 ± 0.22 μM

Table 1. Binding Affinities for PIP-box and RIR motifs to PCNA and Rev1-CT

NMR temperatures selected to obtain the best signal resolution; ITC temperature selected for comparison with literature values; Inconsistencies between NMR and ITC for PCNA binding can be attributed to deuterium labelling which weakens hydrophobic interactions.[10]

Results

Pol η PIP1/PCNA binding was not detectable by NMR or ITC, consistent with it being dispensable for PCNA interaction *in vivo*.[1] Pol κ PIP/PCNA binding was not detectable by NMR or ITC, despite being required for PCNA stimulated DNA synthesis *in vivo*.[11] ***However,*** this peptide did demonstrate affinity for Rev1-CT by NMR. Similarly, by ITC Pol η RIR1 had detectable low affinity for PCNA, along with tight affinity for Rev1-CT by NMR. Pol D3 RIR also showed measurable affinity for both PCNA and Rev1-CT. Notably, Pol η PIP2, the second strongest PCNA binder also exhibited affinity for Rev1-CT. Conversely, Pol ι PIP, the strongest PCNA binder demonstrated no affinity for Rev1-CT.

Conclusions

Our trends in binding affinities (Kd) show that PIP-box motifs are selective for PCNA, while RIR motifs are selective for Rev1-CT. Polι PIP did not bind Rev1-CT, but features YL instead of the FF seen in all RIR motifs. Indeed PIP-box motifs that do bind Rev1-CT feature FF. Nevertheless, only 2 RIR motifs had detectable binding to PCNA. Thus, while it may not be accurate to rebrand all PIP-box and RIR motifs as a single class in the context of TLS, it is clear that motifs with dual affinities do exist. From this study we have identified three new "dual binders" Pol η RIR1, Pol η PIP2 and Pol D3 RIR, and we have re-confirmed that Pol κ PIP binds Rev1-CT.

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Supplementary Slides

Interactions of PIP and RIR Motifs in TLS Polymerases with PCNA and Rev1-CT

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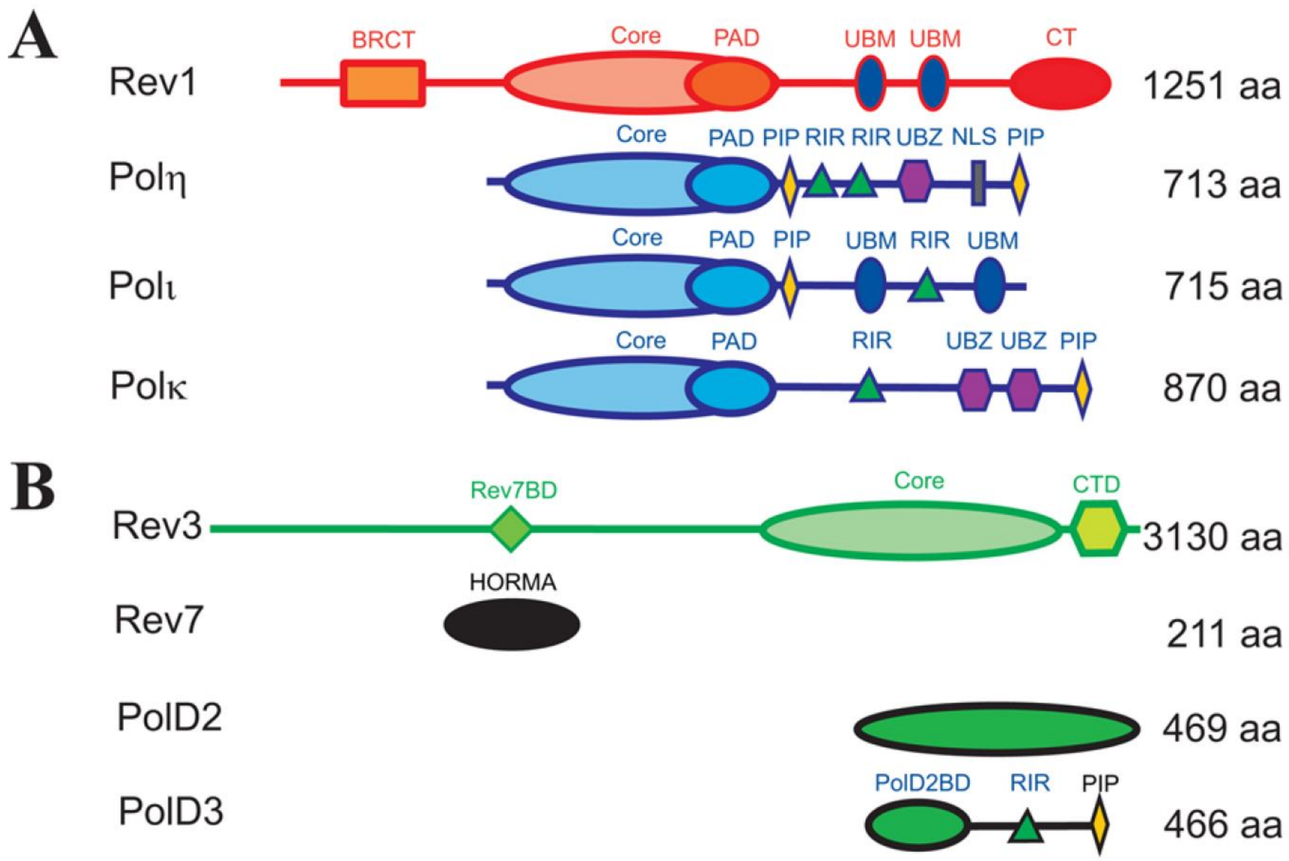

Figure S1. Domains of the TLS Polymerases (Pustovalova, 2016). A) Structural domains of the Y-Family polymerases: Rev1, pol η, pol ι and pol κ. The core domain comprises the palm, finger and thumb domains that make DNA contacts. A polymerase associate domain or "PAD" UBM/UBZ are ubiquitin binding domains. RIR (Rev1-interacting region) bind to Rev1-CT. PIP (PCNA interacting protein) boxes bind to the IDCL of PCNA. B) Subunits of B-Family polymerase: pol ζ, including Rev3, Rev7, PolD2 and PolD3.

The lower fidelity of these polymerases can be attributed to both their lack of a 3'-5' proofreading exonuclease domain (seen in pol δ and ε) and to fewer contacts with DNA and incoming nucleotide, resulting from a larger active site and stubbier finger and thumb domains. Their lower fidelity leads to increased mutation rate and is another way the TLS polymerases contribute to cancer progression.

Each TLS polymerase has a characteristic DNA lesion that it replicates across and while generally error prone, demonstrate high replication fidelity across these specific lesions. It is thought that this property is mediated by the PAD or "little finger" domain, which forms extra DNA contacts in the region of the damage site.

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Peptide (region)	Motif	Sequence	K_d	K_d	K_d
hPolη-(474-491)	RIR1	KKATTSLES**FF**QKAAERQ	NBD	127.40 ± 16.22 μM	6.28 ± 0.10 μM
hPolη-(481-491)	RIR, t-NT	-------ES**FF**QKAAERQ	NBD	NBD	23.91 ± 0.27 μM
hPolη-(474-485)	RIR, t-CT	KKATTSLES**FF**Q------	NBD	NBD	185.12 ± 2.24 μM
hPolκ-(558-575)	RIR	PLEMSHKKS**FF**DKKRSER	NBD	NBD	9.55 ± 0.10 μM
hPolκ-(565-575)	RIR, t-NT	-------KS**FF**DKKRSER	NBD	NBD	8.97 ± 0.15 μM
hPolκ-(558-569)	RIR, t-CT	PLEMSHKKS**FF**D------	NBD	NBD	78.86 ± 0.72 μM

Figure S2. Importance of Helix Residues for RIR Binding.

When binding PCNA, PIP-box motifs form an α-helix N-terminally to the main binding residues (ex. YL/FL or FF). This helix is necessary to orient the two residues into the binding pocket and form additional contacts with the IDCL. A similar property is seen in RIR motifs binding to Rev1-CT, however the α-helix forms C-terminally to the FF.

With this in mind, we sought to test the hypothesis that by deleting the N-terminal or C-terminal helix forming residues in certain peptides, we could “enhance” the propensity for having PIP-box or RIR character, i.e. precluding the formation of a helix on one side of the binding residues or the other, thereby promoting binding affinity to either PCNA or Rev1-CT respectively. To test this we designed 2 truncations for Pol η RIR1 and Pol κ RIR: 7 N-terminal residues (t-NT) or 6 C-terminal residues (t-CT).

In the case of both t-CT peptides, loss of the 6 C-terminal residues led to a large decrease in affinity for Rev1-CT. While Kd values increased 8 fold for Pol κ RIR and 30 fold for Pol η RIR1, binding was still detectable around the 70-200 μM range by NMR. This finding underscores the importance of the α-helix in promoting peptide interaction with Rev1-CT and the significance of the FF motif for mediating the bulk of the interaction. However, loss of these residues did not result in increased affinity for PCNA in either case, suggesting that in the case of RIR motif binding to the IDCL of PCNA formation of the α-helix is not sufficient. Indeed, of the two peptides only Pol η RIR1 binds PCNA indicating that the residues peripheral to the FF are likely important when forming additional contacts to the binding surface.

In the case of the t-NT peptides, loss the 7 N-terminal residues did not severely impact Rev1-CT binding affinity. The Kd for Pol η RIR1 increased 4 fold (thus mildly weaker binding), while the Kd for Pol κ RIR actually saw a small increase from 9.55 μM to 8.97 μM. On the other hand loss of those residues in Pol η RIR1 abolished any detectable binding to PCNA and likely further precluded any increase in affinity for Pol κ RIR.