Biochemical Characterization of Human Exonuclease 1 Protein-protein Interactions

by

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Dennis Thiele

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Biochemistry in the Graduate School of Duke University

2016
ABSTRACT

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Abstract

Human Exonuclease 1 (Exo1) plays important roles in numerous DNA metabolic/repair pathways including DNA mismatch repair, DNA double strand break repair, Okazaki fragment maturation. The nuclease activity of Exo1 is tightly regulated in vivo. The regulation of Exo1 in different pathways is achieved by interactions with different protein partners. The focus of this dissertation will be on characterization of Exo1 interactions with traditional protein partners and providing experimental evidences for new Exo1 interactions.

Molecular cloning, biochemical assays, collaborative nuclear magnetic resonance and X-ray crystallography have been employed to study Exo1 interactions with protein partners. This work contains: (i) the experimental evidence for new Exo1 interactions, and (ii) the detailed characterization of Exo1 interactions with PCNA, MLH1 and MutSα/β.

Taken together, the research progress presented in this dissertation further advances our understanding of the traditional Exo1 interaction network and may provide new insight into the function and regulation of Exo1.
Dedication

This thesis is dedicated to my greatest youth.
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<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CEST</td>
<td>Chemical exchange saturation transfer</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyclin-binding</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>DSBR</td>
<td>Double strand break repair</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary nonpolyposis colon cancer</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence spectroscopy</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>MALS</td>
<td>Multi-angle light scattering</td>
</tr>
<tr>
<td>MIP</td>
<td>MutL-interacting protein</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PIP</td>
<td>PCNA-interacting protein</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small angle X-ray scattering</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</table>
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1. Introduction

1.1 Overview of Exo1 and its biological roles

Human exonuclease 1 (Exo1) is a 5’ to 3’ structure-specific nuclease of RAD2/XPG nuclease family that plays important roles in DNA mismatch repair (MMR) [1] and DNA double strand break repair (DSBR) [2] as well as Okazaki fragment maturation [3], telomere maintenance [4] and rescuing stalled replication folks [5]. Exo1 consists highly conserved N-terminal catalytic domain (Exo1 1-352) and a long C-terminus (353-846) [6]. Most of the Exo1 protein interactions and post-translation modifications are mapped to the C-terminus (Figure 1) [7] [8] and this C-terminus is predicted to be highly disordered from the primary sequence.

There are two isoforms of Exo1, Exo1a and Exo1b, due to alternative splicing [9]. The last 43 residues were missing in Exo1a and the last residue of Exo1a was phenylalanine which is different to Exo1b at the same position [10]. So far, people have not identified any functional difference between these two isoforms.

Exo1 recognizes nicked, gapped, recessed, blunt and flapped DNA substrate, and has both 5’ to 3’ exo- and endonucleolytic activities [11]. The crystal structure of Exo1 N-terminal catalytic domain (1-352) has been solved and a unified mechanism of nucleotide cleavage in RAD2/XPG nuclease family has been proposed [6].
Figure 1: Exo1 interaction network summarized. Interaction interfaces of Exo1 with MSH2 (dark blue), MSH3 (hot pink), MLH1 (pink) are presented in solid rectangles. The well-defined Exo1 peptides, including PCNA-interacting protein box (PIP-box), MutL-interacting protein box (MIP-box) and cyclin-binding motif (Cy-motif) are highlighted in arrowed box. The residues phosphorylated by CDK1/2 are indicated by red solid circles.

1.2 Overview of RAD2/XPG nuclease family

Exo1 is a member of RAD2/XPG structure-specific nuclease family that are involved in a variety of DNA repair pathways. This family contains four proteins which all have different substrate specificities: Exo1 which is a 5’ to 3’ exonuclease mainly responsible for recessed DNA cleavage in DNA mismatch repair; flap endonuclease 1 (FEN1) which cleaves flapped DNA to process Okazaki fragments during DNA replication; flap endonuclease GEN homolog 1 (GEN1) which plays a key role in resolving Holliday junctions during DNA double strand break repair; and xeroderma pigmentosum complementation group G (XPG) which recognizes bubble-structured DNA and is important for gapped DNA formation during DNA nucleotide excision repair [12].
The N-terminal catalytic domain is highly conserved among all these proteins from both primary sequence and tertiary structure, while the C-terminal regions are diverse [13]. People have identified many of the protein partners of FEN1 [14] and XPG [15], and these interactions are important for the nuclease activity regulation in their corresponding pathways. No protein partners of GEN1 has been identified to the best of our knowledge. This protein-interaction nature and sequence variance of the C-terminus are probably the determinants of nuclease pathway choice that differentiate them from each other.

1.3 Exo1 functions and regulations by interactions with protein partners via its C-terminal region

Because Exo1 could hydrolyze a broad spectrum of DNA substrates with high efficiency, the activity of Exo1 must be tightly regulated inside cell to prevent unwanted DNA resection. Most of these regulations are achieved by interactions with protein partners.

A major role of Exo1 identified so far is to remove DNA mismatch match lesion using its exonuclease activity during DNA mismatch repair (Figure 2). To specifically remove DNA mismatch match lesion, Exo1 forms complex with MutSα (MSH2/MSH6 heterodimer) or MutSβ (MSH2/MSH3 heterodimer). MutSα primary binds to single base substitution and dinucleotide insertion-deletion loops (IDL) with high specificities and MutSβ recognize longer insertion-deletion loops precisely [16]. In addition, MutSα/β are
found to confer Exo1 processivity in an ATP-dependent manner. Therefore, with even only one nick at the 5’ upstream of the DNA mismatch, Exo1 in complex with MutSα/β can processively remove all the nucleotides from the nick on the erroneous DNA strand until all the mismatched nucleotides were removed [17]. Exo1 interacts with the common MSH2 subunit of MutSα/β via its C-terminus. Exo1 N-terminal catalytic domain alone failed to be activated by MutSα [6]. The interaction interface to MSH2 has been roughly mapped to 603-846 and Exo1 129-390 interaction with MSH3 has also been suggested in a previous study [18]. MutLα (MLH1/PMS2) is also an important component in DNA mismatch repair because it generates nicks around the mismatch which is used by Exo1 in the later repair process. Exo1 directly interacts with MLH1 subunit via two distinct binding sites: MutL-interacting protein box (MIP-box) at the middle region of Exo1 and Exo1 C-terminus. Although MutLα (MLH1/PMS2 heterodimer) or MLH1 has been shown to slightly inhibit Exo1 activity in vitro [17] [19]. In DNA mismatch repair, MutLα enhances Exo1 specificity toward heteroduplex DNA over homoduplex DNA in an in vitro mismatch-provoked assay [17].
Figure 2: Mechanism of DNA mismatch repair. Adapted from Modrich, 2006 [1].
Exo1 has also been implicated in the homologous recombination (HR) of DNA double strand break repair (Figure 3). The first step of this repair pathway is to generate long single-stranded DNA end used for pairing with the DNA of sister chromatin. Exo1 is one of the nuclease that is found to perform this “long-range” resection process. RecQ-like helicase BLM, one of the key components in DNA double strand break repair, recruits Exo1 to blunt-end DNA via direct physical interaction [2]. Four residues at Exo1 C-terminal region have been identified to be constantly phosphorylated by CDKs in S/G2 phase (Figure 1) [13]. The phosphorylated Exo1 will be recruited to double strand break foci by direct interaction with BRCA1 which is a key factor of nucleofilament formation during homologous recombination. Mutation of these Exo1 phosphorylation sites greatly abolishes the formation of single-stranded DNA. PCNA, the DNA metabolic hub protein, directly interacts with Exo1 at its C-terminal PCNA interacting protein box (PIP-box). This interaction also confers processivity to Exo1 in DNA end resection [20].

Exo1 also plays roles in resolving the stalled replication fork by DNA resection. PCNA, the key hub protein in DNA replication, may also recruit Exo1 to this pathway [21]. To prevent over-resection, 14-3-3 competes PCNA on Exo1, although the 14-3-3 interaction interface is mapped to the middle region of Exo1 (508-750) which does not overlap with the PIP-box [22].
Figure 3: Mechanism of DNA double strand break repair. Adapted from Panier and Boulton, 2014 [23].
Moreover, Exo1 seems to have structural and enzymatic functions in mitotic recombination. Mitotic recombination is similar to homologous recombination in DNA double strand break repair. Exo1 firstly has to generate single-stranded DNA to initiate the recombination using its nucleolytic activity. It also helps to resolve double Holliday junctions generated during mitotic recombination. This function requires Exo1 interaction with MutLγ (MLH1/MLH3 heterodimer) and is independent of Exo1 endonuclease activity [24].

1.4 Functional overlap of Exo1 and other RAD2/XPG nuclease family members

Although all four RAD2/XPG nuclease family members are thought to play distinct roles in different pathways, they do share protein partners and have similar functions in some aspects.

Exo1 and Fen1 corresponding genes in yeast (exo1 and rad27) are synthetic lethal [25]. This suggests that Exo1 and FEN1 probably have complementary roles in different pathways. Although FEN1 is thought to be the critical nuclease in Okazaki fragment maturation during DNA replication, Exo1 contains RNase H activity and is also able to remove DNA/RNA flaps as FEN1 in Okazaki fragment maturation [3]. Both FEN1 and Exo1 contains a PIP-box at the C-terminus binding to DNA processing hub protein PCNA, which is also a key component during DNA replication [14]. In a recent study, MutSα has also been shown to facilitate FEN1 removing mismatch lesions, suggesting a
complementary role of FEN1 to Exo1 in DNA mismatch repair [26]. Both Exo1 and FEN1 nuclease activities can be stimulated by RecQ helicase BLM and WRN [2] [27] [28] [29]. BLM and WRN activate FEN1 via the short C-terminus of FEN1 [28] but where BLM and WRN bind on Exo1 has not been characterized.

XPG, like Exo1 and FEN1, also has a PIP-box at the C-terminus binding to PCNA. The disruption of this interaction failed to reconstitute nucleotide excision repair in an in vivo reconstitution assay [30].

1.5 Study motivations

Because it seems that there are many proteins bound to Exo1 at Exo1 C-terminus, we asked whether these proteins bind to the same binding site at Exo1 C-terminus, whether the protein partners competitively or cooperatively bind to Exo1 C-terminus, whether we can design mutations to selectively disrupt Exo1 interactions only to certain protein partners, and how these combination of interaction contributes to Exo1 activity regulation and pathway choices.

Moreover, because Exo1 and other RAD2/XPG family member, in particular FEN1, share so many protein partners, we wonder whether there are new specific motifs on Exo1 and FEN1 for these protein partners, such as MutSα, BLM and WRN.
2. Biochemical evidences of replication protein A (RPA) and Exo1 interaction

2.1 Introduction

Replication protein A (RPA) is one of the single-stranded DNA binding hub proteins involved in various DNA metabolic pathways. It regulates DNA processing indirectly by displacing proteins from DNA or directly by protein-protein interactions. In DNA mismatch repair, RPA and Exo1 are both critical components. While Exo1 is responsible for the DNA mismatch removal, RPA increases Exo1 specificity and terminate repair process presumably by displacing Exo1 from single-stranded DNA (Figure 2) [17]. No direct interaction between RPA and Exo1 has been observed to date [31]. Here in this study, we found several experimental evidence suggesting that RPA directly interacts with Exo1 at both Exo1 N-terminus and C-terminus.

2.1 Results and conclusions

2.1.1 RPA directly interacts with Exo1 at both Exo1 N-terminus and C-terminus.

We firstly conducted Far-western analysis and found that RPA interacts with full-length Exo1. Using different Exo1 fragments, at least two Exo1 regions have been identified interacting with RPA (Figure 4).

In the Far-western analysis, both Exo1 1-450 and Exo1 1-514 show strong binding signal to RPA while Exo1 1-352 does not show too much binding signal, suggesting that
there is at least one interaction interface to RPA at Exo1 N-terminus 353-450. Both Exo1 452-846 and Exo1 705-846 also show strong binding signal to RPA, suggest that there is also at least one interaction interface to RPA at Exo1 C-terminus 705-846. If I tried to break Exo1 705-846 further to Exo1 705-803 and Exo1 771-846, no binding signals to RPA were observed. It is probably because that Exo1 fragments become too small and the interaction interface was not exposed well on the membrane.

2.1.2 RPA-Exo1 interaction at Exo1 N-terminus

To refine the RPA interaction interface at Exo1 N-terminus, we used two Exo1 1-450 variants, internal deleted Exo1 1-450 Δ402-424 and internal scrambled Exo1 1-450 scr 423-446 (Exo1 423-446 sequence was scrambled from AELSEDDLLSQYSLSFRTKKTKKNS to FLYKQLTSESNKADSKESSLSDK), to see whether the interaction to RPA could be interrupted in Far-western analysis (Figure 5).

We found that Exo1 1-450 scr 423-446 shows similar binding signal to RPA compared to Exo1 1-450 while Exo1 1-450 Δ402-424 shows weaker binding signal and is comparable to Exo1 1-352. This suggests that the RPA interaction interface at Exo1 N-terminus is probably at Exo1 402-424.
Figure 4: Far-western analysis of RPA interactions with Exo1 fragments. (A) Far-western analysis result. The indicated amount of each Exo1 fragment was spotted on a nitrocellulose membrane and incubated with 0.6 μM RPA at 4 degree for 3.5 hours. RPA was detected immunochemically using Anti-RPA70 antibody (NA13 from CALBIOCHEM. Now produced by EMD). It should be noted that Exo1 1-401 has been found proteolysed in SDS-PAGE gels in the followed up studies. (B) Exo1 constructs used in this study.
Figure 5: Far-western analysis of RPA interactions with Exo1 1-450 variants. The indicated amount of each Exo1 variants was spotted on a nitrocellulose membrane and incubated with 0.6 μM RPA at 4 degree for 3.5 hours. RPA was detected immunochemically using Anti-RPA70 antibody (NA13 from CALBIOCHEM. Now produced by EMD).
2.1.3 RPA-Exo1 interaction at Exo1 C-terminus

Regarding the RPA interaction interface at Exo1 C-terminus, we collected $^{15}$N-$^1$H HSQC spectra for $^{15}$N-Exo1 705-846 in the presence and absence of RPA (Figure 6). Although most of the peaks overlap, several well-separated peaks vanished in $^{15}$N-$^1$H HSQC spectrum upon RPA titration. These peaks appear to match the peaks shown in the $^{15}$N-$^1$H HSQC spectrum of $^{15}$N-Exo1 771-803 which is K796, N797, F798 and G799 (Figure 25). The vanished backbone peaks clearly support that Exo1 705-846 interacts with RPA and the RPA interaction interface is probably at least within Exo1 771-803.

2.1.4 RPA-Exo1 interactions on RPA

Because RPA is a multi-subunit protein complex consisting RPA70, RPA32 and RPA14. All three RPA subunits have been reported to interact with several different protein partners [32]. So, we would like to determine where Exo1 binds on RPA.

We firstly resolved the three RPA subunits using SDS-PAGE, transferred the proteins to nitrocellulose membrane, renatured the proteins on the membrane and did Far-western analysis using Exo1 N-terminus 1-514 and Exo1 C-terminus 705-846 as prey protein (Figure 7). Exo1 705-846 shows strong binding signal to RPA70, but no binding signal to RPA32 and RPA14 (Figure 7 middle panel). Exo1 1-514 shows relative weak binding signal to RPA70, but no binding signal to RPA32 and RPA14 (Figure 7 right panel). These results suggest that RPA70 is the major binding target of Exo1.
Figure 6: $^{15}\text{N}-^1\text{H}$ HSQC of $^{15}\text{N}$-Exo1 705-846 in the absence and presence of RPA. The $^{15}\text{N}-^1\text{H}$ HSQC spectra of 85 μM $^{15}\text{N}$-Exo1 705-846 in the absence (red spectrum) and presence of 85 μM RPA (blue spectrum) in buffer 25 mM HEPES-NaOH, pH 7.0; 100 mM NaCl; 1 mM TCEP were overlapped. The key vanished residues are inferred from the peak assignment of Exo1 771-803 (see Materials and Methods).
Figure 7: Far-western analysis of RPA subunit interactions with Exo1 fragments. RPA subunits (RPA70, RPA32 and RPA14) were resolved on 12% SDS-PAGE (left panel), transferred to nitrocellulose membrane and renatured (see Materials and Methods). The membranes are incubated with 0.6 μM Exo1 705-846 (middle panel) and 0.6 μM Exo1 1-514 (right panel) followed by Anti-Exo1 C-terminus (WH0009156M1, Sigma-Aldrich) and Anti-Exo1 N-terminus (sc56387, Santa Cruz) antibody detection, respectively. The Anti-Exo1 C-terminus and Anti-Exo1 N-terminus antibodies used in this study does not react with any RPA subunit (data not shown).
**2.2 Discussion**

As a key DNA metabolic hub protein, RPA interact with other DNA processing proteins mainly via three different sites, RPA70 N-terminal domain (RPA70N), RPA70 A and B domain (RPA70AB) and RPA32 C-terminal domain (RPA32C) [33].

In collaboration with Walter Chazin Lab at Vanderbilt University, we mapped the Exo1 interaction interface at RPA side using NMR spectroscopy. Using our purified Exo1 705-846 and Exo1 1-450, Walter Chazin Lab tested their interactions with RPA70 N-terminal domain (RPA70N) and RPA32 C-terminal domain (RPA32C) where most of the RPA protein partners bind. They found that Exo1 1-450 interacts with RPA32C weakly but no interactions have been observed for Exo1 705-846 with RPA70N and RPA32C, and Exo1 1-450 with RPA70N.

This result is a little bit surprising because we did not observe Exo1 1-450 and Exo1 705-846 interactions with RPA32 in Far-western analysis. The “RPA32C target interaction motifs” from SMARCAL1, TIPIN, XPA, UNG2 and RAD52 have been summarized in a previous study from Walter Chazin Lab [34]. Sequence alignment of our Exo1 1-450, Exo1 705-846, Exo1 402-424 and Exo1 771-803 with these “RPA32C target interaction motifs” does not suggest any sequence of Exo1 N-terminus or C-terminus similar to these “canonical consensus sequence”. However, it should be noted that these Exo1 sequences are highly conserved among different species (Figure 8).
Figure 8: Sequence alignment of Exo1 from different species. The sequences of Exo1 from different species were obtained from UniProt (http://www.uniprot.org/). The sequences were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and formatted with ESPript 3 (http://escripte.ibcp.fr/).
In addition, Exo1 1-450 and Exo1 705-846 did not show interaction with the canonical RPA binding site RPA70N. However, together with the Far-western analysis data of Exo1 and RPA70 subunits interaction (Figure 7), it probably suggests that Exo1 1-450 and Exo1 705-846 interact with the rest domains of RPA70, namely RPA70A, RPA70B and RPA70C domains.

It should be noted that XPG, one of the other XPG/RAD2 family members, interact with RPA [35]. This recruitment of XPG to the DNA damage spot by RPA is probably important for bubble-structured DNA excision in DNA nucleotide excision repair.

For Exo1 and RPA interaction, the biological implication might not be obvious as XPG and RPA interactions. In DNA mismatch repair assay in vitro, RPA terminates the repair process preventing Exo1 over-resection. *E. coli* single-stranded DNA binding protein SSB can also replace RPA in the assay to terminate the repair process [17]. Therefore, the role of RPA in DNA mismatch repair seems to only displace Exo1 by coating onto the single-stranded DNA after mismatch removal. It is interesting that the direct physical Exo1 and RPA interaction we observed does not seem to fully support this “displacing model” proposed in the literature.

However, in a recent single-molecule fluorescence imaging study on Exo1 regulations by single-stranded DNA binding proteins, the authors suggested that Exo1
regulation by RPA is likely due to two nonexclusive mechanisms: direct competition to single-stranded DNA and weak direct physical interaction [36]. Our findings of direct Exo1 and RPA interaction seems to support their conclusions.

Finally, it should be pointed out that Exo1 705-846, potentially Exo1 771-803, interacts with many other protein partners, such as PCNA, MLH1, MutSα/β and Exo1 N-terminus. The characterization of these interactions are introduced in the following chapters.

**2.3 Future directions**

**2.3.1 Determine the binding affinity of Exo1 and RPA interactions.**

Exo1 and RPA interaction seems to be a weak interaction. In size-exclusion chromatography experiments, neither Exo1 1-450 nor Exo1 705-846 co-migrate with RPA (data not shown). In GST-pull down experiments, GST-Exo1 705-803 was not able to pull-down RPA (data not shown).

However, because Exo1 and RPA interact via multiple sites, a combination of several weak interactions may result in a relative strong interaction. Therefore, it is necessary to determine the binding affinity of Exo1 and RPA using full-length Exo1 instead of Exo1 fragments.
2.3.2 Further map the interaction interface on both Exo1 and RPA sides.

Preliminary NMR studies show that the Exo1 C-terminus residues got perturbed upon RPA titration are corresponding to the residues inside Exo1 771-803. We happen to have this $^{15}$N-Exo1 771-803 in the lab. Therefore, the $^{15}$N-$^1$H HSQC spectra of Exo1 771-803 in the absence and presence of RPA can be collected to refine the RPA interaction interface at Exo1 C-terminus.

Exo1 402-424 has been suggested to interact with RPA. To further confirm this, there are two approaches. The first approach is to purchase a synthetic fluorescein-labeled peptide covering Exo1 402-424 and test its interaction with RPA using fluorescence anisotropy. The second approach is to express and purify $^{15}$N-labeled Exo1 peptide covering 402-424 and test its interaction with RPA using NMR spectroscopy.

On the RPA side, Exo1 1-450 and Exo1 705-846 should be further tested interaction with $^{15}$N-labeled RPA70A, RPA70B and RPA70C domains using NMR spectroscopy at Walter Chazin Lab.
3. Biochemical characterization of Exo1 N-terminus and C-terminus interaction

3.1 Introduction

Yuqian Shi, a graduate student in the Lorena Beese Lab, first identified that the Exo1 C-terminus interacts with Exo1 N-terminus using Far-western analysis and crosslinking. The binding site at Exo1 C-terminus seems to be located within Exo1 771-803. Given the fact that Exo1 is regulated by numerous proteins, such as PCNA, MLH1 and MutSα, via the interaction at the C-terminus (Figure 1), this N-terminus and C-terminus interaction might be important for us to understand the mechanism of Exo1 regulation.

3.2 Results and conclusions

3.2.1 Exo1 N-terminus interaction interface at Exo1 C-terminus

To determine the Exo1 N-terminus interaction interface at Exo1 771-803, we employed NMR spectroscopy to determine the binding interface. $^{15}$N-$^1$H HSQC spectra of $^{15}$N-Exo1 771-803 in the absence and presence of Exo1 1-352 were collected and overlapped (Figure 9). Exo1 N792, W795, K796, N797, F798 and G799 got disappeared upon Exo1 1-352 titration, suggesting that these residues contribute to the interaction with Exo1 N-terminus.
Figure 9: $^{15}$N-$^1$H HSQC of $^{15}$N-Exo1 771-803 in the absence and presence of Exo1 1-352. The $^{15}$N-$^1$H HSQC spectra of 100 μM $^{15}$N-Exo1 771-803 in the absence (red spectrum) and presence of 100 μM Exo1 1-352 (blue spectrum) in buffer 25 mM HEPES-NaOH, pH 7.0; 90 mM NaCl; 10 mM KCl; 5 mM MgCl₂; 0.1% Glycerol; 1 mM TCEP were overlapped. The key vanished residues are determined from the peak assignment of Exo1 771-803 (see Materials and Methods).
3.2.2 The effect of Exo1 C-terminus on Exo1 N-terminus functions

In mismatch-provoked repair assay, MutSα confers processivity of Exo1 presumably by interaction with Exo1 C-terminus [17] [6]. Exo1 C-terminus interacts with both Exo1 N-terminus and MutSα, but whether Exo1 C-terminus binds MutSα and Exo1 N-terminus simultaneously or not remains unknown. If Exo1 1-352, Exo1 C-terminus and MutSα form ternary complex, MutSα should still be able to confer processivity of Exo1. We therefore tested Exo1 1-352, Exo1 C-terminus and MutSα in mismatch-provoked excision assay (Figure 10).

For Exo1 full-length, as expected, it is activated by MutSα on 5’-GT heteroduplex DNA substrate excision (Figure 10A lane 9 and 13). However, it should be noted that titration of Exo1 452-846 up to 1264 fmol did not show a decrease in Exo1 full-length activation by MutSα (Figure 10A lane 13-16, Figure 10B purple curve). The reasons to this might be complicated. It might be due to that Exo1 C-terminus binds to DNA substrate (data not shown) and RPA, or even more Exo1 C-terminus are required in titration in order to see a decrease in Exo1 full-length activation.
**Figure 10: MutSα-stimulated Exo1 excision assay.** (A) Activation of Exo1 1-352 and full-length in the presence and absence of MutSα was scored as a function of increasing concentration of Exo1 452-846. The reaction contains 12 fmol Exo1 1-352 or 10 fmol Exo1 full-length, 12 fmol 5’-GT heteroduplex DNA plasmid substrate, 900 fmol RPA with indicated amount of MutSα and Exo1 452-846. After incubation at 37 degree for 5 minutes, reaction products were digested using NheI and ClaI and separated by electrophoresis on a 1% agarose gel. Excised gapped molecules resistant to cleavage by NheI and are indicated by arrows. (B) Extents of excision on 5’-GT heteroduplex DNA substrate. The quantification is based on the gel shown in (A).
For Exo1 1-352, titration of Exo1 452-846 in the absence of MutSα does not affect Exo1 1-352 activity significantly (Figure 10A lane 1-4, Figure 10B blue curve). In the presence of MutSα, the titration of Exo1 452-846 does not activated Exo1 1-352 processivity (Figure 10A lane 5-8, Figure 10B red curve). It should also be noted that the Exo1 1-352 that I purified seems to be more active than Exo1 full-length, contradicting to the data published previously that Exo1 1-352 and Exo1 full-length are equally active in the absence of MutSα [6].

Taken together, from this experiment, we found that Exo1 C-terminus did not affect Exo1 N-terminus activity both in the absence and presence of MutSα. We also did not conclude that Exo1 C-terminus binds MutSα and Exo1 N-terminus simultaneously.

3.2.3 Mutagenesis studies of Exo1 N-terminus and C-terminus interaction

Because we have identified the residues on Exo1 C-terminus interacting with Exo1 N-terminus using NMR spectroscopy, we would like to design mutations to disrupt this interaction. Because there are two hydrophobic residues W795 and F798 of Exo1 771-803 interacting with Exo1 N-terminus as shown in 15N-1H HSQC experiment (Figure 9), if the interaction is hydrophobic, mutation of these two residues to alanine will likely disrupt the interaction.

His-NusA-tagged Exo1 771-803 wild-type and W795A/F798A mutant were expressed, purified and used in Far-western analysis to probe the interactions with Exo1
Purified His-NusA was used as control.

In Far-western analysis, His-NusA-tagged Exo1 1-803 shows strong binding signals to Exo1 1-450, MutSαΔ341, MutSβΔ162 and weak binding signals to Exo1 1-352, RPA (Figure 11 middle panel). Mutation of Exo1 W795 and F798 to alanine abolishes the interactions to MutSαΔ341 and MutSβΔ162, but the interaction to Exo1 1-450 does not seem to be affected (Figure 11 right panel).

This experiment suggests that W795 and F798 are probably the key residues interacting with MutSα and MutSβ (potentially MSH2 subunit) but not with Exo1 N-terminus.
Figure 11: Far-western analysis of Exo1 771-803 variants interactions with protein partners. The indicated amount of each protein was spotted on a nitrocellulose membrane and incubated with 0.23 μM His-NusA, His-NusA tagged Exo1 771-803 wild-type and W795A/F798A mutant at 4 degree for 3.5 hours, respectively. His-NusA, His-NusA tagged Exo1 771-803 wild-type and W795A/F798A mutant were detected immunochemically using Anti-His antibody (Novagen 70796).
3.3 Additional observations

The evidence of this novel Exo1 N-terminus and C-terminus interaction may provide us new insight to understand how Exo1 is regulated in the cell. Here are some additional observations that supports this interaction.

Preliminary fluorescence anisotropy experiments show nearly stoichiometric binding between fluorescein-labeled Exo1 705-803 and Exo1 1-352. To eliminate the possibility that the fluorophore contributes to the interaction, we used unlabeled Exo1 705-803 to compete fluorescein-labeled Exo1 705-803 from Exo1 1-352 (Figure 12). The fluorescence anisotropy decreases as the titration of unlabeled Exo1 705-803 into labeled Exo1 705-803 bound to Exo1 1-352, but it was not able to reach the initial unbound anisotropy within the concentration range of unlabeled Exo1 705-803 we used. The significance of this observation needs further investigation.

Additional cross-linking and SAXS experiments conducted by Yuqian Shi also support that Exo1 C-terminus 705-846 interacts with Exo1 1-352. Taken together, we think there is direct interaction between Exo1 N-terminus and C-terminus.
Figure 12: Fluorescence anisotropy of unlabeled Exo1 705-803 competition with fluorescein-labeled Exo1 705-803 binding to Exo1 1-352. 6.5 nM fluorescein-labeled Exo1 705-803 was firstly titrated with Exo1 1-352 in buffer 25 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 1 mM TCEP followed by the titration of unlabeled Exo1 705-803. The fluorescence anisotropy was measured and the experiment was done only once. The data was fit using the one site binding equation with the presence of inhibitor accounting for ligand/inhibitor depletion.
In this study, the Exo1 N-terminus interaction interface at Exo1 C-terminus was determined using NMR spectroscopy. However, the C-terminus interaction interface at Exo1 N-terminus remains unknown. Knowing where Exo1 C-terminus binds on Exo1 N-terminus is very important because this interaction may affect the conformational change of Exo1 N-terminus during its nucleolytic cleavage and the enzyme activity. Therefore, we might need some other experiment techniques to determine the binding site.

We have once suspected that Exo1 C-terminus binds to or close to Exo1 N-terminus DNA binding site. Therefore, we collected the $^{15}$N-$^1$H HSQC spectra of $^{15}$N-Exo1 771-803 titrated with Exo1 1-352 D173A (catalytically dead mutant) in complex with 5'-recessed DNA substrate. No difference was observed between the $^{15}$N-$^1$H HSQC spectra of $^{15}$N-Exo1 771-803 titrated with Exo1 1-352 wild-type and $^{15}$N-Exo1 771-803 titrated with Exo1 1-352 D173A in complex with DNA substrate (data not shown). This probably suggests that Exo1 771-803 binding site is not located at Exo1 1-352 DNA binding site.
3.4 Future directions

3.4.1 Mutagenesis to disrupt Exo1 N-terminus and C-terminus interaction

NMR spectroscopy experiment suggested a small region at Exo1 771-803 binding to Exo1 1-352. Only 7 residues of Exo1 771-803 were shown perturbation upon Exo1 1-352 titration. Since the mutation of W795 and F798 to alanine does not disrupt the interaction in Far-western analysis, mutations of all these 7 residues to alanine might be the next thing to try to see whether the interaction is disrupted or not.

3.4.2 Determination of Exo1 C-terminus interaction interface at Exo1 N-terminus

$^{15}$N-$^1$H HSQC of $^{15}$N-Exo1 1-352 and titrated it with Exo1 771-803 provided us some weak evidence that Exo1 771-803 binds to the C-terminus of Exo1 1-352. It is feasible to delete several residues from the C-terminus of Exo1 1-352 and test the interaction of this protein to $^{15}$N-Exo1 771-803 in $^{15}$N-$^1$H HSQC experiment. If the hypothesis is true, the Exo1 771-803 HSQC peaks got perturbed upon Exo1 1-352 titration previously should not be perturbed in this experiment.
4. PCNA, MLH1, MutSα/β, RPA and Exo1 N-terminus share the same binding site on Exo1 C-terminus.

4.1 Introduction

In previous studies, people have roughly mapped different Exo1 and protein partner interactions to Exo1 C-terminus (Figure 1), but they did not refine the interaction interface and understand how these protein partners bind, namely cooperative binding or competitive binding. Using different Exo1 fragments, we were able to refine the interaction interface at Exo1 C-terminus to traditional Exo1 protein partners, such as PCNA, MLH1 and MutSα/β, and some “new” protein partners, such as RPA and Exo1 N-terminus. This provides us helpful information to further understand how Exo1 is regulated in different pathways.

4.2 Results and conclusions

4.2.1 PCNA interaction interface at Exo1 C-terminus

PCNA interacts with Exo1 via a conserved PIP-box (Exo1 788-795) at Exo1 C-terminus [7]. We also determined and confirmed the PCNA interaction interface on Exo1 771-803 using NMR spectroscopy (Figure 13). It turns out that Exo1 residue 786-803 are strongly perturbed upon PCNA titration, suggesting a relative larger interaction interface than PIP-box.
Figure 13: $^{15}$N-$^1$H HSQC of $^{15}$N-Exo1 771-803 in the absence and presence of PCNA. The $^{15}$N-$^1$H HSQC spectra of 100 μM $^{15}$N-Exo1 771-803 in the absence (red spectrum) and presence of 100 μM PCNA (blue spectrum) in buffer 25 mM HEPES-NaOH, pH 7.0; 90 mM NaCl; 10 mM KCl; 5 mM MgCl$_2$; 0.1% Glycerol; 1 mM TCEP were overlapped. The key vanished residues are determined from the peak assignment of Exo1 771-803 (see Materials and Methods).
4.2.2 MLH1 interaction interface at Exo1 C-terminus

Because both PCNA and MLH1 interact with Exo1 C-terminus [7], we wondered whether MLH1 and PCNA share the same binding site on Exo1 C-terminus or not. A GST-pull down experiment using GST-tagged Exo1 705-803, MLH1-CTD (467-756) and PCNA has been conducted (Figure 14). From the result, it shows that PCNA competes MLH1-CTD pre-bound to GST-tagged Exo1 705-803 (Figure 14 lane 3-5), and MLH1-CTD also competes PCNA pre-bound to GST-tagged Exo1 705-803 (Figure 14 lane 6-8). This experiment strongly suggests that PCNA and MLH1-CTD competitively bind to the same region at Exo1-C-terminus.
Figure 14: GST-Exo1 pull-down with MLH1 and PCNA. 0.5 μM GST-Exo1 705-803 was incubated with Glutathione Sepharose in the presence of 1 μM MLH1-CTD and an increasing concentration of PCNA from 0 to 5 μM (lane 3-5). Reversely, 0.5 μM GST-Exo1 705-803 was incubated with Glutathione Sepharose in the presence of 1 μM PCNA and an increasing concentration of MLH1-CTD from 0 to 5 μM (lane 6-8). The proteins pull-down by Glutathione Sepharose was resolved by SDS-PAGE, stained with Comassie, verified and quantified by Anti-MLH1-CTD antibody (sc-166625, Santa Cruz) and Anti-PCNA (sc-56, Santa Cruz) antibody.
Given the sequence similarity between PIP-box and MIP-box (Figure 1), we wondered whether Exo1 PIP-box is also a MIP-box that binds to MLH1-CTD. To approach this, we tried to determine MLH1-CTD interaction interface on Exo1 771-803 using NMR spectroscopy. It turns out that upon MLH1-CTD titration, almost all the $^{15}$N-Exo1 771-803 peaks were vanished (Figure 15). Given the experiment was done in a good way and no evidence of protein aggregation and precipitation was observed, it is likely that MLH1-CTD has an even larger interaction interface beyond Exo1 771-803. Therefore, Exo1 PIP-box might not necessary be a MIP-box binding to the small crystallographically well-defined MIP-box binding pocket on MLH1-CTD [37]. To test this hypothesis, synthetic fluorescein-labeled MIP-box peptide was titrated with MLH1-CTD and the fluorescence anisotropy was measured. The fluorescence anisotropy increases as MLH1-CTD was titrated. Then Exo1 C-terminus 705-846 was titrated into MIP-box and MLH1-CTD complex and the fluorescence anisotropy just kept increasing and finally got saturated (data not shown). This experiment did not show any competition of Exo1 MIP-box and Exo1 705-846 at MLH1-CTD and suggests that MLH1-CTD may have two distinct binding sites for Exo1 MIP-box and Exo1 C-terminus, respectively.

Some Exo1 C-terminus mutants, such as GST-Exo1 705-803 N792A/E793A, H778A/H779A were tested interaction with MLH1-CTD in a GST pull-down assay
compared to GST-Exo1 705-803 wild-type. However, neither of the Exo1 mutants shows disruption of binding to MLH1-CTD (data not shown).
Figure 15: $^{15}$N-HSQC of $^{15}$N-Exo1 771-803 in the absence and presence of MLH1-CTD. The $^{15}$N-HSQC spectra of 100 μM $^{15}$N-Exo1 771-803 in the absence (red spectrum) and presence of 200 μM MLH1-CTD (blue spectrum) in buffer 25 mM HEPES-NaOH, pH 7.0; 90 mM NaCl; 10 mM KCl; 5 mM MgCl$_2$; 0.1% Glycerol; 1 mM TCEP were overlapped. The key vanished residues are determined from the peak assignment of Exo1 771-803 (see Materials and Methods).
4.2.3 MutSα/β interaction interface at Exo1 C-terminus

Previously, it has been identified that MutSα and MutSβ common subunit MSH2 interacts with Exo1 at C-terminal region from 603 to 846 (Figure 1). Exo1 alterations (P640S, G759E and P770L) found in atypical HNPCC patients shows reduced interaction to MSH2 [38]. Because Exo1 isoform a (1-803) and Exo1 isoform b (1-846) shows no difference in activation by MutSα/β in mismatch repair assay, we designed an Exo1 C-terminal peptide 705-803 to probe Exo1 interactions with MutSα/β.

We firstly labeled Exo1 705-803 with fluorescein on its intrinsic cysteines. One preliminary fluorescence anisotropy experiments show that fluorescein-labeled Exo1 705-803 binds to MutSβΔ223 and the binding is also nearly stoichiometric. Unlabeled Exo1 705-803 was able to almost completely compete labeled Exo1 705-803 from MutSβΔ223 (Figure 16A). Because the binding between labeled Exo1 705-803 and MutSβΔ223 is nearly stoichiometric, I was not able to determine the $K_d$ of labeled Exo1 705-803 and MutSβΔ223 accurately in principal. However, the $K_d$ of labeled Exo1 705-803 and MutSβΔ223 (240pM) and the $K_i$ of unlabeled Exo1 705-803 and MutSβΔ223 (230pM) from the fitting are actually quite close, suggesting that the fluorophore does not contribute to the interaction of Exo1 705-803 and MutSβΔ223 and the interaction is extremely tight.
We then tested used unlabeled Exo1 771-803 to compete labeled Exo1 705-803 from MutSβΔ223 (Figure 16B). From the data and the fitting, we found that the $K_d$ of labeled Exo1 705-803 and MutSβΔ223 is 520 pM and the $K_i$ of unlabeled Exo1 771-803 and MutSβΔ223 is 11 nM, suggesting that Exo1 771-803 still binds to MutSβΔ223 very tightly but additional MutSβ interaction interface might be located in a region before Exo1 residue 771.
Figure 16: Fluorescence anisotropy of unlabeled Exo1 705-803/771-803 competition with fluorescein-labeled Exo1 705-803 binding to MutSβΔ223. 6.5 nM fluorescein-labeled Exo1 705-803 was firstly titrated with MutSβΔ223 in buffer 25 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 1 mM TCEP followed by the titration of unlabeled Exo1 705-803/771-803. The fluorescence anisotropy was measured and the experiment was done in triplicates. The data was fit using the one site binding equation with the presence of inhibitor accounting for ligand/inhibitor depletion. (A) The $K_d$ of labeled Exo1 705-803 and MutSβΔ223 is 240 pM and the $K_i$ of unlabeled Exo1 705-803 and MutSβΔ223 is 230 pM. (B) The $K_d$ of labeled Exo1 705-803 and MutSβΔ223 is 520 pM and the $K_i$ of unlabeled Exo1 771-803 and MutSβΔ223 is 11 nM.
With this information, Shivesh Kumar, a postdoctoral fellow in the Lorena Beese Lab, has co-crystallized Exo1 765-803 with MutSβΔ162, and Exo1 771-803 with MutSαΔ341 and solved the crystal structures of them. We observed the electron densities of Exo1 fragments binding to MSH2 subunits of MutSα/β. However, due to low resolution of the crystal structure, we were not able to unambiguously determine the key residues of Exo1 C-terminus contributing MSH2 interactions.

Shivesh Kumar designed two mutants that were predicted to disrupt Exo1 C-terminus and MSH2 interaction based on Exo1 765-803 and MutSβ complex crystal structure: L791A/L794A and F798A/F800A mutants. In a Far-western analysis that Shivesh Kumar conducted, His-NusA-tagged Exo1 765-803 L791A/L794A and F798A/F800A mutants showed decreased binding to MutSαΔ341 and MutSβΔ162 compared to His-NusA-tagged Exo1 765-803 wild-type (data not shown). In my Far-western blot, His-NusA-tagged Exo1 771-803 W795A/F798A also shows decreased binding to MutSαΔ341 and MutSβΔ162 compared to His-NusA-tagged Exo1 771-803 wild-type (Figure 11). This suggests that one or several residues of Exo1 L791, L794, W795, F798 and F800 are the key residues contributing to MSH2 interaction.

Based on this information, I designed Exo1 771-803 W795A and F798A single point mutant and conducted Far-western analysis to test their interactions with MutSα/β (Figure 17). It turns out that His-NusA tagged Exo1 771-803 F798A shows as weak
binding to MutSαΔ341 and MutSβΔ162 as His-NusA tagged Exo1 771-803 W795A/F798A double mutant, while His-NusA tagged Exo1 771-803 W795A shows much stronger binding. This observation suggests that F798 is more significant than W795 in the MSH2 interaction.
Figure 17: Far-western analysis of Exo1 771-803 mutant interactions with protein partners. The indicated amount of each protein was spotted on a nitrocellulose membrane and incubated with 0.6 μM His-NusA tagged Exo1 771-803 W795A/F798A, W795A, F798A mutants at 4 degree for 3.5 hours, respectively. His-NusA tagged Exo1 771-803 W795A/F798A, W795A, F798A mutants were detected immunochemically using Anti-His antibody (Novagen 70796).
4.3 Discussion

From the data of Exo1 C-terminus interactions with PCNA, MLH1, MutSα/β, RPA and Exo1 N-terminus, we found that all the interactions seem to happen at Exo1 771-803. Although we have only demonstrated the binding competition between PCNA and MLH1-CTD at Exo1 C-terminus (Figure 14), it is very likely that all these proteins competitively binding to the same region at Exo1 C-terminus.

This immediately raised the interest of Exo1 recruitment, activation and regulation mechanisms in different pathways.

First of all, the recruitment of Exo1 to different pathways is necessary as the first step of DNA resection. The enzyme should be recruited to the place where it is really needed. The Exo1 C-terminus region, Exo1 771-803 in particular, serves as the probe for the DNA processing signal proteins. For example, MutSα/β could be the recruitment signal for Exo1 in DNA mismatch repair. PCNA could be the recruitment signal for Exo1 in Okazaki fragment maturation, stalled replication fork and DNA double strand break repair.

Some sliding molecules, such as MutSα/β and PCNA, could also serve as the processive factors that activate Exo1. Some stationary molecules, such as MLH1 and RPA, might serve as the inhibitory factors that deactivate Exo1.
To terminate the Exo1 functions in different pathways, one of the possible ways is to remove the C-terminus of Exo1 C-terminus by proteolysis. Caspase 3 has been found to specifically cleave Exo1 at residue 514, preventing Exo1 repair functions during apoptosis [39]. It is unclear whether there are some other proteolysis events of Exo1 in pathways other than apoptosis.

Actually, Exo1 771-803 is not the first disordered polypeptide people found to interact with multiple structurally distinct protein partners. p53 C-terminal peptide 374-388 has been found to interact with at four different proteins, including S100ββ, sirtuin, CBP and cyclin A2, and the crystal structures of four different complexes are even available [40]. In these four complex crystal structures, p53 374-388 forms all three major secondary structure types: α helix when associating with S100ββ, β sheet with sirtuin, and distinct irregular structures with CBP and cyclin A2. It is also likely that Exo1 771-803 adopts different secondary structure types when bound to different protein partners.
5. Zn\(^{2+}\) enhances Exo1 and PCNA interaction

5.1 Introduction

Zn\(^{2+}\) is an essential trace element and it plays important biological roles in regulating enzyme activity, signal transduction, gene expression and DNA/RNA metabolisms [41]. Zn\(^{2+}\) deficiency affects DNA repair [38]. The transportation of Zn\(^{2+}\) to different cellular compartments, including nucleus, is tightly regulated and potentially serves as a “secondary messenger” for cellular processes [42]. Here we discovered that Zn\(^{2+}\) enhances Exo1 and PCNA interaction. This may have some implications in identifying the new roles of Zn\(^{2+}\) and Exo1 in DNA repair.

5.2 Results and conclusions

5.2.1 Crystal structure of Exo1 C-terminus and PCNA

Our lab has solved the crystal structure of Exo1 C-terminus 771-803 in complex with PCNA (Figure 18). In the crystal structure, three Exo1 771-803 peptides are bound to one PCNA homotrimer. Two Exo1 771-803 adopts canonical PIP-box conformation while the third one is in a distinct Zn\(^{2+}\) mediated binding mode. The Zn\(^{2+}\) is coordinated by PCNA H44, water mediated Exo1 N792 and E793, and PCNA D58 from symmetry related molecules. Similar PCNA-PIP-box peptide binding modes are also observed in PCNA-polymerase κ PIP-box peptide crystal structure [43]. So we wondered whether Zn\(^{2+}\) plays any role in regulating Exo1 C-terminus and PCNA interaction.
Figure 18: Crystal structure of PCNA in complex with Exo1 771-803. (A) Overview of the crystal structure of PCNA in complex with Exo1 771-803. One PCNA homotrimer (subunit A, B and C) binds to three Exo1 771-803 peptides (X, Y and Z). The Zn$^{2+}$ is depicted as grey sphere. (B) The interaction details of PCNA and Exo1 771-803. The Zn$^{2+}$ is depicted as green sphere.
5.2.2 Zn$^{2+}$ enhances Exo1 C-terminus and PCNA interaction

We immediately tested the binding affinity of Exo1 771-803 and PCNA in the presence and absence of Zn$^{2+}$. We firstly chose fluorescence anisotropy binding assay to measure the binding affinity between Exo1 771-803 and PCNA. We labeled fluorescein at the N-terminal amine of Exo1 771-803 using NHS-Fluorescein (ThermoFisher Scientific 46410). The labeled ligand was then titrated with PCNA in the presence and absence of 20 μM Zn$^{2+}$ and the fluorescence anisotropy was measured (Figure 19).

From the data, we can see that the dissociation constant (Kd) of Exo1 771-803 dramatically increased from 13.2 μM in the absence Zn$^{2+}$ of to 1.7 μM in the presence of 20 μM Zn$^{2+}$, which is around 10-fold enhancement. Therefore, we think that Zn$^{2+}$ enhances Exo1 C-terminus and PCNA interaction.

Additional fluorescence anisotropy experiment without triplicates using different concentrations of Zn$^{2+}$ shows that the dissociation constant of Exo1 C-terminus and PCNA interaction is Zn$^{2+}$ concentration dependent (Figure 20). When Zn$^{2+}$ concentration is only 1 μM, the enhancement is negligible. When Zn$^{2+}$ concentration is 500 μM, the Kd becomes only several nanomolar.
Figure 19: Fluorescein-labeled Exo1 771-803 and PCNA interaction in the presence and absence of Zn$^{2+}$. 10 nM fluorescein-labeled Exo1 771-803 was titrated with PCNA in the presence and absence of 20 μM Zn$^{2+}$ in buffer 25 mM HEPES-NaOH, pH 7.0; 90 mM NaCl; 10 mM KCl; 5 mM MgCl$_2$; 0.1% Glycerol; 1 mM TCEP and the fluorescence anisotropy was measured. The experiment was repeated in triplicates and the data was fit using the equation of one site binding equation accounting for ligand depletion. The $K_d$ of Exo1 771-803 and PCNA in the absence of Zn$^{2+}$ is 13.2 ± 3.4 μM and the $K_d$ of Exo1 771-803 and PCNA in the presence of 20 μM Zn$^{2+}$ is 1.7 ± 0.3 μM.
Figure 20: Fluorescein-labeled Exo1 771-803 and PCNA interaction in the presence and absence of different concentrations of Zn$^{2+}$. 10 nM fluorescein-labeled Exo1 771-803 was titrated with PCNA in the presence and absence of different concentrations of Zn$^{2+}$ in buffer 25 mM HEPES-NaOH, pH 7.0; 90 mM NaCl; 10 mM KCl; 5 mM MgCl$_2$; 0.1% Glycerol; 1 mM TCEP and the fluorescence anisotropy was measured. The experiment was done only once and the data was fit using the equation of one site binding equation accounting for ligand depletion. The data was plotted in log scale. The apparent $K_d$ of Exo1 771-803 and PCNA in the absence and in the presence of 20 μM, 100 μM, 500 μM Zn$^{2+}$ are 11.6 ± 5.2 μM, 1.2 ± 0.1 μM, 480 ± 40 nM and 60 ± 10 nM, respectively.
Moreover, we also performed size-exclusion chromatography experiment to see whether Zn$^{2+}$ enhances Exo1 C-terminus and PCNA interaction. Exo1 771-803 and PCNA does not co-migrate during size-exclusion in the absence of Zn$^{2+}$ (Figure 21A, read curve; Figure 21B, left penal). This is probably because that Exo1 771-803 and PCNA is a weak interaction based on the dissociation constant that we measured.

When there is 200 µM Zn$^{2+}$ present in Exo1 771-803 and PCNA before injection and 20 µM Zn$^{2+}$ present during size-exclusion, Exo1 771-803 and PCNA were able to co-migrate (Figure 21A, blue curve; Figure 21B, right penal). This evidence strongly supports that Zn$^{2+}$ enhances the interaction of Exo1 C-terminus and PCNA. It should also be mentioned that there is no aggregated protein peaks observed in the chromatographs.

Finally, in order to test whether this Zn$^{2+}$ coordination is a strong coordination or not, Exo1 771-803 and PCNA were incubated in the presence of 200 µM Zn$^{2+}$ before injection but no Zn$^{2+}$ was present during size-exclusion. It turned out that Exo1 771-803 failed to co-migrate with PCNA (Figure 21A, green curve; Figure 21B, middle penal) in the case. This result suggests that, although Zn$^{2+}$ enhances the interaction of Exo1 C-terminus and PCNA, the coordination of Zn$^{2+}$ is not very stable.
Figure 21: Size-exclusion chromatography of Exo1 771-803 and PCNA in the presence and absence of Zn$^{2+}$. 500 µL 50 µM PCNA (in terms of monomer) and 70 µM Exo1 771-803 were incubated in the presence and absence of 200 µM ZnCl$_2$ as indicated for 15 minutes at room temperature and injected into 25 mL Superdex200 column pre-equilibrated with SEC Buffer 25 mM HEPES-NaOH, pH 7.0; 90 mM NaCl; 10 mM KCl; 5 mM MgCl$_2$; 0.1% Glycerol; 1 mM TCEP in the presence and absence of 20 µM ZnCl$_2$ as indicated. (A) Chromatographs of Exo1 771-803 and PCNA during size-exclusion. (B) Elution fractions from size-exclusion applied to SDS-PAGE.
GST-pull down experiments also support Zn\(^{2+}\) enhances the interaction of Exo1 C-terminus and PCNA. In the absence and presence of up to 5 μM Zn\(^{2+}\), GST does not interact with PCNA (Figure 22 left penal lane 2-6). In the absence of Zn\(^{2+}\), GST-Exo1 705-803 binds to PCNA weakly (Figure 22 right panel lane 8). In the presence of increasing concentration of Zn\(^{2+}\), GST-Exo1 705-803 binds to PCNA much stronger (Figure 22 right panel lane 8-11).

Mutations of key Zn\(^{2+}\) binding residues, Exo1 N792, Exo1 E793 and PCNA H44, in the crystal structure of PCNA in complex with Exo1 C-terminus (Figure 18B lower panel) reduce the interaction of Exo1 C-terminus and PCNA. However, the Zn\(^{2+}\) enhancement effect on Exo1 C-terminus and PCNA interaction remains (data not shown). This result suggests that Zn\(^{2+}\) may also mediate the interaction of Exo1 and PCNA outside the canonical PIP-box shown in the crystal structure.
Figure 22: GST pull-down of GST-Exo1 705-803 and PCNA in the presence and absence of Zn²⁺. 0.5 μM GST or GST-Exo1 771-803 was incubated with 1 μM PCNA and pull-down by Glutathione Sepharose in the absence and presence of different concentrations of Zn²⁺ as indicated. The proteins bound to Glutathione Sepharose was analyzed by SDS-PAGE and stained with Commassie-blue.
To identify the key residues of Exo1 that are involved in Zn\(^{2+}\)-mediated interaction, we titrated Zn\(^{2+}\) into \(^{15}\)N-Exo1 771-803 and PCNA complex and collected the \(^{15}\)N-\(^1\)H HSQC spectra (Figure 23). Some peaks start to vanish when the ratio of \(^{15}\)N-Exo1 771-803-PCNA complex and Zn\(^{2+}\) becomes 1:2 and most of the peaks are vanished when the ratio becomes 1:4. There are two possibilities to this phenomenon. One of the explanations is that \(^{15}\)N-Exo1 771-803 forms large aggregates with other components in the solution. The other explanation is that the N-terminus of \(^{15}\)N-Exo1 771-803, which does not interact with PCNA in the absence of Zn\(^{2+}\), interacts with PCNA when Zn\(^{2+}\) is present. To date, we do not have evidence to support the former possibility. \(^{15}\)N-Exo1 771-803 and PCNA in the presence of Zn\(^{2+}\) did not form observable precipitations during the whole \(^{15}\)N-\(^1\)H HSQC experiment (data not shown). Size-exclusion chromatography experiment in the presence of low-concentration Zn\(^{2+}\) does not show aggregation peaks in the chromatographs (Figure 21). Small-angle X-ray Scattering (SAXS) data of Exo1 771-803 and PCNA complex in the presence of stoichiometric amount of Zn\(^{2+}\) did not shown evidence of protein aggregation (data not shown). Therefore, it is likely that the N-terminus of Exo1 771-803 are involved in the interaction with PCNA when Zn\(^{2+}\) is present.
Figure 23: $^{15}$N-$^1$H HSQC of $^{15}$N-Exo1 771-803 and PCNA complex titrated with Zn$^{2+}$. The $^{15}$N-$^1$H HSQC spectra of 100 μM $^{15}$N-Exo1 771-803 and PCNA in buffer 25 mM HEPES-NaOH, pH 7.0; 90 mM NaCl; 10 mM KCl; 5 mM MgCl$_2$; 0.1% Glycerol; 1 mM TCEP (blue) was titrated with 100 μM (orange), 200 μM (green) and 400 μM Zn$^{2+}$ (purple). The $^{15}$N-$^1$H HSQC spectra were collected and overlapped.
It should also be noted that Exo1 771-803 itself is also a metal-binding peptide. Zn²⁺ was titrated into ¹⁵N-Exo1 771-803 and the ¹⁵N-¹H HSQC spectra were collected (Figure 24). Exo1 773-777 and 781-784 peaks got shifted upon Zn²⁺ titration. These residues did not show perturbation upon PCNA titration in the absence of Zn²⁺ (Figure 13). However, when Zn²⁺ was titrated into ¹⁵N-Exo1 771-803 and PCNA complex, these peaks got vanished (Figure 23).
Figure 24: $^{15}$N-$^1$H HSQC of $^{15}$N-Exo1 771-803 titrated with Zn$^{2+}$. The $^{15}$N-$^1$H HSQC spectra of 100 μM $^{15}$N-Exo1 771-803 in buffer 25 mM HEPES-NaOH, pH 7.0; 90 mM NaCl; 10 mM KCl; 5 mM MgCl$_2$; 0.1% Glycerol; 1 mM TCEP (red) was titrated with 100 μM (orange), 200 μM (yellow), 400 μM Zn$^{2+}$ (green), 600 μM Zn$^{2+}$ (cyan) and 1000 μM Zn$^{2+}$ (blue). The $^{15}$N-$^1$H HSQC spectra were collected and overlapped.
5.3 Discussion

Zn\(^{2+}\) seems to be the metal cation specific to enhance Exo1 and PCNA interaction. All the experiments to test the Zn\(^{2+}\) effect to Exo1 and PCNA interaction were done in the presence of 5 mM MgCl\(_2\). Replacement of Zn\(^{2+}\) to Mg\(^{2+}\), Ca\(^{2+}\) and Fe\(^{2+}\) failed to show enhancement of Exo1 and PCNA interaction in GST pull-down assay (data not shown).

PCNA confers Exo1 processivity by direct interaction with Exo1 PIP-box [44]. Given Zn\(^{2+}\) enhances PCNA and Exo1 C-terminus interaction, it will be interesting to know whether Zn\(^{2+}\) has any impacts on the sensitivity and the extents of Exo1 processive cleavage in the presence PCNA.

Size-exclusion chromatography experiment suggests that the Zn\(^{2+}\) coordination by Exo1 and PCNA is not stable (Figure 21). This might provide flexibility for the tightly controlled Zn\(^{2+}\) transportation. If Zn\(^{2+}\) transportation to nucleus is a signal of DNA repair, Exo1 can be “activated” by PCNA immediately in the presence of Zn\(^{2+}\). When DNA is repaired and Zn\(^{2+}\) transportation signals disappear, Exo1 goes back to normal state.

One caveat about the Zn\(^{2+}\) enhanced PCNA and Exo1 interaction is that this enhancement is Zn\(^{2+}\) concentration dependent. Higher Zn\(^{2+}\) concentration causes more enhancement in K\(_d\). Zn\(^{2+}\) concentration lower than 1 μM is not likely to cause significant enhancement for Exo1 and PCNA interaction. Inside certain cellular compartment, if
Zn\textsuperscript{2+} concentration can never reach such concentration, the functional implications of Zn\textsuperscript{2+} on Exo1 discussed above might not be true in cell.

Finally, Exo1 and PCNA does not seem to be the only Exo1 interaction that Zn\textsuperscript{2+} enhances. We have evidence that Zn\textsuperscript{2+} also enhances Exo1 and MLH1-CTD interaction. Therefore, whether these Zn\textsuperscript{2+} enhanced Exo1 interactions are specific and really making biological implications should be carefully examined.
6. Materials and methods

6.1 Protein constructs

All the protein constructs used in this study were summarized in Table 1.

6.2 Site-directed mutagenesis

All the mutant constructs are generated using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacture’s protocol [45]. All the template constructs and primers used are listed in Table 2. All the mutant constructs are sequenced at GENEWIZ.
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### Table 2: Template and primers used for site-directed mutagenesis

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<th>Primer 2 (5’ to 3’)</th>
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6.3 Protein expression and purification

6.3.1 Exo1 1-352 WT and D173A

Expression

Plasmid was transformed into BL21-CodonPlus(DE3)-RIL competent cell (Agilent Technologies) and plated on LB plate with ampicillin and chloramphenicol. A single colony was picked and inoculated into 100 mL LB medium with ampicillin and chloramphenicol. The culture was shaken at 150 rpm overnight at 37 degree. 20 mL of the overnight culture was inoculated into 2 L LB medium with ampicillin and chloramphenicol. The culture was shaken at 150 rpm at 37 degree until OD600 reaches 0.6 to 0.8. Reduce the temperature to 15 degree for 15 minutes and induce the cell with 1 mM IPTG overnight. The E. coli cells were harvested and frozen at -80 degree for long-term storage.

Purification

The E. coli cell were thawed, re-suspended in IEX-A buffer 25 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 10 mM KCl; 1 mM EDTA; 5% Glycerol; 5 mM DTT and cracked using microfluidizer. The cell lysate was clarified by centrifugation and applied onto SP sepharose HP column (GE) pre-equilibrated with IEX-A buffer. The column was then washed with the same buffer to remove the unbound proteins and eluted with a gradient to IEX-B buffer 25 mM HEPES-NaOH, pH 7.5; 600 mM NaCl; 10 mM KCl; 1
mM EDTA; 5% Glycerol; 5 mM DTT in 10 column-volume. The elution fractions were checked by SDS-PAGE and the fractions containing corresponding Exo1 were pooled. The protein solution was then diluted with Dilution buffer 25 mM HEPES-NaOH, pH 7.5; 10 mM KCl; 1 mM EDTA; 5% Glycerol; 5 mM DTT to match the conductivity of IEX-A buffer and applied onto Q sepharose HP column followed by Heparin sepharose HP column pre-equilibrated with IEX-A buffer. The columns were then washed with the same buffer to remove the unbound proteins. The Q sepharose HP column was removed and the Heparin sepharose HP was eluted with a gradient to IEX-B buffer in 15 column-volume. The elution fractions were checked by SDS-PAGE and the fractions containing corresponding Exo1 were pooled. The protein solution was then concentrated and applied onto a 120mL Superdex200 column pre-equilibrated with GF buffer 25 mM HEPES-NaOH, pH 7.5; 125 mM NaCl; 10 mM KCl; 0.1% Glycerol; 1 mM TCEP. The fractions from the column were checked by SDS-PAGE and the fractions containing corresponding Exo1 were pooled, concentrated to 10 to 20 mg/mL, frozen using liquid nitrogen and stored at -80 degree. The yield is at least 5 mg per liter growth.

6.3.2 Exo1 1-450 and its variants

The expression and purification method is the same to Exo1 1-352 WT method, except that the protein purity is sufficient before running Superdex200 column. Therefore, the protein was pooled after Heparin sepharose HP column, concentrated to
10 to 20 mg/mL, frozen using liquid nitrogen and stored at -80 degree. The yield is at least 10 mg per liter growth.

6.3.3 Exo1 452-514, 452-846, 705-846, Exo1 705-803, Exo1 765-803, Exo1 771-803 and their variants

Expression

Plasmid was transformed into BL21-CodonPlus(DE3)-RIL competent cell (Agilent Technologies) and plated on LB plate with kanamycin and chloramphenicol. A single colony was picked and inoculated into 100 mL LB medium with kanamycin and chloramphenicol. The culture was shaken at 150 rpm overnight at 37 degree. 20 mL of the overnight culture was inoculated into 2 L LB medium with kanamycin and chloramphenicol. The culture was shaken at 150 rpm at 37 degree until OD600 reaches 0.6 to 0.8. Reduce the temperature to 18 degree for 15 minutes and induce the cell with 1 mM IPTG overnight. The E. coli cells were harvested and frozen at -80 degree for long-term storage.

For 15N-labeled Exo1 C-terminus, the 20 mL of the overnight LB culture was inoculated into 1 L M9 medium (1 L M9 Medium: 6.0 g Na2HPO4, 3.0 g KH2PO4, 0.5 g NaCl, 1.0 g 15NH4Cl supplemented with 2 mL 1 M MgSO4, 1 mL 0.1 M CaCl2, 0.5 mL 0.1% Thiamine and 20 mL 20% D-Glucose) with kanamycin and chloramphenicol. The culture was shaken at 150 rpm at 37 degree until OD600 reaches 0.6 to 0.8. Reduce the
temperature to 18 degree for 15 minutes and induce the cell with 1 mM IPTG overnight. The E. coli cells were harvested and frozen at -80 degree for long-term storage.

For $^{15}$N-Lysine-labeled Exo1 C-terminus, the 20 mL of the overnight LB culture was inoculated into 1 L non-isotope M9 medium (1 L M9 Medium: 6.0 g Na$_2$HPO$_4$, 3.0 g KH$_2$PO$_4$, 0.5 g NaCl, 1.0 g NH$_4$Cl supplemented with 2 mL 1 M MgSO$_4$, 1 mL 0.1 M CaCl$_2$, 0.5 mL 0.1% Thiamine and 20 mL 20% D-Glucose) with kanamycin and chloramphenicol. The culture was shaken at 150 rpm at 37 degree until OD600 reaches 0.6 to 0.8. Reduce the temperature to 18 degree for 15 minutes, add 0.1 g $^{15}$N-lysine (L-lysine:2HCl, alpha-15N NLM-153-0.25, Cambridge Isotope Laboratories, Inc) and induce the cell with 1 mM IPTG overnight. The E. coli cells were harvested and frozen at -80 degree for long-term storage.

For $^{15}$N,$^{13}$C-labeled Exo1 C-terminus, the expression protocol is almost the same to $^{15}$N-labeled Exo1 C-terminus, except that we use $^{13}$C-D-Glucose in M9 Medium instead of normal D-Glucose.

**Purification**

The E. coli cell were thawed, re-suspended in Lysis buffer 50 mM HEPES-NaOH, pH 8.0; 300 mM NaCl; 10 mM KCl; 10 mM Imidazole; 5% Glycerol; 5 mM BME and cracked using microfluidizer. The cell lysate was clarified by centrifugation and applied onto Ni-NTA Agarose column (Qiagen) pre-equilibrated with Lysis buffer. The column
was then washed with Wash buffer 50 mM HEPES-NaOH, pH 8.0; 300 mM NaCl; 10mM KCl; 20 mM Imidazole; 5% Glycerol; 5 mM BME to remove the unbound proteins and elute with Elution buffer 50 mM HEPES-NaOH, pH 8.0; 100 mM NaCl; 10mM KCl; 400 mM Imidazole; 5% Glycerol; 5 mM BME. The amount of NusA-tagged Exo1 was estimated by measuring OD280 using NanoDrop (Thermo Scientific). The protein solution was supplemented with TEV protease with a ratio of 1:200 to Nus-tagged Exo1, incubated at room temperature for 6 hours followed by 4 degree overnight.

The protein solution was then diluted with Dilution buffer 25 mM HEPES-NaOH, pH 7.5; 10 mM KCl; 1 mM EDTA; 5% Glycerol; 5 mM DTT to match the conductivity of IEX-A buffer 25 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 1 mM EDTA; 1 mM TCEP and applied onto Q sepharose HP column followed by SP sepharose HP column pre-equilibrated with IEX-A buffer. The Q sepharose HP column was removed and the SP sepharose HP was eluted with a gradient to IEX-B buffer 25 mM HEPES-NaOH, pH 7.5; 600 mM NaCl; 1 mM EDTA; 1 mM TCEP in 15 column-volume. The elution fractions were checked by SDS-PAGE and the fractions containing corresponding Exo1 were pooled. The purity of protein is usually greater than 99% based on the coomassie-stained gel.

The yield is at least 0.5 mmol per liter growth for each of the Exo1 C-terminus protein.
The yields are at least 0.5 mmol per liter growth for each of the Exo1 C-terminus protein and the yields of all isotope labeled Exo1 C-terminus proteins expressed from M9 medium are almost the same to unlabeled Exo1 C-terminus proteins expressed from LB medium.

6.3.4 PCNA WT and H44A

T7-hPCNA WT plasmid was obtained from Modrich Lab at Duke University and its source is at Stillman Lab at Cold Spring Harbor Laboratory [46]. PCNA H44A mutant was generated using QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) based on PCNA WT construct. PCNA WT and PCNA H44A purification protocol are the same.

Expression

Plasmid was transformed into Rosetta™ 2(DE3) Singles™ Competent Cells (EMD-Millipore) and plated on LB plate with carbenicillin. A single colony was picked and inoculated into 100 mL LB medium with carbenicillin. The culture was shaken at 150 rpm overnight at 37 degree. 20 mL of the overnight culture was inoculated into 1 L LB medium with carbenicillin. The culture was shaken at 150 rpm at 37 degree until OD600 reaches 0.8. Induce the cell with 1mM IPTG for 4 hours at 37 degree. The E. coli cells were then harvested and frozen at -80 degree for long-term storage.

Purification
The E. coli cell were thawed, re-suspended in IEX-A Buffer 25 mM Tris-HCl, pH 7.4; 200 mM NaCl; 1 mM EDTA; 0.01% NP-40; 10% Glycerol; 1mM DTT containing SIGMAFAST™ Protease Inhibitor Cocktail Tablets, EDTA-Free (Sigma-Aldrich) and cracked using microfluidizer. The cell lysate was clarified by centrifugation and applied onto HiTrap Q sepharose HP column pre-equilibrated with IEX-A Buffer. The column was then washed sequentially with 10 column volumes of IEX-A Buffer and was eluted with a gradient to IEX-B buffer 25 mM Tris-HCl, pH 7.4; 700 mM NaCl; 1 mM EDTA; 0.01% NP-40; 10% Glycerol; 1mM DTT in 15 column-volume. The elution fractions were checked by SDS-PAGE and the fractions containing corresponding Exo1 were pooled. The protein solution was then supplemented with 3.5 M ammonium sulfate solution to match the conductivity of Phenyl-A Buffer 25 mM HEPES-NaOH, pH 7.4; 1.2 M NaCl; 0.01% NP-40; 1 mM DTT.

The elution fractions were checked by SDS-PAGE and the fractions containing corresponding Exo1 were pooled. The protein solution was then diluted with Dilution buffer 25 mM HEPES-NaOH, pH 7.5; 10 mM KCl; 1 mM EDTA; 5% Glycerol; 5 mM DTT to match the conductivity of IEX-A buffer and applied onto Q sepharose HP column followed by Heparin sepharose HP column pre-equilibrated with IEX-A buffer. The columns were then washed with the same buffer to remove the unbound proteins. The Q sepharose HP column was removed and the Heparin sepharose HP was eluted with a
gradient to IEX-B buffer in 15 column-volume. The elution fractions were checked by SDS-PAGE and the fractions containing corresponding Exo1 were pooled. The protein solution was then concentrated and applied onto a 120mL Superdex200 column pre-equilibrated with GF buffer 25 mM HEPES-NaOH, pH 7.5; 125 mM NaCl; 10 mM KCl; 0.1% Glycerol; 1 mM TCEP. The fractions from the column were checked by SDS-PAGE and the fractions containing corresponding Exo1 were pooled, concentrated to 10 to 20 mg/mL, frozen using liquid nitrogen and stored at -80 degree. The yield is around 10 mg per liter growth.

6.3.5 RPA

RPA WT plasmid was obtained from Modrich Lab at Duke University and it was originally cloned by Wold Lab at University of Iowa [47].

Expression

Plasmid was transformed into One Shot® BL21(DE3) Chemically Competent Cell (ThermoFisher Scientific) and plated on LB plate with ampicillin. A single colony was picked and inoculated into 100 mL LB medium with ampicillin. The culture was shaken at 150 rpm overnight at 37 degree. 20 mL of the overnight culture was inoculated into 1 L TB medium with ampicillin. The culture was shaken at 150 rpm at 37 degree until OD600 reaches 0.6 to 0.8. Induce the cell with 0.3 mM IPTG for 2.5 hours at 37 degree. The E. coli cells were then harvested and frozen at -80 degree for long-term storage.
Purification

The E. coli cell were thawed, re-suspended in HI Buffer 30 mM HEPES-NaOH, pH 7.8; 0.25 mM EDTA; 10% Glycerol; 0.01% (v/v) NP-40 containing 1 mM DTT, 1 mM PMSF and SIGMAFAST™ Protease Inhibitor Cocktail Tablets, EDTA-Free (Sigma-Aldrich) and cracked using microfluidizer. The cell lysate was clarified by centrifugation and applied onto HiTrap BLUE HP column pre-equilibrated with HI Buffer containing 50 mM KCl and 1 mM DTT. The column was then washed sequentially with 10 column volumes of HI Buffer containing 50 mM KCl, 0.8 M KCl, 0.5 M NaSCN and 1.5 M NaSCN with 1 mM DTT, respectively. RPA was eluted in the 1.5 M NaSCN Wash fraction.

Immediately dialyze the 1.5 M NaSCN Wash fraction against HI Buffer containing 1.5 M NaCl, 20% (w/v) sucrose and 4 mM DTT for 4 hours. Then dialyze the protein against IEX-A Buffer 25 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 10% Glycerol and 1 mM TCEP overnight. Remove the precipitated protein if necessary by centrifugation and load the protein solution onto HiTrap Heparin HP column pre-equilibrated with IEX-A Buffer. The column was washed with IEX-A Buffer for at least 5 column volume and eluted with a gradient to IEX-B Buffer 25 mM HEPES-NaOH, pH 7.5; 600 mM NaCl; 10% Glycerol and 1 mM TCEP in 15 column volume. The elution fractions were checked by SDS-PAGE, the fractions containing corresponding RPA were
pooled, concentrated, frozen in liquid nitrogen and stored at -80 degree. The yield is around 2 mg per liter growth.

6.3.6 MLH1-CTD

Expression

Plasmid was transformed into BL21-CodonPlus(DE3)-RIL competent cell (Agilent Technologies) and plated on LB plate with streptomycin and chloramphenicol. A single colony was picked and inoculated into 100 mL LB medium with streptomycin and chloramphenicol. The culture was shaken at 150 rpm overnight at 37 degree. 20 mL of the overnight culture was inoculated into 2 L LB medium with streptomycin and chloramphenicol. The culture was shaken at 150 rpm at 37 degree until OD600 reaches 0.6 to 0.8. Reduce the temperature to 18 degree for 15 minutes and induce the cell with 1 mM IPTG overnight. The E. coli cells were harvested and frozen at -80 degree for long-term storage.

Purification

The E. coli cell were thawed, re-suspended in Lysis buffer 50 mM HEPES-NaOH, pH 7.5; 250 mM NaCl; 10mM KCl; 10mM MgCl₂; 10 mM Imidazole; 10% Glycerol; 5 mM BME and cracked using microfluidizer. The cell lysate was clarified by centrifugation and applied onto Ni-NTA Agarose column (Qiagen) pre-equilibrated with Lysis buffer. The column was then washed with Wash buffer 50 mM HEPES-NaOH, pH 7.5; 250 mM
NaCl; 10mM KCl; 10mM MgCl₂; 25 mM Imidazole; 10% Glycerol; 5 mM BME to remove the unbound proteins and elute with Elution buffer 50 mM HEPES-NaOH, pH 7.5; 250 mM NaCl; 10mM KCl; 10mM MgCl₂; 300 mM Imidazole; 10% Glycerol; 5 mM BME. The amount of His-tagged MLH1-CTD was estimated by measuring OD280 using NanoDrop (Thermo Scientific). The protein solution was supplemented with TEV protease with a ratio of 1:200 to His-tagged MLH1-CTD while dialyzed against 1.5 L Dialysis buffer 50 mM HEPES-NaOH, pH 7.5; 250 mM NaCl; 10 mM KCl; 10 mM NaCl; 10mM MgCl₂; 5% Glycerol; 10 mM BME at 4 degree overnight.

Pour the protein solution onto cleaned Ni-NTA column pre-equilibrated with Wash buffer and collect the FT. Wash the column with more Wash buffer to collect more tag-cleaved protein. The tag-cleaved protein solution was then diluted with Dilution buffer 50mM HEPES-NaOH, pH 7.5; 10 mM KCl; 10 mM MgCl₂; 1 mM EDTA; 5% Glycerol; 1 mM DTT to match the conductivity of IEX-A buffer 50 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 10 mM KCl; 10 mM MgCl₂; 1 mM EDTA; 5% Glycerol; 1 mM DTT and applied onto Q sepharose HP column pre-equilibrated with IEX-A buffer. The Q sepharose HP column was then eluted with a gradient to IEX-B buffer 50 mM HEPES-NaOH, pH 7.5; 600 mM NaCl; 10 mM KCl; 10 mM MgCl₂; 1 mM EDTA; 5% Glycerol; 1 mM DTT in 15 column-volume. The elution fractions were checked by SDS-PAGE and the fractions containing corresponding MLH1-CTD were pooled. The protein solution
was then concentrated and applied onto a 120mL Superdex200 column pre-equilibrated with GF buffer SEC buffer 50 mM HEPES-NaOH, pH 7.5; 500 mM NaCl; 10 mM KCl; 10 mM MgCl₂; 0.1 mM EDTA; 0.1% Glycerol; 1 mM DTT. The fractions from the column were checked by SDS-PAGE and the fractions containing corresponding MLH1-CTD were pooled, concentrated to 10 to 20 mg/mL, frozen using liquid nitrogen and stored at -80 degree. The yield is at least 10 mg per liter growth.

6.4 Nuclear magnetic resonance (NMR) spectroscopy in collaboration with Pei Zhou Lab

6.4.1 Protein sample preparation

All the isotope labeled protein expression and purifications were described in the previous sections. Before NMR experiment, all the proteins were dialyzed against certain buffer extensively using Slide-A-Lyzer™ MINI Dialysis Devices (ThermoFisher Scientific). The proteins were then mixed and supplemented with 10% D₂O before experiment.

6.4.2 ¹⁵N-¹H HSQC experiment

All the ¹⁵N-¹H HSQC spectra of samples specified were collected by Dr. Qinglin Wu at Pei Zhou Lab on Bruker 600MHz/700MHz and Agilent 800MHz NMR spectrometers at 25 degree using the standard setup reported by Schleucher and his co-workers in 1994 [48]. All the data were processed by Dr. Qinglin Wu at Pei Zhou Lab using NMRPipe [49] and analyzed by Lei Mao using SPARKY [50].
6.4.3 Protein sequence assignment

400 μL 1.25 mM $^{15}$N,$^{13}$C-labeled Exo1 771-803 in NMR Buffer 25 mM HEPES-NaOH, pH 7.5; 90 mM NaCl; 10 mM KCl; 5mM MgCl$_2$; 0.1% Glycerol; 1 mM TCEP is used in all the NMR triple resonance experiments for Exo1 771-803 protein backbone assignment. HA(CACO)NH, HA(CA)NH, HN(CO)CA, HNCA, HN(COCA)CB, HN(CA)CB, HN(CA)CO, HNCO spectra were obtained on Bruker 700MHz spectrometer at 25 degree. These four pairs of experiments were carried out by sparsely sampled scheme and processed by NMRPipe [49] and SCRUB [51]. All the data were collected by Dr. Qinglin Wu at Pei Zhou Lab and analyzed by Lei Mao using SPARKY [50]. The sequence assignment analysis was conducted by Lei Mao using PACES (Pei Zhou Lab, Duke University) and confirmed by Dr. Pei Zhou at Pei Zhou Lab (Figure 25).
Figure 25: $^{15}$N-$^1$H HSQC Peak assignment of Exo1 771-803.
6.5 Fluorescence anisotropy

6.5.1 Peptide labeling

Exo1 629-803, Exo1 705-803 and Exo1 705-846 were labeled with fluorescein on the cysteine.

Exo1 C-terminus proteins were dialyzed against 300 mL Reaction Buffer (20 mM PBS, pH 7.2; 150 mM NaCl) for one hour three times using a 7 KDa Slide-A-Lyzer™ MINI Dialysis Devices (ThermoFisher Scientific 69560). Prepare fresh 1 mM fluorescein-5-maleimide (ThermoFisher Scientific 62245) solution in Reaction Buffer from powder. Mix the protein and dye solution with a cysteine/dye ratio of 1:2, 1:4 and 1:8 and react for 40 minutes at room temperature in dark. Stop the reaction by supplementing DTT and incubate at room temperature for another 30 minutes. Pour the reaction product onto self-packed small disposable SP sepharose column pre-equilibrated with IEX-A Buffer 25 mM HEPES-NaOH, pH 7.5; 125 mM NaCl; 10 mM KCl; 0.1% Glycerol; 1 mM TCEP and wash the column with IEX-A Buffer extensively to remove the unbound free dye. Elute the labeled protein from the column with IEX-B Buffer 25 mM HEPES-NaOH, pH 7.5; 600 mM NaCl; 10 mM KCl; 0.1% Glycerol; 1 mM TCEP. Run SDS-PAGE to check the labeling and purification. Measure the protein concentration and the number of fluorophores labeled on the protein using the manufacture’s protocol [52].
For some of the Exo1 C-terminus proteins which do not have intrinsic cysteines, such as Exo1 771-803 and Exo1 765-803, we labeled these proteins on its N-terminal amine.

Exo1 C-terminus proteins were dialyzed against 300 mL Reaction Buffer (25 mM HEPES-NaOH, pH 7.0) for one hour three times using a 7 KDa Slide-A-Lyzer™ MINI Dialysis Devices (ThermoFisher Scientific 69560). Prepare fresh 1 mM NHS-Fluorescein (ThermoFisher Scientific 46410) solution in Reaction Buffer from powder. Mix the protein and dye solution with a protein/dye ratio of 1:2 and react for one hour at room temperature in dark. Stop the reaction by supplementing Tris-HCl solution and incubate at room temperature for another 10 minutes. Pour the reaction product onto self-packed small disposable SP sepharose column pre-equilibrated with IEX-A Buffer 25 mM HEPES-NaOH, pH 7.5; 125 mM NaCl; 10 mM KCl; 0.1% Glycerol; 1 mM TCEP and wash the column with IEX-A Buffer extensively to remove the unbound free dye. Elute the labeled protein from the column with IEX-B Buffer 25 mM HEPES-NaOH, pH 7.5; 600 mM NaCl; 10 mM KCl; 0.1% Glycerol; 1 mM TCEP. Run SDS-PAGE to check the labeling and purification. Measure the protein concentration and the number of fluorophores labeled on the protein using the manufacture’s protocol [53].

Specifically for Exo1 629-803 labeling reaction, 10 μM Exo1 629-803 are incubated with 80 μM (Batch 1), 160 μM (Batch 2) and 320 μM (Batch 3) fluorescein-5-maleimide in
500 μL Reaction Buffer, respectively (Figure 26A). For Exo1 705-803 labeling reaction, 10 μM Exo1 705-803 are incubated with 40 μM (Batch 4), 80 μM (Batch 5) and 160 μM (Batch 6) fluorescein-5-maleimide in 500 μL Reaction Buffer, respectively (Figure 26B). After the ligand labeling and purification, the labeled ligand was tested interaction with MLH1-CTD. All the labeled ligands showed increased fluorescence anisotropy upon MLH1-CTD titration. Exo1 629-803 from Batch 2 and Exo1 705-803 from Batch 5 was chosen to be used for all the interaction studies.
Figure 26: Fluorescence labeling of Exo1 629-803 and Exo1 705-803. Protein samples from 20 minutes in the reaction, 40 minutes in the reactions, wash fraction from purification and elution fraction from purification in Exo1 629-803 labeling Batch 1, 2 and 3, and Exo1 705-803 labeling Batch 4, 5 and 6 are applied to SDS-PAGE. After SDS-PAGE, the gel was excited by UV and the image was captured (right panel) followed by staining with Commassie (left panel). (A) Fluorescence labeling of Exo1 629-803. (B) Fluorescence labeling of Exo1 705-803.
6.5.2 Fluorescence anisotropy binding assay

Nanomolar Fluorophore-labeled Exo1 C-terminus was titrated with protein partners in FA Buffer 25 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 1 mM TCEP and the fluorescence intensity and fluorescence anisotropy was measured on Beacon 2000 (PanVera).

6.5.3 Data analysis

The binding curve was fit in GraphPad 6.0 using the self-programmed equation of one site binding equation accounting for ligand depletion [54]. Namely, the equation of one site binding equation accounting for ligand depletion is:

Equation 1: One site binding equation accounting for ligand depletion

\[
B = \frac{R_T + L_T + K_d - \sqrt{(R_T + L_T + K_d)^2 - 4R_T L_T}}{2}
\]

Where B is the concentration of receptor-bounded ligand, \(R_T\) is the total concentration of receptor, \(L_T\) is the total concentration of ligand, \(K_d\) is the dissociation constant remains to fit.

The competition binding curve was fit in GraphPad 6.0 using the self-programmed equation of one site binding equation with the presence of inhibitor accounting for ligand/inhibitor depletion [55]. Namely, the equation of one site binding equation with the presence of inhibitor accounting for ligand/inhibitor depletion is:
Equation 2: One site binding equation with the presence of inhibitor accounting for ligand/inhibitor depletion

\[
B = \frac{(R_T + L_T + K_d + \frac{K_d}{K_i} I) - \sqrt{(R_T + L_T + K_d + \frac{K_d}{K_i} I)^2 - 4R_T L_T}}{2}
\]

Where B is the concentration of receptor-bounded ligand, \( R_T \) is the total concentration of receptor, \( L_T \) is the total concentration of ligand, \( K_d \) is the dissociation constant of ligand and receptor, \( K_i \) is the dissociation constant of inhibitor and receptor, and I is the total concentration of inhibitor.

6.6 Pull-down experiment

0.5 μM GST or GST-tagged protein and other proteins are incubated with 12.5 μL Glutathione Sepharose beads (GE Healthcare) in 250 μL Binding Buffer 25 mM HEPES-NaOH, pH 7.5; 90 mM NaCl; 10 mM KCl; 5mM MgCl₂, 0.1% Glycerol; 1 mM TCEP in a 500 μL tube at room temperature while rotating for one hour. The beads were spun down and washed with 400 μL Binding Buffer three times. Finally, the beads were supplemented with 25 μL 1X Laemmli Sample Buffer (Bio-rad) and boiled for 5 minutes. The beads were spun down and the supernatant was taken for SDS-PAGE analysis.

6.7 Far-western analysis

Picomole proteins was applied onto the Nitrocellulose Membrane (Bio-rad). The membrane was then dried at room temperature for 5 minutes followed by blocking
using Blocking Buffer (1X TBST Buffer with 5% milk) for one hour. 0.4 μM protein of interest was then added into the Blocking Buffer. The membrane was transfer to 4 degree and incubated for 3.5 hours. The Blocking Buffer was then removed and the membrane was washed with 1XTBST for 5 minutes three times. The membrane was incubated with primary antibody in Blocking Buffer either at room temperature for one hour or at 4 degree overnight. The Blocking Buffer was then removed and the membrane was washed with 1XTBST for 5 minutes three times. The membrane was incubated with corresponding secondary antibody (alkaline phosphatase conjugated) in Blocking Buffer at room temperature for one hour. The Blocking Buffer was then removed and the membrane was washed with 1XTBST for 5 minutes three times. Finally, the blot was visualized using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega).

A Far-western analysis protocol variant is that instead of applying proteins directly onto the nitrocellulose membrane, the proteins were separated using SDS-PAGE and transferred to nitrocellulose membrane using standard transfer protocol of Western Blot. The proteins transferred has to be renatured before the following Far-western analysis steps described above [56]. Basically, the membrane was treated with 8 M urea and 1% BME in FW buffer 20 mM Tris–HCl, pH 7.5; 60 mM NaCl; 10 mM MgCl₂; 0.1 mM EDTA; 5% glycerol and 0.02% NP-40. Proteins adsorbed to the membrane were
renatured by incubation in 10 sequential 2-fold dilutions of urea in FW buffer. Incubate the membrane in each dilution for 5 minutes.

6.8 Analytical size-exclusion chromatography

Protein or proteins were prepared and incubated for 15 minutes. 500 μL protein solution was injected into a 15 mL superdex200 size-exclusion column (GE Healthcare). The chromatograph was recorded and the elution fractions were collected for SDS-PAGE analysis.

6.9 Isothermal titration calorimetry (ITC) Experiment

Before ITC experiment, all the proteins were dialyzed against certain buffer using Slide-A-Lyzer™ MINI Dialysis Devices (ThermoFisher Scientific). The proteins were titrated in a VP-ITC or ITC-200 (GE Healthcare) instrument according to the manufacture’s protocol.

6.10 Mismatch-provoked excision assay

The mismatch-provoked assay was conducted according to the protocol published by Modrich Lab [17]. Briefly, mismatch-provoked assay substrate was prepared from f1MR phage as described previously by Jochen Genschel from Modrich Lab [16]. The protein components were diluted as necessary into 25 mM HEPES-KOH, pH 7.6; 200 mM KCl; 10% Glycerol; 1 mM DTT; 2 mg/mL BSA. Mismatch-provoked reaction with purified proteins were assembled by mixing proteins indicated in 10 μL
Dilution Buffer and 10 μL of 40 mM Tris-HCl, pH 7.6; 3 mM ATP; 2 mM glutathione; 10 mM MgCl₂; 0.1 mg/mL BSA and 10 ng/μL DNA substrate. The reaction was incubated at 37 degree for 5 minutes. Quickly stop the reaction by adding 70 μL of Stop Buffer 7 mM Tris-HCl, pH 7.6; 0.6 mg/mL glycogen; 0.18% SDS. Add 30 μL H₂O and 50 μL to extract DNA for 5 minutes. The mixture was spun at maximum speed for 5 minutes and the supernatant above the phenol layer was taken to 10 μL 3M NaOAc and 300 μL EtOH. Ethanol precipitate DNA for 30 minutes at room temperature and solution was spun at maximum speed at room temperature for 20 minutes. The supernatant was then removed and the DNA pellet was washed with 1 mL 70% EtOH for 5 minutes twice. The DNA pellet was then dried on bench at room temperature for 5 minutes. 10 μL H₂O and 3 μL restriction enzyme stock (0.7 U/μL NheI, 0.7 U/μL ClaI, 1mg/mL BSA in NEB Buffer 2 (New England BioLabs Inc)). The DNA was digested at 37 degree for one hour. The DNA products were analyzed on 1% TAE agarose gel and stained with 2 μg/mL EtBr solution.
7 References


