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The DHX9 helicase interacts with human DNA Polymerase δ4 and stimulates its activity in D-loop extension synthesis

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Abstract

The extension of the invading strand within a displacement loop (D-loop) is a key step in homology directed repair (HDR) of doubled stranded DNA breaks. The primary goal of these studies was to test the hypotheses that 1) D-loop extension by human DNA polymerase δ4 (Pol δ4) is facilitated by DHX9, a 3' to 5' motor helicase, which acts to unwind the leading edge of the D-loop, and 2) the recruitment of DHX9 is mediated by direct protein-protein interactions between DHX9 and Pol δ4 and/or PCNA. DNA synthesis by Pol δ4 was analyzed in a reconstitution assay by the extension of a 93mer oligonucleotide inserted into a plasmid to form a D-loop. Product formation by Pol δ4 was monitored by incorporation of [α-^{32}P]dNTPs into the 93mer primer followed by denaturing gel electrophoresis. The results showed that DHX9 strongly stimulated Pol δ4 mediated D-loop extension. Direct interactions of DHX9 with PCNA, the p125 and the p12 subunits of Pol δ4 were demonstrated by pull-down assays with purified proteins. These data support the hypothesis that DHX9 helicase is recruited by Pol δ4/PCNA to facilitate D-loop synthesis in HDR, and is a participant in cellular HDR. The involvement of DHX9 in HDR represents an important addition to its multiple cellular roles. Such helicase-polymerase interactions may represent an important aspect of the mechanisms involved in D-loop primer extension synthesis in HDR.

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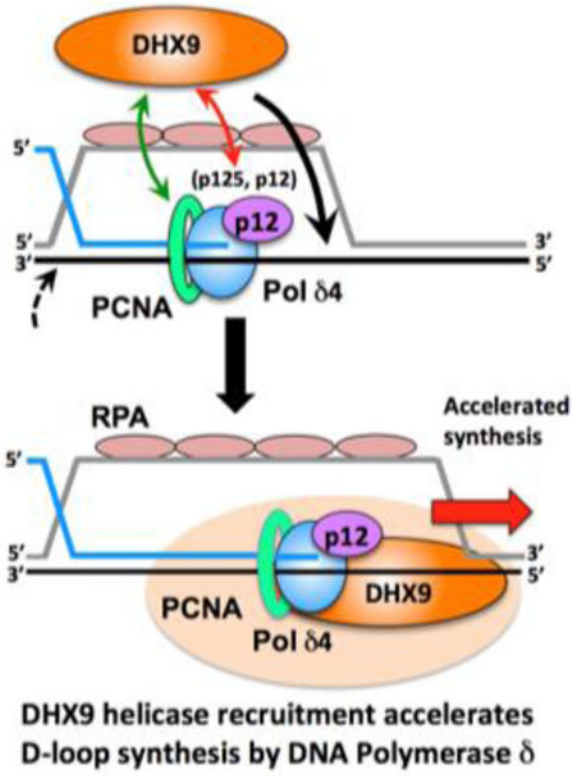
The authors declare that there are no conflicts of interest.

Graphical Abstract



Keywords

DHX9 helicase; DNA Polymerase δ; D-loop synthesis; DNA double-strand break repair; Homologous recombination; Helicase-polymerase interaction

1. Introduction.

The replication of the genome by exquisitely accurate DNA polymerases is critical for the maintenance of genomic stability. In eukaryotes, DNA polymerases Pol δ [1–4] and Pol ε [5–7] replicate genomic DNA. There is a “division of labor” in that Pol δ is primarily involved in lagging strand synthesis, while Pol ε is involved in leading strand synthesis [5]. Pol δ also has major roles in gap filling in DNA repair processes (NER, BER, MMR) and in D-loop extension during HDR (homology directed repair) of DSBs (double-strand breaks) [1–4,8].

Extension of the invading strand in the D-loop is a key step in HDR of DSBs. Once a D-loop is created, the 3’ end of the invading strand forms what is a primer on a template strand. The extension of this primer in the D-loop by DNA polymerases has been variously referred to as displacement synthesis, homology associated DNA synthesis, or simply D-loop extension. D-loop extension is involved in several HDR subpathways: synthesis dependent strand annealing (SDSA), double-Holliday Junction (dHJ) repair [9–15], and Break-induced replication (BIR), which is utilized for repair of single-ended DSBs [16,17].

BIR is also involved in responses to replication stress from a number of sources that include stalled replication forks, under-replicated DNA at common fragile sites (CFS) [18], G4 quadruplex structures and damaged telomeres [12,19,20]. This convergence through multiple avenues of replication stress that can cause potential harm to genomic stability is intimately connected to HDR [21].

It has been generally thought that Pol δ (rather than Pol ε) is the replication polymerase involved in HDR. Studies in yeast have shown that Pol δ (yPol δ), rather than Pol ε, is the dominant polymerase involved in D-loop extension [9,11,22]. This is consistent with the observations that yeast Pol δ, but not Pol ε, exhibits the ability for strand displacement [23] which is involved in the process of D-loop extension [9].

In human cells, there are two physiologically important forms of Pol δ. Human DNA polymerase δ is a heterotetrameric enzyme (Pol δ4) composed of the catalytic subunit (p125), and three non-catalytic subunits, p50 and p68, and p12. These four subunits are encoded by the *POLD1*, *POLD2*, *POLD3,* and *POLD4* genes, respectively. The p12 subunit plays an important role in the regulation of human Pol δ activity [1–4]. Its transient degradation leaves behind an intact trimeric form of Pol δ (Pol δ3) consisting of the p125, p50 and p68 subunits. This occurs in response to UV, alkylating agents and replication stress under the control of ATR [24], as well during the cell cycle, where p12 is degraded on entry to S-phase and is restored during G2/M [25,26]. Pol δ3 and Pol δ4 exhibit different functional capabilities: Pol δ3 is an intrinsically more accurate polymerase than Pol δ4. It is adapted for Okazaki fragment processing in concert with Fen1 [27], and for gap-filling in excision repair, while Pol δ4 is adapted for synthesis through more complex templates and is required for HDR [1–4,28].

The CryoEM structure of the Pol δ4/PCNA complex shows that the C-terminal region of p12 (residues 42–107) forms a cuboidal structure that is wedged between p125 and p50, and allows structural insights into the basis for the differential functional characteristics of Pol δ4 and Pol δ3 [29]. The N-terminal 41 residues are not resolved in the p12 structure, indicating conformational flexibility. Its orbit of movement makes it feasible for the N-terminally located PIP box of p12 (residues 4–11) [30] to interact with PCNA [29]. The roles of Pol δ4 are less well defined, but it has been established through studies of CRISPR/Cas9 knockout of the *POLD4* gene in cultured cells that it is required for HDR [1,28]. The p12-knockout cells were HDR-deficient as they exhibited a ca. 60% reduction in repair of I-SceI induced DSBs in the DR-GFP plasmid reporter assay, and were highly sensitized to DNA damaging agents and PARP inhibitors [1,28].

Studies of human Pol δ4 in a plasmid based D-loop assay system have demonstrated that it is capable of D-loop extension [31,32], but the Pol δ3 form has not been studied in relation to its ability to perform D-loop extension. The inference from the p12-KO studies is that Pol δ3 is unable or less able to perform D-loop extension. Pol δ3 has little or no strand displacement activity [27], and this is a plausible basis for predicting a lack of function in D-loop synthesis [27]. Also, strand displacement by Pol δ4 is much slower than synthesis on an unimpeded template [27]. We have suggested that the D-loop synthesis by Pol δ4 might be slow and be facilitated by helicases [1]. Studies of yeast Pol δ (yPol δ) using a plasmid

based D-loop reconstitution assay [10,33] demonstrated a requirement for Pif1, a 5' to 3' helicase, for efficient D-loop synthesis [22,34]. Pif1 binds to PCNA and this interaction is critical for its recruitment and participation in BIR [10,22,33,34]. Studies of human Pol δ4 in a plasmid based D-loop assay have demonstrated that it is capable of D-loop extension [31,32], but thus far no biochemical evidence for helicase participation in this process in a human reconstitution system has been reported.

In the hypothesis we proposed for a role of helicases in facilitating D-loop synthesis we emphasized the importance of their abilities to interact with Pol δ4 and/or PCNA [1]. Such protein-protein interactions would drive the affinity of the helicase for recruitment to the leading edge of the D-loop, bearing in mind that helicases have a broad and complex repertoire of activities that could also act to disassemble the D-loop to terminate D-loop extension [35]. In this model (Fig. 1), recruitment of a helicase (shown here as DHX9) to the site of D-loop extension (rather than to a site that leads to D-loop dissolution) is driven by protein-protein interactions with Pol δ4 and PCNA. This ability would be essential for helicase recruitment to the appropriate site of action.

DHX9 was chosen as a candidate for a potential role in D-loop extension because it has a long history of involvement with mammalian Pol δ functions. DHX9, together with Pol δ and other replication proteins, were bound on immobilized PCNA columns [36], and was also associated with Pol δ purified by immunoaffinity chromatography and subsequent gel filtration (Supplementary Material, Table SI). DHX9 is a multifunctional RNA/DNA 3' to 5' helicase, originally identified as an RNA helicase (RNA Helicase A) [37], and then as Nuclear DNA Helicase II [38,39]. It has been implicated in transcription, translation, microRNA biogenesis, RNA processing and transport as well as in maintaining genomic stability in DNA replication (reviewed in [40,41]). Its substrate specificity overlaps with those of the Bloom and Werner helicases, and include complex DNA structures, D-loops, R-loops as well as triplex DNA and RNA or DNA G-quadruplexes. A role for DHX9 in the DDR (DNA Damage Response) of DSBs formed during transcription through the recruitment of BRCA1 was also reported [41].

We provide evidence that DHX9 has a direct involvement in the process of DSB repair through the stimulation of Pol δ4-mediated DNA synthesis in a reconstitution assay. We also show evidence for protein-protein interactions between DHX9 with Pol δ4 via the p125 and p12 subunits, and between DHX9 and PCNA.

2. Materials and Methods.

2.1. Proteins.

Pol δ4 and Pol δ3 were expressed in Sf9 insect cells using the Multibac baculovirus vector system [42,43] and purified to near homogeneity as previously described [44]. The Multibac vectors allowed expression of Pol δ4 and Pol δ3 in a cassette system so that single baculovirus vectors containing the desired combination of subunits can be expressed. This system has advantages over the use of mixtures of baculoviruses containing individual subunits which could result in formation of different subassemblies of Pol δ [42,43]. None of the subunits were his-tagged as we have observed that tagging of the p68 and p12

subunits may compromise the behavior of Pol δ4. Preparations of Pol δ4 were monitored for the presence of p12, which can be lost during purification and result in the generation of the Pol δ3 form. PCNA was expressed in *E. coli* [45,46]. N-terminally his-tagged human DHX9 was expressed in insect cells using the pReceiver-I01a vector supplied by GeneCopoeia. The protein was purified using a column of Ni-NTA beads, followed by Mono-Q column chromatography (Fig, S1, Supplementary Material).

GST-p12 was expressed in *E. coli* and purified on glutathione beads [30]. Un-tagged p125 subunit was expressed in insect cells, and purified by immunoaffinity chromatography [44]. RPA was expressed and isolated as described [47]. Rad51 was generously provided by Drs. K. Bernstein and S. Hengel, University of Pennsylvania School of Medicine, and RFC by Dr. G.D. Bowman, Johns Hopkins University.

2.2. D-loop reconstitution assay.

The system used was essentially that described by Sneeden et al. [32]. A 93mer oligonucleotide (25 nM) was inserted into negatively supercoiled pUC19 (2.7kb) plasmid (25 nM) by incubation with Rad51 (100 nM), RPA (100 nM) and ATP (1 mM) in the presence of Ca^{2+} to induce insertion of the primer into the plasmid [32]. PCNA (100 nM) was then loaded onto the primer terminus in the D-loop with RFC (20nM) and RPA (100 nM). Reactions were initiated by addition of Pol δ4 (or Pol δ3) with or without DHX9 at the indicated concentrations after addition of dNTPs (0.5 mM). The ionic strength of the final reaction buffer (25 mM Tris-HCl, pH 7.4) was brought to physiological levels by the addition of KCl (150 mM). DNA synthesis was assayed by incorporation of [α-^{32}P]dATP into unlabeled 93mer/plasmid substrate. The extension products were resolved by denaturing alkaline 1.2% agarose gel electrophoresis, and visualized by phosphorimaging. D-loop extension was alternatively monitored by examination of the relaxation of the plasmid substrate on native agarose gel electrophoresis [22,31,32]. In this case, a [^{32}P]5'- end labeled 93mer primer was used for insertion into the plasmid and unlabeled dNTPs were used [32]. Experiments were repeated at least twice.

3. Results.

3.1. Plasmid based reconstitution assay systems for assay of D-loop extension by DNA polymerases.

Several biochemical reconstitution plasmid based systems have been developed for D-loop extension. These systems have provided important insights into D-loop extension synthesis as they allow the operation of a dynamic D-loop, as demonstrated by studies of yeast Pol δ D-loop synthesis [10,22,33,34]. Similar plasmid systems have been used to examine D-loop extension by human Pol δ4 prepared in our laboratory [31] and by human Pol δ4 containing tagged subunits [32]. These circular plasmids are topologically constrained and are negatively supercoiled. The latter topology favors stable insertion of D-loops [31,32,48]. Rad51 recombinase is used to form a nucleofilament with a complementary primer that mimics the invading strand, and is inserted into the plasmid with ancillary factors. In the yeast system, Rad51 and Rad54 were used [22], while Rad51 and Hop2-Mnd1 were used in the system described by Sebesta et al. [31]. The system we used is based on the 2.7kb

pUC19 plasmid as described by Sneeden et al. [32]. This method does not require the use of Rad54. A [^{32}P]5'-end labeled 93mer is inserted into negatively supercoiled pUC19 DNA by Rad51 and RPA in the presence of calcium to form a D-loop. PCNA is then loaded with RFC with the addition of ATP. We will refer to this as the DLPlasmid, noting that the PCNA loaded primer/template is the appropriate substrate for Pol δ. The reactions were initiated by the addition of Pol δ4, DHX9 and dNTP substrates. Two gel electrophoresis methods have been used to follow the reactions. The first is native or non-denaturing agarose gel electrophoresis. D-loop extension of a [^{32}P]5'-end labeled primer is monitored by the appearance of a slower moving band representing the relaxed plasmid. The second method uses denaturing gel electrophoresis in which the end labeled radiolabeled products are separated by size. This provides information on the distribution of extended primers according to size. For our purposes we utilized an unlabeled 93mer primer and labeled [α–^{32}P]dATP incorporation for analysis of the products to allow measurement of DNA synthesis. This is in keeping with other assays for Pol δ activity where total DNA synthesis is measured by incorporation of [α–^{32}P]dNTPs [44,49].

3.2. Pol δ4 exhibits a robust activity in D-loop extension in the reconstituted plasmid based D-loop assay.

The time course of DNA synthesis by Pol δ4 (20 nM) was examined by incorporation of [α–^{32}P]dATP using DL-Plasmid loaded with unlabeled 93mer (25 nM). The concentration of PCNA used was 100 nM, and RPA was 100 nM (Fig. 2). In this experiment (and in Figs. 3 and 4) the products were examined by electrophoresis on alkaline denaturing gels. These conditions release the reaction products from the plasmids. The results show that the product size distribution falls in a range of 2 – 5 kb (Fig. 2A). This is also evident in subsequent experiments shown in Figs. 3 and 4, where the distribution is seen to be more narrowly centered around a range of 2.5 – 3.5 kb. (There was little evidence of smaller products that would indicate stalling sites, with the proviso that very small products < 0.5 kb might not be detected because of the smaller amount of radioactive label incorporated.) This is noteworthy as this range is close to the size of plasmid substrate (2.7 kb). Similar observations for formation of products close to the plasmid size by Pol δ4 have been made [31]. Analysis by 2-D gel electrophoresis [31] have shown that the products synthesized by Pol δ4 can range from the size of the plasmid of 2.7 kb to higher products > 10 kb. The formation of the longer products was suggested to occur when the displaced strand is dissociated upon reaching the plasmid length, following which rolling circle synthesis occurs [32]. However, we have not observed that Pol δ4 exhibits a capacity for rolling circle synthesis on singly primed ssM13 substrates, as the products formed reached the plasmid size of 7 kb [44].

The efficient accumulation of the major products is reminiscent of the behavior of Pol δ4 during processive synthesis on singly primed ssM13 DNA (7 kb) to form a full-length product [44,49]. Here, we should comment on the apparent "processivity" during synthesis on the ssM13 substrate. In the absence of PCNA, Pol δ4 is a distributive enzyme. With PCNA, Pol δ4 is processive, i.e. capable of multiple rounds of dNTP incorporation in a single binding event to the primer/template. The synthesis observed on M13 has been demonstrated to be due to multiple binding events and not a single binding event [50].

In vivo live-cell single-molecule tracking of association of PCNA and Pol δ to genomic DNA has shown that Pol δ synthesis involves multiple rounds of ordered assembly and disassembly from PCNA during DNA synthesis [51].

It has been previously reported that rates of synthesis by human Pol δ4 in D-loop synthesis is comparable to that on conventional substrates [31,32]. This is somewhat unexpected based on the findings that strand displacement by Pol δ4 is notably slower than synthesis on unimpeded templates [27]. However, this behavior in the plasmid system may reflect the behavior of a dynamic D-loop as well as topological behavior of the plasmid substrate [32] (see Discussion 4.3).

3.3. DHX9 stimulates Pol δ4 in D-loop primer extension.

3.3.1. Time course of the effects of DHX9.—The effects of DHX9 (40 nM) on Pol δ4 (20 nM) were examined over a time course of 0 to 30 min (Fig. 3A). DHX9 clearly stimulated D-loop extension by Pol δ4, as observed by increase in the reaction products. The sizes of these extension products fall in a range of 2.5–3.5 kb (Fig. 3A). (It is noted that the observed intensity of the radioactive products in the left panel is not comparable with that of Fig. 2, which was performed with a different preparation of [α–^{32}P]dATP.) Quantitation of the data (Fig. 3B) shows that the stimulation is significant, reaching about 5 fold at the longest time point. A brief lag in activation at the10 minute time point was observed (Fig. 3B).

3.3.2. Concentration dependence of the effects of DHX9.—The effects of DHX9 concentration on D-loop extension by Pol δ4 are shown in Fig. 4. A fixed concentration of Pol δ4 (20 nM) and a fixed early time point (15 min, cf. Fig. 3) was used. The concentration of DHX9 was varied from 0 to 80 nM. The data again shows that DHX9 stimulates D-loop extension. It is noted that the stimulation observed (Fig. 4B) is more modest (ca. 2 fold) than that observed from the later time points of the course (Fig. 3B). Nevertheless, the data of Figs. 3 and 4 provide evidence that DHX9 is capable of stimulating D-loop extension by Pol δ4. Noted also is that the experiment in Fig. 4 was performed with the same preparation of substrates and proteins as that of Fig. 3. The ability of DHX9 to stimulate D-loop extension was reproducible and observed over multiple experiments.

Parenthetically, it is noted that the reaction appears to be plateauing by the 40 – 80 nM concentration range (Fig. 4B). While more detailed analysis is needed, such experiments would provide a titration analysis that would allow an estimate of DHX9 affinity that would provide insights into whether this is within a low nM range. Such information would be relevant to assessment of whether DHX9 might operate to stimulate Pol δ4 under physiological conditions.

3.4. Analysis of DHX9/Pol δ4 mediated D-loop extension by relaxation of the DL-plasmid on non-denaturing gels.

The plasmid substrates are negatively supercoiled and migrate faster than the relaxed form of the plasmids on native (non-denaturing) gel electrophoresis (Section 3.1). During the course of D-loop extension, positive supercoils are generated by DNA synthesis and

relaxation of the plasmid takes place [32]. This leads to appearance of a slower migrating band. Thus, these assays monitor the topological changes of the plasmid as a consequence of DNA synthesis associated with the D-loop. The relaxed plasmids are associated with extension products of different sizes, and also dissociated long extension linear products [31,32]. It has been noted that nicking of the plasmid substrates can also lead to product synthesis unassociated with D-loop synthesis [32]. Therefore, we also examined the effects of DHX9 on the Pol δ4 reaction by its effects on the relaxation of the plasmid. This was done to verify that the analysis of mass DNA synthesis in the data of the preceding experiments is occurring by D-loop extension.

The experiments were performed under similar conditions as in Fig. 4, where the same fixed time point was used with increasing concentrations of DHX9. The differences were that a [^{32}P]5'-end-labeled primer and unlabeled dNTPs were used as the substrates, and the electrophoresis was performed under non-denaturing (native) conditions in agarose gels (Fig. 5). With Pol δ4 alone, a small amount of the relaxed form ("R") is produced (Fig. 5A). The addition of increasing DHX9 is seen to stimulate the formation of the relaxed form with the reciprocal loss of the supercoiled form (Fig. 5A). The quantitation of the formation of the relaxed plasmids is shown in Fig. 5B. It is noted that this concentration dependence curve correlates well with that for the analysis of product formation by [α-^{32}P]dATP incorporation (Fig. 4B). This provides confirmation that the DNA synthesis observed in the prior experiments (Figs. 2–4) is occurring in the physical context of the plasmid D-loop. It is noted also that the plasmid relaxation assay does not directly provide information of total DNA synthesis as dos the prior experiments which utilized labeled dNTPs.

The plasmid D-loop reconstitution assay system has been invaluable in revealing the behavior of Pol δ extension in a system that allows operation of a mobile D-loop [32]. These studies revealed the constraints of topological stress on D-loop synthesis, and have indicated that the extension of D-loop in the plasmid environment is complex [32]. It was estimated that a short amount of synthesis by Pol δ4 would generate sufficient supercoiling to stall further synthesis, and indeed transient stalling was observed before synthesis continued. The facility of Pol δ4 synthesis was attributed to the effects of RPA, which expedited the extrusion of the 5' end of the elongating primer, which then allows the operation of a migrating D-loop that counteracts the supercoiling of the synthesis by Pol δ4 [32] (see Discussion, 4.4).

3.5. Protein-protein interactions in the recruitment/coupling of DHX9 with Pol δ/PCNA in D-loop extension.

Central to our hypothesis for a role of DHX9 in HDR is that protein-protein interactions of DHX9 with Pol δ4/PCNA at the primer/terminus plays an important role in its recruitment to the junction of the leading edge of the D-loop to form a functional complex (Fig. 1). Using purified proteins, direct interactions of DHX9 with the p125 catalytic subunit and the p12 subunit of Pol δ4 were demonstrated by Ni-NTA (Fig 6A) and glutathione bead (Fig 6B) pull down assays, respectively. Additional evidence for a direct interaction of p125 with DHX9 is shown in an interaction mapping experiment, in which overlapping fragments of the p125 subunit were tested for their interaction with DHX9 (Fig. S4, Supplementary

Material). The p68 and p50 subunits did not exhibit interactions with DHX9 in pull down assays (not shown). A direct interaction of DHX9 with PCNA, the other key component of D-loop extension system, was shown by Ni-NTA bead pull down assays (Fig. 6C).

Next we examined whether DHX9 interacted with intact Pol δ complexes, *viz,* Pol δ4 and Pol δ3. Both contain the p125 subunit but the Pol δ4 also contains the p12 subunit. (This experiment was performed since interactions of isolated subunits do not directly demonstrate that these are the intact complexes). The ability of DHX9 to pull down the Pol δ complexes was demonstrated by immunoblotting for the p125, p12 and p68 subunits. DHX9 pulled down the Pol δ4 complex as shown by the presence of p68 as well as the p125 and p12 subunits (Fig. 6D). DHX9 also interacted with the Pol δ3 complex (Fig. 6E) as shown by the pull down of p68 and the p125 subunits. Thus, both Pol δ3 and Pol δ4 complexes interact with DHX9. The ability of the Pol δ4 complex to interact with DHX9 through p125 interaction shows that p12 interaction is dispensable. This does not preclude the possibility that p12 interaction is also operative and functionally important in the case of Pol δ4. This is supported by the observations that Pol δ4, but not Pol δ3, is stimulated by DHX9 (Fig. 4 and Fig. S3). It is likely that the addition of a protein-protein interaction between p12 and DHX9 would enhance the affinity of the latter for Pol δ4.

Recent advances in the solution of the human Pol δ4/PCNA/DNA replication complex as well as its complex with Fen1 [29], have provided a structural basis for understanding how they can function as complexes in Okazaki fragment processing [27,29]. The structure of the Pol δ4/PCNA/Fen1 complex shows that the positioning of Pol δ4 on PCNA is such that it spatially accommodates Fen1 which has access to the flap structure. These structures suggest the feasibility of a similar model in which DHX9 and Pol δ might be accommodated on PCNA.

4. Discussion.

4.1. DHX9 stimulates Pol δ4 in a reconstituted system for D-loop synthesis, and provides a mechanistic basis for its participation in HDR.

Our studies show that DHX9 stimulates Pol δ4 synthesis in a reconstituted plasmid assay system and exhibits the requisite protein-protein interactions with PCNA and Pol δ4 that could drive its recruitment to Pol δ/PCNA at the leading edge of the D-loop. This provides support for the interaction of DHX9 with Pol δ/PCNA that could act functionally in a coordinated manner in D-loop extension. These studies provide biochemical evidence that the DHX9 helicase plays an important role in the process of D-loop extension by Pol δ4 in human cells. These findings support the hypothesis proposed in Fig. 1, which stresses the role of recruitment of DHX9 to the Pol δ4/PCNA complex to accelerate D-loop extension.

A key question is whether our *in vitro* findings are relevant *in vivo*. The concept that DHX9 action is mechanistically involved in DNA loop extension by Pol δ4 is strongly supported by evidence from *in vivo* studies that DHX9 is required for HDR [41]. It was demonstrated that siRNA depletion of DHX9 in cultured cells led to a 50–70% reduction in HDR repair using a pDR-GFP reporter assay. DHX9 depleted cells exhibited two hallmarks of HDR deficiency, *viz*. increased sensitivity to DNA damaging agents and PARP inhibitors. It was

concluded that DHX9 acted by an as yet unknown direct mechanistic manner [41]. These findings are highly similar to those observed by knockout of p12 which leads to loss of Pol δ4 [1,28]. Thus, these findings support an *in vivo* role for DHX9 that involves its ability to facilitate Pol δ4 mediated D-loop extension. Combined depletion of DHX9 and BRCA1 had similar effects on HDR, indicating they functioned in the same pathway [41].

In yeast, it has been well established that the Pif1 5' to 3' helicase facilitates Pol δ D-loop extension in BIR [10,22,33,34], and more recently, cellular evidence for the participation of mammalian Pif1 has been reported, although its mechanistic involvement in a reconstitution system has not yet been established [52]. The involvement of both 5' to 3' and 3' to 5' helicases in mammalian D-loop extension remain to be further elucidated.

4.2. Additional roles of DHX9 in Pol δ4 functions.

Because of its multifunctional roles in RNA and DNA metabolism, there has been significant interest in the role of DHX9 in genomic instability as it relates to tumorigenesis, cancer and diseases originating from microsatellite instability [40,53–55]. The role of DHX9 as a potential therapeutic target due to its overexpression in many tumors has been recently reviewed [54]. Considerations of the roles DHX9 in maintenance of genomic stability should now factor in its role in HDR through its mechanistic involvement in D-loop extension. Many helicases have been implicated in homologous recombination processes [56,57], and this raises questions of whether other helicases may also be involved in cooperation with DHX9 and/or Pol δ4 in D-loop extension.

As noted in the Introduction, DHX9 helicase is also capable of dissolution of complex DNA structures that include triplex DNA and RNA or DNA G-quadruplexes [40,58–60]. DHX9 could play an important role during D-loop extension by removing structural blocks to Pol δ4 synthesis. This is potentially a significant component of its functions. Interestingly, DHX9 has been reported to interact with Pol η, and to facilitate Pol η synthesis through G4-structures [61].

Studies of Pol δ4 in various *in vitro* assays have raised a conundrum in that it is readily stalled at secondary structures and complex templates [62,63]. This stalling is also observed in Pol δ4 synthesis on singly primed ssM13 substrates [49,50]. We would predict that DHX9 could play a key role in facilitating Pol δ4 function in this context. As a precedent, it is noted that PDIP46, which binds to both Pol δ4 and PCNA, has been shown to significantly accelerate Pol δ4 synthesis on ssM13 templates [49]. Thus, DHX9 could emerge as an important partner in Pol δ4 functions in cellular DNA synthesis. This could include synthesis involving telomeres [64] or common fragile sites [18]. Further research from this perspective may provide new information on the cellular roles of Pol δ4.

In addition to facilitating synthesis across complex template structures, we speculate that DHX9 may also affect the processivity of Pol δ4. An instructive study comes from single molecule analysis of T4 holoenzyme and helicase [65] using molecular tweezers and hairpin sequences as a substrate [66] where functional coupling of the T4 polymerase and helicase leads to rapid and processive synthesis [65]. This may be relevant for understanding the

potential functions of DHX9 in BIR, which is still poorly understood [16], particularly in the mechanisms for synthesis of long tracts of DNA.

4.3. Pol δ3 is defective in D-loop extension as demonstrated by biochemical D-loop extension assays.

Our studies provide further biochemical evidence for a separation of functions between Pol δ4 and Pol δ3, where Pol δ4 has the primary role in HDR. As noted in the Introduction, Pol δ3, unlike Pol δ4, lacks strand displacement activity. This was demonstrated by the use of both gapped and flap-containing oligonucleotide substrates in a study of Okazaki fragment processing. It was also noted that strand displacement by Pol δ4 was slower than on unimpeded templates [27]. We have used a static D-loop oligonucleotide substrate [67] to compare the abilities of Pol δ3 and Pol δ4 to perform D-loop synthesis (Fig. S2, Supplementary Material). The results show that Pol δ3 is ineffective in D-loop synthesis, in contrast to Pol δ4.

The ability of Pol δ3 to perform D-loop extension was examined using the plasmid based assay. In this experiment, the effects of varying concentrations of DHX9 on Pol δ3 was examined in parallel with the experiment shown in Fig. 4 for Pol δ4. The results (Fig. S3, Supplementary Material) show that Pol δ3 exhibits very little activity by comparison to Pol δ4, and is not affected by DHX9. These data provide biochemical support for our findings from cellular evidence in p12-KO cells that Pol δ3 is insufficient to maintain HDR activity [1,28].

The evidence that Pol δ3 and Pol δ4 have clearly distinct functions in HDR highlights the importance of the existence of two forms of Pol δ in higher eukaryotes, that impacts their roles in its central functions in DNA replication and DNA repair. Since the balance of their activities is based on their interconversion by controlled degradation of the p12 subunit, it is noted that this process is also under cell cycle control and the response to DNA damage [1,28].

4.4. Comments on the behavior of Pol δ4 in the plasmid based assay

Unlike Pol δ3 (see above), Pol δ4 exhibited a robust activity in the plasmid based assay, indicating that the expectation that a slower rate of strand displacement was not a limiting factor in D-loop extension by Pol δ4. However, as previously noted (Results 3.2, 3.3), rates of DNA synthesis by Pol δ4 in the plasmid system have indicated that synthesis rates are comparable to that with primed linear substrates [31,32]. The basis for this is not fully understood, but is likely due to the dynamic nature of the D-loop, which is subject to topological stresses that are influenced by a number of factors. One likely factor that may permit dynamic movement of the D-loop to facilitate Pol δ4 synthesis is RPA, which has been proposed to elicit extrusion of the lengthening primer at the lagging end of the D-loop to permit the migration of the D-loop [32]. RPA has a dynamic interaction with ssDNA, and with the junctions of forked structures. RPA can zip or unzip these structures [68]. Application of external torsion strain has been shown to open forked structures in the presence of RPA [69]. This property may underlie the proposed role of RPA [32].

Conclusions

These studies demonstrate that DHX9 stimulates D-loop synthesis by human Pol δ4, and exhibits protein-protein interactions with PCNA and Pol δ4. The findings support a functional coupling between DHX9 and Pol δ4 and support the hypothesis for involvement of helicase-Pol δ4 interaction as an operative system for D-loop extension in human cells. Our results show that the plasmid-based assay could serve as a test bed for identification of other helicases that might facilitate D-loop extension. Given the numerous helicases that could function in this capacity, identification of candidate helicases could be facilitated by screening for those which exhibit protein-protein interactions with Pol δ4 and or PCNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights.

- DHX9 helicase facilitates D-loop extension synthesis in double-strand break repair
- DHX9 stimulates DNA synthesis by Pol δ4 in the D-loop
- DHX9 exhibits direct protein-protein interactions with Pol δ4 and PCNA
- DHX9 is mechanistically involved in double-strand break repair
- This new role of DHX9 adds to its potential as a target for cancer therapeutics

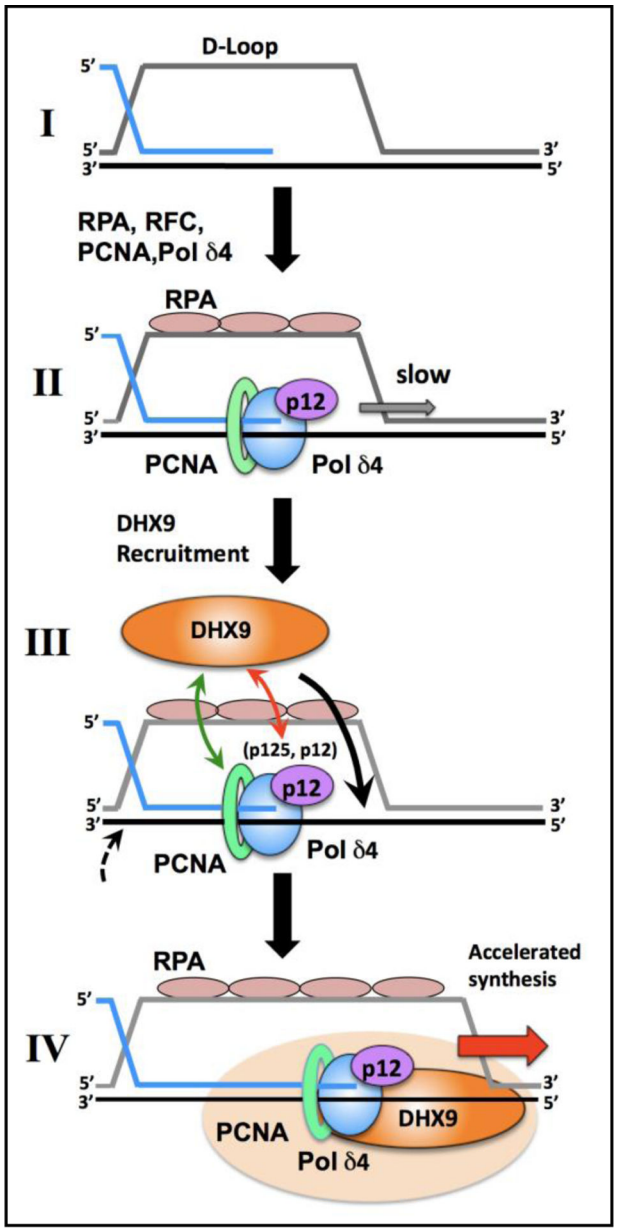

Fig. 1. Hypothesis for DHX9 participation in D-loop extension by Pol δ4.

I. Diagrammatic representation of a D-loop with an inserted invading strand to form a 3' primer end for polymerase action. **II.** RFC loads PCNA and Pol δ4 to the primer end, which permits D-loop extension. **III.** DHX9 is recruited to the PCNA/Pol δ4/primer-template. This is driven by protein-protein interactions between: DHX9 and PCNA (green arrow); DHX9 and Pol δ4 via the p125 and p12 subunits (red arrow); DHX9 and the leading edge of the D loop (black arrow). Also shown in "III" is an alternative site of action of DHX9 (dashed arrow) that would lead to dissolution of the D-loop. **IV.** Recruitment of DHX9 leads to accelerated D-loop extension. Note that recruitment of DHX9 to act at the leading edge of the D-loop (shaded oval) would be energetically favored over recruitment to the position shown in "III" (dashed arrow) which would lead to dissolution of the D-loop.

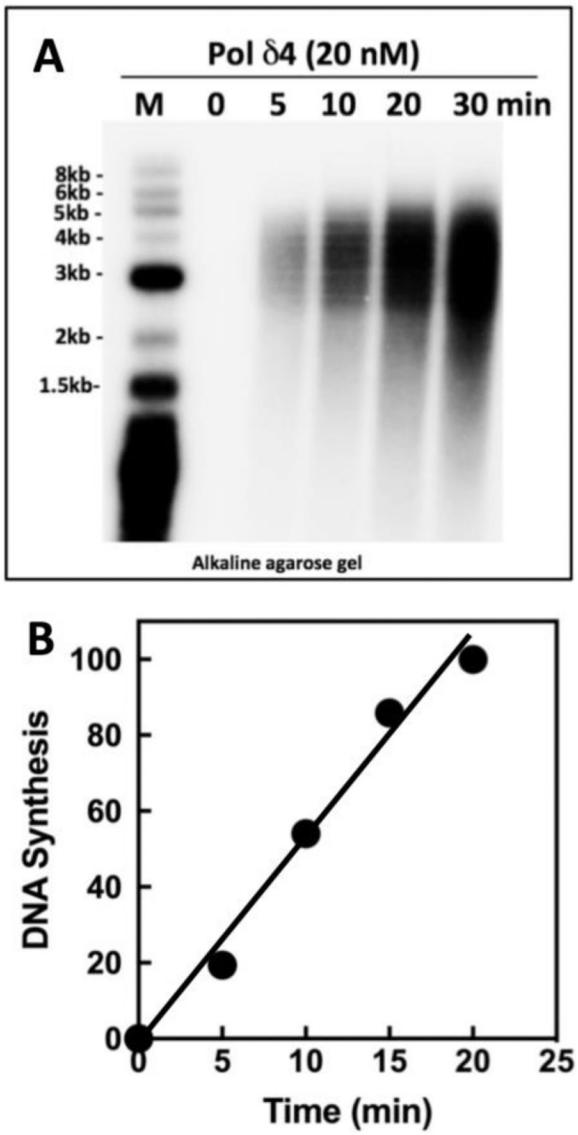

Fig. 2. Time course of DNA synthesis by Pol δ4 in the D-loop-plasmid assay.

Unlabeled 93mer was used to form D-loops in the pUC19 plasmid and was then loaded with PCNA by RFC. D-loop extension synthesis by Pol δ4 (20 nM) was followed by incorporation of [α−^{32}P]dATP into the 93mer primer. **A**. Reaction products were analyzed by electrophoresis on 1.2% alkaline agarose gels and visualized by phosphorimaging. Positions of molecular weight markers are indicated on the left. **B**. Reaction products were quantified and shown as arbitrary units of DNA synthesis.

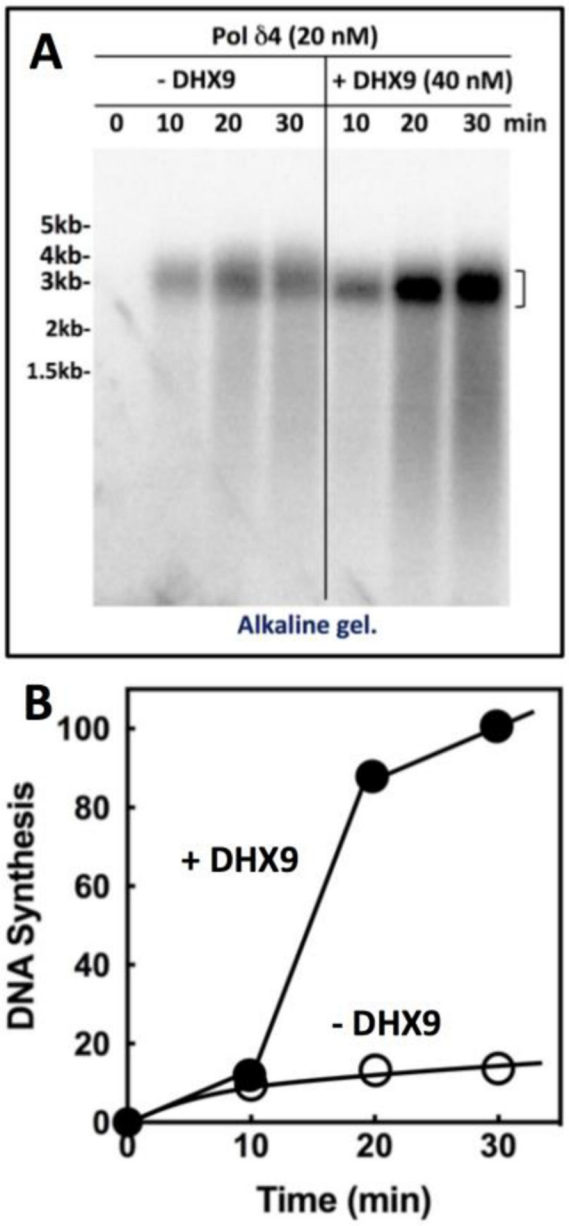

Fig. 3. Time course of DHX9 stimulation of DNA synthesis at a fixed concentration of Pol δ4.

Conditions were as in Fig. 2. Unlabeled 93mer was used to form D-loops into the DL-plasmid substrate. D-loop extension synthesis by Pol δ4 (20 nM) in the absence and presence of DHX9 (40 nM) was monitored as in Fig. 2 by incorporation of [α−^{32}P]dATP into the 93mer primer. **A.** Products were analyzed by electrophoresis on 1.2% alkaline agarose gels and visualized by phosphorimaging. Positions of molecular weight markers are indicated on the left side of the gel image. **B.** Reaction products in the region shown by the bracket on the right in panel A were quantified and shown as arbitrary units of DNA synthesis (open circles, in the absence of DHX9; solid circles, in the presence of DHX9).

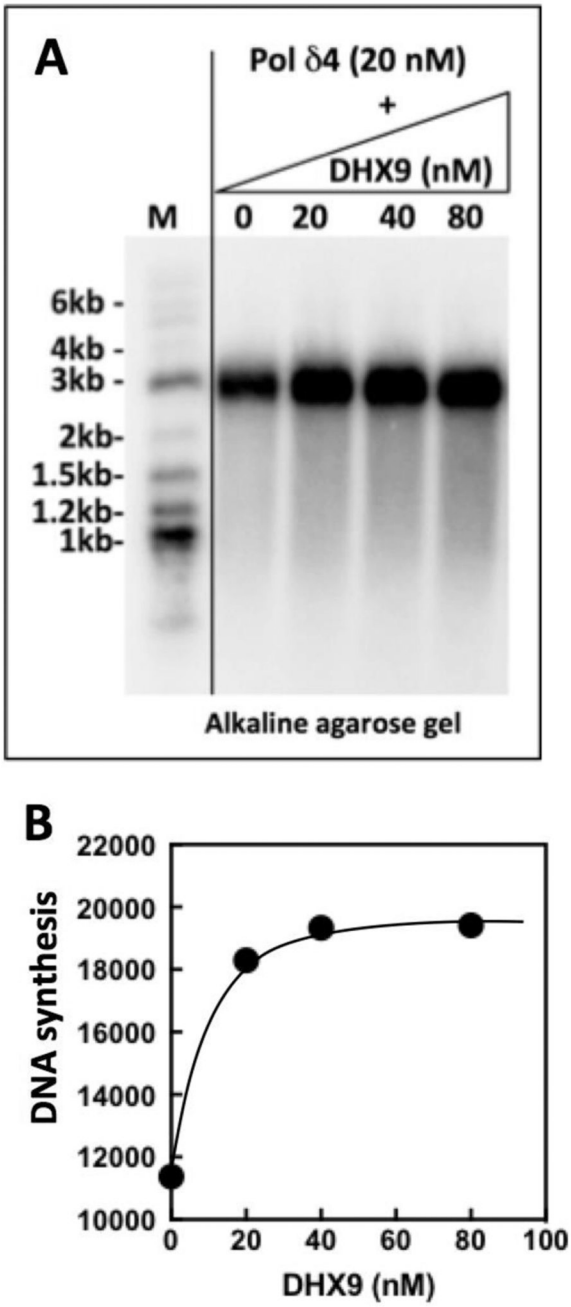

Fig. 4. Concentration dependence of the effects of DHX9 on DNA synthesis by Pol δ4.

Conditions of the assay were as in Fig. 3, except that a fixed time point (15 min) was used and the DHX9 concentrations were increased from 0 – 80 nM. **A.** The reactions were monitored by incorporation of [α−^{32}P]dATP into the 93mer primer and analyzed by electrophoresis on 1.2% alkaline agarose gels and visualized by phosphorimaging. **B.** Quantitation of the major product was expressed as arbitrary units of DNA synthesis.

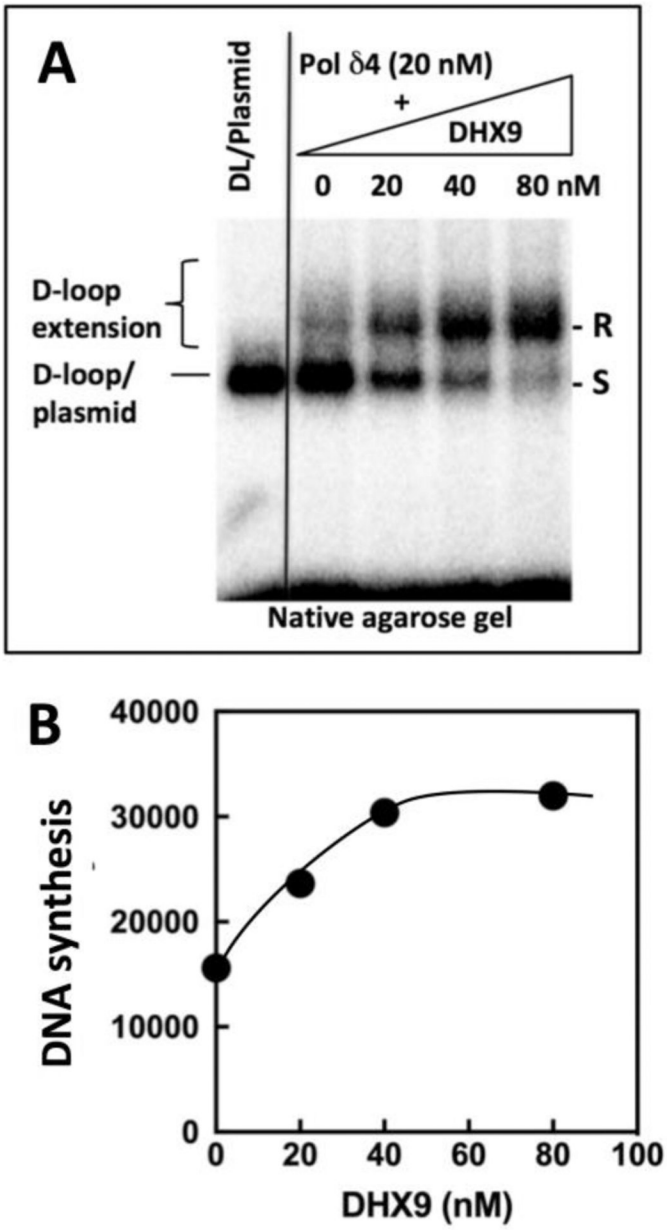

Fig. 5. DHX9 stimulates D-loop synthesis by Pol δ4 as monitored by the relaxation of the DL-plasmid.

The assays were performed as described for Fig. 4, with the difference that a [^{32}P]end-labeled 93mer was used and unlabeled dNTPs were used as the substrate. A fixed time point (15 min) was used and the DHX9 concentrations were increased from 0 – 80 nM as in Fig. 4. **A.** The reaction products were analyzed on native non-denaturing agarose gels. “D-loop/ Plasmid” refers to the plasmid preloaded with the labeled primer and PCNA. “S” refers to the negatively supercoiled plasmid and “R” refers to the relaxed forms of the plasmid. **B.** Quantitation of the “R” region expressed as arbitrary units.

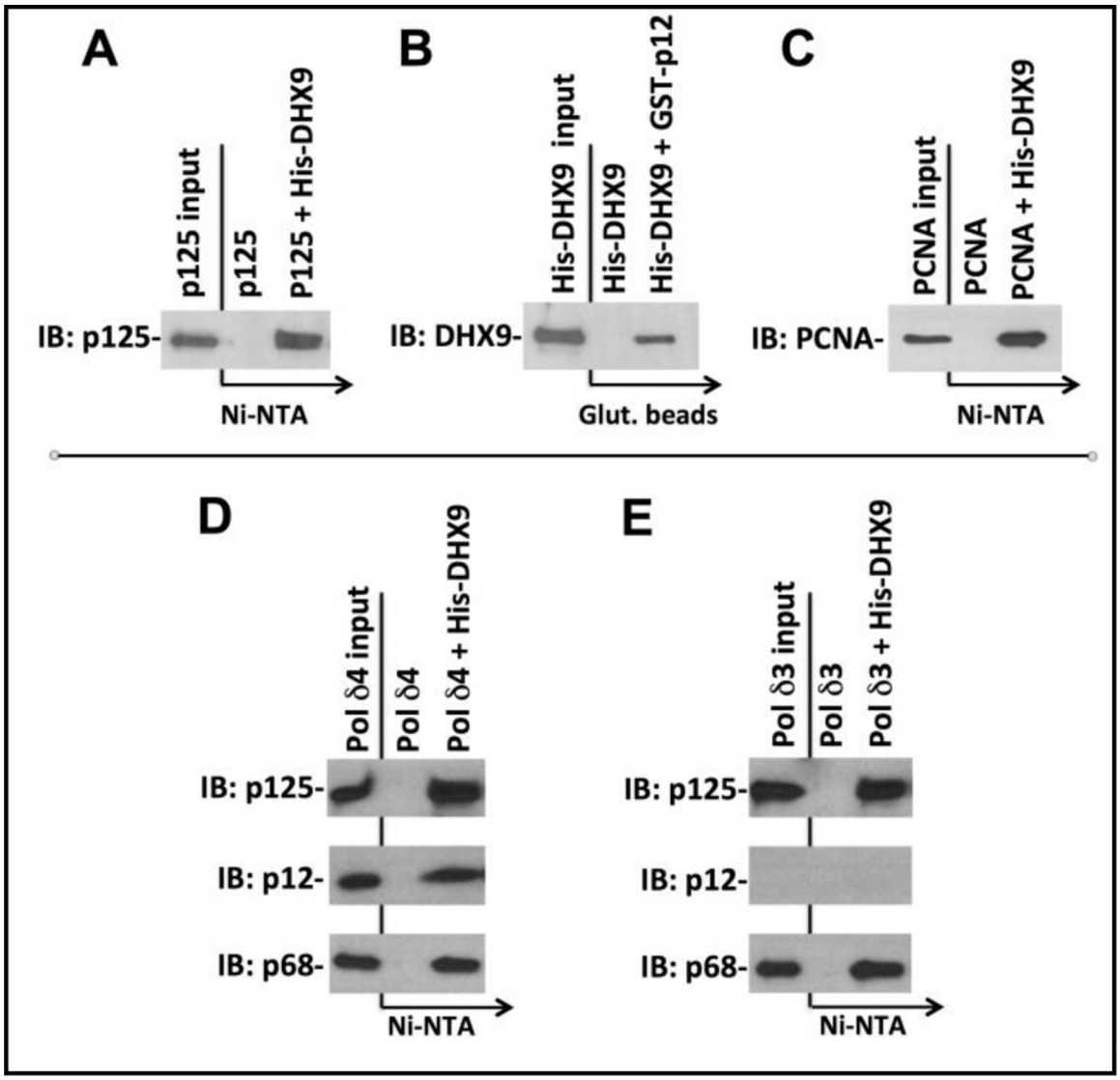

Fig. 6. Interaction of DHX9 with the p125 catalytic subunit and with the p12 subunit as well as the intact Pol δ4 and Pol δ3 complexes.

A. Interaction between his-DHX9 and the p125 subunit using Ni-NTA pull-downs with his-DHX9. **B.** Interaction of his-DHX9 and GST-p12 using glutathione beads. **C.** Interaction of his-DHX9 and PCNA using Ni-NTA pull-downs **D.** His-DHX9 was used to pull down purified Pol δ4 complex. The pull-downs were immunoblotted for p125 and p12, well as with p68 to indicate pull down of the complexes. **E.** His-DHX9 was used to pull down purified Pol δ3 complex. The pull-downs were immunoblotted for p125 and with p68 to indicate pull down of the complexes.