Harmonin homology domains of human RTEL1 interact with replication protein A and DNA

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ABSTRACT

Regulator of telomere elongation helicase 1 (RTEL1) is an Fe-S cluster containing helicase that plays important roles in telomere DNA maintenance, DNA repair, and genome stability. It is a modular protein comprising a helicase domain, two tandem harmonin homology domains 1 & 2 (HHD1 and HHD2), and a RING domain. The RING domain interacts with telomere DNA binding protein TRF2 and helps in the recruitment of RTEL1 to the telomeres while the helicase domain disassembles telomere DNA t/D-loop and unwinds G-quadruplexes. The novel HHDs are predicted to mediate protein-protein interactions. Mutations in RTEL1 are associated with Hoyeraal-Hreidarsson syndrome and familial pulmonary fibrosis. Several of these mutations are found in the HHDs of RTEL1. In this study, we have carried out the structural characterization of HHDs of RTEL1. We report that human RTEL1 interacts and co-localizes with the replication protein A (RPA) in the cell. Using NMR spectroscopy and ITC experiments, we have shown that the HHD2 of RTEL1 interacts directly with the winged-helix 32C domain of RPA as well as DNA using an overlapping surface. These results establish an interplay among RTEL1, RPA, and DNA that has implications for understanding the role of RTEL1 in DNA replication, repair, and recombination processes.

INTRODUCTION

The regulator of telomere elongation helicase 1 (RTEL1) is a Fe-S cluster and DEAH motif-containing DNA helicase with ATP-dependent 5' to 3' helicase activity (Estep and Brosh, 2018; Uringa et al., 2011). RTEL1 plays essential roles in telomere maintenance, DNA repair, meiotic recombination, and genome-wide replication (Sarek et al., 2015; Uringa et al., 2012; Uringa et al., 2011; Vannier et al., 2012; Vannier et al., 2014). It is a modular protein consisting of an N-terminal helicase domain followed by two tandem harmonin homology domains 1 & 2 (HHD1 and HHD2), a PCNA-interacting protein-box (PIP-box) motif, and a C-terminal C4C4 type RING domain (Vannier et al., 2014) (Figure 1A). RTEL1 is an anti-recombinase and executes non-crossovers by promoting synthesis-dependent strand annealing pathway of homologous recombination through disassembling D-loop intermediates during the DNA repair and meiotic recombination processes (Barber et al., 2008; Uringa et al., 2011). RTEL1 interacts with the replisome's proliferating cell nuclear antigen (PCNA) through its PIP-box motif and helps in the genome-wide replication (Sarek et al., 2015; Vannier et al., 2013). The central HHD1 and HHD2 are predicted to mediate protein-protein interactions (Faure et al., 2014). Several mutations in RTEL1 are associated with genetic diseases Hoyeraal-Hreidarsson syndrome (HHS) and familial pulmonary fibrosis (FPF) (Deng et al., 2013; Jullien et al., 2016; Stuart et al., 2015; Vannier et al., 2014; Walne et al., 2013). These diseases are associated with short telomere length in patients resulting in premature aging, bone marrow failure, and predisposition to cancer.

HHDs are the most recent entrant to newly classified αα-hub domain-containing proteins (Colcombet-Cazenave et al., 2021). So far, nine HHDs have been identified in six proteins, viz. Harmonin, Whirlin, Delphilin, PDZD7, CCM2, and RTEL1 (Bugge et al., 2021; Colcombet-Cazenave et al., 2021; Staby et al., 2021). The HHD1 and HHD2 of RTEL1 harbor several mutations associated with HHS and FPF, underscoring their functional importance (Faure et al., 2014). In a recent study, a region encompassing the HHD1 of RTEL1 was shown to interact with SLX4 (Takedachi et al., 2020; Vannier et al., 2012; Wu et al., 2020).

Replication protein A (RPA) is a trimeric protein complex (consisting of RPA70, RPA32, and RPA14 subunits) (Figure 1B) that binds single-stranded DNA (ssDNA) in a sequence non-specific manner (Wold, 1997). ssDNA is an intermediate product in the replication, recombination, and repair pathway (Branzei and Szakal, 2021). RPA interacts physically and functionally with many DNA helicases like BLM, WRN, and FANCJ (Awate and Brosh, 2017; Caldwell and Spies, 2020; Jullien et al., 2016). RPA-dependent enhancement of D-loop disassembly by RTEL1 and its role in controlling the excessive crossing-over during meiosis was previously reported (Youds et al., 2010). However, the evidence of the direct RTEL1–RPA interaction had remained undefined.

Here, we report that human RTEL1 directly interacts and colocalizes with RPA upon DNA damage. Interestingly, this interaction is mediated by the tandem harmonin homology domains of RTEL1. Using NMR chemical shift perturbation experiments, we showed that the HHD2 of RTEL1 interacts exclusively with the winged-helix domain 32C of RPA. We have characterized the HHDs using NMR spectroscopy and solved the X-ray crystal structure of HHD2 of human RTEL1. The structure of HHD2 revealed a unique surface charge potential with positive and negative surfaces. Using NMR spectroscopy and isothermal titration calorimetry (ITC), we have shown that the HHD2 also interacts with DNA using the RPA 32C binding surface. This study established HHD2 as a novel accessory domain of RTEL1 that mediates protein-protein and protein-DNA interactions.

RESULTS

The tandem harmonin homology domains of RTEL1 are connected through an intrinsically disordered linker region.

Although predicted to be consisting of a harmonin-N-like fold using the hydrophobic cluster analysis (Faure et al., 2014), HHD1 and HHD2 of the RTEL1 have not been characterized experimentally for their structures. Bioinformatic analysis of the tandem harmonin homology domains of RTEL1 indicates that a disordered linker region separates HHD1 and HHD2

(Figure S1A). We have purified the ^{15}N-labeled HHD1, HHD2, and HHD1+2 (HHD1-linker-HHD2) (Figure S1B) and recorded the ^{1}H-^{15}N 2D HSQC spectrum for each protein. The spectra of HHD1 and HHD2 overlaid well on the spectrum of HHD1+2 (Figure 1C). The extra cross-peaks arising from the linker region clustered at the centre of the spectrum (~8.2 ppm in the ^{1}H dimension) of HHD1+2, indicating the disordered nature of the linker. These results suggest that individual HHD1 and HHD2 are independently folded domains separated by a disordered linker region. We have recently reported near-complete backbone and sidechain resonance assignments of HHD1 and HHD2 (Kumar et al., 2022).

RTEL1 directly interacts with RPA using the harmonin homology domains

The RPA was shown to enhance *in vitro* D-loop disassembly by RTEL1 (Youds et al., 2010). Furthermore, as per the BioGRID database (Oughtred et al., 2021), RTEL1 is included in the interactome of the RPA-ssDNA complex (Maréchal et al., 2014). However, there is no report of direct interaction between RTEL1 and RPA. Therefore, we investigated for any possible physical and functional interaction between RTEL1 and RPA. First, the HEK293T cells were transfected with pcDNA3.1-N-HA-RTEL1 for transient overexpression of HA-tagged RTEL1. A co-immunoprecipitation experiment was performed, and the results showed that endogenous RPA co-immunoprecipitated with RTEL1, suggesting physical interaction between RPA and RTEL1 (Figure 1D). Since both RPA and RTEL1 have important roles in DNA repair pathways, we treated HeLa cells with hydroxyurea (a DNA damaging agent) and performed immunofluorescence microscopy-based experiment. Upon DNA damage, a subset of endogenous RTEL1 and RPA foci co-localize (Figure 1E), suggesting the functional nature of RTEL1-RPA interaction.

Analysis of the RPA interacting sites on the BLM, WRN, and FANCJ helicases indicates that a motif/domain other than the helicase domain interacts with the RPA (Awate and Brosh, 2017; Kang et al., 2018; Yeom et al., 2019). Since the HHDs in RTEL1 were classified as putative protein-protein interaction domains (Colcombet-Cazenave et al., 2021; Faure et al., 2014), we hypothesized that HHDs could harbor potential RPA binding sites (Figure S1C). To confirm

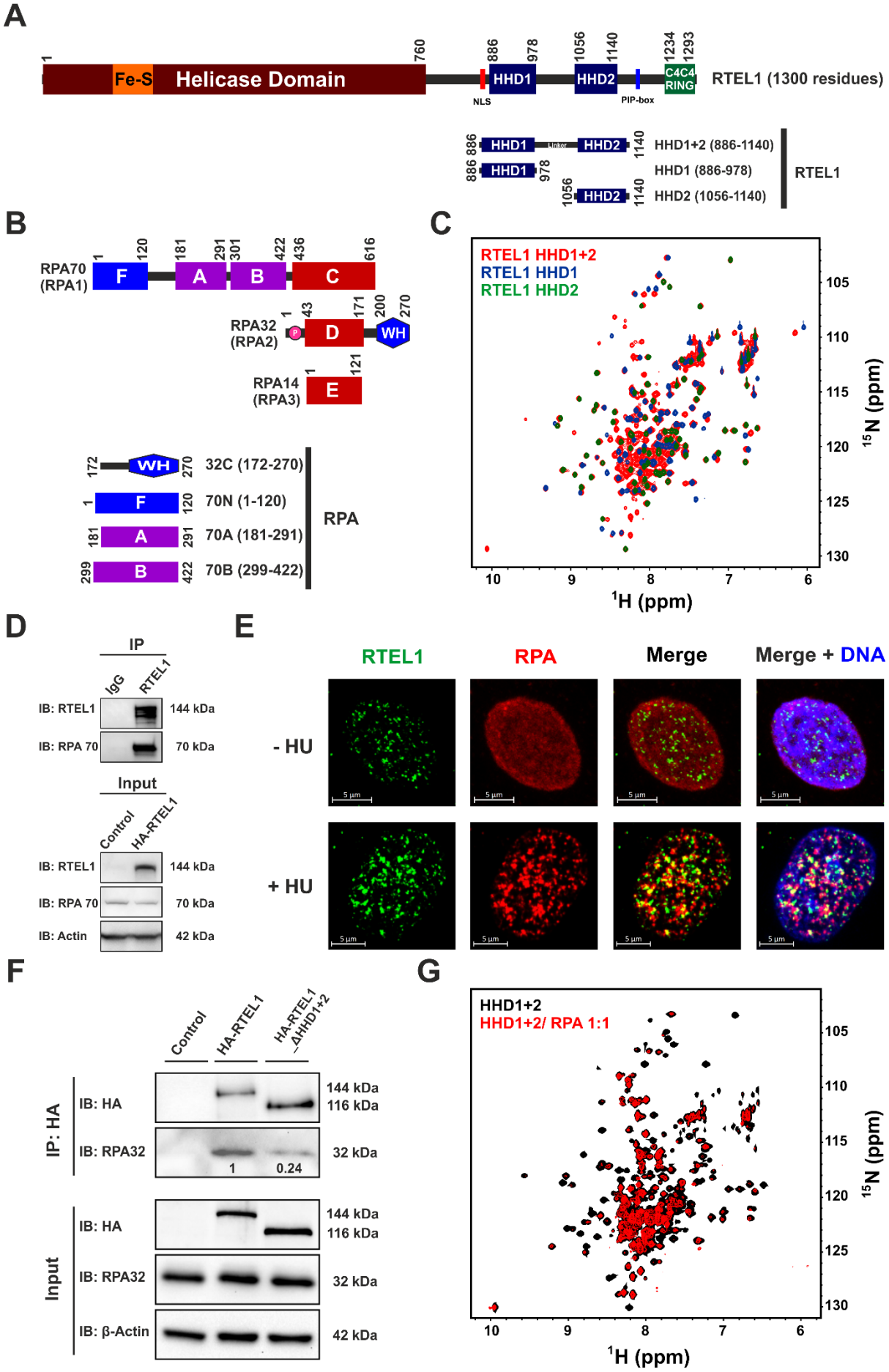
A
Fe-S
Helicase Domain
HHD1
HHD2
C4C4 RING
NLS
PIP-box
RTEL1 (1300 residues)
HHD1+2 (886-1140)
HHD1 (886-978)
HHD2 (1056-1140)
RTEL1
B
RPA70 (RPA1)
RPA32 (RPA2)
RPA14 (RPA3)
F
A
B
C
D
E
WH
32C (172-270)
70N (1-120)
70A (181-291)
70B (299-422)
RPA
C
RTEL1 HHD1+2
RTEL1 HHD1
RTEL1 HHD2
^{15}N (ppm)
^{1}H (ppm)
D
IP
IgG
RTEL1
IB: RTEL1
IB: RPA 70
144 kDa
70 kDa
Input
Control
HA-RTEL1
IB: Actin
42 kDa
E
RTEL1
RPA
Merge
Merge + DNA
- HU
+ HU
F
Control
HA-RTEL1
HA-RTEL1 _ΔHHD1+2
IP: HA
IB: HA
IB: RPA32
144 kDa
116 kDa
32 kDa
1
0.24
Input
IB: β-Actin
G
HHD1+2
HHD1+2/ RPA 1:1
^{15}N (ppm)
^{1}H (ppm)

Figure 1. RTEL1 directly interacts with RPA. (A) Domain organization of human RTEL1. HHD constructs used in this work are shown with domain boundaries. **(B)** Domain organization of human RPA trimeric complex (consisting of RPA70, RPA32, and RPA14). Various constructs of RPA used in this study are shown with their domain boundaries. **(C)** Overlay of ^{1}H-^{15}N HSQC spectra of HHD1 (blue), HHD2 (green), and HHD1+2 (red). **(D)** Whole-cell extracts of HEK293T cells overexpressing HA-tagged human RTEL1 were immunoprecipitated (IP) with mentioned antibodies. The immunocomplexes (*upper panel*) and input (*bottom panel*) were analyzed through immunoblotting (IB) with indicated antibodies. **(E)** Representative immunofluorescence images showing co-localization of endogenous RTEL1 and RPA in the nucleus of HeLa cells. Cells were untreated (*upper panel*) or treated (*bottom panel*) with 4 mM Hydroxyurea (HU) for 48 h, paraformaldehyde fixed, and immunostained for RTEL1 (green) and RPA (red). Nuclear DNA (blue) was counterstained with Hoechst 33342. **(F)** Representative immunoblot showing co-immunoprecipitation of RPA with HA-RTEL1 and HA-RTEL1_ΔHHD1+2. Whole-cell extracts of HEK293T cells overexpressing HA-tagged human RTEL1 and its deletion construct RTEL1_ΔHHD1+2 were immunoprecipitated (IP) using an anti-HA antibody. The immunocomplexes (*upper panel*) and input (*bottom panel*) were analyzed through immunoblotting (IB) with indicated antibodies. RTEL1 and RPA interaction reduced by about 75% upon deletion of the HHD1+2 domain. Data were analyzed from two independent biological experiments using ImageJ software. The normalized value of RPA is mentioned below the respective bands. **(G)** Overlay of ^{1}H-^{15}N TROSY HSQC spectra of ^{15}N-labelled HHD1+2 in the absence (black) and presence (red) of RPA complex (at 1:1 molar ratio).

this hypothesis, we overexpressed the HHD1+2 deletion construct of RTEL1 (pcDNA3.1-N-HA-RTEL1-ΔHHD1+2) in HEK293T cells. Deletion of HHD1+2 resulted in a significant reduction of co-immunoprecipitated RPA (Figure 1F), suggesting the HHDs of RTEL1 may interacts with RPA.

Protein-protein interactions in the cell are often weak and transient. NMR chemical shifts are extremely sensitive to changes in the environment. Therefore, weak but biologically relevant interactions can be studied using NMR chemical shift perturbations (Vaynberg and Qin, 2006). The ^{15}N-labeled HHD1+2 was titrated with human RPA. We observed resonance peak broadening and chemical shift perturbations (CSP) of several residues, suggesting a direct interaction between HHD1+2 and RPA (Figure 1G and Figure S1D).

RPA interacts with HHD2 of RTEL1 through its winged-helix domain 32C

Protein and DNA binding roles have been assigned to different domains of RPA. The 70N, 70A, 70B, and 32C domains of RPA interact with partner proteins (Caldwell and Spies, 2020)

(Figure 1B). We took an NMR spectroscopy-based titration approach to map the interaction between HHDs of RTEL1 and different domains of RPA. Uniformly ^{15}N-labeled samples of 70N, 70A, 70B, and 32C domains of RPA (Figure S1D) were titrated with the HHD1+2 domain of RTEL1. No significant CSPs were observed in the spectra of 70N, 70A, and 70B (Figure S2A-2C), while we observed distinct CSPs in the spectrum of 32C upon titration with HHD1+2 (Figure S2D). The reverse titrations (^{15}N-labelled HHD1+2 titrated with RPA 32C) also showed distinct CSPs in the spectrum of HHD1+2 (Figure S2E).

In subsequent experiments, individual HHD1 and HHD2 were titrated with RPA 32C. No significant CSPs were observed in the spectrum of HHD1 (Figure S2F), however distinct CSPs of several residues were observed in the case of HHD2 (Figure 2A). We also performed a reverse NMR titration of ^{15}N-labelled 32C with HHD2 (Figure 2B). Again, we observed distinct CSPs in several residues of 32C. Interestingly, these are the same set of residues of 32C, which were perturbed upon titration with HHD1+2 (Figure S2D). Based on these results, we conclude that the HHD2 domain of RTEL1 interacts with the 32C domain of RPA.

Structural basis of RTEL1 HHD2 and RPA 32C interaction

To understand the structural basis of RPA 32C and RTEL1 HHD2 interaction, we solved the structure of HHD2 at a resolution of 1.6 Å (Table S1) using the X-ray crystallography (STAR Methods). The structure revealed that HHD2 consists of five helices, H1-H5, connected by a short loop. All the helices are mainly α-helical (3.6_{13}) except the N-and C-termini of H4 and the N-terminal of H5, which consists of short 3_{10} helices (Figure 2C and 2D). The secondary structures match well with the NMR chemical shift indexing-based secondary structures (Kumar et al., 2022). The overall topology of RTEL1 HHD2 is similar to the N-terminal domain of the human Harmonin (Faure et al., 2014). Evolutionarily conserved residues are mainly

present in the core region, stabilizing the structure (Figure 2C-2E). The electrostatic surface potential of HHD2 revealed a distinct pattern of positively and negatively charged surfaces (Figure 2F).

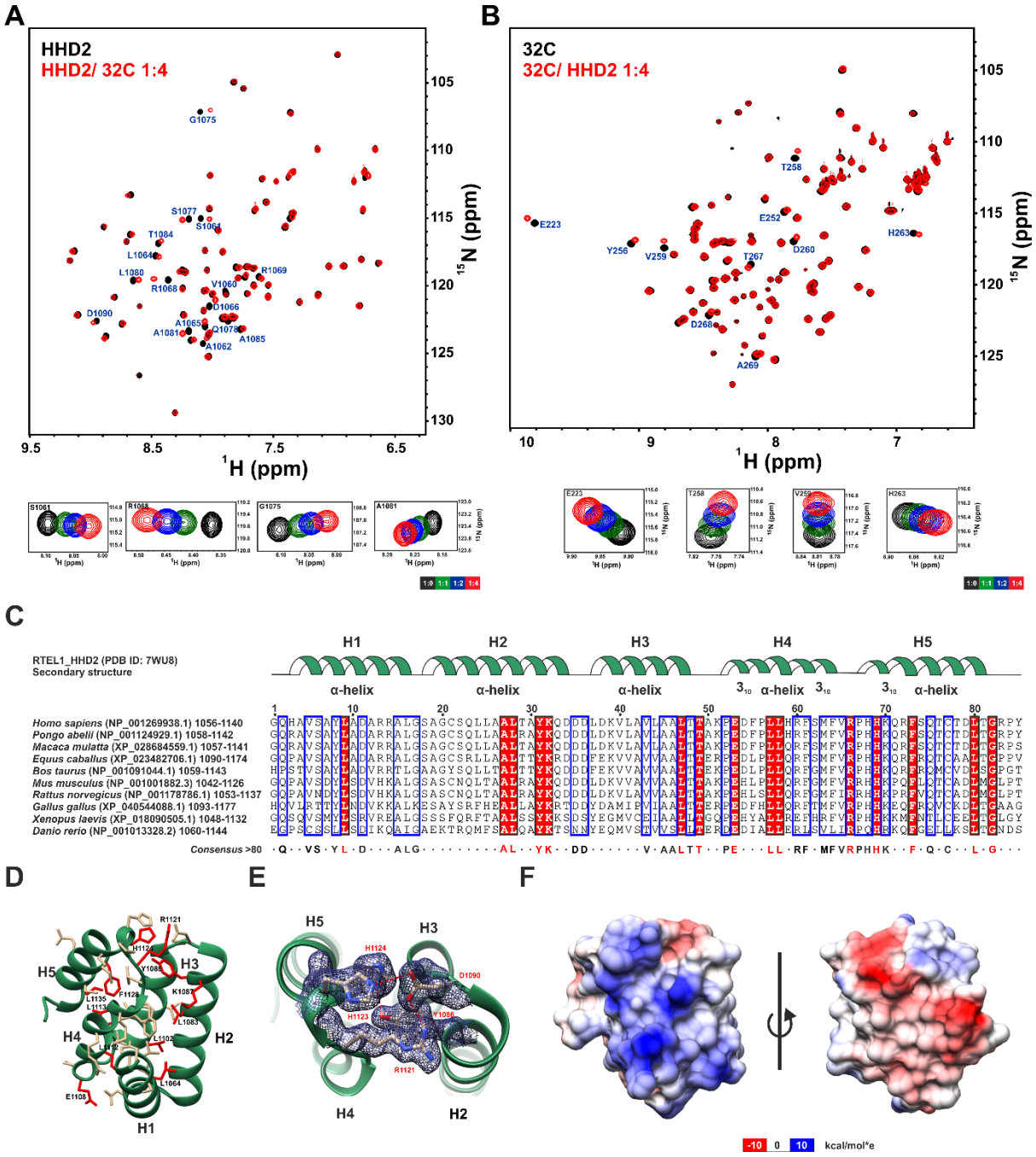
A
HHD2
HHD2/ 32C 1:4
B
32C
32C/ HHD2 1:4
C
RTEL1_HHD2 (PDB ID: 7WU8)
Secondary structure
H1
H2
H3
H4
H5
α-helix
Homo sapiens (NP_001269938.1) 1056-1140
Pongo abelii (NP_001124929.1) 1058-1142
Macaca mulatta (XP_028684559.1) 1057-1141
Equus caballus (XP_023482706.1) 1090-1174
Bos taurus (NP_001091044.1) 1059-1143
Mus musculus (NP_001001882.3) 1042-1126
Rattus norvegicus (NP_001178786.1) 1053-1137
Gallus gallus (XP_040544088.1) 1093-1177
Xenopus laevis (XP_018090505.1) 1048-1132
Danio rerio (NP_001013328.2) 1060-1144
Consensus >80
D
E
F
kcal/mol*e

Figure 2. Structure of HHD2 domain of RTEL1. **(A)** Overlay of ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled HHD2 in the absence (black) and presence (red) of RPA 32C (at 1:4 molar ratio). Residues that showed large CSPs are labelled (*upper panel*). ^{1}H-^{15}N cross-peaks trajectory of representative residues S1061, R1068, G1075, and A1081 of HHD2 upon titration with RPA 32C at indicated molar ratios (*bottom panel*). **(B)** Overlay of ^{1}H - ^{15}N HSQC spectra of ^{15}N-labelled 32C in the absence (black) and presence (red) of HHD2 (at 1:4 molar ratio). Residues that showed large CSPs are labeled (*upper panel*). ^{1}H -^{15}N cross-peaks trajectory of representative residues E223, T258, V259, and H263 of 32C upon titration with HHD2 at indicated molar ratios (*bottom panel*). **(C)** Multiple sequence alignment of HHD2 of RTEL1 proteins from different vertebrate species. The secondary structure corresponding to the HHD2 of human RTEL1 is depicted at the top (green ribbon). The consensus sequence, including the conserved residues (red), is shown at the bottom. **(D)** 1.6 Å crystal structure of HHD2 domain of human RTEL1. Sidechains of evolutionarily conserved residues are shown as stick models (fully conserved residues and partially conserved residues are shown in red and gold, respectively). **(E)** Electron density map of a representative region consisting of conserved residues Y1086, D1090, R1121, H1123, and H1124. One salt bridge (D1090 OD1 – R1121 NH1) and two H-bonds (Y1086 OH – H1124 ND1 and D1090 OD2 – H1123 NE2) are shown (red dash line). **(F)** Unique surface electrostatic potential map of HHD2. One surface (consisting of H1 and H2 helices) of the protein is positively charged (blue), while the other surface (consisting of H2, H3, and H5) is negatively charged (red).

Our recently deposited chemical shifts of HHD2 (BMRB entry 51077) (Kumar et al., 2022) were used to map the RPA 32C binding surface on the HHD2 crystal structure. The observed CSPs in the titration of ^{15}N-labelled HHD2 with 32C (Figure 2A) were quantified and plotted (Figure 3A). The HHD2 residues that showed perturbation more than the average CSPs upon 32C binding were mainly present in the N-terminal helices H1 and H2 (Figure 3B).

Similarly, the observed CSPs in the titration of ^{15}N-labelled 32C with HHD2 (Figure 2B) were quantified and plotted (Figure 3C) to map the HHD2 binding surface on the RPA 32C domain. A previously determined structure (PDB ID 1DPU) and deposited chemical shifts (BMRB entry 4460) of RPA 32C were used for the analysis (Mer et al., 2000a; Mer et al., 2000b). The residues that showed perturbation more than the average CSPs upon HHD2 binding were mainly present in the C-terminal β-sheet and the adjacent loops of 32C (Figure 3D). Interestingly, this region of 32C has been shown to interact with several proteins involved in the DNA repair and replication processes (Figure S2G and S2H) (Ali et al., 2010; Arunkumar et al., 2005; Dueva and Iliakis, 2020; Feldkamp et al., 2014; Jumper et al., 2021; Mer et al., 2000a).

Electrostatic surface potential analysis revealed that the positively charged surface of HHD2 (Figure 3B) interacts with the negatively charged surface of 32C (Figure 3D). To get molecular insight into this interaction, we generated the NMR CSPs data-driven HADDOCK model of the HHD2–32C complex (Figure 3E and Table S2). The largest cluster (cluster 1) contains 79 models, out of the 153 final clustered models, with the best HADDOCK score (-78.6 ± 3.0) and Z-score (-1.9), suggesting that the selected HADDOCK cluster is of good quality. Therefore, we chose this cluster to represent the HHD2–32C complex. A closer inspection of the selected model showed that hydrophobic and ionic interactions stabilize the HHD2–32C complex

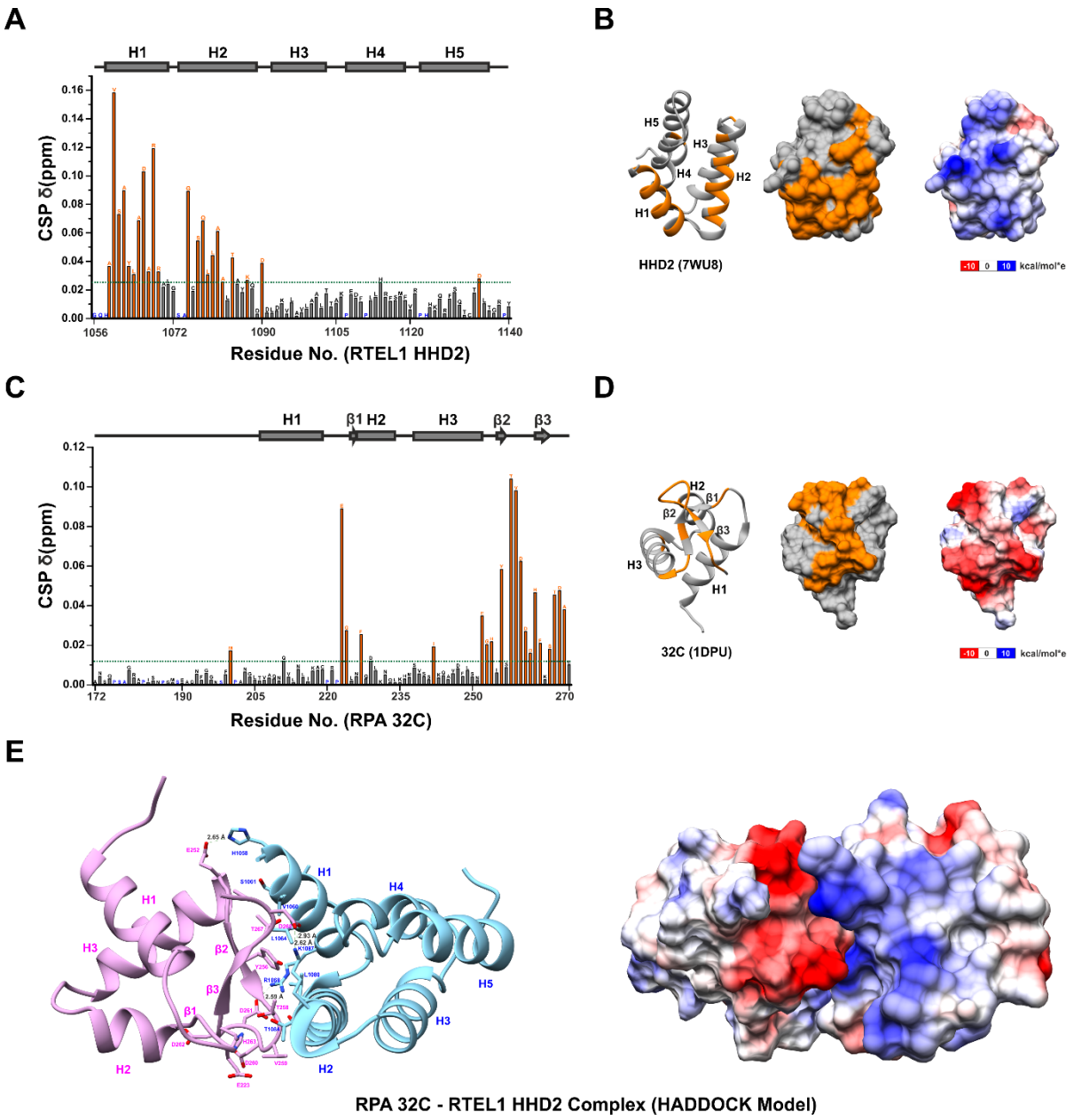

Figure 3. The binding interface of RTEL1-RPA interaction. (A) Quantification of CSPs in HHD2 upon titration with RPA 32C (at 1:4 molar ratio). Residues with more than average CSP (green dash line) are marked as an orange bar and considered as significantly perturbed residues. Proline and unassigned residues are marked in blue. The secondary structure corresponding to HHD2 is shown at the top. **(B)** Significantly perturbed residues (orange) are marked on the structure of HHD2 (ribbon - left and surface - middle forms). Most of these residues lie on the positively charged surface (right) of HHD2. **(C)** Quantification of CSPs in 32C upon titration with HHD2 (at 1:4 molar ratio). Residues with more than average CSP (green dash line) are marked as an orange bar and considered as significantly perturbed residues. Proline and unassigned residues are marked in blue. The secondary structure corresponding to 32C is shown at the top. **(D)** Significantly perturbed residues (orange) are marked on the structure of 32C (ribbon - left and surface - middle forms). Most of these residues lie on the negatively charged surface (right) of 32C. **(E)** HADDOCK model of RTEL1 HHD2 – RPA 32C complex with indicated interface residues. Positively (blue) and negatively (red) charged interacting surfaces of HHD2 and 32C, respectively, are shown.

(Figure 3E). Upon complex formation, the total buried surface area is 1322.7 ± 29.4 A^2 (Table S2).

HHD2 of RTEL1 interacts with DNA.

The HRDC domain in WRN and BLM helicases regulates its helicase activity through binding with DNA (Estep and Brosh, 2018; Kitano, 2014). We hypothesize that HHDs of RTEL1 may have similar functions and can potentially interact with DNA.

We performed NMR titrations of individual HHDs using a 22-mer ssDNA (ssDNA-22) and a 22 base-paired double-stranded DNA (dsDNA-22). There were no significant CSPs in the ^{1}H-^{15}N HSQC spectra of HHD1 upon ssDNA titrations (Figure S3A). In the case of dsDNA titrations, we observed only small CSPs of some HHD1 residues (Figure S3B). These results suggest that HHD1 of RTEL1 either does not bind ssDNA (ssDNA-22) or weakly binds dsDNA (dsDNA-22).

Interestingly, in the case of the titrations of HHD2 with DNA, we observed distinct CSPs upon the addition of both ssDNA and dsDNA. HHD2 residues that showed large CSPs upon ssDNA (Figure 4A and 4B) and dsDNA binding (Figure S3C) were mapped to the helices H1, H2, and H4 of the HHD2 structure (Figure 4C and 4D; and Figure S3D and S3E).

To further understand the HHD2–DNA interaction, we performed isothermal titration calorimetry (ITC) experiments. The ITC results revealed that HHD2 interacts with both ssDNA

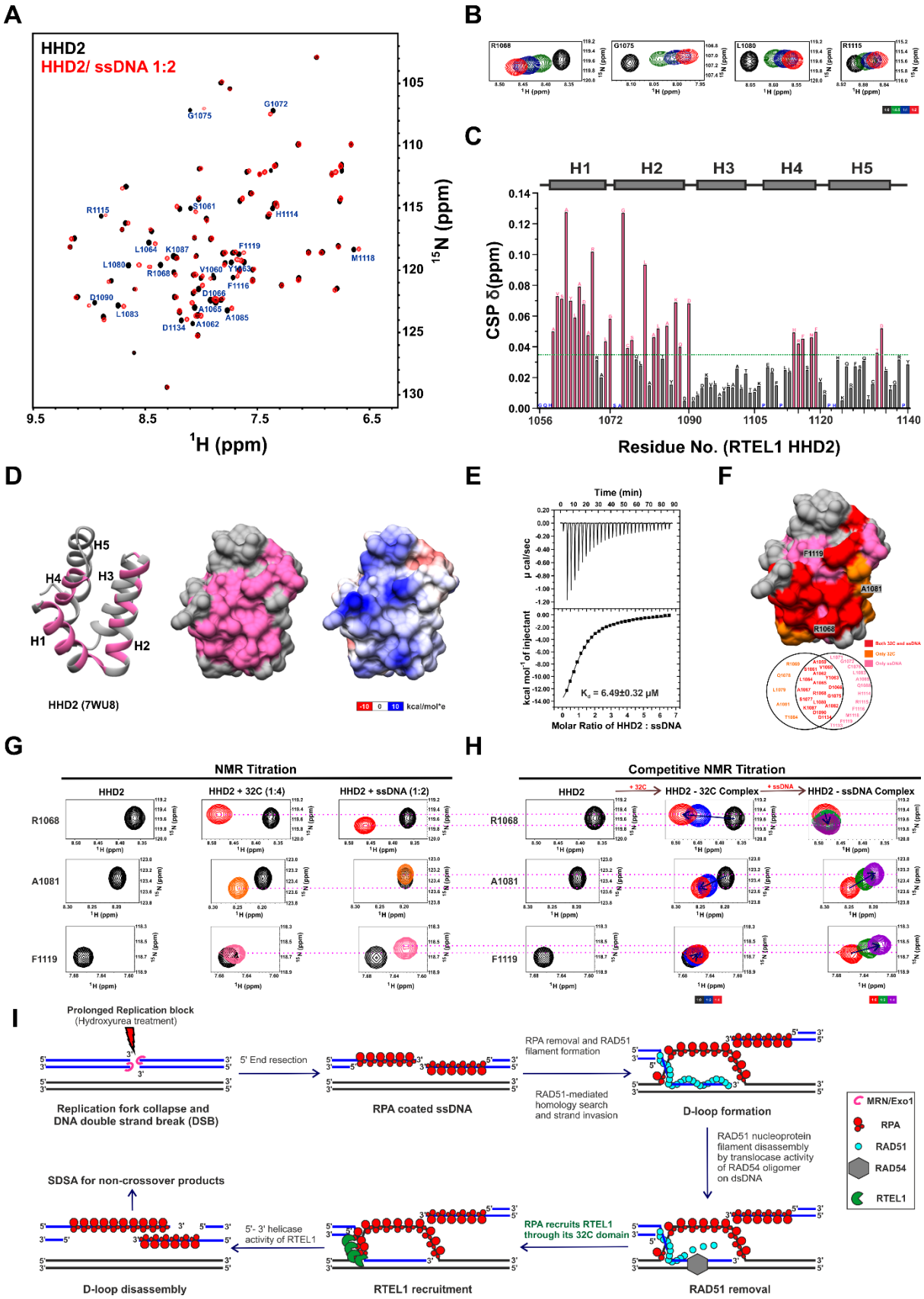

Figure 4. The interplay among RTEL1, RPA, and DNA. (A) Overlay of ^{1}H -^{15}N HSQC spectra of HHD2 in the absence (black) and presence (red) of ssDNA-22 (at 1:2 molar ratio). Residues that showed large CSPs are labelled. **(B)** ^{1}H-^{15}N cross-peaks trajectory of representative residues R1068, G1075, L1080, and R1115 of HHD2 upon titration with ssDNA-22 at indicated molar ratios. **(C)** Quantification of CSPs in HHD2 upon titration with ssDNA-22 (at 1:2 molar ratio). Residues that showed more than average CSP (green dash line) are marked as a pink bar and considered as significantly perturbed residues. Proline and unassigned residues are marked in blue. The secondary structure of HHD2 is shown at the top. **(D)** Significantly perturbed residues (pink) are marked on the structure of HHD2 (ribbon – left and surface – middle form). Most of these residues lie on the positively charged surface (right) of HHD2. **(E)** Raw and fitted ITC binding isotherms for the interaction of HHD2 with ssDNA-22. The equilibrium K_d obtained upon fitting the raw data is mentioned. **(F)** A subset of significantly perturbed residues of HHD2 upon binding to only RPA 32C (orange), only ssDNA (pink), and both 32C and ssDNA (red) are shown. One representative residue from each subset is denoted on the surface-filled structure of HHD2. **(G)** ^{1}H-^{15}N HSQC cross-peaks trajectory of representative residues R1068, A1081, and F1119 of HHD2 upon individual titration with RPA 32C and ssDNA-22 at indicated molar ratios. **(H)** Competitive NMR titration of ^{15}N-HHD2 with RPA 32C and ssDNA. Arrow indicates the direction of movement of the cross-peaks upon titration at indicated molar ratios. NMR titration of the HHD2–32C complex with DNA showed that the cross-peak of A1081 comes back to the free HHD2/HHD2-DNA complex position while the cross-peaks of F1119 and R1068 follow the path towards the HHD2-DNA complex. **(I)** Proposed model for RPA-mediated recruitment of RTEL1 to perform efficient D-loop disassembly. Prolonged replication block by hydroxyurea treatment eventually leads to DNA double-strand break (DSB). MRN/Exo1 (pink) complex executes 5' end resection to generate the 3' overhang, which quickly gets coated by RPA (red). RAD51 (cyan) nucleoprotein filament invades the homologous dsDNA to form D (displacement)-loop. RAD54 (grey) translocase activity helps in the removal of RAD51. RPA, present on the displaced ssDNA, recruits the RTEL1 (green) on D-loop through its 32C domain. The 5'-3' helicase activity of RTEL1 helps in the disassembly of the D-loop and thus directs the recombination intermediate to follow the synthesis-dependent strand-annealing (SDSA) pathway of homologous recombination to generate the non-crossover products.

(Figure 4E) and dsDNA (Figure S3F and S3G) in enthalpically driven binding with dissociation constants (K_d) of 6.49±0.32 μM and 4.67±0.32 μM, respectively (Table S3). These results unequivocally showed that HHD2 of RTEL1 interacts with DNA. This is the first report of an HHD binding to DNA.

RPA 32C and DNA compete for the same binding site on RTEL1 HHD2

We observed that the RPA 32C binding surface overlaps with the DNA binding surface in the HHD2 structure (Figure 4F). RPA 32C binds mainly to the solvent-exposed surface of helix H1 and H2, while DNA binds in the pocket formed by helix H1, H2, and H4, suggesting HHD2 adapts to bind DNA and 32C on the same surface. We hypothesize that 32C and DNA may

bind competitively to HHD2. We performed an NMR-based competitive experiment to address this hypothesis and recorded a ^{1}H-^{15}N HSQC spectrum of ^{15}N-labeled HHD2 with 32C. The HHD2–32C complex was then titrated with the ssDNA-22. The addition of DNA resulted in resonance peaks coming to the position of the HHD2–DNA complex, thus suggesting the competitive displacement of 32C by the DNA (Figure 4G and 4H).

DISCUSSION

In an *in vitro* assay, RTEL1 was shown to preferentially dissociate the D-loops with 3' invasion. Interestingly, the efficient unwinding required the presence of the RPA (Youds et al., 2010). However, the mechanism of RPA-dependent enhancement of D-loop disassembly by RTEL1 had remained unknown. Here, we have shown that the HHD2 of RTEL1 interacts directly with the 32C domain of RPA. We have observed that the deletion of the HHD1+2 region from RTEL1 significantly reduced but didn't abolish the interaction with RPA (Figure 1E). Since both RPA and RTEL1 are multi-domain proteins, there might be other interaction sites in addition to the 32C–HHD2 binding site. We have also shown that upon DNA damage RTEL1 and RPA co-localizes in the cell. Based on these results we propose a model describing the role of RPA in the recruitment of RTEL1 at the D-loop (Figure 4I). RPA is present on the displaced ssDNA of the D-loop structure (Eggler et al., 2002; Li and Heyer, 2008). The RPA 32C and RTEL1 HHD2 interaction will enable the recruitment of RTEL1 at the ss-dsDNA junction of the invading strand of the D-loop. A conformational change in RPA (Pokhrel et al., 2019) upon interaction with RTEL1 will expose the ssDNA, making it available for RTEL1 binding (Figure 4H). This will allow RTEL1 to unwind the DNA at ss-ds DNA junction using its 5’-3’ DNA helicase activity (Figure 4I).

HHDs belong to a newly classified group of αα-hub domains found in several proteins (Colcombet-Cazenave et al., 2021). The αα-hub domains typically consist of 3-5 α-helices. Hub proteins mediate fidelity in signalling and larger protein complexes. HHDs consist of five α-helices proposed to act mainly as a protein-protein interaction domain. The HHDs are associated with the PDZ domain in Harmonin, Whirlin, Delphilin, and PDZD7, with the PID

domain in CCM2, and with the helicase and RING domains in RTEL1 (Figure S4A), suggesting that HHDs have evolved to provide context-dependent functions in different proteins (Bugge et al., 2021; Colcombet-Cazenave et al., 2021; Staby et al., 2021).

Although the structure of HHDs in these proteins bears high structural similarity (Figure S4B) (Delhommel et al., 2018; Jumper et al., 2021; Lin et al., 2021; Wang et al., 2015; Yan et al., 2010), the functions of these proteins are diverse and entirely unrelated (Colcombet-Cazenave et al., 2021). The Harmonin and CCM2 HHDs interact with partner proteins (Colcombet-Cazenave et al., 2021). The mouse PDZD7 HHD-region interacts with lipids (Lin et al., 2021). Here, we report the DNA and protein binding properties of the HHD2 domain of RTEL1. Therefore, HHDs display the remarkable functional plasticity to interact with diverse biomolecules (e.g., protein, lipids, and DNA).

The HHD1 and HHD2 domains of RTEL1 fold into a common globular bundle of five helices. However, the amino acids on the domain surfaces are poorly conserved, yielding distinct surface properties. The isoelectric point (pI) of HHD1 and HHD2 is 6.31 and 8.73, respectively (Figure S4C). HHD1 has an acidic surface and a largely neutral opposite surface. However, HHD2 consists of distinct basic and acidic surfaces (Figure S4D), suggesting that these two tandem domains may have distinct roles in RTEL1.

HHD1 was shown to interact with SLX4 (Takedachi et al., 2020). Here, we have characterized the RPA as the first interacting partner of the HHD2 domain. The HHD2 interacts with the 32C domain of RPA and DNA using a common surface (helices H1 and H2). Interestingly, equivalent helices in the Harmonin and CCM2 HHDs were reported as the protein-binding sites (Colcombet-Cazenave et al., 2021). Like many RPA interacting proteins, the HHD2 of RTEL1 interacts with the conserved binding surface of RPA 32C (Figure S2G and S2H). Therefore, we conclude that the HHD2 of RTEL1 is a unique hub domain capable of mediating protein-protein and protein-DNA interactions.

Interestingly, the HRDC domain of RecQ DNA helicases is also all-helical (consisting of five α-helices and one 3_{10}-helix) like the HHDs of the RTEL1 (Kitano, 2014). The HRDC domains interact with DNA, facilitating the recruitment of these helicases to the site of DNA repair (Croteau et al., 2014). The HRDC–DNA interaction confers specificity and modulates the G-quadruplex unwinding activity of REQ helicase in *E. coli* (Teng et al., 2020). Based on the results from this study, we suggest that the HHDs could provide the HRDC equivalent roles in RTEL1.

Since RTEL1 HHD2 interacts with both ssDNA and dsDNA with similar affinity (Table S3), we postulate that HHD2 has the potential to bind at the ss-dsDNA junction formed during DNA metabolism processes in the cell. We have shown the competitive binding of RPA 32C and DNA on the HHD2 of RTEL1 (Figure 4H). This interplay of RPA-RTEL1-DNA interactions will have implications for understanding the mechanism of the role of RTEL1 in the DNA replication, repair, and recombination processes.

METHODS

Plasmid construction

DNA sequences corresponding to HHD1 (residues 886-978) and HHD2 (residues 1056-1140) were cloned in the pET-28a vector as described earlier (Kumar et al., 2022). The DNA sequences encoding the tandem harmonin homology domains (HHD1+2) (residues 886–1140) of human RTEL1 (Uniprot identifier Q9NZ71- 6) were PCR amplified using human RTEL1 cDNA clone as a template and subcloned into *E. coli* expression vector pET-28a (+) between NdeI and XhoI restriction endonuclease sites.

The DNA sequences encoding the 70N (residues 1–120 of RPA1), 70A (residues 181–291 of RPA1), 70B (residues 299–422 of RPA1), and 32C (residues 172–270 of RPA2) domains of human RPA were PCR amplified using p11d-tRPA plasmid as a template (Addgene plasmid

102613) (Henricksen et al., 1994) and subcloned into *E. coli* expression vector pET-15b between NdeI and BamHI restriction endonuclease sites.

The forward and reverse primer sets for each construct are listed in the supplementary Table S4. Phusion High-Fidelity DNA polymerase (NEB) was used for PCR amplification of each construct. The recombinant plasmids were amplified in *E. coli* DH5α cells and isolated through the Miniprep Kit (Qiagen). All plasmids encode a hexa-histidine (6xHis) purification tag at the N-terminus of the protein sequence.

Protein expression and purification

RTEL1 HHD1, HHD2, and HHD1+2

RTEL1 HHD1 and HHD2 domains were expressed and purified as described previously (Kumar et al., 2022). In summary, the following protocol was followed for the purification of HHD1, HHD2, and HHD1+2. Plasmids containing HHD1, HHD2, and HHD1+2 were individually transformed into *E. coli* Rosetta (DE3) cells. Cells were grown in either the LB (for unlabeled protein) or M9 minimal media (for uniformly ^{15}N-labeled protein for NMR) in the presence of Kanamycin and Chloramphenicol antibiotics. ^{15}N NH_4Cl (1g/L of the media) was used as sole sources of nitrogen in case of minimal media preparation. Protein expression was induced at OD_{600} of 0.8-1 by adding 1 mM of IPTG, and the culture was incubated at 20°C for 18 h. Cells were harvested and lysed using sonication under lysis buffer (50 mM Tris pH 8 at 4°C, 500 mM NaCl, 10 % Glycerol, 0.02 % NaN_3). EDTA-free Protease Inhibitor Cocktail tablets (Roche) and 1 mM PMSF (Roche) were added before lysing the resuspended cells through sonication. The lysate was subjected to centrifugation at 13,000 rpm for 60 min at 4°C and the supernatant was collected. 0.1% (v/v) polyethyleneimine (PEI) precipitation followed by another round of centrifugation was carried out to remove any nucleic acid contamination in the case of HHD2 and HHD1+2.

For Ni^{2+}–NTA affinity chromatography, the supernatant was loaded on the His Trap FF column (GE) pre-equilibrated with lysis buffer. Extensive column wash was performed with wash buffer

(50 mM Tris pH 8 at 4°C, 500 mM NaCl, 20 mM Imidazole, 10 % Glycerol, 0.02 % NaN_3) to remove the non-specifically bound proteins. 6xHis-tagged proteins were eluted with imidazole in the elution buffer (50 mM Tris pH 8 at 4°C, 500 mM NaCl, 250 mM Imidazole, 10 % Glycerol, 0.02 % NaN_3).

For performing the ion-exchange chromatography, Ni^{2+}–NTA eluted proteins were subjected to buffer exchange in buffer A (20 mM Tris pH 7.4 at 4°C, 50 mM NaCl, 2 mM DTT, 5% glycerol, 0.02% NaN_3) and loaded on the ion-exchange column (HiTrap Q or HiTrap Heparin column, GE) pre-equilibrated with buffer A. The bound protein was eluted with a linear gradient of 1M NaCl containing buffer B (20 mM Tris pH 7.4 at 4°C, 1 M NaCl, 2 mM DTT, 5% glycerol, 0.02% NaN_3).

Finally, size-exclusion chromatography (SEC) was carried out on a Superdex 75 column pre-equilibrated with the NMR buffer (20 mM Tris-HCl pH 7.4 at 25°C, 50 mM NaCl, 2 mM DTT, 0.02% NaN_3), or crystallization buffer (20 mM Tris-HCl pH 7.4 at 25°C, 250 mM NaCl, 5% Glycerol, 2 mM DTT, 0.02% NaN_3), or ITC buffer (10 mM Potassium phosphate pH 6.5 at 25°C, 50 mM KCl, 0.02% NaN_3) depending on the intended use of the protein for the subsequent experiments.

SDS-PAGE analysis was performed to ascertain the purity, molecular weight (relative to protein standard), and integrity of the purified proteins (Figure S1B). Proteins were concentrated using centrifugal filter unit (3 kDa MWCO, Merck Millipore) at 3500 rpm and 4°C. The HHD2 samples were concentrated at 15°C, owing to its less solubility at 4°C. The concentrations of the protein were calculated by ultraviolet (UV) absorbance at 280 nm using a UV spectrophotometer (Eppendorf) and the calculated molar extinction coefficients of HHD1, HHD2, and HHD1+2.

Full-length RPA trimeric complex

RPA complex was expressed and purified as per the protocol from M.S. Wold lab (Binz et al., 2006; Henricksen et al., 1994). p11d-tRPA (123) plasmid was transformed into *E. coli*

BL21(DE3) strain. Cells were grown in the presence of Ampicillin antibiotics. Protein expression was induced at OD_{600} of 0.7-0.8 by adding 0.3 mM of IPTG, and the culture was incubated at 37°C for 3 h. Protein purification was performed through Affi-gel blue (Bio-rad), Hydroxyapatite (Bio-rad), and Q-column (GE). 1.5 M NaSCN, 80 mM KPO_4, and 300 mM KCl buffer were used to elute the RPA (Figure S1D) from Affi-Gel blue, Hydroxyapatite, and Q-column, respectively. Finally, dialysis of RPA was carried out in the NMR buffer containing 100 mM NaCl (20 mM Tris-HCl pH 7.4 at 25°C, 100 mM NaCl, 2 mM DTT, 0.02% NaN_3).

RPA 70N, 70A, 70B, and 32C

RPA 70N, 70A, 70B, and 32C domains were expressed and purified as described previously (Bhattacharya et al., 2004; Bochkareva et al., 2005; Lee and Park, 2016; Mer et al., 2000b) with a few modifications. Plasmids carrying coding DNA for different RPA domains were transformed into *E. coli* BL21 (DE3) (for 70N domain) or BL21 (DE3) pLysS strains. Cells were grown in either the LB (for unlabeled protein) or M9 minimal media (for uniformly ^{15}N labeled protein for NMR) in the presence of Ampicillin / Ampicillin and Chloramphenicol antibiotics. ^{15}N NH_4Cl (1g/L of the media) was used as sole source of nitrogen in case of minimal media preparation. Protein expression was induced at OD_{600} of 0.8-1 by adding 1 mM of IPTG, and the culture was incubated at 37°C for 4 h (70N incubated at 20°C for 18 h). First step of purification involved His-tag - Ni^{2+}-NTA affinity chromatography for all the domains. In the next step, RPA 70A and 70B were purified using cation exchange (Heparin column). RPA 32C was purified using anion exchange (Q column). All the domains were purified using SEC on a Superdex 75 column at the final step of purification and eluted in the NMR buffer (20 mM Tris-HCl pH 7.4 at 25°C, 50 mM NaCl, 2 mM DTT, 0.02% NaN_3).

SDS-PAGE analysis was performed to ascertain the purity, molecular weight (relative to protein standard), and integrity of the purified proteins (Figure S1D). Proteins were concentrated using centrifugal filter unit (3 kDa MWCO, Merck Millipore) at 3500 rpm and 4°C. The concentrations of the protein were calculated by ultraviolet (UV) absorbance at 280 nm

using a UV spectrophotometer (Eppendorf) and the calculated molar extinction coefficients of 70N, 70A, 70B, and 32C.

Co-immunoprecipitation (Co-IP)

Cell culture and transfection

HEK293T cells were grown in low glucose DMEM supplemented with 10% foetal bovine serum and an antibiotic-antimycotic mix. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator. For transfection, cells were grown till 70–80% confluence and the plasmids (pcDNA3.1(+)-N-HA empty vector, pcDNA3.1(+)-N-HA-RTEL1, and pcDNA3.1(+)-N-HA-RTEL1-ΔHHD1+2) were transfected using Lipofectamine® 2000 reagent (Invitrogen) according to the manufacturer's protocol. 48 h post-transfection the cells were harvested.

Preparation of cell lysates

Cells were harvested from culture dishes using a cell scraper and lysed with ice-cold cell lysis buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF and 1X protease inhibitor cocktail). The lysates were cleared by centrifugation at 12,000 rpm for 10 min at 4°C, and the supernatant was collected in a fresh tube.

Immunoprecipitation, electrophoresis, and immunoblotting

Protein lysates were quantified using the Bradford assay, and 500-750 µg lysate was pre-cleared with Protein G agarose beads (Roche) for 1 hour at 4°C with rotation at 5 rpm in a rotator. Pre-cleared lysates were incubated with 3 µg of specific antibody overnight at 4°C with rotation at 5 rpm in a rotator. The immunoprecipitated complexes were captured on protein G agarose beads. After brief centrifugation, the supernatant was discarded, and beads were washed thrice with ice-cold PBS (1X) at 500 rpm and 4°C for 30 sec. After boiling the beads at 95°C for 5 min in 2X Laemmli sample buffer with 5% β-mercaptoethanol (Bio-rad), the immunoprecipitated protein was resolved on 10% SDS-PAGE gels and then transferred onto 0.45 µm PVDF membrane (GE) by overnight wet transfer. The blot was blocked with 5%

skimmed milk solution (prepared in TBST, Tris-buffered saline supplemented with 0.05% Tween 20) for 1 h at room temperature, followed by incubation at 4°C overnight with a specific primary antibody prepared in 5% BSA or skimmed milk in TBST (dilution range: 1:500 to 1:1,000). Bound antibodies on the blot were probed with HRP-conjugated secondary antibody at RT for 1 h. Between each step, the blots were washed thrice for three minutes each with TBST. Chemiluminescence was detected using ECL western blotting substrate (Bio-rad), and the images were acquired using a chemiluminescence imager (Bio-rad).

Immunofluorescence microscopy

HeLa cells were grown on sterilized glass cover slips placed inside a 12-well plate. 4 mM Hydroxyurea (Sigma) was added to the 60% to 80% confluent cultures for 48 h before harvesting. The cells were fixed using 4% paraformaldehyde for 10–15 min at room temperature. Permeabilization was carried out with 0.2% Triton X-100 for 5 min at room temperature followed by incubation with blocking buffer (5% BSA in PBS containing 0.1% Tween 20) for 60 min. The cells were incubated overnight at 4°C with indicated primary antibodies (prepared in the blocking buffer at dilutions ranging from 1:50–1:150) followed by incubation with species-specific Alexa Fluor-conjugated secondary antibodies for 1 h at room temperature. The nuclei were stained with Hoechst 33342 by incubation for 15 min at room temperature. The cells were washed twice with 1X PBS between each step. Finally, the cells were mounted on a clean glass cover slide using the Fluoromount-G Aqueous Mounting Medium and the images were acquired using Zeiss LSM 880 confocal microscope. The images were further processed and analysed with ZEN 3.5 (Blue edition).

Crystallization and X-ray structure determination

The SEC fractions of RTEL1 HHD2 were concentrated up to ~7 mg/ml and used in the crystallization trials. Initial screening was conducted using Crystal Screen HT (Hampton Research) in 72-well oil immersion plates. The rod-like crystals of HHD2 appeared within two days at 4°C in many conditions of Crystal Screen as well as in the native crystallization buffer (20 mM Tris-HCl pH 7.4 at 25°C, 250 mM NaCl, 5% Glycerol, 2 mM DTT, 0.02% NaN_3) of

HHD2. Later, the HHD2 crystals were grown in the native buffer condition using the hanging drop vapour diffusion method.

The X-ray diffraction data sets were collected at ESRF beamline ID23-1 at Grenoble (France) using the PILATUS 6M pixel-array detector (DECTRIS Ltd., Switzerland). High-resolution data set was collected at an energy of 12.7 keV (**λ** = 0.976 Å). The crystals diffracted up to a maximum resolution of 1.49 Å. The diffraction data sets were processed using XDSAPP (Sparta et al., 2016). The RTEL1 HHD2 structure was solved at 1.6 Å resolution by using the Molecular Replacement with Phaser (McCoy et al., 2007), employing coordinates of the Alpha fold model of RTEL1 HHD2 (AF-Q9NZ71-F1) (Jumper et al., 2021) as the search model. Coot (Emsley and Cowtan, 2004), PHENIX (Adams et al., 2010), and REFMAC5 (Murshudov et al., 2011) were used for iterative model building and refinement. The R_{work} and R_{free} of the final model are 0.182 and 0.211, respectively. The quality of the final model was assessed using Coot (Emsley and Cowtan, 2004), the Molprobity server (Chen et al., 2010), and the wwPDB validation server (Young et al., 2017). Structural figures were generated using UCSF Chimera (Pettersen et al., 2004). The data statistics are presented in Table S1.

DNA sample preparation

The DNA oligos (ssDNA-22 and csDNA-22) were purchased from Sigma-Aldrich in the lyophilised form. Both oligos are 22-mer ssDNA having complementary sequences to each other (Table S4). The stock solutions of DNA samples were prepared by dissolving the lyophilised oligos separately in the required amount of NMR buffer (20 mM Tris-HCl pH 7.4 at 25°C, 50 mM NaCl, 2 mM DTT, 0.02% NaN_3), and ITC buffer (10 mM Potassium phosphate pH 6.5 at 25°C, 50 mM KCl, 0.02% NaN_3) for performing the NMR and ITC titrations, respectively.

ssDNA-22 sample was used in the case of titration experiments with ssDNA. Samples of dsDNA-22 were made by mixing the equimolar amount of both the oligos, followed by incubation at 95°C for 10 min. The sample was finally reannealed by snap cooling on ice for 1 h.

Solution NMR spectroscopy

All NMR spectra (2D ^{1}H-^{15}N HSQC and 2D ^{1}H-^{15}N TROSY HSQC) were acquired at 25°C on a Bruker 700 MHz spectrometer equipped with a cryogenic probe or a room temperature probe. Uniformly ^{15}N-labeled protein samples were prepared in the NMR buffer (20 mM Tris-HCl pH 7.4 at 25°C, 50 mM NaCl, 2 mM DTT, 0.02% NaN_3). 10% D_2O (v/v) was added to the sample for the spectrometer deuterium lock. Typically, for the titration experiments, protein samples concentration was kept at 150 μM (in case of cryoprobe) or 250 μM (room temperature probe).

The NMR data were processed using Bruker Topspin and analysed using the NMRFAM-SPARKY software (Lee et al., 2015).

The chemical shift perturbations (CSPs) were analysed as combined amide chemical shift changes with equation 1: $\Delta\delta_{NH}(ppm) = [(\Delta\delta^{1}H)^{2} + (\Delta\delta^{15}N/5)^{2}]^{1/2}$, where the chemical shift changes in the ^{1}H and ^{15}N dimensions are denoted by $\Delta\delta^{1}H$ and $\Delta\delta^{15}N$ respectively.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) experiments were performed using a VP-ITC instrument (MicroCal, USA) at 25°C. The protein and DNA samples were prepared in ITC buffer (10 mM Potassium phosphate pH 6.5 at 25°C, 50 mM KCl, 0.02% NaN_3) and thoroughly degassed before experiments.

The sample cell was filled with 10 μM of the DNA and titrated with 300 μM of the RTEL1 HHD2. 30 injections of the titrant (10 μl per injection) were performed with a spacing of 3 min between each injection. A control experiment (titration of HHD2 into the buffer; Figure S3G) was performed to know the heat of dilution of protein into the buffer. The integrated heat data was fitted for one-site binding model using Origin software provided by the manufacturer. The Thermodynamic parameters of HHD2 and DNA interactions are presented in Table S3.

Bioinformatic analysis

Disordered linker region between HHD1 and HHD2 domain was predicted through VSL2B (Obradovic et al., 2005) and IUPred3 (Erdős et al., 2021) servers. Multalign (Corpet, 1988) tool was used to perform the sequence alignment of the RTEL1 HHD2 domain from different vertebrate species. 32C binding peptide sequences were aligned with the help of Clustal Omega (Sievers et al., 2011). UCSF Chimera (Pettersen et al., 2004) was used for performing the secondary structure-based sequence alignment of different HHDs.

HADDOCK Modelling

The NMR chemical shift perturbations (CSPs) data-driven docking of RTEL1 HHD2 and RPA 32C domain was performed through HADDOCK2.2 webserver with easy interface (van Zundert et al., 2016). In case of HHD2 domain A1059, V1060, S1061, A1062, Y1063, L1064, A1065, D1066, A1067, R1068, R1069, G1075, S1077, Q1078, L1079, L1080, A1081, A1082, T1084, K1087, D1090, and D1134 were selected as active residues and for 32C domain E223, G224, F227, I242, E252, G253, H254, Y256, T258, V259, D260, D261, D262, H263, F264, S266, T267, D268, and A269 were selected as active residues based on their perturbations above-average CSPs. Apart from these residues, other surrounding residues were considered as passive residues.

Here, HADDOCK clustered 153 structures in 9 cluster(s), which represents 76 % of the water-refined models HADDOCK generated (Table S2). The maximum number of models considered for clustering is 200.

After docking the best-docked clusters are ranked according to their HADDOCK score. The ranking of the clusters is based on the average score of the top 4 members of each cluster. The score of the water-refined model is calculated as:

HADDOCK score = 1.0 × Evdw +0.2 × Eelec +1.0 × Edesol +0.1 × Eair

Where Evdw = intermolecular Van der Waals energy, Eelec = intermolecular electrostatic energy, Edesol = empirical desolvation energy, Eair = Restrain energy

The Z-score of a cluster indicates how many standard deviations from the average this cluster is in terms of score. The more negative HADDOCK score and Z-score of a cluster indicate better quality.

Quantification and statistical analysis

Quantification of co-immunoprecipitation data was performed through ImageJ software (Schneider et al., 2012). The quantified value and number of independent experiments are mentioned in the corresponding figure legend.

DATABASE DEPOSITIONS

The atomic coordinates and structure factors for the RTEL1 HHD2 have been deposited in the Protein Data Bank under PDB accession code 7WU8.

ABBREVIATIONS

RTEL1, a regulator of telomere elongation helicase 1; HHD, harmonin homology domain; RPA, replication protein A; NMR, nuclear magnetic resonance; ITC, isothermal titration calorimetry; CSP, chemical shift perturbation

AUTHOR CONTRIBUTIONS

M.S. conceived the research; M.S. and N.K. designed the experiments. N.K. performed molecular cloning, protein purification, NMR, and ITC titration experiments. M.G. performed cloning, initial purification and initial acquisition of the NMR spectrum of HHD1+2. N.K. and U.R. performed crystallization experiments; N.K., U.R., and M.S. solved and analyzed the X-ray structure. A.T., N.K., and N. R. S. performed the co-immunoprecipitation and immunofluorescence microscopy experiments. M.S. and N.K. wrote the manuscript with inputs from all other authors; all authors reviewed the final manuscript.

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COMPETING INTERESTS

The authors declare that there are no competing interests associated with the manuscript.

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