SUPPLEMENTARY ONLINE MATERIAL

HARP Is an ATP-driven Annealing Helicase

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Materials and methods

Protein purification

Human HARP (SMARCAL1) cDNA was isolated by RT-PCR from HeLa cell RNA, and sequenced to verify its integrity. The cDNA was then subcloned into pFastBac1 (Invitrogen) with an N-terminal FLAG-tag. Mutant HARP cDNAs were generated by overlapping PCR with mutations in the primers. Baculovirus stocks were generated and amplified according to the Bac-to-Bac system protocol (Invitrogen). Infected Sf9 cells (1 L) were washed two times with 1xPBS and resuspended in 20 mL Lysis buffer [20 mM Hepes (K^+), pH 7.6, 0.5 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% (v/v) glycerol, 0.01% (v/v) NP-40, 1 mM DTT, 0.2 mM PMSF, 0.5 mM benzamidine, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin]. Cells were homogenized with approximately 40 strokes over a period of 30 min with a Wheaton Dounce homogenizer (B pestle) on ice. The extract was centrifuged for 10 min at 14000 x *g*, and the supernatant was transferred to a new tube and centrifuged again for 5 min. The supernatant was then incubated with 200 μL of washed anti-FLAG (M2) resin (Sigma) for 4 hours at 4°C. The resin was washed in batch two times with Lysis buffer (25 mL each) followed by two times with Wash buffer (25 mL each) [Lysis buffer containing 0.1 M KCl. The protein was recovered by three consecutive incubations of the resin with 200 μL of Wash buffer containing 0.20 mg/mL FLAG peptide (Sigma). Fractions were pooled and applied to a 1 mL CM-Sepharose column (Pharmacia), washed with 5 mL Wash buffer, and eluted with Elution buffer [Lysis buffer containing 0.3 M KCl]. The purified protein was then dialyzed into Wash buffer, frozen in liquid nitrogen, and stored at –80˚C. The purified HARP proteins are shown in figs. S1 and S10.

Human RPA was synthesized and purified essentially as described (*S1*), except that 5% (v/v) glycerol was substituted for inositol and a Source 15Q column was used instead of a MonoQ column. After purification, RPA was dialyzed into 10 mM Hepes (K^+), pH 7.6, buffer containing 30 mM KCl, 0.1 mM EDTA, 15% (v/v) glycerol, 0.01% (v/v) NP-40, 1 mM DTT, and

0.2 mM PMSF. The purified RPA is shown in fig. S13. *Drosophila* topoisomerase I catalytic domain (ND423) was synthesized and purified as described (*S2*). The purified protein is shown in fig. S13. Human BRG1 (*S3*), *Drosophila* ACF (*S2*), and Rad54 (*S4*) were synthesized and purified as described previously.

Gel mobility shift assays

The binding of HARP proteins to single-stranded DNA, double-stranded DNA, and fork DNA were carried out with 30-mer oligonucleotides that are similar but not identical to those used in a study of RAD2 family proteins (*S5*). The sequences of the oligonucleotides are as follows.

Top: 5'-CCAGTGAATTGTTGCTCGGTACCTGCTAAC

Bot-ds: 5'-GTTAGCAGGTACCGAGCAACAATTCACTGG

Bot-fork: 5'-GACATTTGATACCGAGCAACAATTCACTGG

ss: 5'- $d(CT)_{15}$ (which does not form hairpin or duplex structures)

The Bot-ds oligonucleotide is complementary to the Top oligonucleotide, and the Bot-fork oligonucleotide is identical to the Bot-ds oligonucleotide except for nine base pairs at the 5' end. The Top oligonucleotide was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB), and then annealed to the Bot-ds or the Bot-fork oligonucleotide to create the double-stranded and fork substrates, respectively. Increasing concentrations of purified HARP (0, 0.05, 0.1, 0.2, and 0.4 nM final concentrations) were combined with radiolabelled oligonucleotide probe (0.9 nM final concentration) in Binding buffer [20 mM Hepes (K^+), pH 7.6, 0.1 M KCl, 5 mM $MgCl_2$, 3% (v/v) glycerol, 0.25 mg/ml BSA, 0.05 mM EDTA, 0.5 mM DTT, 0.01% (v/v) NP-40] in a total volume of 20 μL, and then incubated for 30 minutes at room temperature. The samples were loaded into a 7% (w/v) polyacrylamide 0.5x TBE gel (16 X 18 cm, 1mm thick), subjected to electrophoresis in 0.5x TBE for 3 hours, dried, and exposed to film.

The gel shift assays with the bubble DNA (Fig. 2B) were carried out as follows. Where indicated, RPA (3 nM) was incubated with the radiolabeled substrate for 10 min at 22˚C. A 10-fold molar excess of unlabeled 60 nt $d(CT)_{30}$ competitor single-stranded DNA was added along with HARP (2 nM), where noted, and the reactions were incubated for another 20 min at 22˚C. Reactions also contained either 1.5 mM ATP or 1.5 mM UTP (as the –ATP control). The resulting products were resolved in a 5% polyacrylamide gel with 0.5x TBE buffer. The oligonucleotides used in these experiments are as follows.

Top: 5'-AATTCGAGCGAGTGTTTCACGTTCCGCCAGGGTCTGTAGGAGTTCTCTTGCCAGCA
TTTCGTTTGGATACGGCTTCACCTTACCGA

Bottom: 5'-TCGGTAAGGTGAAGCCGTATCCAAACGCCTCTGAAATCCTCTCGAAAACTTGTG
GTGAAGCGGAACGTGAAACACTCGCTCGAATT

For gel shift assays using RPA and HARP on single-stranded DNA (fig. S6), 90-nt and 24-nt oligonucleotides were used. Their sequences are as follows:

90-nt oligonucleotide: 5’-GAACCTGCTAACGACGATGAACTACGACCAGTAATCTAACGCG
AATGACGACTAACTAATGCTGTATGAAAGGACCCAACGCCAGTGAAT

24-nt oligonucleotide: 5’-AGCGGATAACAATTTCACACAGGA

ATPase assays

ATPase assays were carried out with the same oligonucleotides that were used in the DNA-binding assays, except that the oligonucleotides were not radiolabelled. The double-stranded and fork oligonucleotides were annealed prior to use. Increasing concentrations of oligonucleotides (0, 1.25, 2.5, 5, and 15 nM final concentrations) were combined with purified HARP (8 nM final concentration) in Binding buffer in a final volume of 10 μL, and the reactions

were incubated for one hour at 30˚C. For reactions containing RPA, the indicated oligonucleotides (15 nM) were used with 8 nM HARP and 8 nM (1:1 molar ratio of RPA:HARP) or 24 nM (3:1 molar ratio of RPA:HARP) of RPA, where noted. An aliquot (1 μL) of each reaction was applied to a PEI cellulose plate (Selecto Scientific), and the samples were subjected to thin-layer chromatography with 1 M formic acid, 0.5 M LiCl for 40 minutes. The plates were dried and quantitated by using a phosphorimager (Molecular Dynamics). Each experiment was performed in triplicate, and the mean and standard deviation for each condition were determined and displayed in a graph by using GraphPad Prism software. For the ATPase assays shown in Figs. 1B, 3C, 3D, and S7, the ATP concentrations were 40 nM of [γ-^{32}P]ATP and 100 nM of cold ATP. To determine the kcat of HARP, ATPase reactions were performed with HARP (10 nM) at saturating concentrations of fork DNA (20 nM) and ATP (0.4 mM) along with 10 nM [γ-^{32}P]ATP in Binding buffer at 30˚C. Time points were taken every 30 sec for the first 4 minutes, during which the reaction rate is linear. The data are reported as mean +/– SD (N = 4).

Annealing helicase assays

Supercoiled pGIE-0 plasmid DNA (*S6*) (0.3 μg; 3.2 kbp) was combined with purified RPA (2.5 μg) or corresponding buffer control in TE buffer in a final volume of 20 μL, and then incubated for 45 min at 37˚C to allow binding of RPA to the DNA. Purified recombinant *Drosophila* topoisomerase I catalytic domain (130 ng; 3.5 μL) was added to allow relaxation of the DNA and additional RPA binding, and each sample was incubated for an additional 10 minutes at 37˚C. To the resulting RPA-unwound DNA, 2.5 μL of Annealing Helicase buffer [100 mM Tris-HCl, pH 7.9, 0.2 M KCl, 20 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, and either 15 mM ATP or 15 mM UTP (as a control for the absence of ATP)] was added. Next, purified HARP, Rad54, ACF, or BRG1 (to 300 nM final concentration) or corresponding buffer control was added to a final volume of

27 μL, and each reaction was incubated at 30˚C for 15 minutes. The reactions were terminated by the addition of 100 μL of Stop buffer [20 mM EDTA (Na^+), pH 8, 0.2 M NaCl, 1% (w/v) SDS, 0.25 mg/mL glycogen]. The resulting DNA was deproteinized by phenol extraction and precipitated with ethanol. The reaction products were resolved by electrophoresis in 0.8% (w/v) agarose in 0.5x TAE buffer, and then visualized by staining with ethidium bromide.

Two notes that pertain to these assays are as follows. First, although we typically generate the partially unwound RPA-bound DNA substrate by using supercoiled plasmid DNA, the RPA-unwound DNA can also be prepared with relaxed plasmid DNA. The results of the annealing helicase assays with HARP are the same whether supercoiled or relaxed plasmid DNA is used as the starting material (data not shown). Second, we often observe that the addition of HARP to the RPA-unwound DNA in the absence of ATP results in a small but detectable increase in DNA supercoiling (compare, for example, Fig. 2A lane 3 versus lane 7). This effect may be due to the stabilization of the fork DNA at the ends of each bubble by the binding of HARP in the absence of ATP.

Triple-helix strand-displacement assays

The probe for the triple-helix strand-displacement assay was generated by annealing a ^{32}P-labeled $d(TC)_{20}$ oligo to a plasmid containing a 40-bp d(GA):d(TC) tract (*S7*). Reactions were carried out with Rad54 or HARP as described previously for Rad54 (*S8*), except that the reactions were performed in 20 mM Tris-acetate, pH 7.9, 50 mM KOAc, 10 mM MgOAc, 1 mM DTT, and 3 mM ATP. For the triple-helix strand-displacement assays, we used baculovirus-synthesized, His-tagged human HARP.

Helicase Assays

Helicase assays with partial duplex DNA were performed as described previously (see, for example, *S9*). DNA oligonucleotides used in this assay contain a 25-nt region complementary to single-stranded ΦX174 phage DNA and a 10-nt overhang either on the 3'- or 5'- side. The oligonucleotide sequences are: CATCATCTTGATTAAGCTCATTAGGCGTACATGTC (3'-overhang) and CGTACATGTCCATCATCTTGATTAAGCTCATTAGG (5'-overhang). These oligonucleotides (18 nM) were 5'-end labeled with [γ-^{32}P]ATP and annealed to single-stranded ΦX174 phage DNA (NEB; 25 nM) to form a partial duplex probe. Recombinant 6xHis-tagged bacterial RecQ (3 nM) or purified HARP (3 nM or 12 nM) was incubated with 1 nM probe in binding buffer in the presence or absence of 1.5 mM ATP for 30 minutes at 30˚C. Reactions were terminated with 20 mM EDTA, 1% SDS, and 10 nM cold oligonucleotide, and the resulting products were resolved on a 10% polyacrylamide gel (0.5x TBE buffer), which was then dried and exposed to film.

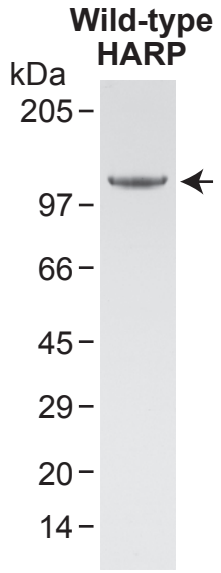

Figure S1. Purification of wild-type human HARP protein.

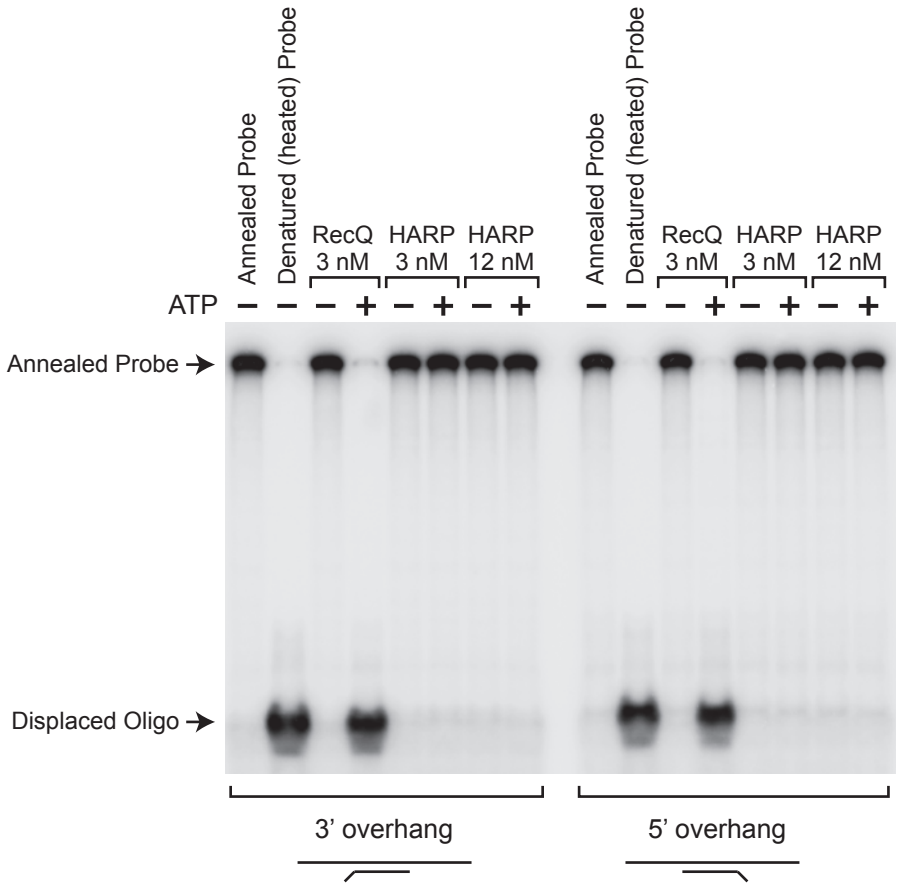

Figure S2. HARP does not exhibit helicase activity. Helicase assays were performed with partial duplex DNA substrates, as indicated, containing a radiolabeled oligonucleotide annealed to single-stranded ΦX174 DNA. Purified HARP or RecQ were incubated with the DNA substrates in the absence or presence or ATP. The products were resolved by electrophoresis in a 10% polyacrylamide gel.

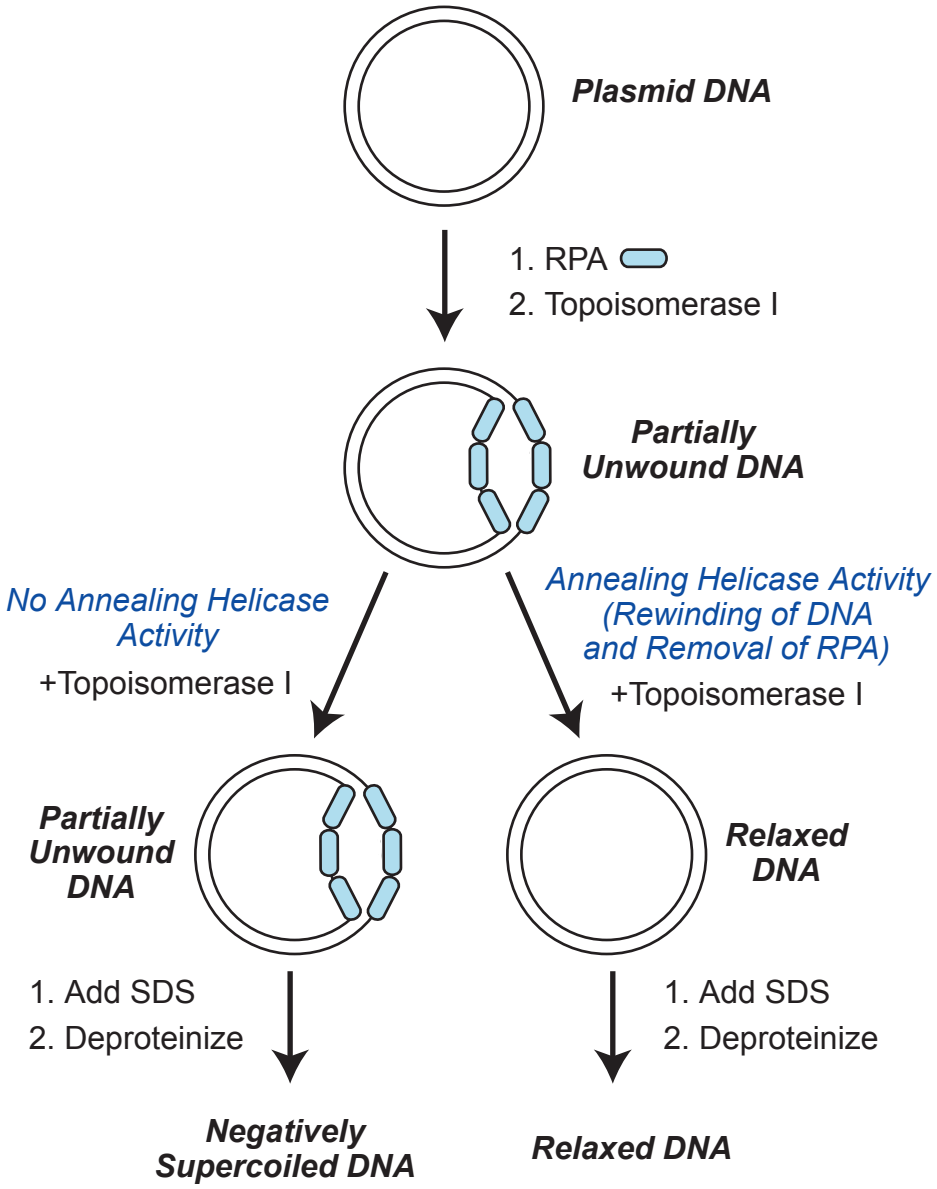

Figure S3. Schematic diagram of the annealing helicase assay. First, partially unwound DNA with stably-bound RPA is generated by incubating plasmid DNA with purified RPA and topoisomerase I. If the partially unwound DNA is treated with SDS (to inactivate enzymes, such as topoisomerase I) and deproteinized, then the resulting DNA would be negatively supercoiled due to the unwinding of the DNA by the RPA. However, if an annealing helicase rewinds the partially unwound DNA in the presence of topoisomerase I, the resulting DNA would be relaxed prior to SDS treatment and deproteinization.

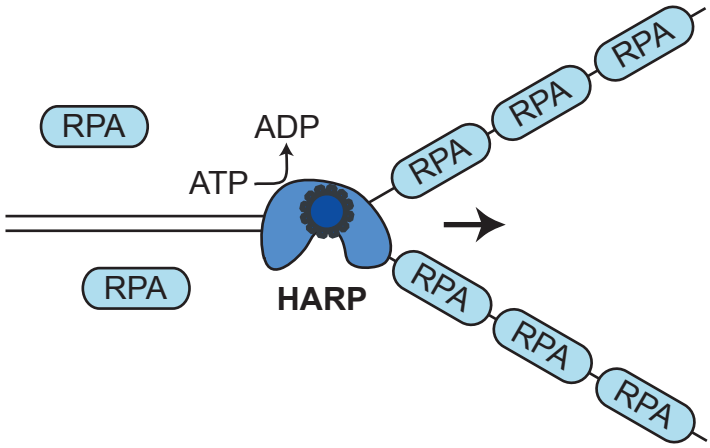

Figure S4. Depiction of HARP as an ATP-driven annealing helicase.

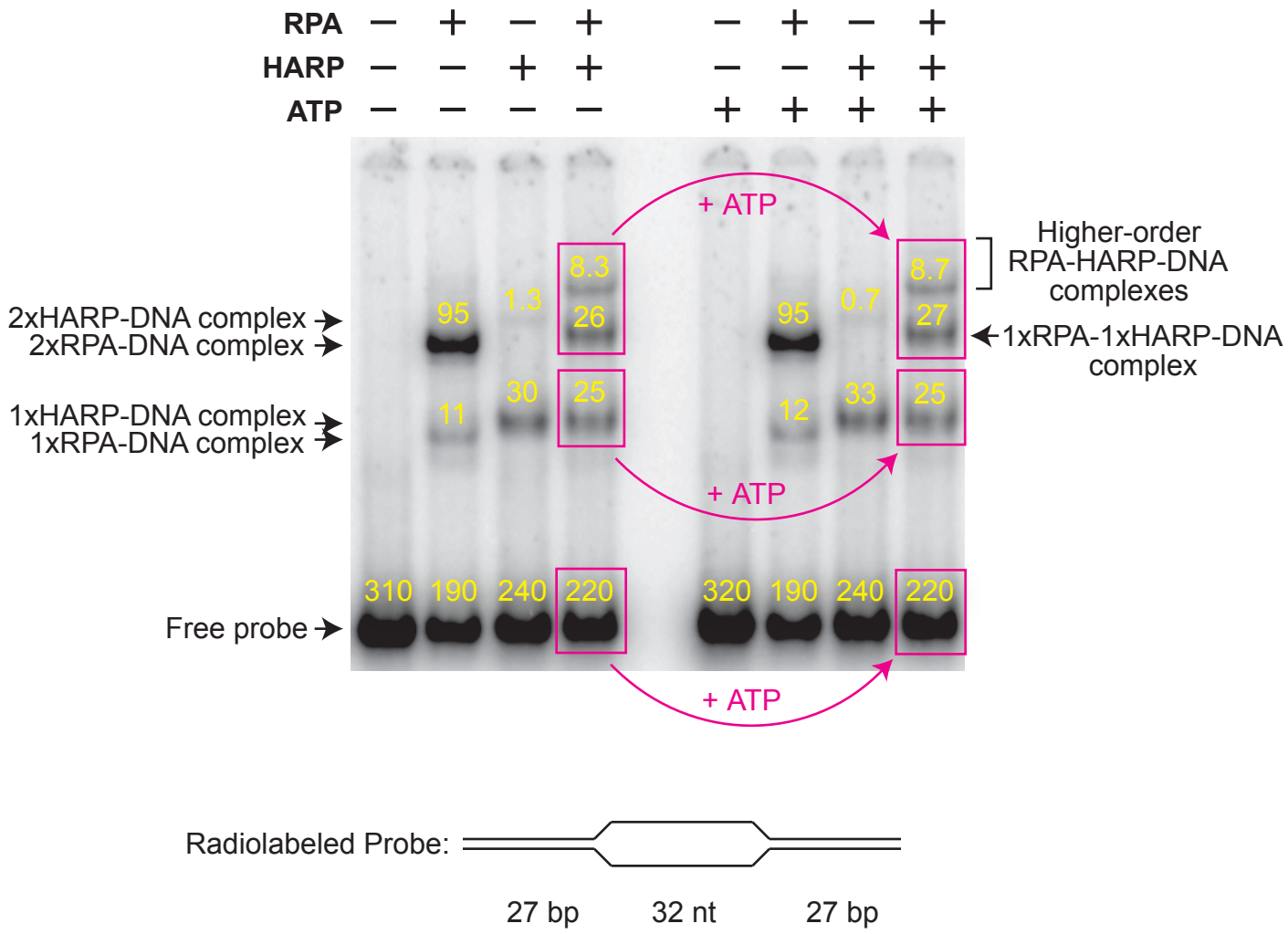

Figure S5. HARP does not displace RPA from DNA in an ATP-dependent manner. The bands in Fig. 2B of the main text were quantitated by using a phosphorimager, and the relative amounts of each band (in arbitrary units) are indicated. Upon addition of ATP, there is neither a decrease in RPA-HARP-DNA complexes nor an increase in HARP-DNA complexes or Free Probe, as would be expected if HARP catalyzes the ATP-dependent removal of RPA from DNA.

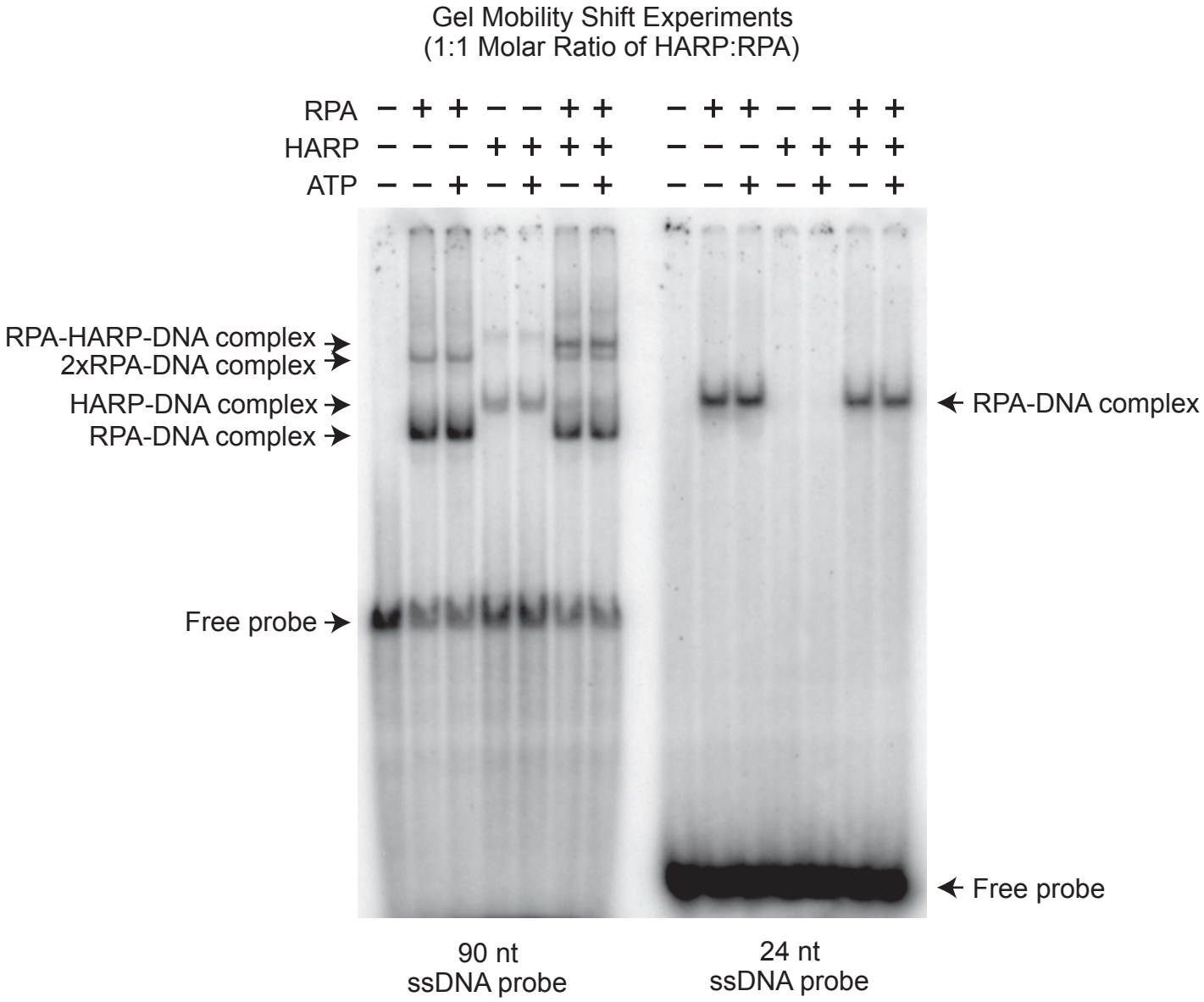

Figure S6. HARP does not displace RPA from DNA in an ATP-dependent manner. Gel mobility shift assays were performed with a 90 nt or a 24 nt radiolabeled single-stranded DNA probe. Where indicated, RPA (2 nM) was incubated with the radiolabeled DNA for 10 min at 22˚C. A 10-fold molar excess of an unlabelled competitor oligonucleotide (with the same sequence as the corresponding labeled probe) was added along with HARP (2 nM), where noted, and the reactions were incubated for another 20 min at 22˚C. Reactions contained either 1.5 mM ATP or 1.5 mM UTP (as the –ATP control). The resulting products were resolved in a 6% polyacrylamide gel with 0.5x TBE buffer. Disruption of the RPA-DNA complex is not observed upon addition of HARP. With the 90 nt probe, a ternary HARP-RPA-DNA complex is formed. The binding of HARP to the 90 nt probe is probably due to the formation of hairpin structures (and hence, fork DNA) in the longer 90 nt probe DNA. HARP does not appear to bind to the shorter 24 nt probe.

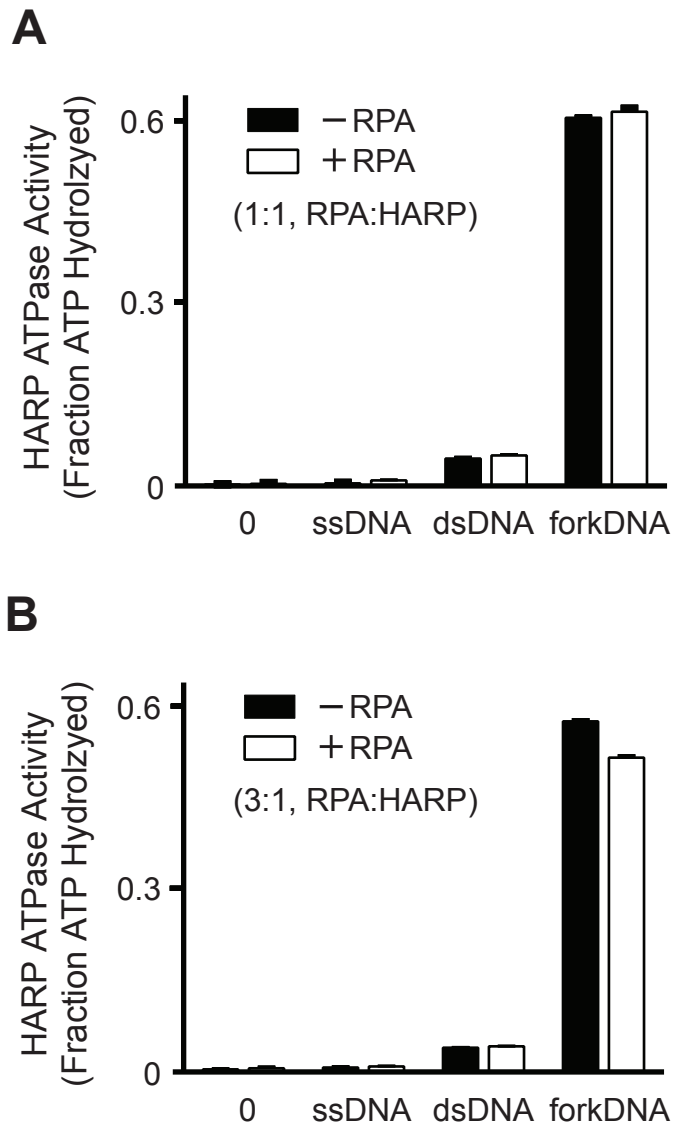

Figure S7. The ATPase activity of HARP is not stimulated by RPA. ATPase reactions were carried out with HARP and the indicated DNA substrates in the presence or absence of RPA, as noted. (**A**) 1:1 molar ratio of RPA:HARP. (**B**) 3:1 molar ratio of RPA:HARP. Error bars represent SD ($N = 3$).

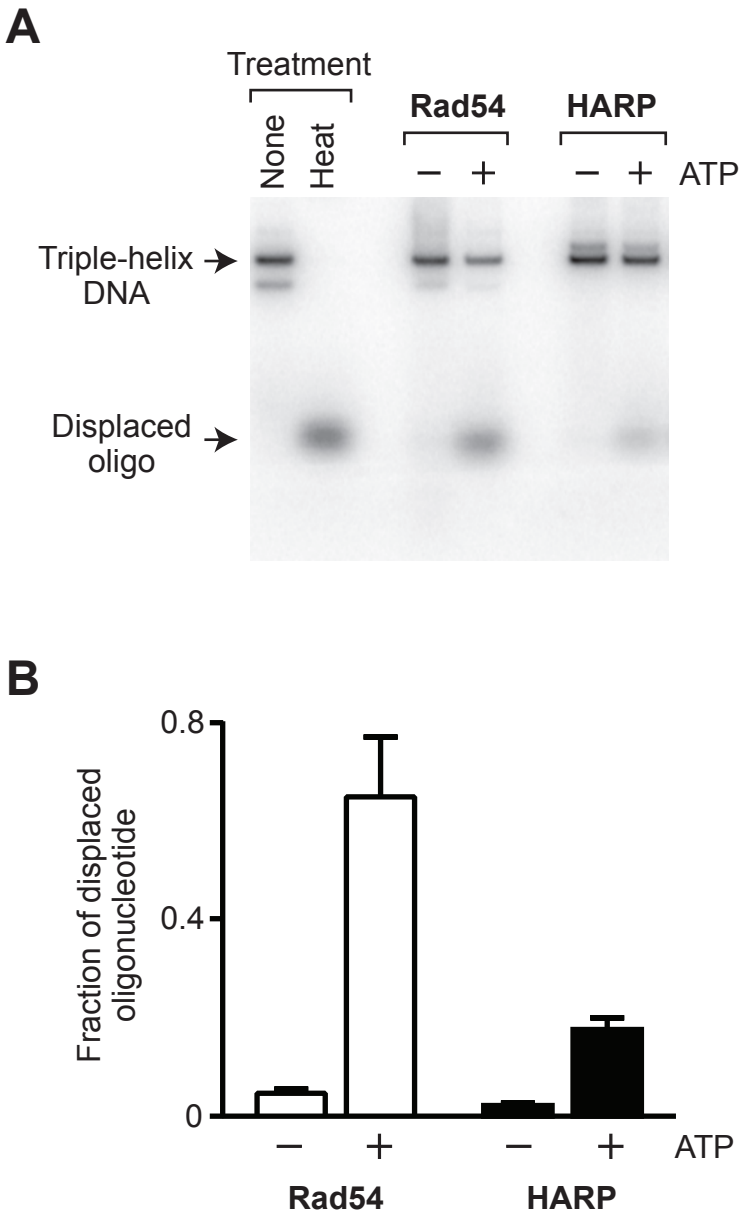

Figure S8. Triple-helix strand-displacement activity of Rad54 and HARP. Triple-helix DNA was prepared with a radiolabelled oligonucleotide, and then incubated with Rad54 or HARP. (**A**) Analysis of triple-helix strand displacement by agarose gel electrophoresis. Purified Rad54 and purified HARP were each used at 200 nM final concentration. To provide a reference for the location of the dissociated oligonucleotide, a sample of the triple-helix DNA was heat-treated to dissociate the labeled oligonucleotide (”Heat”). (**B**) Quantitation of triple-helix strand-displacement activity from three independent experiments. Rad54 and HARP were each used at 200 nM final concentration. Error bars represent SD ($N = 3$).

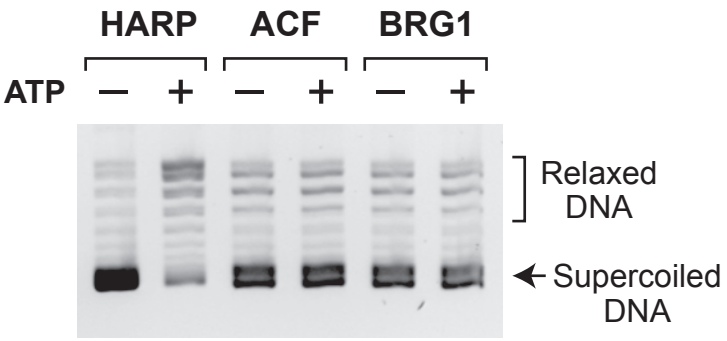

Figure S9. Neither ACF nor BRG1 exhibit annealing helicase activity. Annealing helicase assays were performed, as in Fig. 2 of the main text, with equimolar concentrations (300 nM) of HARP, ACF, or BRG1 proteins. All reactions contained plasmid DNA, RPA, and topoisomerase I. UTP was used as a control for the absence of ATP.

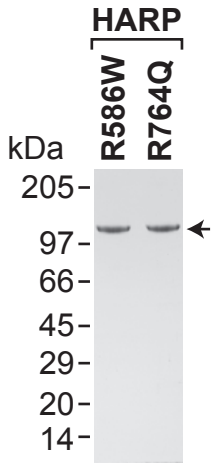

Figure S10. Purification of mutant HARP proteins.

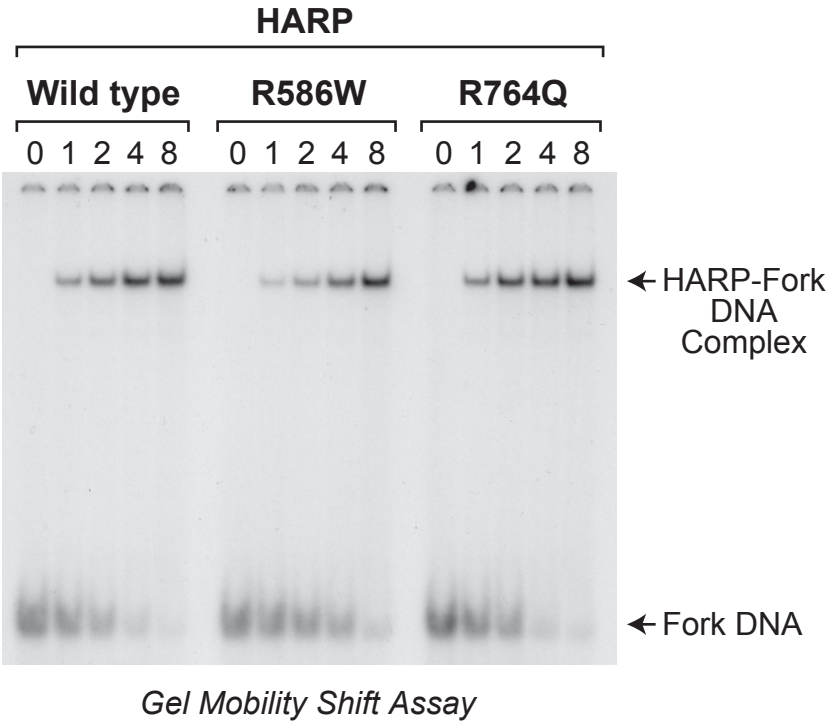

Figure S11. Direct comparison of the DNA-binding activities of wild-type and mutant HARP proteins. Gel mobility shift analyses were carried out, as in Fig. 1A, with fork DNA. The concentrations of each of the HARP proteins are 0, 0.05, 0.1, 0.2, and 0.4 nM.

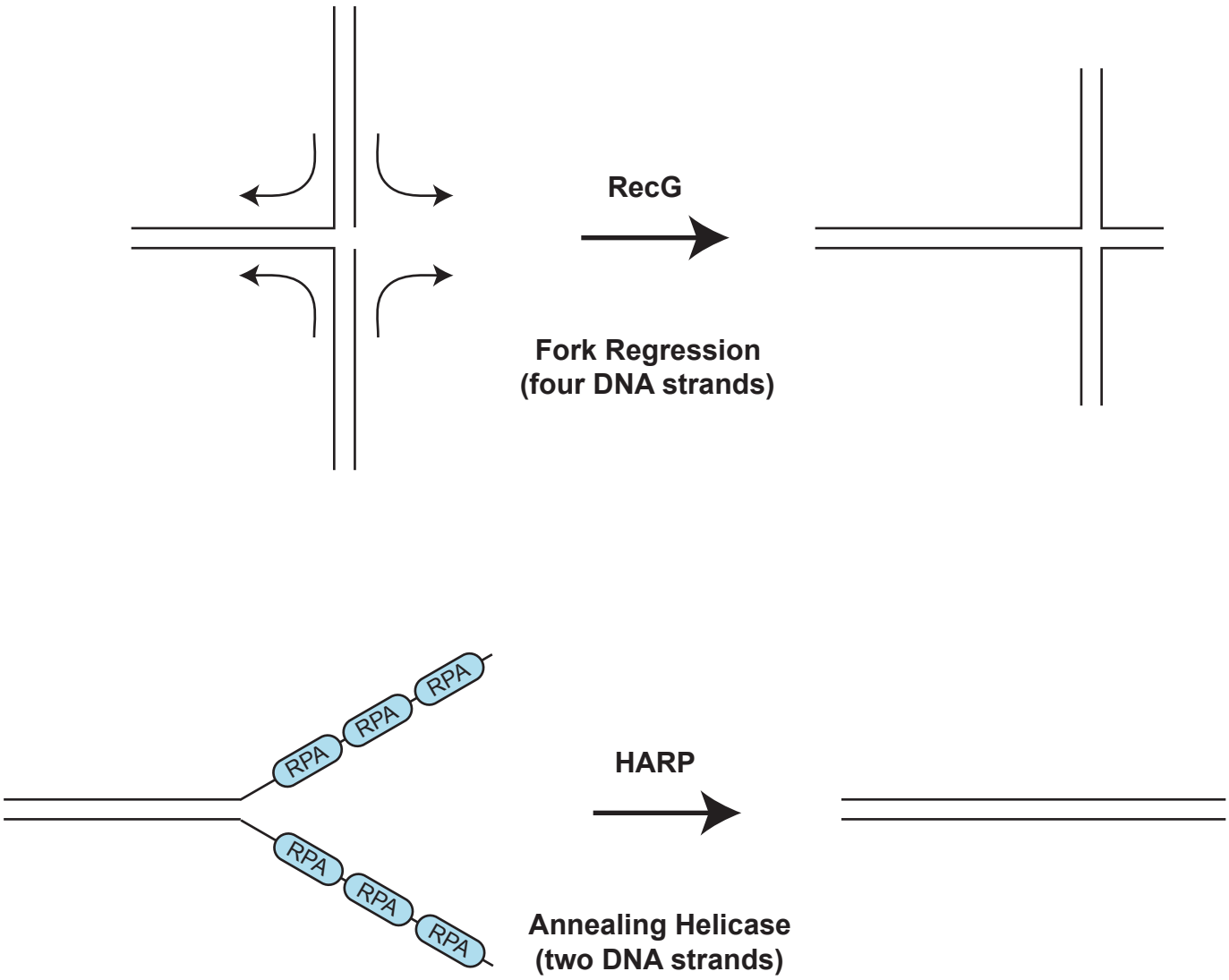

Figure S12. HARP annealing helicase activity is distinct from fork regression activity, such as by RecG, Rad5, and Bloom's Syndrome helicases.

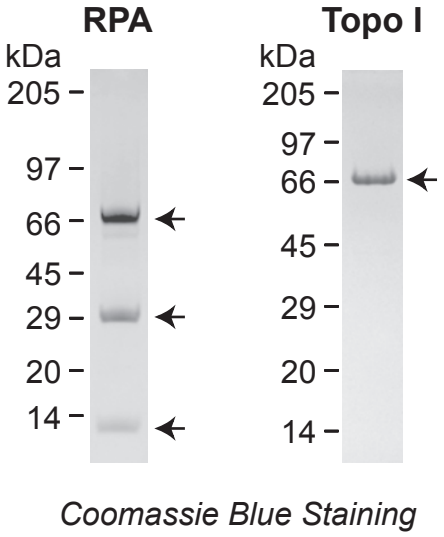

Figure S13. Purification of recombinant human replication protein A (RPA) and recombinant *Drosophila* topoisomerase I catalytic domain. Note that RPA is a heterotrimeric protein. The proteins were subjected to SDS-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue R-250.

References

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