SUPPLEMENTAL INFORMATION

Table S1. DNA oligos and substrate designs, Related to Figures 1, 2, and 3

Oligos	Sequence (5'→3')
A1	TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTT
A2	AAGCCACTGCAGGTCGACTCTAGAGGATCTCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTA
A3	CCAATTGTACTGCCGAGCTCGAATTCACTGGCCGTCGGTGACACGTCGTTAAAAGGTCTCTAACGTTCACCTGG
A4	CCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTA
A5	AAGCCACTGCAGGTCGACTCTAGAGGATCTCCGGGTA
A6	GAGTCGACCTGCAGTGGCTT
A7	AAGCCACTGCAGGTCGACTC
A8	TACGTTGTAAAACGACGGCC
A9	GGCCGTCGTTTTACAACGTA
A10	AGGTCTCGACTAACTCTAGTCGTTGTTCCACCCGTCCACCCGACGCCACCTCCTG
A11	GCAGGAGGTGGCGTCGGGTGGACGGGATTGAAATTTAGGCTGGCACGGTCG
A12	GCAGGAGGTGGCGTCGGGTGGACGGGTGGAACAACGACTAGAGTTAGTCGAGACCTATTGAAATTTAGGCTGGC ACGGTCG
A13	TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTT CTGCAGTGGCTT
A14	GTGACACGTCGTTAAAAGGTCTCTAACGTTCACCTGGAAGCCACTGCAGGTCGACTCTAGAGGATCTCCGGGTA CAATCTCTAGTG
A15	CCAGGTGAACGTTAGAGACCTTTTAACGACGTGTCAC
A16	GTGACACGTCGTTAAAAGGTCTCTAACGTTCACCTGGAAGCCACTGCAGGTCGACTCTAGAGGATCTCCGGGTA
A17	CCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTAGTGACACGTCGTTAAAAGGTCTCTAACGTTCACCTGG
A18	GGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATTAAGGGCGACACGGAAATGTTGAATACTCAT ACTCTTC

(*^{32}P labeling; Numbers are the lengths of oligos in nt; Green, annealing area)

1. Substrates for annealing of fully complementary ssDNA

```
                                                    74
A1: *5′-TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTT

A2:      ATGCAACATTTTGCTGCCGGTCACTTAAGCTCGAGCCATGGGCCTCTAGGAGATCTCAGCTGGACGTCACCGAA-5′
                                                    74
```

2. Substrates for annealing of 3'-overhangs

```
                          37                        37
A1: *5′-TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTT
A4:     ATGCAACATTTTGCTGCCGGTCACTTAAGCTCGAGCC-5′
A15:                                                                5′-CCAGGTGAACGTTAGAGACCTTTTAACGACGTGTCAC
A16:                                      ATGGGCCTCTAGGAGATCTCAGCTGGACGTCACCGAAGGTCCACTTGCAATCTCTGGAAAATTGCTGCACAGTG-5′
                                                    37                                  37
```

3. Substrates for annealing of 3'-matched/without dsDNA

```
                          37                        37
A1: *5′-TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTT

A16:                                      ATGGGCCTCTAGGAGATCTCAGCTGGACGTCACCGAAGGTCCACTTGCAATCTCTGGAAAATTGCTGCACAGTG-5′
                                                    37                                  37
```

4. Substrates for annealing of 3'-overhangs with heterogeneous 3'-tails (red)

```
                          37                        37                        12
A13: *5′-TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTTCTGCAGTGGCTT
A4:      ATGCAACATTTTGCTGCCGGTCACTTAAGCTCGAGCC-5′
A15:                                                                5′-CCAGGTGAACGTTAGAGACCTTTTAACGACGTGTCAC
A14:                                GTGATCTCTAACATGGGCCTCTAGGAGATCTCAGCTGGACGTCACCGAAGGTCCACTTGCAATCTCTGGAAAATTGCTGCACAGTG-5′
                                    12                  37                                  37
```

5. Substrates for annealing of center-matched ssDNA (3'-overhang control)

```
                          37                                37
A13: *5′-TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTTCTGCAGTGGCTT

A14:                                GTGATCTCTAACATGGGCCTCTAGGAGATCTCAGCTGGACGTCACCGAAGGTCCACTTGCAATCTCTGGAAAATTGCTGCACAGTG-5′
```

6. Substrates for annealing of 5’-overhangs

```
                          37                                37
A1:    TTCGGTGACGTCCAGCTGAGATCTCCTAGAGGCCCATGGCTCGAGCTTAAGTGACCGGCAGCAAAATGTTGCAT-5′*
A5: 5′-AAGCCACTGCAGGTCGACTCTAGAGGATCTCCGGGTA
A15:                                                                  CACTGTGCAGCAATTTTCCAGAGATTGCAAGTGGACC-5′
A17:                                    5′-CCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTAGTGACACGTCGTTAAAAGGTCTCTAACGTTCACCTGG
                                                                  37                                37
```

7. Substrates for annealing of 3’-matched/without dsDNA

```
                          37                                37
A1:    TTCGGTGACGTCCAGCTGAGATCTCCTAGAGGCCCATGGCTCGAGCTTAAGTGACCGGCAGCAAAATGTTGCAT-5′*

A17:                                    5′-CCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTAGTGACACGTCGTTAAAAGGTCTCTAACGTTCACCTGG
                                                                  37                                37
```

8. Substrates for annealing of 5’-overhangs heterogeneous 5’-tails (red)

```
                          37                      25        12
A1:    TTCGGTGACGTCCAGCTGAGATCTCCTAGAGGCCCATGGCTCGAGCTTAAGTGACCGGCAGCAAAATGTTGCAT-5′*
A5: 5′-AAGCCACTGCAGGTCGACTCTAGAGGATCTCCGGGTA
A15:                                                                  CACTGTGCAGCAATTTTCCAGAGATTGCAAGTGGACC-5′
A3:                           5′-CCAATTGTACTGCCGAGCTCGAATTCACTGGCCGTCGGTGACACGTCGTTAAAAGGTCTCTAACGTTCACCTGG
                                    12                25                                37
```

9. Substrates for annealing of center-matched ssDNA (5’-overhangs control)

```
                          37                      25        12
A1:    TTCGGTGACGTCCAGCTGAGATCTCCTAGAGGCCCATGGCTCGAGCTTAAGTGACCGGCAGCAAAATGTTGCAT-5′*

A3:                           5′-CCAATTGTACTGCCGAGCTCGAATTCACTGGCCGTCGGTGACACGTCGTTAAAAGGTCTCTAACGTTCACCTGG
                                    12                25                                37
```

10. Substrates for strand exchange with a splayed arm

The labeled oligo A10 anneals with A11 to create a splayed arm (blue). Cold A12 is used to compete with A11 for strand exchange (Red: ssDNA tail; the exchange area is underlined).

```
                                          30                                25
A10:                        *5′-AGGTCTCGACTAACTCTAGTCGTTGTTCCACCCGTCCACCCGACGCCACCTCCTG
A11:                              GCTGGCACGGTCGGATTTAAAGTTAGGGCAGGTGGGCTGCGGTGGAGGACG-5′
                                          25                                25

A12: GCTGGCACGGTCGGATTTAAAGTTATCCAGAGCTGATTGAGATCAGCAACAAGGTGGGCAGGTGGGCTGCGGTGGAGGACG-5′
          25                                          55
```

11. Substrates for strand exchange with a 3’-overhang and a blunt-end dsDNA (Red: ssDNA; the exchange area is underlined)

```
                          37                                17                  20
A1: *5′-TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTT
A4:     ATGCAACATTTTGCTGCCGGTCACTTAAGCTCGAGCC-5′

A6:                                                                   5′-GAGTCGACCTGCAGTGGCTT
A7:                                                                   3′-CTCAGCTGGACGTCACCGAA
```

12. Substrates for strand exchange with a 5’-overhang and a blunt-end dsDNA (Red: ssDNA; the exchange area is underlined)

```
                    20                  17                                37
A1: *5′-TACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTCTAGAGTCGACCTGCAGTGGCTT
A5:                                       ATGGGCCTCTAGGAGATCTCAGCTGGACGTCACCGAA-5′

A8:  5′-TACGTTGTAAAACGACGGCC
A9:  3′-ATGCAACATTTTGCTGCCGG
```

Supplemental Figure Legends:

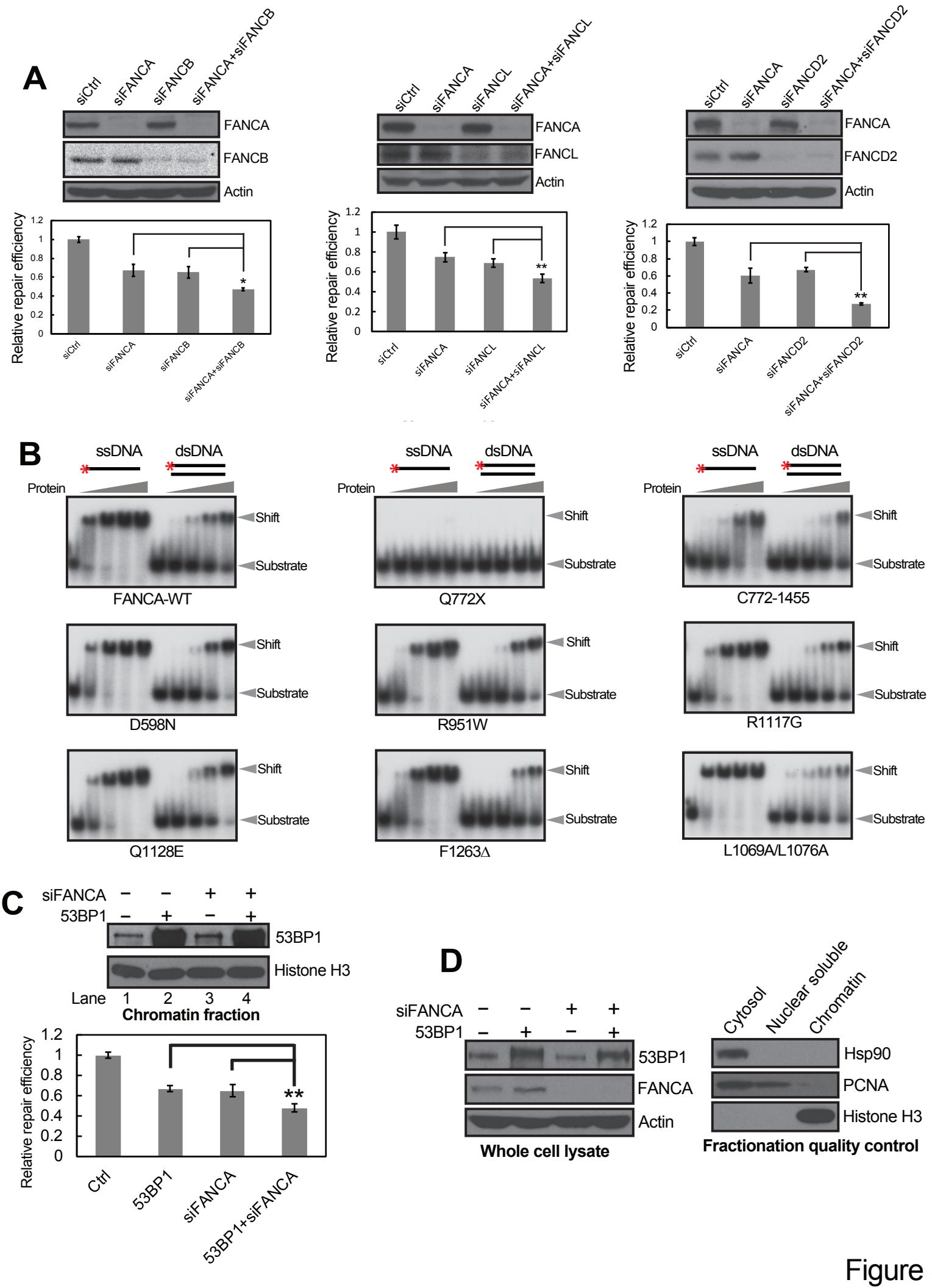
Figure S1 Related to Figures 1 and 4. **(A)** Double knockdowns of FANCA and FANCB, FANCA and FANCL, and FANCA and FANCD2 show a role of FANCA in the single-strand annealing subpathway of DSB repair independently of the FA core complex and FANCD2 in U2OS cells measured by a GFP reporter assay (see experimental procedures). Top panels, Western blots. Bottom panels, relative repair efficiency normalized to siRNA control siCtrl (** indicates p values of <0.01 when compared to either single knockdown; * indicates p values of <0.05 when compared to either single knockdown; experiments were independently repeated 4 times). Error bar, standard deviation. In the FANCD2 panel, we aimed to show the overall level of FANCD2 by running a regular 10% gel until the dye is close to the bottom of the gel. Under this condition, the expected spontaneous FANCD2 monoubiquitination (doublet) in lane 1 is indiscernible. **(B)** Purified genetic variants of FANCA: C-terminal truncation (Q772X), N-terminal truncation (C772-1455), D598N, R951W, R1117G, Q1128E, F1263Δ, and L1069A/L1076A were analyzed for DNA binding activity using an electronic mobility shift assay. The concentrations of FANCA-WT and its variants are 0, 8, 16, 32, and 64 nM (calculated as monomer). The reactions were resolved in 40-min non-denaturing gel electrophoresis (see experimental procedures). Shifted bands indicate protein-DNA interactions. **(C)** FANCA plays a role in SSA independently of 53BP1. Top panel, Western blots of chromatin 53BP1 in FANCA knockdown and/or 53BP1 overexpression cells after chromatin fractionation. Histone H3 is the loading control; Bottom panel, SSA repair efficiency of FANCA knockdown and/or 53BP1 overexpression cells by a GFP reporter assay. (** indicates p values of <0.01 when compared to either FANCA knockdown or 53BP1 overexpression; experiments were independently repeated 4 times). **(D)** Input and fractionation quality control for the 53BP1 experiment. Western blots of 53BP1 and FANCA in FANCA knockdown and/or 53BP1 overexpression whole cell lysates (left panel) and fractionation quality controls of cytosol, nuclear soluble, and chromatin preps measured by Hsp90 (expected to present in cytosol only), PCNA (expected to present in both cytosol and nucleus), and histone H3 (expected to present in chromatin only).

Figure S2. Related to Figures 1 and 2. **(A)** FANCA catalyzes single-strand annealing. Time course incubations (0, 1, 2, 5, 10, 20, 40 min) with (bottom panel) or without (top panel) 30 nM FANCA on the fully complementary ssDNA substrates. Red asterisks (*) indicate γ-^{32}P labeling by T4 polynucleotide kinase. **(B)** Quantification of the single-strand annealing activity on different 3'-overhang substrates. Schematic representations of annealing substrates are shown on the left. Experiments were independently repeated 3 times. Error bar, standard deviation. **(C)** Quantification of the single-strand annealing activity on different 5'-overhang substrates. Schematic representations of annealing substrates are shown on the left. Experiments were independently repeated 3 times. Error bar, standard deviation. **(D)** Titration of the purified human FANCA or human RAD52 (10, 20, 30, and 40 nM calculated as monomers) on fully complementary ssDNA oligos 74 nt in length; **(E)** a 3'-overhang with a central homologous sequence stretch flanked by a dsDNA; and **(F)** a 5'-overhang with a central homologous sequence stretch flanked by a dsDNA region for strand annealing. **(G)** Titration of the purified FANCA and RAD52 (10, 20, 30, and 40 nM calculated as monomers) on a pre-annealed splayed arm structure; **(H)** a dsDNA structure with partial 5'-end resection of one strand and a short blunt-ended dsDNA with full homology to the uncovered flap of the invading DNA structure (FANCA

or RAD52 at 10, 20, 30, 40, and 50 nM calculated as monomers); and **(I)** a dsDNA structure with partial 3'end resection of one strand and a short blunt-ended dsDNA with full homology to the uncovered flap of the invading DNA structure for strand exchange (FANCA or RAD52 at 10, 20, 30, 40, and 50 nM calculated as monomers). Quantification of strand annealing or exchange activity is represented by % product below the gel. Red asterisks (*) indicate ^{32}P labeling.

Figure S3. Related to Figures 1 and 2. **(A)** FANCA catalyzes single-strand annealing in the presence of RPA. Titration of the purified RPA (10, 20, 30, and 40 nM) on the fully complementary ssDNA oligos 74 nt substrate in the presence of 10 nM of FANCA (calculated as dimer). FANCA RPA premix indicates that both proteins were added to the reaction mixture at the same time. FANCA coated RPA 2nd indicates that FANCA was added 40 minutes before RPA for pre-incubation at RT. RPA coated FANCA 2nd indicates that RPA was added 40 minutes before FANCA for pre-incubation at RT. The calculated molar ratio of FANCA:RPA for each lane is shown at the bottom. **(B)** FANCA forms homodimer. SDS-PAGE analysis of FANCA and FANCA-L1069A/L1076A fractions from Superdex-200. Column calibration is shown at the top. Arrows indicate the sizes of standard proteins. Fractions in red are protein peaks (highlighted in red rectangles). Protein markers in kilodaltons are indicated on the left. **(C)** Top panel: Titration of the purified FANCA-WT and FANCA-L1069A/L1076A mutant (10, 20, 30, and 40 nM calculated as monomers) on fully complementary ssDNA oligos 74 nt in length. Quantification of strand annealing activity is represented by % product below the gel. Repeated 3 times independently. Error bars, standard deviation. Bottom panel: Titration of the purified FANCA-WT and FANCA-L1069A/L1076A mutant (10, 20, 30, and 40 nM calculated as monomers) on a pre-annealed splayed arm structure for strand exchange. Quantification of strand exchange activity is represented by % product below the gel. Repeated 3 times independently. Error bar, standard deviation.

Figure S4. Related to Figures 1 and 2. FANCG, but not FANCB, stimulates FANCA-mediated strand annealing and exchange. **(A)** SDS-PAGE analysis of the purified human FANCG and FANCB overexpressed in insect cells. Molecular weight marker in kDa is shown on the left. Titration of the purified FANCG (5, 10, 20, and 30 nM) in the presence (Lanes 4-8) or absence (Lanes 1-3 and 9-12) of FANCA on **(B)** perfectly complementary ssDNAs (10 nM FANCA), **(C)** 3'-perfectly matched overhangs (5 nM FANCA), **(D)** 3'-mismatched overhangs (15 nM FANCA), **(E)** 5'-perfectly matched overhangs (5 nM FANCA), and **(F)** 5'-mismatched overhangs (15 nM FANCA). Titration of the purified FANCG (5, 10, 20, and 30 nM) in the presence (Lanes 4-8) or absence (Lanes 1-3 and 9-12) of FANCA on **(G)** a pre-annealed splayed arm structure (10 nM FANCA) showing strand exchange activity, **(H)** a dsDNA structure with partial 5'-end resection of one strand and a short blunt-ended dsDNA with full homology to the uncovered flap of the invading DNA structure (20 nM FANCA); and **(I)** a dsDNA structure with partial 3'end resection of one strand and a short blunt-ended dsDNA with full homology to the uncovered flap of the invading DNA structure (20 nM FANCA). Titration of the purified FANCB (5, 10, 20, and 30 nM) in the presence (Lanes 4-8) or absence (Lanes 1-3 and 9-12) of FANCA on **(J)** perfectly complementary ssDNAs (10 nM FANCA), and **(K)** on a pre-annealed splayed arm structure (20 nM FANCA) showing strand exchange activity. Quantification of SA or SE activity is represented by % product below each gel**.** Red asterisks (*) indicate ^{32}P labeling. All experiments were independently repeated 3 times.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Protein
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C772-1455
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C
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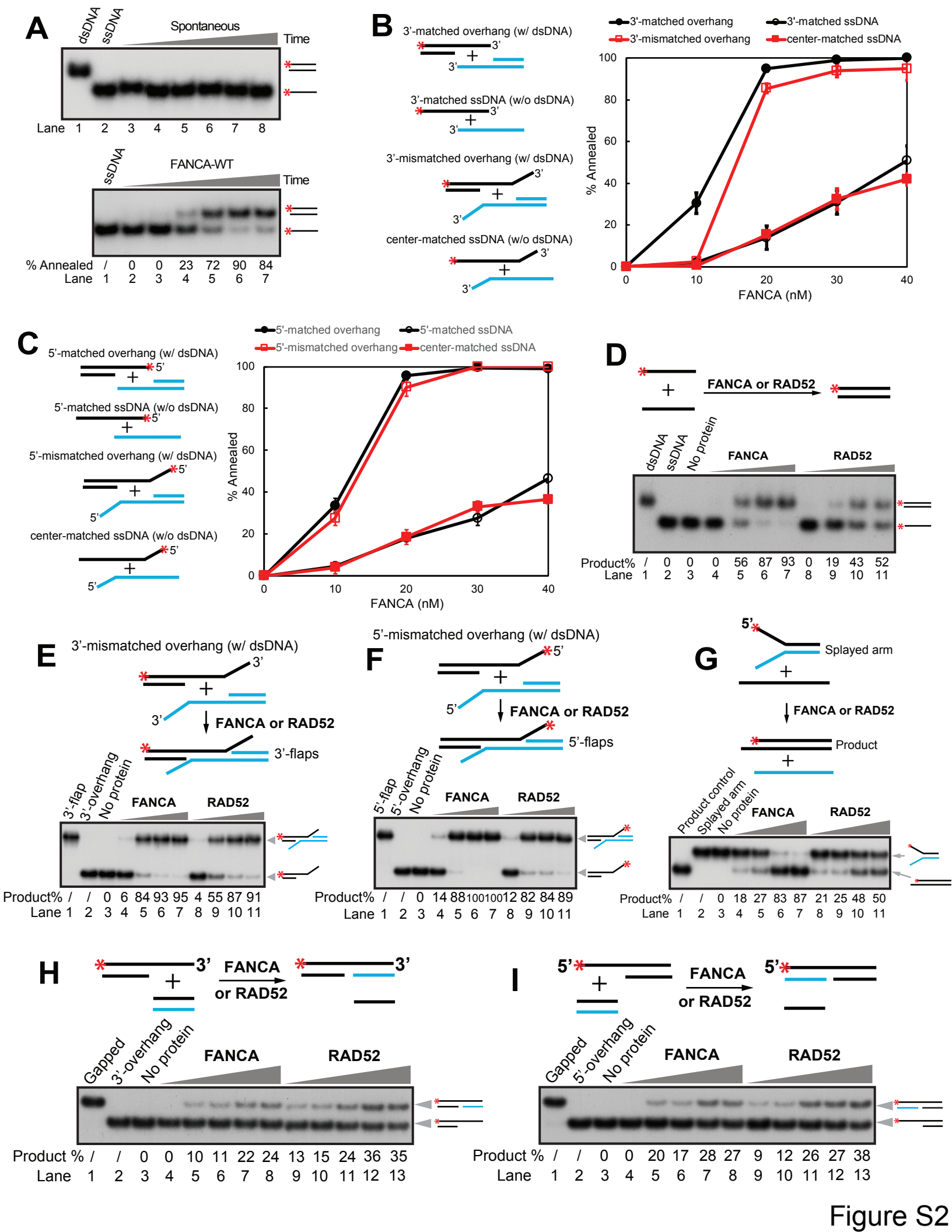
53BP1
Histone H3
Lane 1 2 3 4
Chromatin fraction
Ctrl
53BP1+siFANCA
D
Whole cell lysate
Cytosol
Nuclear soluble
Chromatin
Hsp90
PCNA
Fractionation quality control

Figure S1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ssDNA
Spontaneous
Time
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ssDNA
FANCA-WT
Time
% Annealed / 0 0 23 72 90 84
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B
3'-matched overhang (w/ dsDNA)
3'-matched ssDNA (w/o dsDNA)
3'-mismatched overhang (w/ dsDNA)
center-matched ssDNA (w/o dsDNA)
3'-matched overhang
3'-matched ssDNA
3'-mismatched overhang
center-matched ssDNA
% Annealed
FANCA (nM)
C
5'-matched overhang (w/ dsDNA)
5'-matched ssDNA (w/o dsDNA)
5'-mismatched overhang (w/ dsDNA)
center-matched ssDNA (w/o dsDNA)
5'-matched overhang
5'-matched ssDNA
5'-mismatched overhang
center-matched ssDNA
% Annealed
FANCA (nM)
D
FANCA or RAD52
dsDNA
ssDNA
No protein
FANCA
RAD52
Product% / 0 0 0 56 87 93 0 19 43 52
Lane 1 2 3 4 5 6 7 8 9 10 11
E
3'-mismatched overhang (w/ dsDNA)
FANCA or RAD52
3'-flaps
3'-flap
3'-overhang
No protein
FANCA
RAD52
Product% / / 0 6 84 93 95 4 55 87 91
Lane 1 2 3 4 5 6 7 8 9 10 11
F
5'-mismatched overhang (w/ dsDNA)
FANCA or RAD52
5'-flaps
5'-flap
5'-overhang
No protein
FANCA
RAD52
Product% / / 0 14 88 100 100 12 82 84 89
Lane 1 2 3 4 5 6 7 8 9 10 11
G
Splayed arm
FANCA or RAD52
Product
Product control
Splayed arm
No protein
FANCA
RAD52
Product% / / 0 18 27 83 87 21 25 48 50
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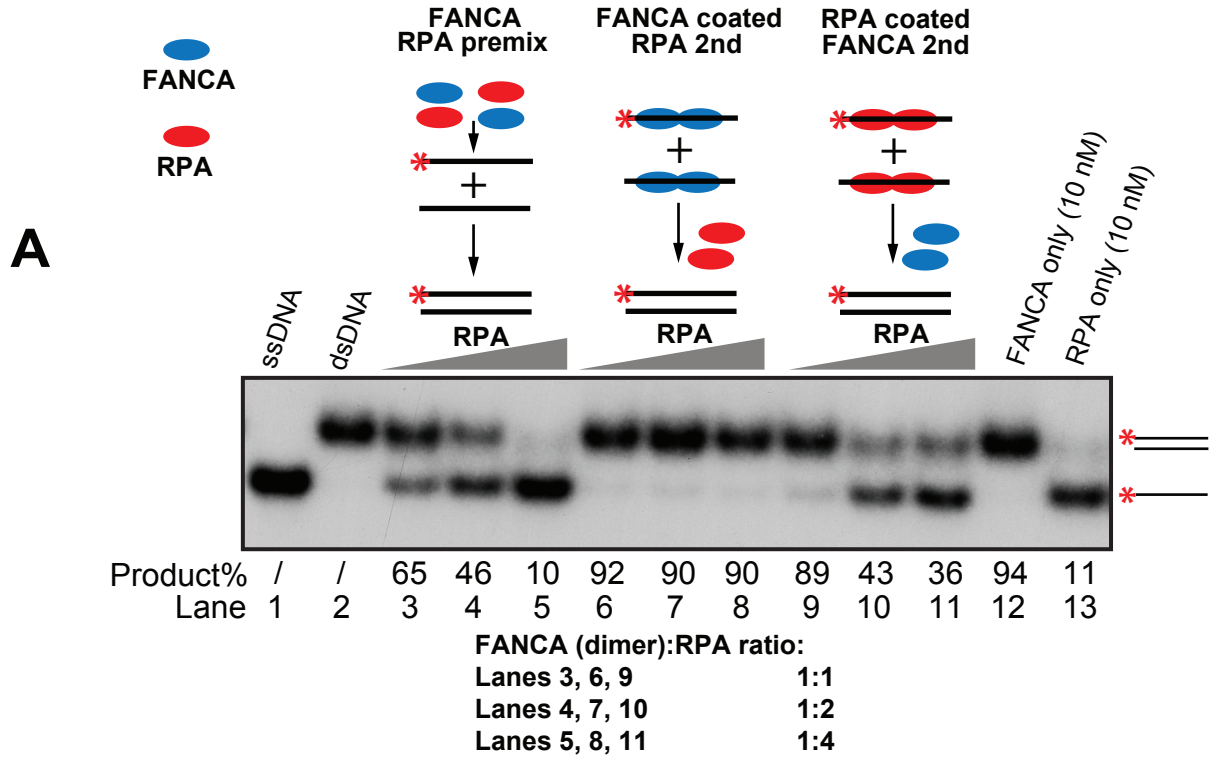
Figure S2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RPA 2nd
RPA coated
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dsDNA
RPA
RPA
RPA
FANCA only (10 nM)
RPA only (10 nM)
Product% / / 65 46 10 92 90 90 89 43 36 94 11
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13
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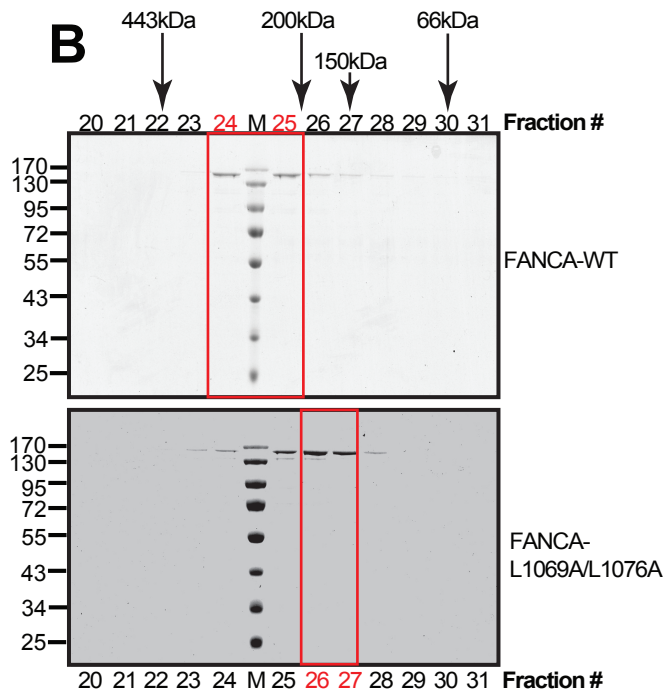
Lanes 4, 7, 10 1:2
Lanes 5, 8, 11 1:4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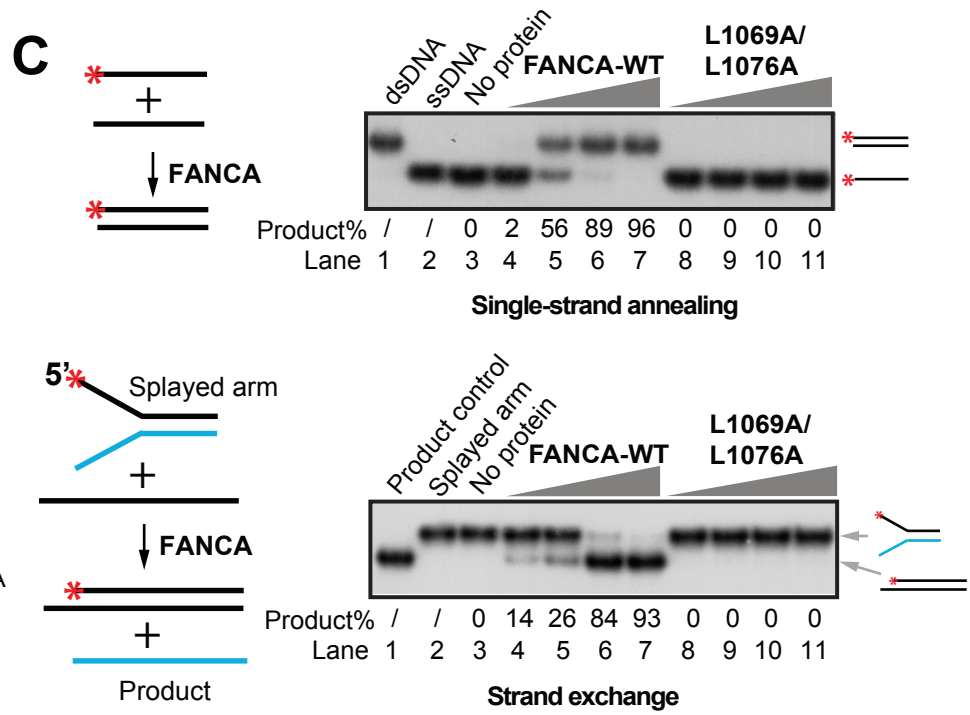
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Single-strand annealing
5'
Splayed arm
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Product
Product control
Splayed arm
No protein
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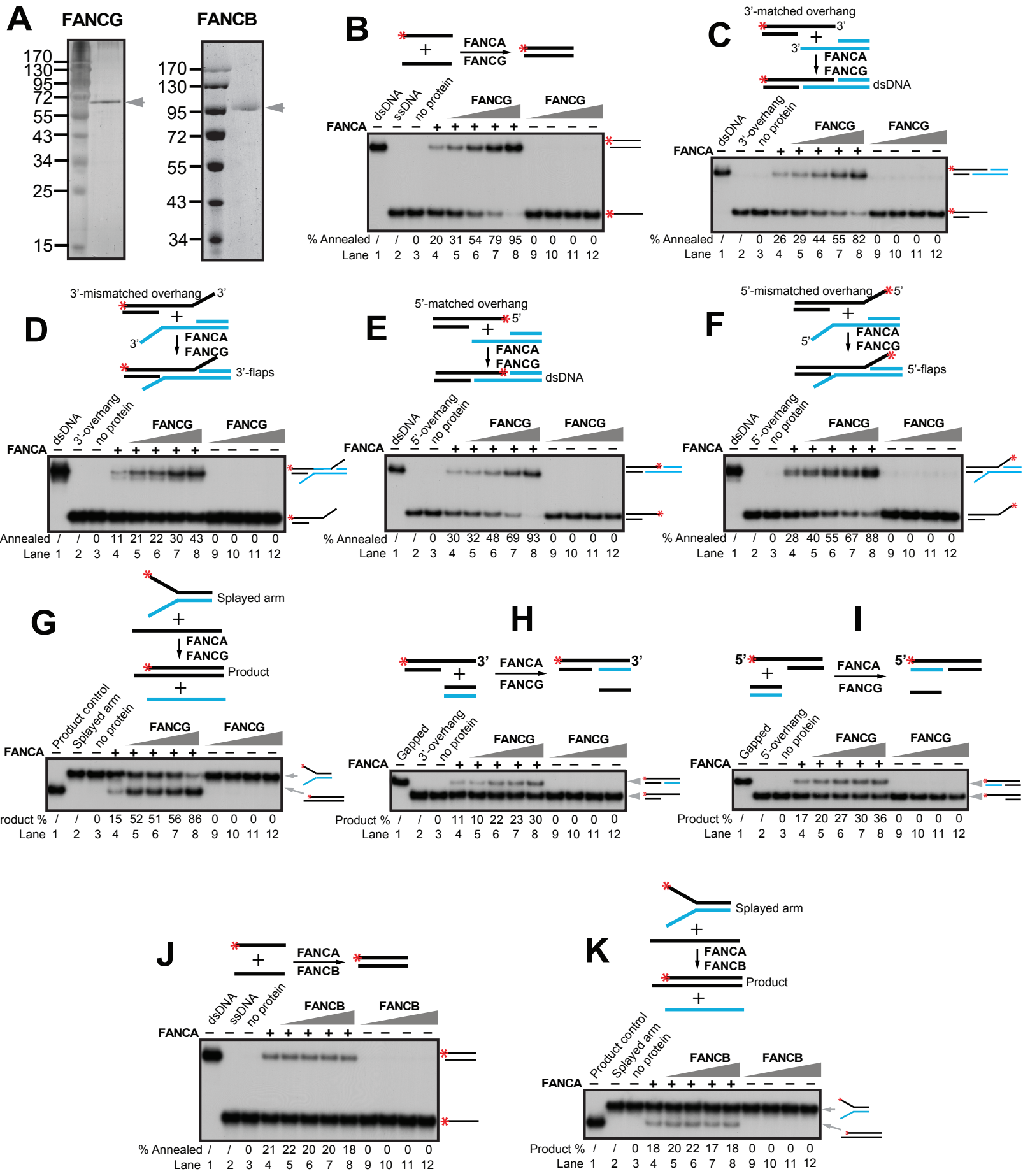
Strand exchange

Figure S3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% Annealed / / 0 28 40 55 67 88 0 0 0 0
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G
Splayed arm
Product
Product control
Splayed arm
no protein
Product % / / 0 15 52 51 56 86 0 0 0 0
Lane 1 2 3 4 5 6 7 8 9 10 11 12
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Gapped
Product % / / 0 11 10 22 23 30 0 0 0 0
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Product % / / 0 17 20 27 30 36 0 0 0 0
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J
FANCB
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K
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Lane 1 2 3 4 5 6 7 8 9 10 11 12

Figure S4