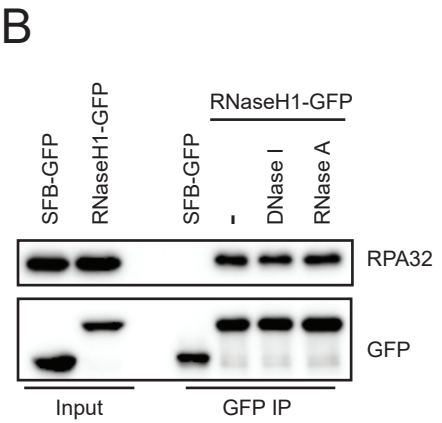
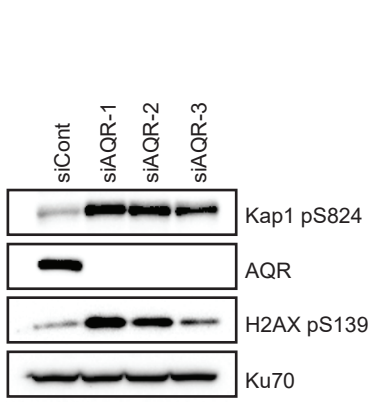
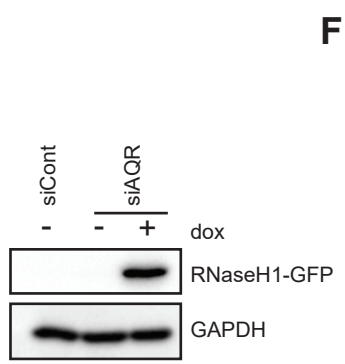
Figure S1

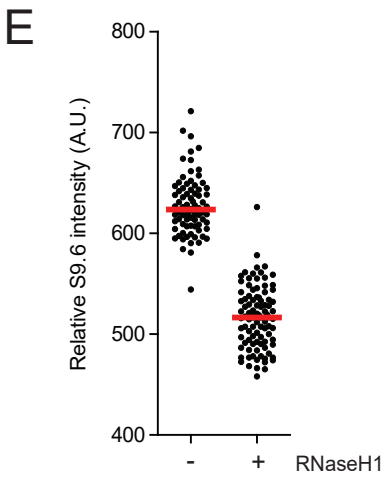
A

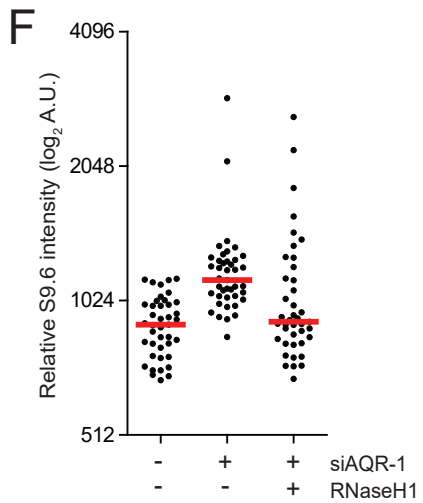
Proteins	# of peptides
RPA1	238
RPA2	45
RPA3	14
ATR	73
ATRIP	14
SMARCAL1	69
RFWD3	8
CDC5L	13
SPF27	7
PRP19	5
PRL1	2
RNaseH1	2

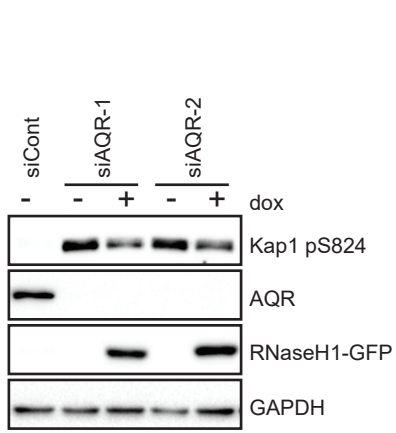


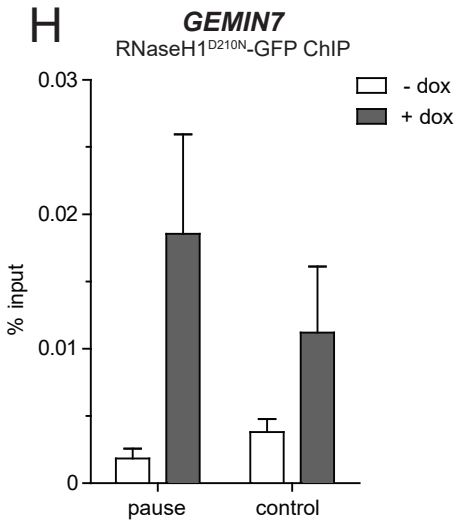


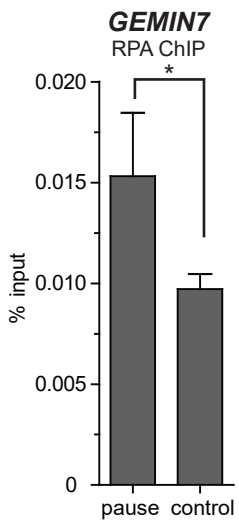












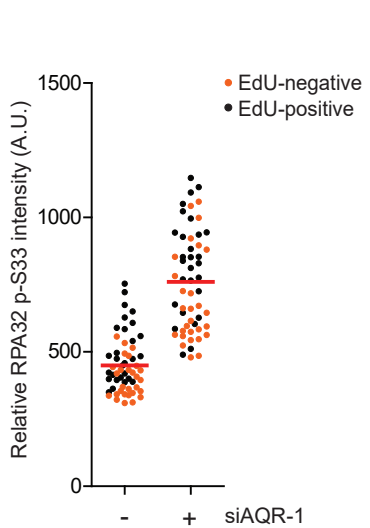


Figure S2

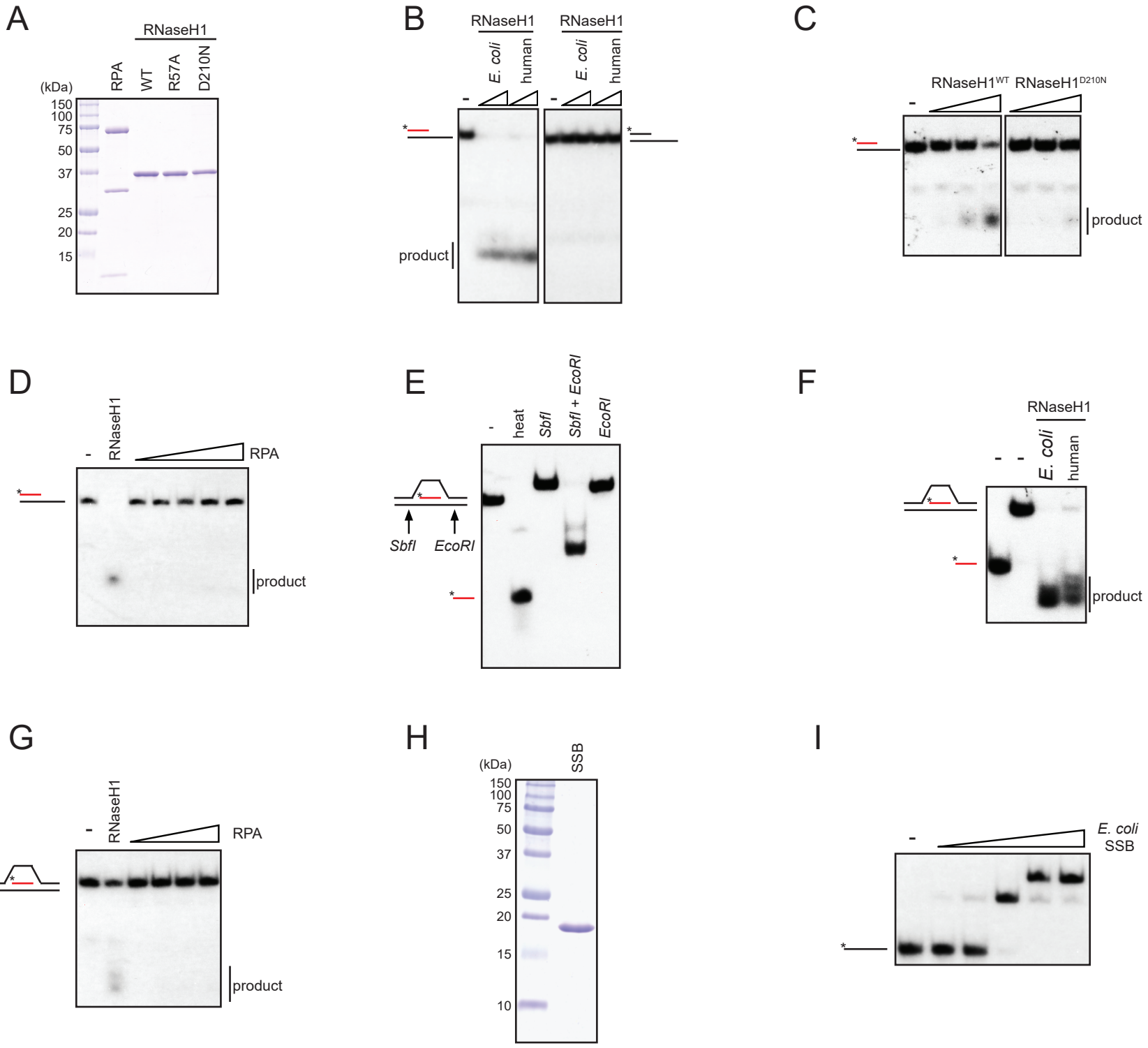
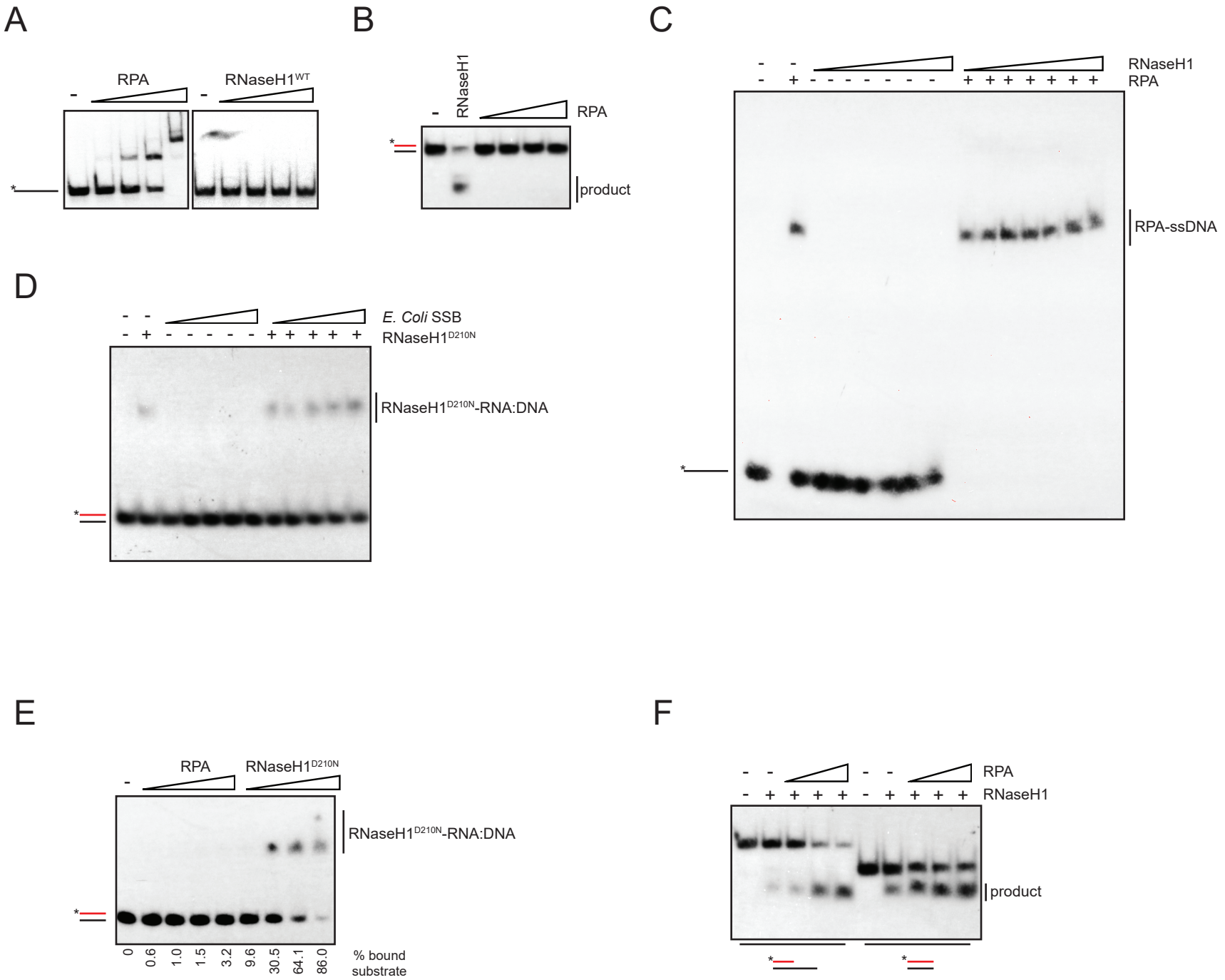

A
RNaseH1
(kDa)
RPA
WT
R57A
D210N
150
100
75
50
37
25
20
15
B
RNaseH1
RNaseH1
E. coli
human
E. coli
human
-
product
C
RNaseH1WT
RNaseH1D210N
-
product
D
-
RNaseH1
RPA
product
E
-
heat
SbfI
SbfI + EcoRI
EcoRI
SbfI
EcoRI
F
RNaseH1
-
-
E. coli
human
product
G
-
RNaseH1
RPA
product
H
(kDa)
SSB
150
100
75
50
37
25
20
15
10
I
-
E. coli
SSB

Figure S3


A
RPA
RNaseH1WT
B
RNaseH1
RPA
product
C
RNaseH1
RPA
RPA-ssDNA
D
E. Coli SSB
RNaseH1D210N
RNaseH1D210N-RNA:DNA
E
RPA
RNaseH1D210N
RNaseH1D210N-RNA:DNA
0 0.6 1.0 1.5 3.2 9.6 30.5 64.1 86.0
% bound substrate
F
RPA
RNaseH1
product

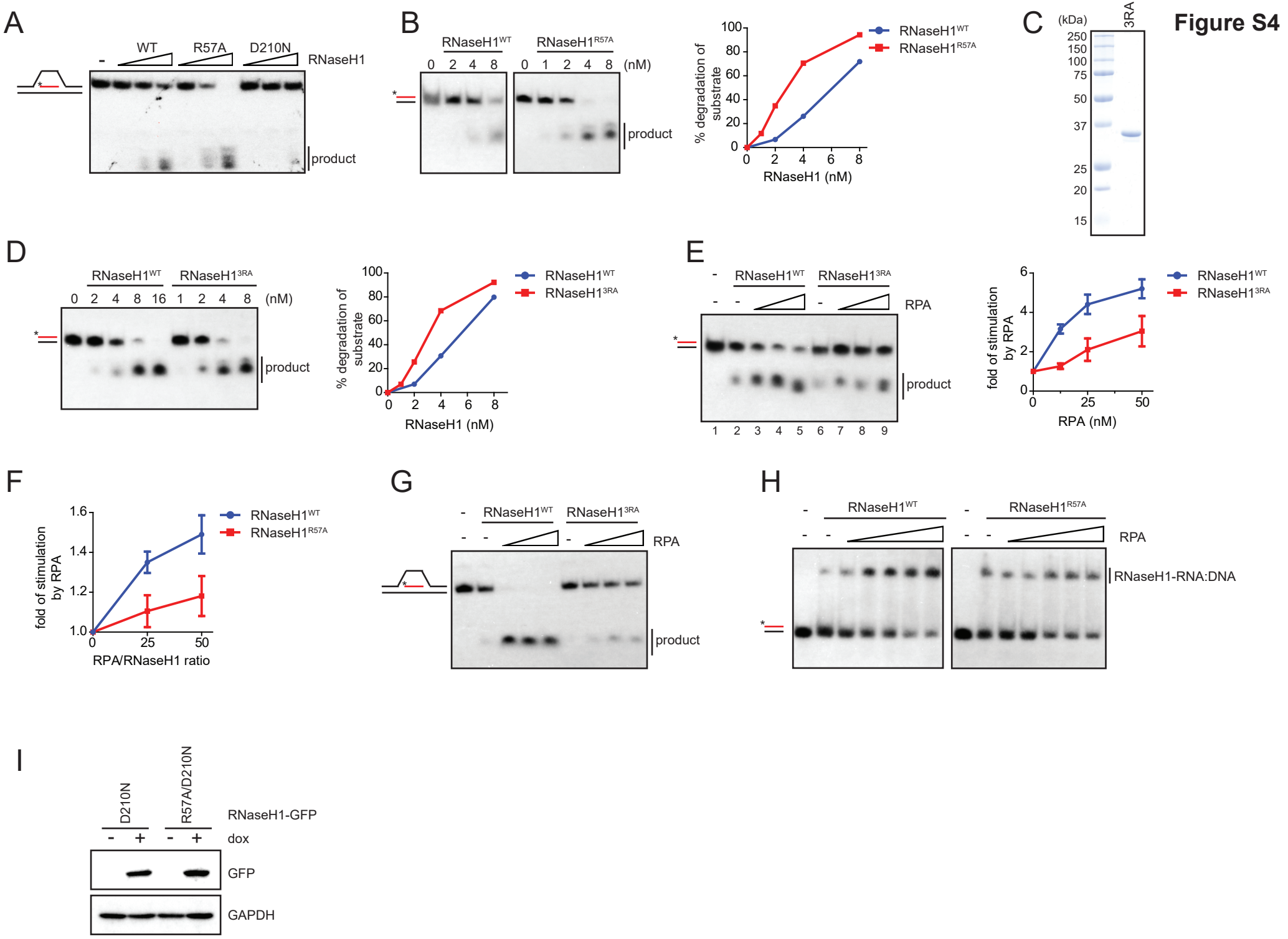
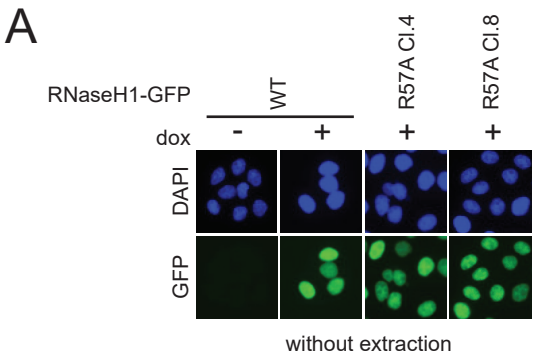
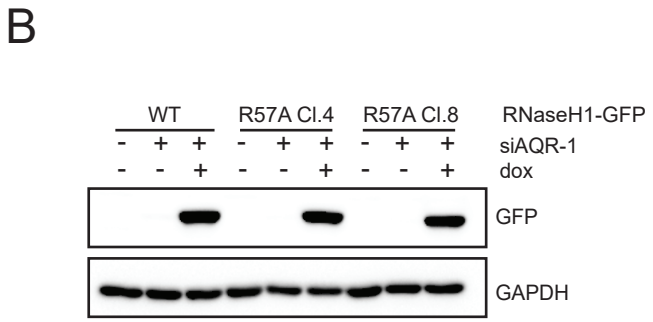
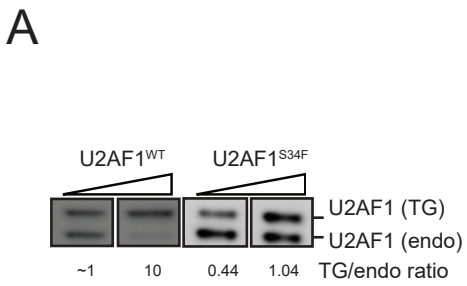
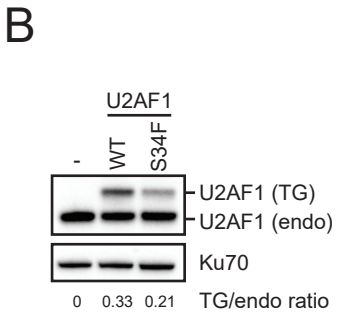


Figure S4
A
WT
R57A
D210N
RNaseH1
product
B
RNaseH1WT
RNaseH1R57A
0 2 4 8
0 1 2 4 8 (nM)
product
% degradation of substrate
RNaseH1 (nM)
C
(kDa)
3RA
250
150
100
75
50
37
25
20
15
D
RNaseH1WT
RNaseH13RA
0 2 4 8 16 1 2 4 8 (nM)
product
% degradation of substrate
RNaseH1 (nM)
E
RNaseH1WT
RNaseH13RA
RPA
product
1 2 3 4 5 6 7 8 9
fold of stimulation by RPA
RPA (nM)
F
RNaseH1WT
RNaseH1R57A
fold of stimulation by RPA
RPA/RNaseH1 ratio
G
RNaseH1WT
RNaseH13RA
RPA
product
H
RNaseH1WT
RNaseH1R57A
RPA
RNaseH1-RNA:DNA
I
D210N
R57A/D210N
RNaseH1-GFP
dox
GFP
GAPDH


A
RNaseH1-GFP
WT
R57A Cl.4
R57A Cl.8
dox
- + + +
DAPI
GFP
without extraction


B
WT
R57A Cl.4
R57A Cl.8
RNaseH1-GFP
- + + - + + - + +
siAQR-1
- - + - - + - - +
dox
GFP
GAPDH


A
U2AF1WT
U2AF1S34F
U2AF1 (TG)
U2AF1 (endo)
~1
10
0.44
1.04
TG/endo ratio


B
U2AF1
-
WT
S34F
U2AF1 (TG)
U2AF1 (endo)
Ku70
0
0.33
0.21
TG/endo ratio

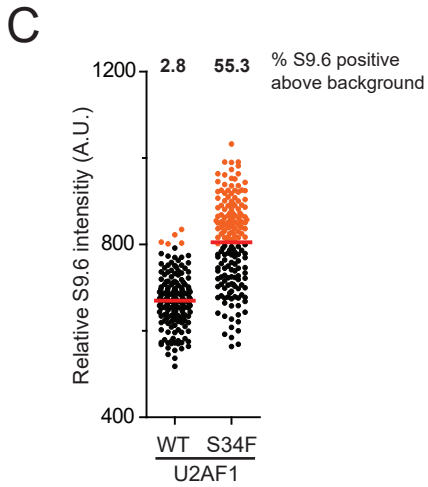

C
1200
800
400
2.8
55.3
% S9.6 positive above background
Relative S9.6 intensity (A.U.)
WT
S34F
U2AF1

Fig. S1, related to Fig 1. RNaseH1 suppresses R loop-induced DNA damage in AQR knockdown cells. (A) A list of selected proteins identified from the proteomics screen using RPA-ssDNA as bait (Maréchal et al., 2014). (**B**) RNaseH1-GFP or SFB-GFP was expressed in HEK293T cells and immunoprecipitated using anti-GFP antibody. Whole cell lysates (1 mg) were left untreated or treated with either DNaseI (10 units) or RNaseA (1 μg) for 10 min at 37°C prior to immunoprecipitation. 1% of cell lysates were loaded as input. (**C**) HeLa cells were transfected with control or 3 independent AQR siRNAs, cultured for 60 h, and analyzed by western blot with the indicated antibodies. (**D**) HeLa-derivative cells were transfected with the indicated siRNAs and cultured for 60 h. RNaseH1-GFP was induced by doxycycline for 48 h. Cell lysates were analyzed by western blot with the indicated antibodies; related to Fig. 1C. (**E)** RNaseH1-GFP was induced by doxycycline for 60 h. R loop levels in individual cells were analyzed using the S9.6 antibody and quantified (n>80). Red bars in the right panel represent the median S9.6 intensities of the indicated cell populations. (**F**) HeLa-derivative cells were transfected with the indicated siRNAs and cultured for 60 h. RNaseH1-GFP was induced by doxycycline for 48 h. R loop levels in individual cells were analyzed using the S9.6 antibody and quantified (n=40). Red bars in the right panel represent the median S9.6 intensities of the indicated cell populations. (**G**) HeLa-derivative cells were transfected with the indicated siRNAs and cultured for 60 h. RNaseH1-GFP was induced by doxycycline for 48 h. Cell lysates were analyzed by western blot with the indicated antibodies. (**H-I**) HeLa-derived RNaseH1^{D210N}-GFP cells were induced with doxycycline for 48 hours prior to fixation and fragmented. Chromatin was immunoprecipitated using anti-GFP (in **H**) and anti-RPA32 (in **I**). ChIP-qPCR results were analyzed at indicated regions (n=3). Asterisks indicate $p<0.05$. (**J**) HeLa cells were transfected with control or AQR siRNA and cultured for 48 h. S-phase and non-S-phase cells were distinguished by 30-min EdU labeling. Immunofluorescence intensity of individual cells were analyzed with the p-RPA32 antibody (n>150). Red bars represent the mean p-RPA32 intensities of the indicated cell populations.

Fig. S2, related to Fig 2. Characterizations of RNaseH1, RPA, SSB, and various substrates. (**A**) Recombinant human RPA, RNaseH1^{WT}, RNaseH1^{R57A}, and RNH1^{D210N} proteins were expressed in *E. coli*, purified, and analyzed on a polyacrylamide gel (2 μg each) followed by coomassie blue staining. (**B**) Human RNaseH1 (100, 200 nM) or *E. coli* RNaseH1 (0.5, 1 nM) was incubated with the R:D+ssDNA substrate (40 nM) for 10 min (left panel). A substrate containing dsDNA and a ssDNA overhang was used as a control (right panel). (**C**) RNaseH1^{WT} and RNaseH1^{D210N} (4, 8, 16 nM) were incubated with the R:D+ssDNA substrate (50 nM) for 10 min. (**D**) The R:D+ssDNA substrate (25 nM) was incubated with increasing concentrations of RPA (0, 12.5, 25, 50, 100, 200 nM) for 5 min; related to Fig. 2B. (**E**-**F**) Validation of the R loop substrate containing ^{32}P-labeled RNA (in red). Labeled RNA was released from the substrate by heat denaturation, confirming that RNA is incorporated to the R loop by annealing. In **E**, cleavage of the substrate by *SbfI* and *EcoRI* confirms the presence of dsDNA flanking the R loop. In **F**, the cleavage by *E. coli* and human RNaseH1 confirms the presence of an RNA:DNA hybrid in the R loop. (**G**) The R loop substrate was incubated with RNaseH1 and increasing concentrations of RPA (0, 25, 50, 100, 200 nM) for 5 min; related to Fig. 2C. (**H**) Recombinant *E. coli* SSB protein was analyzed on a polyacrylamide gel (2 μg) followed by coomassie blue staining. (**I**) A ^{32}P-labeled 80-nt ssDNA probe (20 nM) was incubated with increasing concentrations of *E. coli* SSB (0, 10, 20, 40, 80, 160 nM) and analyzed by native polyacrylamide gel.

Fig. S3, related to Fig 3. RPA stimulates the binding of RNaseH1 to RNA:DNA hybrids. (**A**) A ^{32}P-labeled 80-nt ssDNA probe (30 nM) was incubated with increasing concentrations of RPA (0, 10, 20, 40, 80 nM) or RNaseH1 (0, 15, 30, 60, 120 nM). Protein-ssDNA complexes were resolved on a native polyacrylamide gel. (**B**) The R:D substrate (25 nM) was incubated with RNaseH1 or increasing concentrations of RPA (12.5, 25, 50, 100 nM) for 5 min; related to Fig. 3A. (**C**) A ^{32}P-labeled 80-mer ssDNA probe (10 nM) was first incubated with RPA (25 nM), then with increasing concentrations of RNaseH1 (25, 50, 100, 200, 400, 800, 1600 nM), and finally analyzed by native polyacrylamide electrophoresis. As a control, the ssDNA probe was also incubated with the same concentrations of RNaseH1 in the absence of RPA. (**D**) The R:D substrate (25 nM) was incubated with RNaseH1^{D210N} (50 nM) and increasing concentrations of *E. coli* SSB (0, 12.5, 25, 50, 100, 200 nM), and analyzed by EMSA. As a control, the R:D substrate was also incubated with *E. coli* SSB in the absence of RNaseH1. (**E**) The R:D substrate (20 nM) was incubated with increasing concentrations of RPA or RNaseH1^{D210N} (0, 20, 40, 80, 160 nM, respectively), and analyzed by EMSA. **(F)** R:D+ssDNA substrate (25 nM) or R:D substrate (25 nM) was incubated with increasing concentrations of RPA (12.5, 25, 50, 100 nM) and RNaseH1^{WT} (2 nM) for 5 min in Buffer A with 50 mM KCl.

Fig. S4, related to Fig 4. RNaseH1^{R57A} is compromised for RPA-mediated stimulation. (**A**) RNaseH1^{WT} (4, 8, 16 nM), RNaseH1^{R57A} (1, 2, 4 nM), and RNaseH1^{D210N} (4, 8, 16 nM) were incubated with the R loop substrate (50 nM) for 10 min. (**B**) RNaseH1^{WT} (2, 4, 8 nM) and RNaseH1^{R57A} (1, 2, 4, 8 nM) were incubated with the R:D substrates (25 nM) for 5 min. Cleavage of the substrate was quantified and shown in the right panel. (**C**) Recombinant human RNaseH1^{3RA} protein was expressed in *E. coli*, purified, and analyzed on a polyacrylamide gel (2 μg) followed by coomassie blue staining. (**D**) RNaseH1^{WT} (2, 4, 8, 16 nM) or RNaseH1^{3RA} (1, 2, 4, 8 nM) were incubated with the R:D substrates (25 nM) for 5 min. Cleavage of the substrate was quantified and shown in the right panel. (**E**) The R:D substrate (25 nM) substrate was incubated with RNaseH1^{WT} (2 nM) or RNaseH1^{3RA} (1.25 nM) and increasing concentrations of RPA (0, 12.5, 25, 50 nM) for 5 min in Buffer A with 50 mM KCl. The cleavage of substrate was quantified and the fold of stimulation by RPA was determined. Data are presented as mean ± SD (n=3). (**F**) The fold of stimulation of RNaseH1^{WT} and RNaseH1^{R57A} by RPA was plotted against RPA/RNaseH1 ratios. Data are presented as mean ± SD (n=3); related to Fig. 4E. (**G**) Increasing concentration of RPA (25, 50 100 nM) and RNaseH1^{WT} (2nM) or RNaseH1^{3RA} (1.25nM) were incubated with R loop (25 nM) for 5 min in buffer A with 50 mM KCl. (**H**) The RNA:DNA probe (20 nM) was incubated with RNaseH1^{WT} (140 nM) or RNaseH1^{R57A} (120 nM) and increasing concentrations of RPA (0, 20, 40, 80, 160, 320 nM) in buffer B with 10 mM EDTA. (**I**) Induction of RNaseH1^{D210N} and RNaseH1$^{R57A/D210N}$ by doxycycline in the stable cell lines was confirmed by western blot; related to Fig. 4F.

Fig. S5, related to Fig 5. Characterizations of the inducible cell lines expressing RNaseH1^{WT} and RNaseH1^{R57A}. (**A**) HeLa-derived inducible cell lines of RNaseH1^{WT} and RNaseH1^{R57A} were treated with doxycycline for 24 h or left untreated. Cells were directly fixed without triton extraction, and analyzed by immunostaining using anti-GFP antibody. (**B**) HeLa-derived inducible cell lines of RNaseH1^{WT} and RNaseH1^{R57A} were transfected with control or AQR siRNA, and treated with doxycycline for 48 h. Expression levels of RNaseH1^{WT} and RNaseH1^{R57A} were analyzed by western blot.

Fig. S6, related to Fig 6. RNaseH1^{R57A} fails to suppress R loops in cells expressing the MDS-associated U2AF1^{S34F} mutant. (A) U2AF1^{WT} and U2AF1^{S34F} were infected in K562 cells. Individual clones were collected and expression levels of both exogenous and endogenous U2AF1 were analyzed by western blot; related to Fig. 6E. (**B-C**) U2AF1^{WT} and U2AF1^{S34F} were infected in HeLa cells. Cell lysates were collected and expression levels of both exogenous and endogenous U2AF1 were analyzed by western blot in **B**. In **C**, R loop levels in individual cells were analyzed with the S9.6 antibody (n=180). Red bars represent the mean S9.6 intensities of the indicated cell populations.