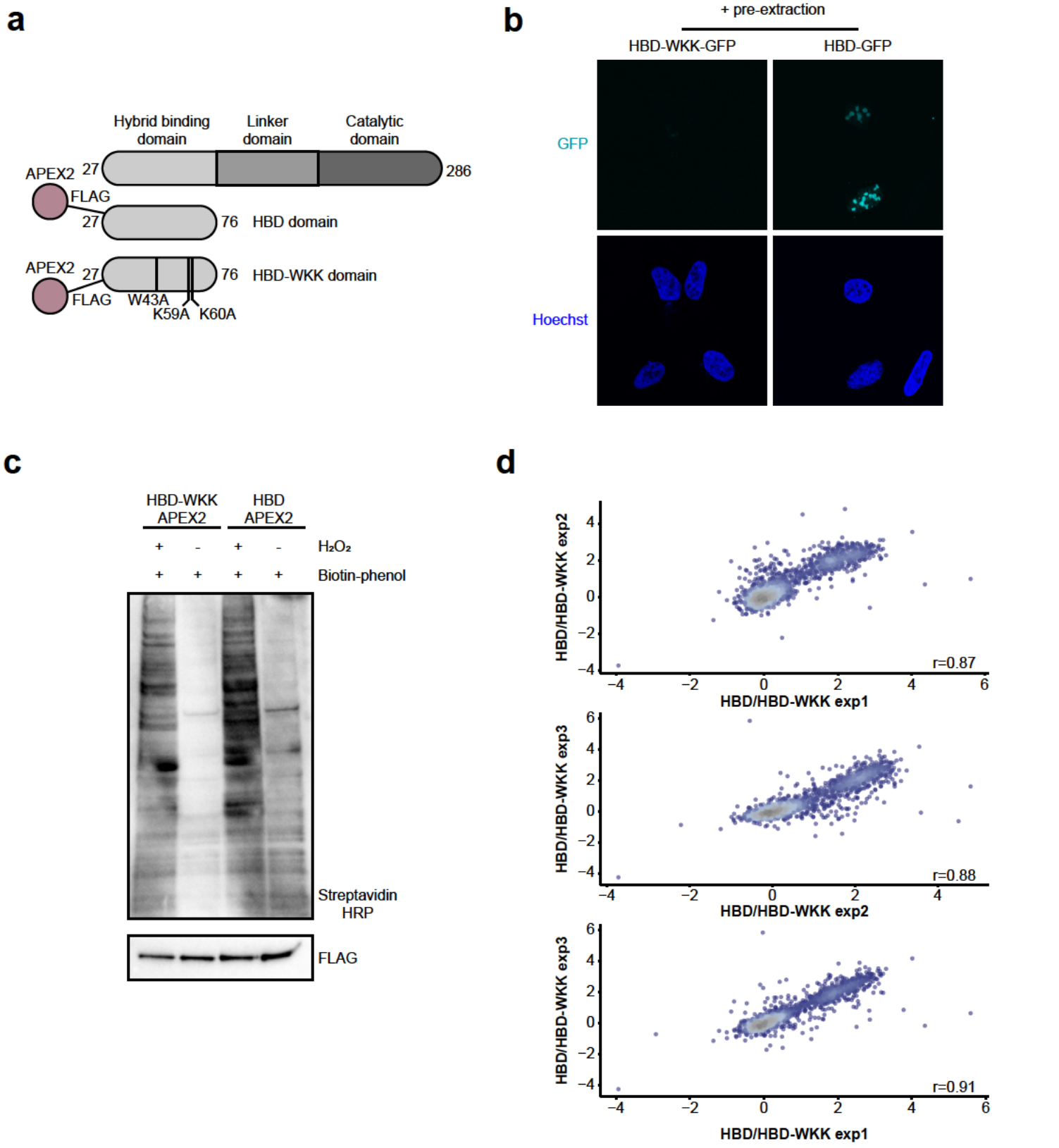
Figure S1


a
Hybrid binding domain
Linker domain
Catalytic domain
APEX2
27
286
FLAG
27
76
HBD domain
APEX2
27
76
HBD-WKK domain
FLAG
W43A
K59A
K60A
b
+ pre-extraction
HBD-WKK-GFP
HBD-GFP
GFP
Hoechst
c
HBD-WKK APEX2
HBD APEX2
+
-
+
-
H₂O₂
+
+
+
+
Biotin-phenol
Streptavidin HRP
FLAG
d
HBD/HBD-WKK exp2
HBD/HBD-WKK exp1
r=0.87
HBD/HBD-WKK exp3
HBD/HBD-WKK exp1
r=0.88
HBD/HBD-WKK exp3
HBD/HBD-WKK exp2
r=0.91

Supplementary Figure 1: RDProx-Mapping R-loop-proximal proteome on native chromatin

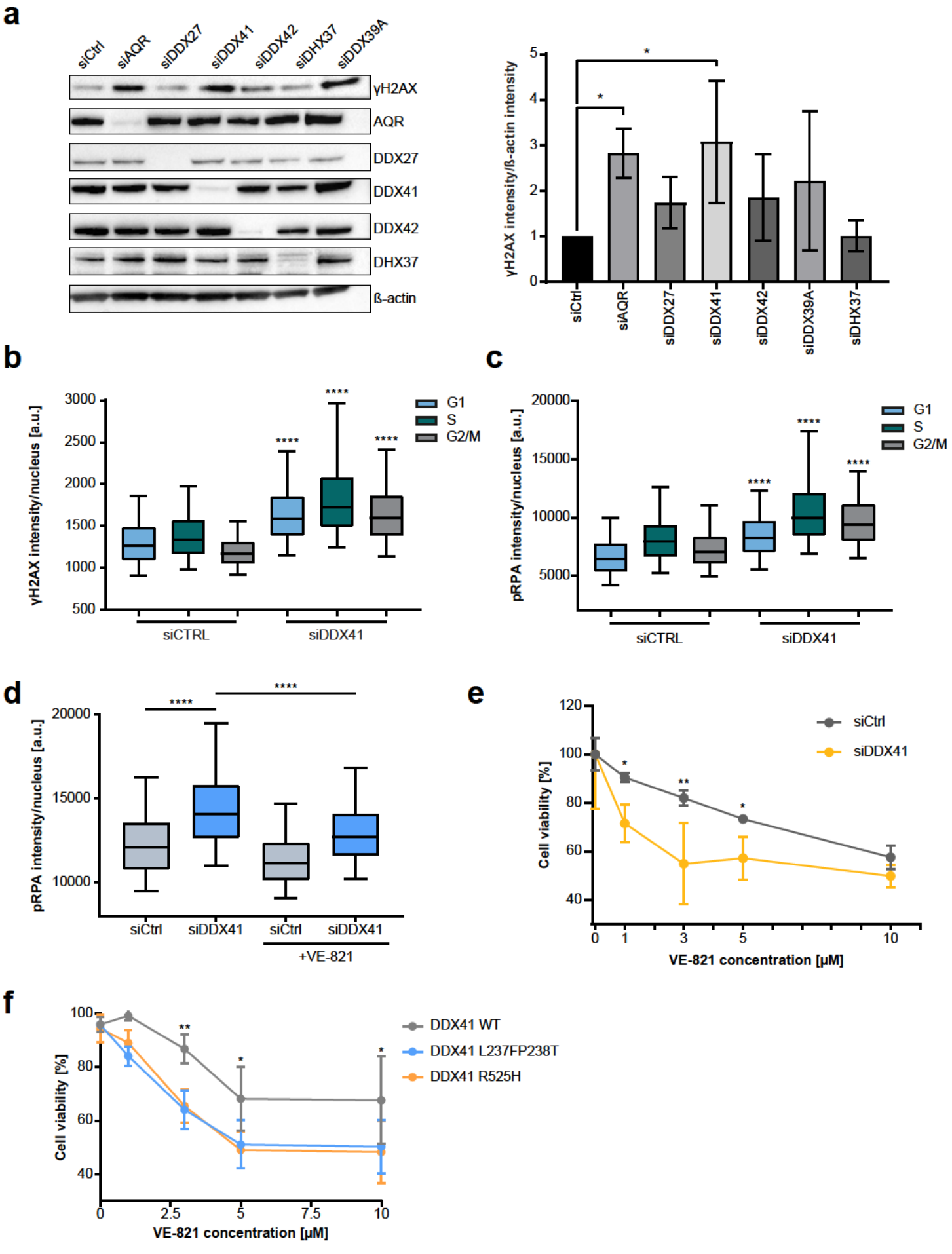
a. Schematic representation of M27-RNaseH1 domains. The wild type version of hybrid binding domain (HBD) of RNaseH1 was cloned as well as the mutant with indicated WKK point mutations to abrogate the binding function. HBD and WKK-HBD were fused to the ascorbate peroxidase APEX2 on the N-terminus. Amino acid positions are indicated by the numbers.

b. Immunofluorescence analysis of the retention of GFP-tagged HBD or WKK-HBD on chromatin after pre-extraction with 0.4% NP-40 for 30 min on ice. The GFP-tagged domains were transiently expressed in U2OS cells for 24 h. Chromatin-bound domains were visualized by retained GFP signal (cyan). DNA was counterstained with Hoechst33342 (blue).

c. Western blot analysis of biotinylated proteins in U2OS whole cell extracts. HBD and WKK-HBD fused to APEX2 and FLAG were expressed for 48 h before inducing biotinylation of proximal proteins with 500 µM biotin-phenol for 2 hours and 1 mM H2O2 for 2 minutes. Induction with H_2O_2 was omitted in control conditions. Streptavidin-HRP was used for the detection of biotinylation. FLAG signal indicates the expression of the fusion constructs.

d. Multi-scatter plots indicating the correlation between the three RDProx SILAC experiments in HEK293T cells. SILAC ratios between the HBD and HBD-WKK conditions of all individual experiments are plotted against each other. Pearson correlation is highlighted below each plot. Coloring indicates the density of the population (blue=less dense, grey= high density).

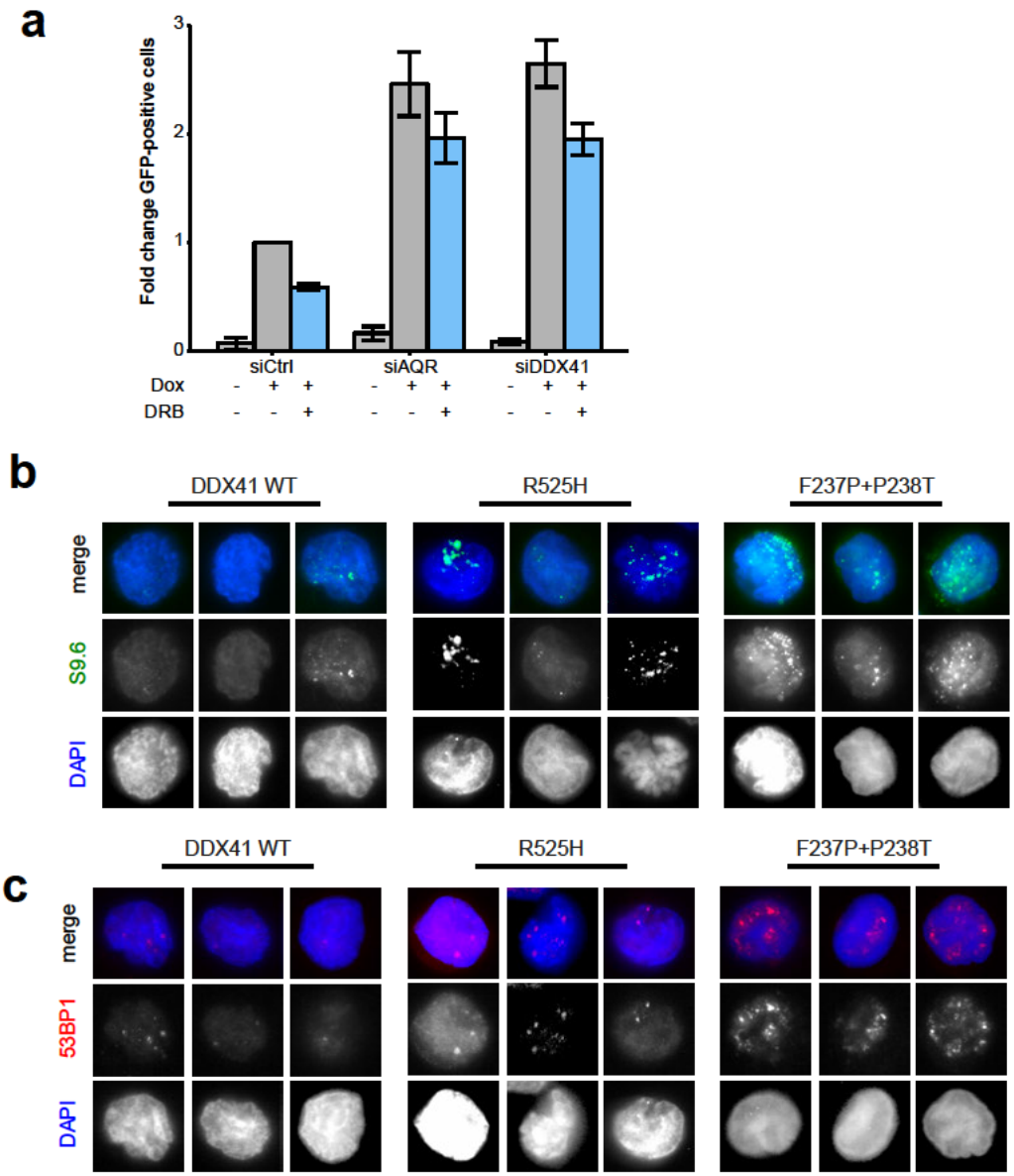
Figure S2


a
siCtrl
siAQR
siDDX27
siDDX41
siDDX42
siDHX37
siDDX39A
γH2AX
AQR
DDX27
DDX41
DDX42
DHX37
ß-actin
γH2AX intensity/ß-actin intensity
b
γH2AX intensity/nucleus [a.u.]
G1
S
G2/M
siCTRL
siDDX41
c
pRPA intensity/nucleus [a.u.]
d
pRPA intensity/nucleus [a.u.]
siCtrl
siDDX41
+VE-821
e
Cell viability [%]
VE-821 concentration [μM]
f
DDX41 WT
DDX41 L237FP238T
DDX41 R525H

Supplementary Figure 2: DDX41 depletion leads to replication stress and double strand breaks

a. Western blot analysis of yH2AX in U2OS cells after transfections with indicated siRNAs. Representative Western blot for yH2AX, ß-actin and knockdown efficiency of the respective helicases (left). Quantification of the ratio between yH2AX intensity and the intensity of the loading control ß-actin (right). The mean of three biological replicates is displayed in the bar plot. Whiskers indicate the standard deviation of the mean. *P-value <0.05, One-Way-ANOVA.
b. Box plot displaying the immunofluorescence analysis of yH2AX after 48 h knockdown with the indicated siRNAs in different cell cycle stages: G1 (blue), S (green), G2/M (grey). Cell cycle phases for each cell were individually identified based on Hoechst staining and EdU incorporation with 10 µM for 30 min. Mean nuclear intensity for each cell was quantified. Black line within the box represents the median and whiskers indicate the 10^{th}-90^{th} percentile. **** P-value <0.0001, One-Way-ANOVA.
c. Same setup as Supplemental Figure 3b: cells were stained for pRPA (Ser33). **** P-value <0.0001, One-Way-ANOVA.
d. Quantification of the immunofluorescence analysis of pRPA (Ser33) in U2OS cells after indicated knockdowns for 48 h. Cells were either treated with DMSO or 10 µM VE-821(ATRi) for 6 h before fixation and subsequent staining. Boxplot is displaying the 10th-90th percentile, black line indicates the median. **** P-value <0.0001, One-Way-ANOVA.
e. Survival curves displaying the results of the cell titer blue assay after knockdown of DDX41 or control knockdown in U2OS cells. Cells were re-seeded 48 h after post-knockdown and challenged with VE-821 in indicated concentrations for 72 h. Cell titer blue reagent was added for 4 h and resulting fluorescence of resorufin was measured as a proxy for cell viability. Mean of three biological replicates is displayed. Whiskers indicate the standard deviation of the mean. *P-value < 0.05, **P-value < 0.01, Two-way ANOVA.
f. Similar to Supplemental Figure 3e: Expression of GFP-tagged DDX41 WT, L237F+P238T or R525H was induced in OCI-AML3 cells using doxycycline for 72 h before re-seeding. Mean of three biological replicates is displayed. Whiskers indicate the standard deviation of the mean. *P-value < 0.05, **P-value < 0.01, Two-way ANOVA.

Figure S3

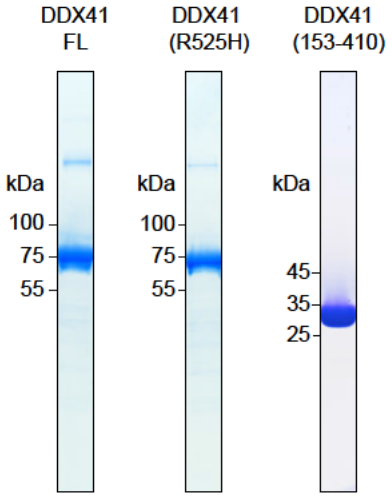

a
Fold change GFP-positive cells
3
2
1
0
siCtrl
siAQR
siDDX41
Dox - + + - + + - + +
DRB - - + - - + - - +
b
DDX41 WT
R525H
F237P+P238T
merge
S9.6
DAPI
c
DDX41 WT
R525H
F237P+P238T
merge
53BP1
DAPI

Supplementary Figure 3: DDX41 opposes R-loop accumulation in HSPCs

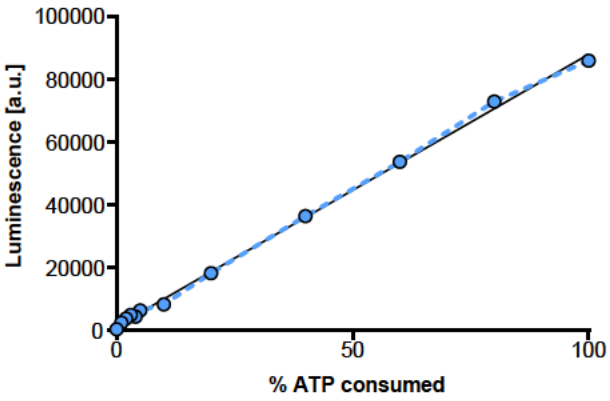
a. Bar plot displays the quantification of the HBD retention assay in U2OS cells analyzed by flow cytometry. Indicated knockdowns were performed for 48 hours. Auto-fluorescence of the cells was determined by treating cells with DMSO instead of doxycycline. Additional cells were treated with 10 μM DRB for 2 hours to inhibit transcription. Number of GFP-positive cells normalized on the control knockdown was quantified. Mean and standard deviation of the mean of three biological replicates are displayed.
b. Representative images of the immunofluorescence analysis of nuclear S9.6 staining (green) in HSPCs after expression of WT DDX41, L237F+P238T or R525H mutant. DNA was counterstained with DAPI (blue).
c. Representative images of the immunofluorescence analysis of nuclear 53BP1 staining (red) in HSPCs after expression of WT DDX41, L237F+P238T or R525H mutant. DNA was counterstained with DAPI (blue).

Figure S4

a


DDX41
FL
DDX41
(R525H)
DDX41
(153-410)
kDa
100
75
55
kDa
100
75
55
kDa
45
35
25

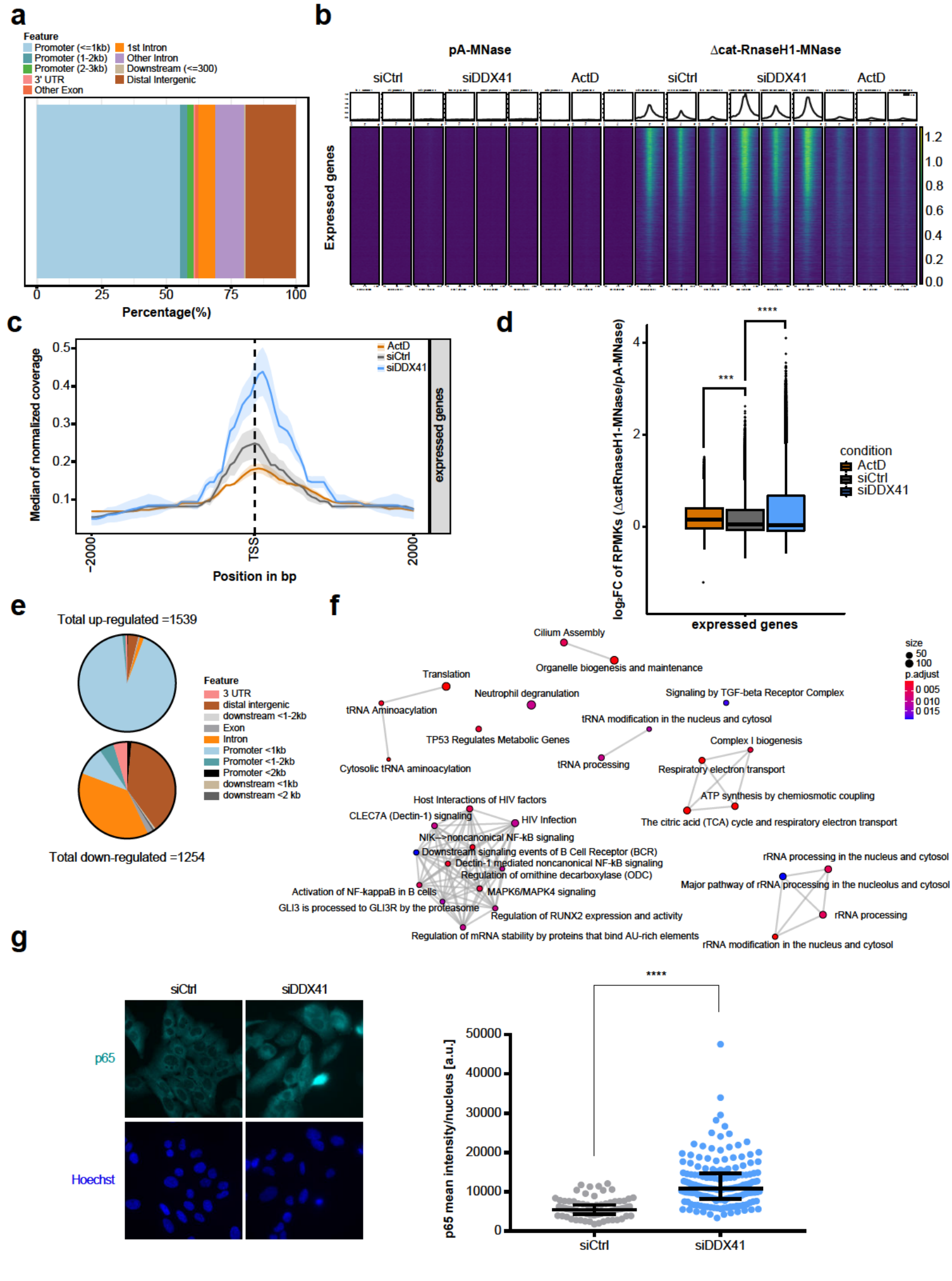
b


Luminescence [a.u.]
100000
80000
60000
40000
20000
0
0
50
100
% ATP consumed

Supplementary Figure 4: DDX41 binds and unwinds RNA-DNA hybrids in *vitro*

a. Coomassie-stained gel of 6his-tagged DDX41 full length, R525H and 153-410 mutant after expression and purification from SF9 insect cells.
b. Standard curve for the interpolation of values obtained from the ADP-Glo assay. ATP and ADP were mixed in various ratios and further processed together with ADP-Glo samples. Activity of the luciferase was increased with increased ADP concentration in a linear manner. a.u. = arbitrary units.

Figure S5


a
Feature
Promoter (<=1kb)
Promoter (1-2kb)
Promoter (2-3kb)
3' UTR
Other Exon
1st Intron
Other Intron
Downstream (<=300)
Distal Intergenic
0
25
50
75
100
Percentage(%)
b
pA-MNase
ΔcatRnaseH1-MNase
siCtrl
siDDX41
ActD
siCtrl
siDDX41
ActD
Expressed genes
1.2
1.0
0.8
0.6
0.4
0.2
0.0
c
Median of normalized coverage
0.5
0.4
0.3
0.2
0.1
ActD
siCtrl
siDDX41
expressed genes
-2000
TSS
2000
Position in bp
d
log2FC of RPMKs (ΔcatRnaseH1-MNase/pA-MNase)
4
2
0
****
***
condition
ActD
siCtrl
siDDX41
expressed genes
e
Total up-regulated =1539
Feature
3 UTR
distal intergenic
downstream <1-2kb
Exon
Intron
Promoter <1kb
Promoter <1-2kb
Promoter <2kb
downstream <1kb
downstream <2 kb
Total down-regulated =1254
f
Cilium Assembly
Organelle biogenesis and maintenance
Translation
Neutrophil degranulation
Signaling by TGF-beta Receptor Complex
tRNA Aminoacylation
tRNA modification in the nucleus and cytosol
TP53 Regulates Metabolic Genes
Complex I biogenesis
Cytosolic tRNA aminoacylation
tRNA processing
Respiratory electron transport
ATP synthesis by chemiosmotic coupling
Host Interactions of HIV factors
CLEC7A (Dectin-1) signaling
HIV Infection
The citric acid (TCA) cycle and respiratory electron transport
NIK–>noncanonical NF-kB signaling
Downstream signaling events of B Cell Receptor (BCR)
Dectin-1 mediated noncanonical NF-kB signaling
Regulation of ornithine decarboxylase (ODC)
rRNA processing in the nucleus and cytosol
Activation of NF-kappaB in B cells
MAPK6/MAPK4 signaling
Major pathway of rRNA processing in the nucleolus and cytosol
GLI3 is processed to GLI3R by the proteasome
Regulation of RUNX2 expression and activity
rRNA processing
Regulation of mRNA stability by proteins that bind AU-rich elements
rRNA modification in the nucleus and cytosol
size
50
100
p.adjust
0.005
0.010
0.015
g
siCtrl
siDDX41
p65
Hoechst
****
p65 mean intensity/nucleus [a.u.]
50000
40000
30000
20000
10000
0
siCtrl
siDDX41

Supplementary Figure 5: DDX41 depletion results in R-loop accumulation at CGI promoters

a. Bar plot displaying the feature distribution of all peaks called by MACS2 in the MapR experiment in three biological replicates from U2OS cells after 48 h knockdown with control siRNA. Consensus peaks were created as peaks which were overlapping in at least 2 samples. Proportion of the individual feature is represented in the indicated colors.
b. Heat maps showing the distribution of the MapR signal of all expressed genes for pA-MNase and ΔcatRnahseH1-MNase conditions after either 4µM Actinomycin D treatment for 6 h or after 48 h control or DDX41 knockdown. MapR signal was sorted based on gene expression level obtained by RNA-seq from high to low. The region +/- 2kB around the TSS is individually displayed for each biological replicate. Metagene profiles are outlined above the associated heat maps.
c. MapR in three biological replicates from U2OS cells after 48 h knockdown with control siRNA, DDX41 siRNA or treatment with 4 µM Actinomycin D for 6 hours. Metagene profiles of MapR signal from +/-2kb around the transcription start site of all expressed genes. Shadows indicate the standard error of the median.
d. The boxplot displays the $\log_2$ fold change of the reads per kilo base per million mapped reads (RPMK) of MapR signal between the ΔcatRNaseH1-MNase and the untargeted pA-MNase obtained +/- 1 kB around the TSS of all expressed genes based on RNA-sequencing. TSS without sufficient MapR signal were excluded (only $\log_2(RPKM)>0$ are taken into account). . Whiskers represent the 10^{th}-90^{th} percentile and the black line indicates the median. ***P-value < 0.001, ****P-value < 0.0001, two-sided paired Welch´s t-test.
e. Feature distribution of the differentially-bound genomic regions in MapR after DDX41 knockdown. Number of differentially bound regions with higher affinity (up, right) and lower binding (down, left) are indicated alongside the respective distribution. Features are color-coded as indicated in the legend.
f. Network of the Reactome pathway enrichment analysis of up-regulated genes after DDX41 knockdown compared to control knockdown based on RNA-seq. All expressed genes based on RNA-seq were used as background. The size of the dots indicates the number of genes contributing to the displayed term. Gradual coloring represents the adjusted p-values.
g. Immunofluorescence analysis of the mean p65 intensity per nucleus after 48 h control knockdown or DDX41 knockdown in U2OS cells. Representative images of p65 staining (cyan) and DNA counterstaining with DAPI are displayed on the left. The mean p65

intensity per nucleus was quantified using Fiji and displayed in a box plot (right). Black line indicates the median of the population and whiskers the 10^{th}-90^{th} percentiles. ****P-value < 0.0001, unpaired, two-sided Welch´s t-test.