Supplementary Information for

Impaired binding affinity of YTHDC1 with METTL3/METTL14 results in R-loop accumulation in myelodysplastic neoplasms with DDX41 mutation

Won Chan Hwang[1,7], Kibeom Park[1,7], Silvia Park[2,3,7], Na Young Cheon[1,7], Ja Yil Lee[1,7], Jong-Mi Lee[4,5], Min Kyung Ju[1], Joo Rak Lee[1], Yong-Rim Kwon[3], Woo-Lam Jo[4,6], Myungshin Kim[4,5*], Yoo-Jin Kim[2,3*], and Hongtae Kim[1*]

[*]Corresponding authors. Email: khtcat@unist.ac.kr, yoojink@catholic.ac.kr, microkim@catholic.ac.kr

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Supplementary Materials and Methods

Cell culture

The human myelogenous leukemia cell line K562 was purchased from American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI1640 (Cytiva, Marlborough, MA) supplemented with 10% FBS (Gibco, Franklin Lakes, NJ) and 1% penicillin/streptomycin (Gibco) in 5% CO_2 in a 37°C incubator.

Plasmids, sgRNAs, siRNAs, and transfection

DDX41 A1, A2, A3, A4, N-term, C-term, and DEAD domain fragment and D1, D2, D3, and D4 deletion mutant expression plasmids were created using GFP-, SFB-, or Myc-tagged mammalian expression vectors. The *DDX41-R525H* and *DDX41-Y259C* point mutants were generated from GFP-tagged *DDX41* using Phusion Site-Directed Mutagenesis Kits (ThermoFisher Scientific). *METTL3*, *METTL14*, and *YTHDC1* genes were purchased from the Korea Human Gene Bank. SFB-Mettl3, SFB-Mettl14, SFB-D1, SFB-D2, and SFB-D3 deletion mutant expression plasmids were created using a SFB-tagged mammalian expression vector. Myc-YTHDC1, D1, D2, D3, D4, D5, D6, and D7 deletion mutant expression plasmids were created using a Myc-tagged expression vector. GFP-METTL3, and GFP-YTHDC1 expression plasmids were created using GFP-tagged mammalian expression vectors. Guide RNA plasmids for human *DDX41* gene were generated by cloning guide sequences into pX330 (plasmid number 42230; Addgene). Target sequences for gene editing were selected using CRISPOR (www.crispor.tefor.net). The sequences of RT-qPCR primers, siRNAs, sgRNAs, and oligonucleotides for CRISPR/Cas9 knock-in are listed in Supplementary Table S3. All transfected plasmids were added to 100 μl cell suspensions and electroporated using a Neon Tranfection System (Thermo Fisher Scientific) at 1450 V for 15 ms over two pulses. Control

and *DDX41* siRNA duplexes used in the present can be found in Supplementary Table 3. siRNAs were transfected into cells using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific). All experiments were performed at 48 hrs after transfection.

Generation of *DDX41* knockout (KO) and knock-in (KI) K562 cells

To generate the *DDX41* KO and *DDX41* KI cell lines, K562 cells were co-transfected with CRISPR/Cas9, double guide RNA targeting *DDX41* or a one-point mutation guide RNA targeting *DDX41,* and donor plasmids using the Neon Transfection system (Thermo Fisher Scientific, Waltham, MA). After 48 h, high mCherry-expressing cells were sorted into 96-well plates using a FACSAria Fusion cell sorter (BD Biosciences, San Jose, CA). *DDX41* KO and KI cell lines were confirmed by targeted sequencing and western blotting for DDX41. Each clone was generated from a single population to avoid clonal heterogeneity.

Immunofluorescence

Immunofluorescence (IF) for S9.6, m6A, and co-localization with γH2AX, RAD51, and YTHDC1 were performed as described previously with minor modifications(1). Briefly, *DDX41* KO or KI cells were pelleted in 15 ml tubes at 1200 rpm for 5 min at 25°C. Media was removed until 0.5 ml media remained and cell pellets were resuspended. Pre-warmed 75 mM KCl solution at 37°C was added to cells in a drop-wise manner while cells were agitated on a vortex. Cells were then incubated at 37°C for 15 min and then 150 µl of freshly made, ice-cold methanol:acetic acid (3:1) were added cells in a drop-wise manner with agitation. Cells were pelleted and supernatants were removed until 0.5 ml media remained. Cells were resuspended in 5 ml of methanol:acetic acid added in a drop-wise manner under agitation. Cells were then fixed on ice for 20 min. Cells were washed once with methanol:acetic acid before being spotted onto slides. Slides were left to dry and immediately treated with pre-extraction buffer (0.1% TritonX-100, 20 mM HEPES-KOH pH 7.9, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose) for

10 min at RT and then washed with 0.5% TritonX-100 in phosphate buffered saline (PBS) for 10 min at RT. After permeabilization, cells were washed with PBS and treated with RNase H1 (50 U/ml, TAKRA, Japan) for 60 min at RT. Slides were rinsed with ice-cold PBS, fixed with 4% Paraformaldehyde (PFA), and finally fixed with ice-cold methanol for 30 min at RT. Slides were blocked with blocking buffer (PBS containing 4% BSA and 0.1% TritonX-100) for 1 h followed by incubation with primary antibodies against S9.6, m6A, γH2AX, RAD51, and YTHDC1 overnight at 4°C. Slides were washed three times with wash buffer (PBS containing 0.1% TritonX-100) and subsequently incubated with secondary antibodies conjugated with fluorophores (Alexa Fluor 594 or Alexa Fluor 488, Thermo Fisher Scientific) and 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Thermo Fisher Scientific) for 1 h at RT. After three washes with wash buffer, slides were mounted using VectaMount AQ Aqueous Mounting Medium (Vector Laboratories, Burlingame, CA). Images were captured and analyzed using a LSM-880 confocal microscope. Nuclear fluorescence intensities were quantified in each sample using ZEN Blue software (Carl Zeiss). Numbers above each sample indicate n values, which are the numbers of nuclei analyzed. Cells with ≥10 overlapping S9.6 and m6A foci were counted as positive and the proportion of positive cells among all cells was determined. More than 100 cells were examined for each sample.

Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were washed with ice-cold PBS and then lysed in NETN buffer (0.5% Nonidet P-40, 20 mM Tris [pH 8.0], 50 mM NaCl, 50 mM NaF, 100 μM Na3VO4, 1 mM dithiothreitol [DTT], and 50 μg/ml phenylmethylsulfonyl fluoride [PMSF]) with benzonase (Enzynomics, M018H) at 4°C for 40 min. Crude lysates were cleared by centrifugation at 14,000 rpm at 4°C for 5 min and supernatants were incubated with protein A-agarose-conjugated primary antibodies, FLAG-M2 affinity gel (Sigma, Cat#A2220), or c-Myc

agarose affinity gel (Sigma, Cat#7470). Immunocomplexes were washed three times with NETN buffer and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed using the antibodies indicated in figure legends. Proteins were visualized using secondary horseradish peroxidase-conjugated antibodies (Enzo Life Sciences, New York, NY) and enhanced chemiluminescence reagent (Thermo Fisher Scientific). Signals were detected using an automated imaging system (ChemiDoc™; Bio-Rad Laboratories, Hercules, CA).

Antibodies

The following antibodies were used in the present study: anti-Flag-HRP (Sigma, A8592), anti-Myc-HRP antibody (Roche, 11814150001), anti-GFP (Clontech, 632380), anti-β-actin antibody (Sigma, A5441), Anti-γH2AX antibodies (Cell signal, #2577, Sigma, 05636), anti-DDX41 antibody (Abnova, H00051428-M01, Altas antibodies, HPA049807), anti-METTL3 antibody (Abclonal, A8370), anti-METTL14 antibody (Sigma-Aldrich, HPA038002), anti-YTHDC1 antibody (Abcam, ab122340), anti-S9.6 antibody (Kerafast, ENH001), anti-m6A antibody (Synaptic Systems, 202003), anti-ATR antibody (Bethyl Laboratories, A300-137A), anti-phosphoATR antibody (S428, Cell signaling, #2853), anti-CHK1(Santa Cruz, SC-8408), anti-phosphoCHK1 (S317, Cell Signaling, #2344), anti-phosphoCHK1 (S345, Cell Signaling, #2341), anti-RPA2 antibody (Bethyl Laboratories, A300-244A), anti-phosphoRPA2 (S33, Bethyl Laboratories, A300-246A), anti-THOC1 antibody (Abcam, ab487), anti-FTO antibody (Abcam, ab126605), anti-BLM antibody (Abcam, ab2179), anti-FANCD2 antibody (Novus Biologicals, NB100-182), RAD51 antibody (Abcam, ab3801), and horseradish peroxidase-conjugated secondary antibodies specific to rabbit (Sigma-Aldrich, A0545) or mouse (Sigma-Aldrich, A9917) IgG.

Laser micro-irradiation and cell imaging

For laser microirradiation, cells were grown on 35 mm glass bottom dishes (SPL, Korea). Hela cells were transfected with plasmids or siRNA for 24 h. Transfected cells were treated with 10 μM BrdU (Sigma-Aldrich) prior to laser microirradiation for 20 h. Laser-scanning confocal microscopy was performed using a Zeiss LSM880 microscope with a 40x W (N.A. 1.2) C-apo objective. DNA damage was induced in live cells (maintained at 37°C in a humidified environment at 5% CO_2) using a 355-nm UVA optically pumped semiconductor laser (Coherent, Genesis, 15 μm/s, 100% power, 20 iterations). For GFP- and mCherry-tagged proteins, time-lapse images were acquired at 10 s time intervals after laser microirradiation. Acquisition and analysis were performed using ZEN software (black edition, Zeiss, Germany).

Cell proliferation assays

5×10^4 of *DDX41* KO or KI cells were plated in 6-well plates. The number of living and dead cells was evaluated by Trypan Blue exclusion every 24 h for 120 h as previously described (2). Cells were then counted every 24 h using a hemocytometer. Each experiment was repeated three times.

Purification of DDX41 constructs

Full-length DDX41, DDX41_ΔHel (helicase c domain deletion), DDX41_ΔDEAD (DEAD domain deletion), DDX41_ΔHel-Y259C, and DDX41-R525H mutants, all of which contain triple FLAG residues at the N-terminus and ten His residues at the C-terminus, were subcloned into pET19b-derived plasmid vectors. Each construct was cultured in Rosetta (DE3) *E. coli* strain (Millipore, 70954; 20 L culture for full-length DDX41 and 10 L culture for others) with 0.1 mg/mL carbenicillin until OD_{600} reached approximately 0.6. Plasmid expression was induced by the addition of 1 mM IPTG. Cells were further incubated at 16°C for 16 hrs. Harvested cells were resuspended in 200 mL lysis buffer (25 mM Tris-HCl [7.5], 100 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1x Halt protease inhibitor

[Thermofisher, 31170724-2]) and then lysed by sonication. Cell lysates were clarified by centrifugation at 30,000 g for 40 min at 4°C. For full-length DDX41, 15 mL of clarified lysates were loaded onto a Talon gravity column (Clontech, 635503) equilibrated with Talon A buffer (50 mM HEPES [7.5], 200 mM NaCl, and 30 mM imidazole). The column was then washed with 100 mL Talon A buffer. Proteins were eluted with Talon B buffer (50 mM HEPES [7.5], 200 mM NaCl, and 500 mM imidazole). In the ÄKTA pure FPLC system (Cytiva), 1 mL of HiTrap Q HP column (Cytiva, 17-1154-01) was equilibrated with QA buffer (25 mM Tris-HCl [7.5], 10% glycerol, 0.5 mM EDTA, and 1 mM DTT). Pooled fractions from the Talon column were loaded onto a Q HP column which was then washed with 100 mL of 10% QB buffer (25 mM Tris-HCl [7.5], 1 M NaCl, 10% glycerol, 0.5 mM EDTA, and 1 mM DTT). Full-length DDX41 proteins were eluted using linear gradient from 10% to 100% QB buffer. Eluates were collected in 1 mL fractions. For DDX41_ΔHel (helicase c domain deletion), DDX41_ΔHel Y259C, and DDX41_ΔHel R525H, 30 mL of clarified cell lysate was loaded onto a MHAP column (Bio-Rad, 1570020) equilibrated with MHAP A buffer (25 mM Tris-HCl [7.5], 100 mM NaCl, 10% glycerol, 0.5 mM EDTA, and 1 mM DTT) using the ÄKTA pure FPLC system (Cytiva). The MHAP column was then washed with 100 mL of 10% MHAP B buffer (25 mM Tris-HCl, 100 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, and 1 M KH_2PO_4; pH adjusted to 6.3 with NaOH). DDX41 constructs were eluted using linear gradient from 10% to 80% MHAP B buffer. Eluates were collected in 5 mL fractions and target protein-containing fractions were pooled. 15 mL of pooled fractions were loaded onto a Talon column equilibrated with Talon A buffer. The column was then washed with 100 mL of Talon A buffer. Proteins were eluted with Talon B buffer. All purified proteins were finally dialyzed against storage buffer (25 mM HEPES [7.5], 300 mM NaCl, 1 mM DTT, 10% glycerol, and 1 mM EDTA) and then snap-frozen in liquid nitrogen for storage at -80°C.

Electrophoretic mobility shift assay (EMSA)

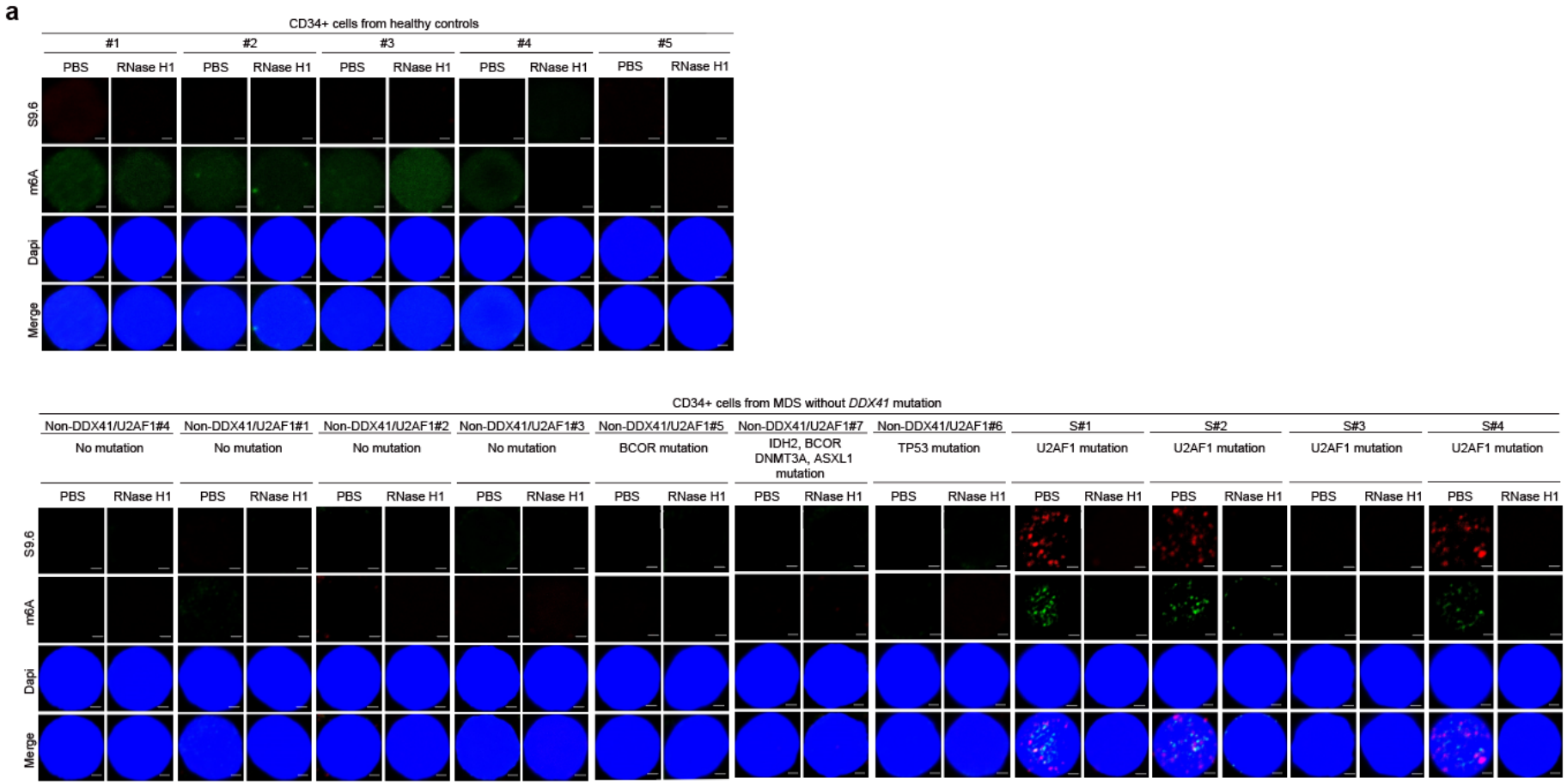
The binding affinity of DDX41 constructs with diverse substrates were measured using EMSA. Various oligomers (Supplementary Table 4) were synthesized (Bioneer, South Korea) and diluted in DDX41 reaction buffer (25 mM Tris-HCl [8.0], 100 mM NaCl) as described in Supplementary Table 5. For annealing, each pair of oligomers was mixed and heated to 95°C followed by slow cooling to 23°C. 10 nM of each annealed DNA substrate was incubated with several types of DDX41 constructs at different concentrations (0, 80, 160, 325, 650, and 1300 nM) in DDX41 reaction buffer in a dark room at 23°C for 30 min. Reactants were run on 5% non-denaturing PAGE in 0.5x TBE and fluorescence signals were measured using Typhoon 2000 imager (Cytiva). The binding ratio was calculated by dividing the intensity of shifted bands by the sum of shifted and non-shifted bands using ImageJ (NIH).

Supplementary materials and methods references

1. Nguyen HD, Yadav T, Giri S, Saez B, Graubert TA, Zou L. Functions of Replication Protein A as a Sensor of R Loops and a Regulator of RNaseH1. Molecular Cell. 2017;65(5):832-47 e4.

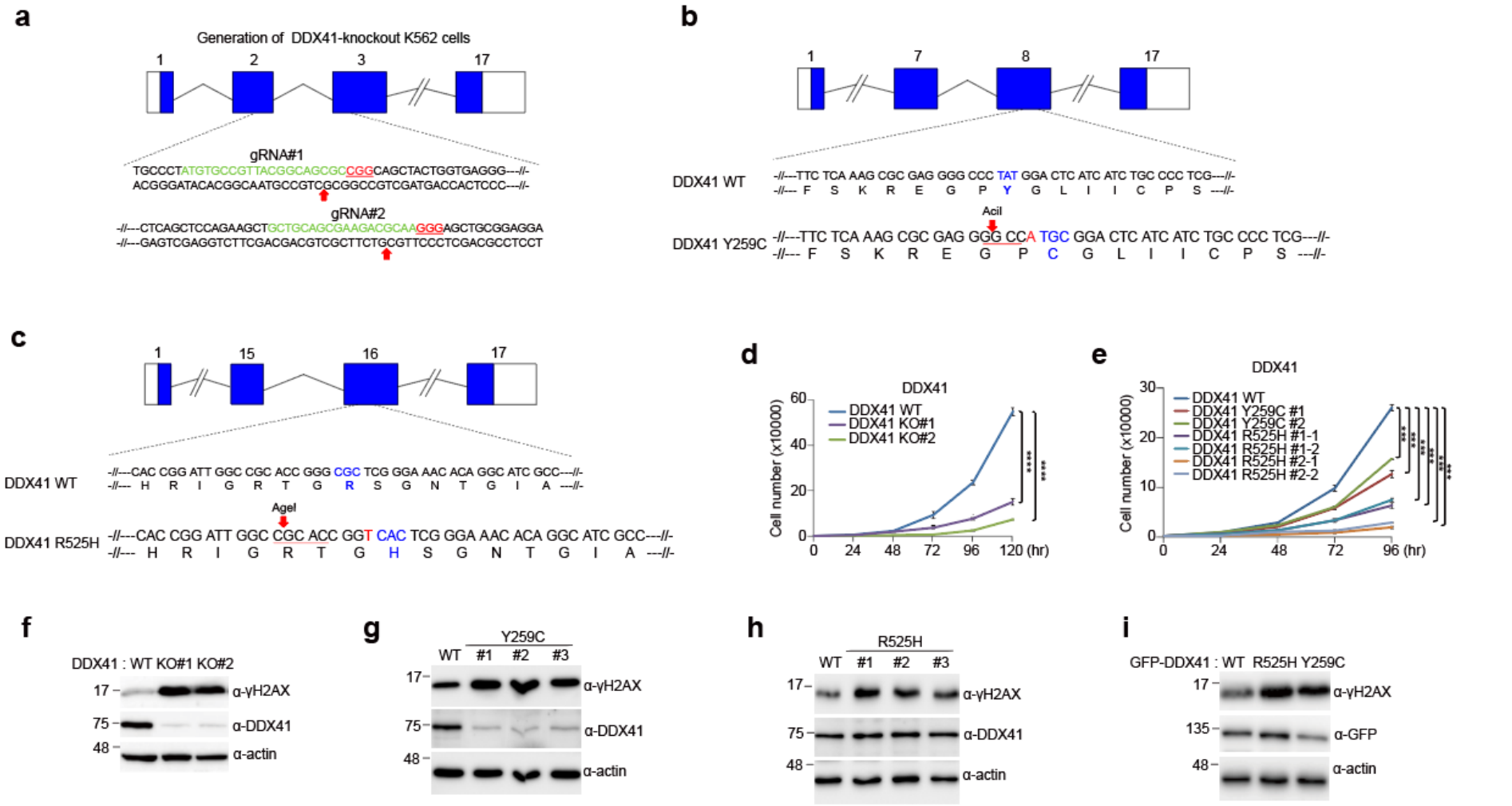
2. Ju MK, Shin KJ, Lee JR, Khim KW, E AL, Ra JS, et al. NSMF promotes the replication stress-induced DNA damage response for genome maintenance. Nucleic Acids Research. 2021;49(10):5605-22.

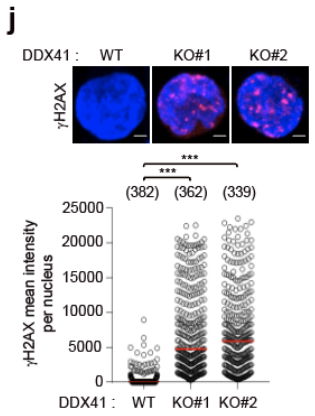
Supplementary Figures

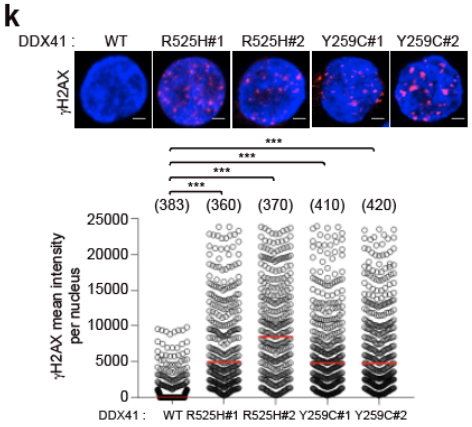


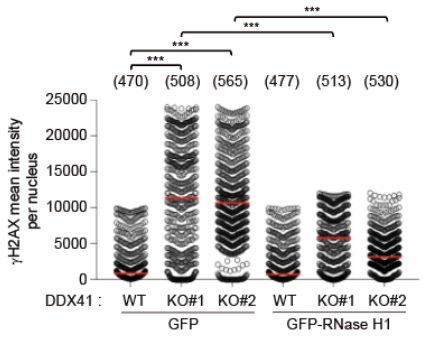
Supplementary Figure S1. Non-DDX41 mutated MDS samples were decreased m6A and R-loop in $CD34^+$ bone marrow cells.

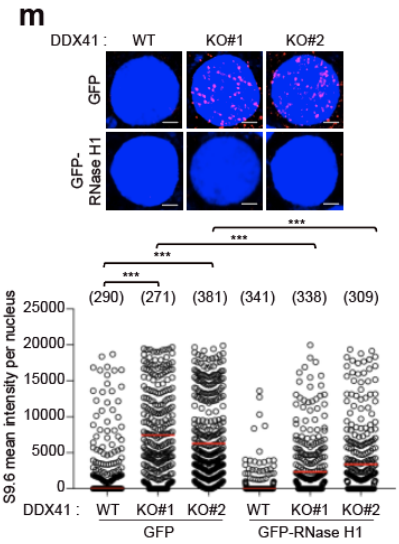
(a) Representative immunofluorescence images of localization m6A and S9.6 in $CD34^+$ cells isolated from MDS BM. Ordinary one-way ANOVA was used to determine the statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). Scale bar, 1 μm.

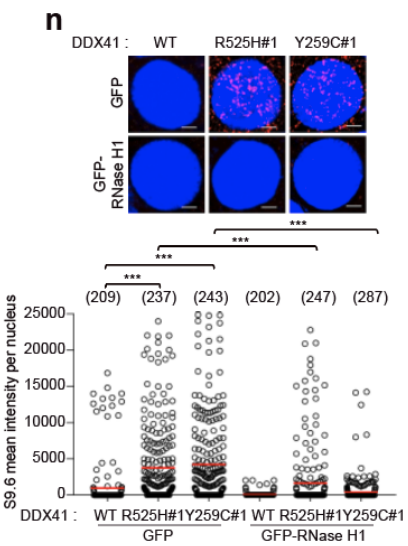

a
Generation of DDX41-knockout K562 cells
1
2
3
17
gRNA#1
TGCCCTATGTGCCGTTACGGCAGCGCCGGCAGCTACTGGTGAGGG---//-
ACGGGATACACGGCAATGCCGTCGCGGCCGTCGATGACCACTCCC---//-
gRNA#2
-//---CTCAGCTCCAGAAGCTGCTGCAGCGAAGACGCAAGGGAGCTGCGGAGGA
-//---GAGTCGAGGTCTTCGACGACGTCGCTTCTGCGTTCCCTCGACGCCTCCT
b
1
7
8
17
DDX41 WT
-//---TTC TCA AAG CGC GAG GGG CCC TAT GGA CTC ATC ATC TGC CCC TCG---//-
-//--- F S K R E G P Y G L I I C P S ---//-
AciI
DDX41 Y259C
-//---TTC TCA AAG CGC GAG GGG CCA TGC GGA CTC ATC ATC TGC CCC TCG---//-
-//--- F S K R E G P C G L I I C P S ---//-
c
1
15
16
17
DDX41 WT
-//---CAC CGG ATT GGC CGC ACC GGG CGC TCG GGA AAC ACA GGC ATC GCC---//-
-//--- H R I G R T G R S G N T G I A ---//-
AgeI
DDX41 R525H
-//---CAC CGG ATT GGC CGC ACC GGT CAC TCG GGA AAC ACA GGC ATC GCC---//-
-//--- H R I G R T G H S G N T G I A ---//-
d
DDX41
DDX41 WT
DDX41 KO#1
DDX41 KO#2
Cell number (x10000)
0
20
40
60
0
24
48
72
96
120 (hr)
****
****
e
DDX41
DDX41 WT
DDX41 Y259C #1
DDX41 Y259C #2
DDX41 R525H #1-1
DDX41 R525H #1-2
DDX41 R525H #2-1
DDX41 R525H #2-2
Cell number (x10000)
0
10
20
30
0
24
48
72
96 (hr)
***
***
***
***
***
f
DDX41 : WT KO#1 KO#2
17
75
48
α-γH2AX
α-DDX41
α-actin
g
Y259C
WT
#1
#2
#3
17
75
48
α-γH2AX
α-DDX41
α-actin
h
R525H
WT
#1
#2
#3
17
75
48
α-γH2AX
α-DDX41
α-actin
i
GFP-DDX41 : WT R525H Y259C
17
135
48
α-γH2AX
α-GFP
α-actin

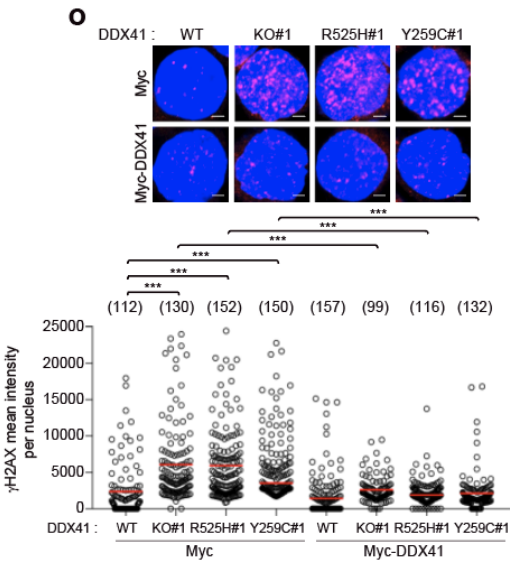

j
DDX41 : WT KO#1 KO#2
γH2AX
***
***
(382) (362) (339)
γH2AX mean intensity per nucleus
25000
20000
15000
10000
5000
0
DDX41 : WT KO#1 KO#2

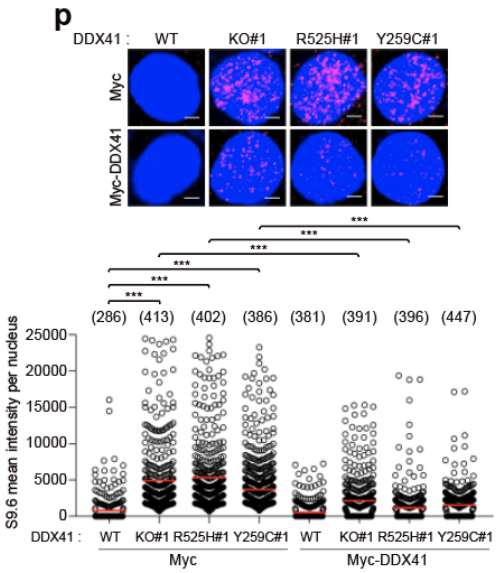

k
DDX41 : WT R525H#1 R525H#2 Y259C#1 Y259C#2
γH2AX
***
***
***
***
(383) (360) (370) (410) (420)
γH2AX mean intensity per nucleus
25000
20000
15000
10000
5000
0
DDX41 : WT R525H#1 R525H#2 Y259C#1 Y259C#2


l
***
***
***
***
(470) (508) (565) (477) (513) (530)
γH2AX mean intensity per nucleus
25000
20000
15000
10000
5000
0
DDX41 : WT KO#1 KO#2 WT KO#1 KO#2
GFP
GFP-RNase H1


m
DDX41 : WT KO#1 KO#2
GFP
GFP-RNase H1
***
***
***
***
(290) (271) (381) (341) (338) (309)
S9.6 mean intensity per nucleus
25000
20000
15000
10000
5000
0
DDX41 : WT KO#1 KO#2 WT KO#1 KO#2
GFP
GFP-RNase H1

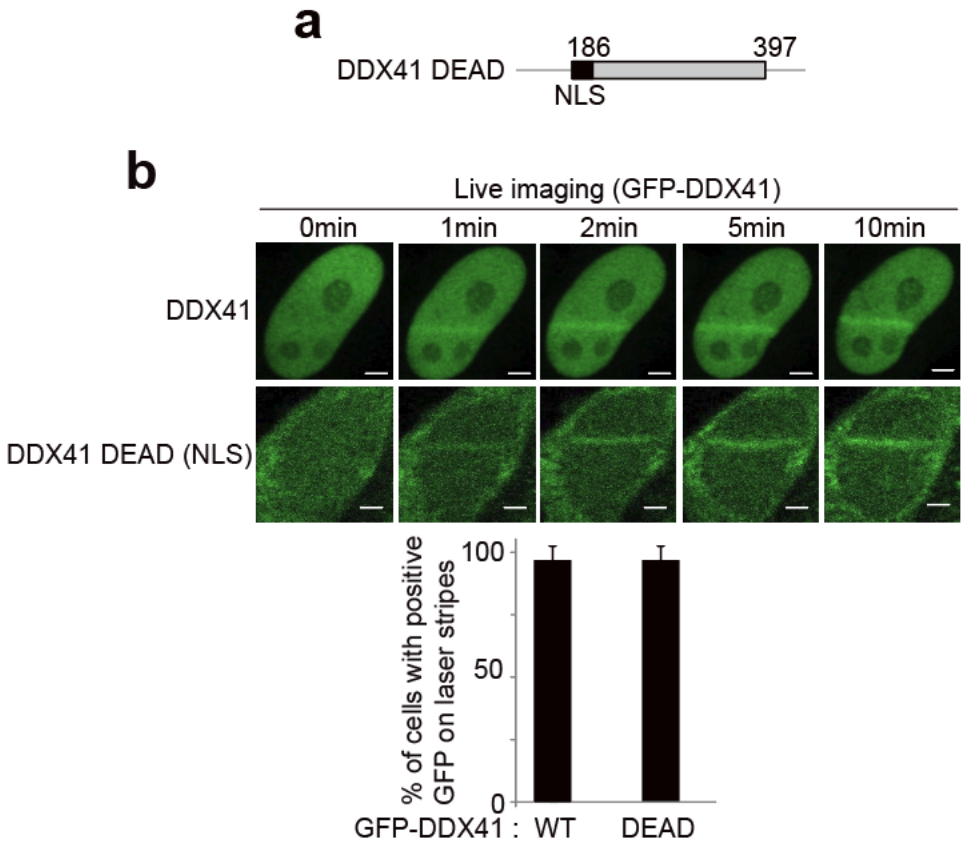

n
DDX41 : WT R525H#1 Y259C#1
GFP
GFP-RNase H1
***
***
***
***
(209) (237) (243) (202) (247) (287)
S9.6 mean intensity per nucleus
25000
20000
15000
10000
5000
0
DDX41 : WT R525H#1 Y259C#1 WT R525H#1 Y259C#1
GFP
GFP-RNase H1


o
DDX41 : WT KO#1 R525H#1 Y259C#1
Myc
Myc-DDX41
***
***
***
***
***
(112) (130) (152) (150) (157) (99) (116) (132)
γH2AX mean intensity per nucleus
25000
20000
15000
10000
5000
0
DDX41 : WT KO#1 R525H#1 Y259C#1 WT KO#1 R525H#1 Y259C#1
Myc
Myc-DDX41


p
DDX41 : WT KO#1 R525H#1 Y259C#1
Myc
Myc-DDX41
***
***
***
***
***
(286) (413) (402) (386) (381) (391) (396) (447)
S9.6 mean intensity per nucleus
25000
20000
15000
10000
5000
0
DDX41 : WT KO#1 R525H#1 Y259C#1 WT KO#1 R525H#1 Y259C#1
Myc
Myc-DDX41

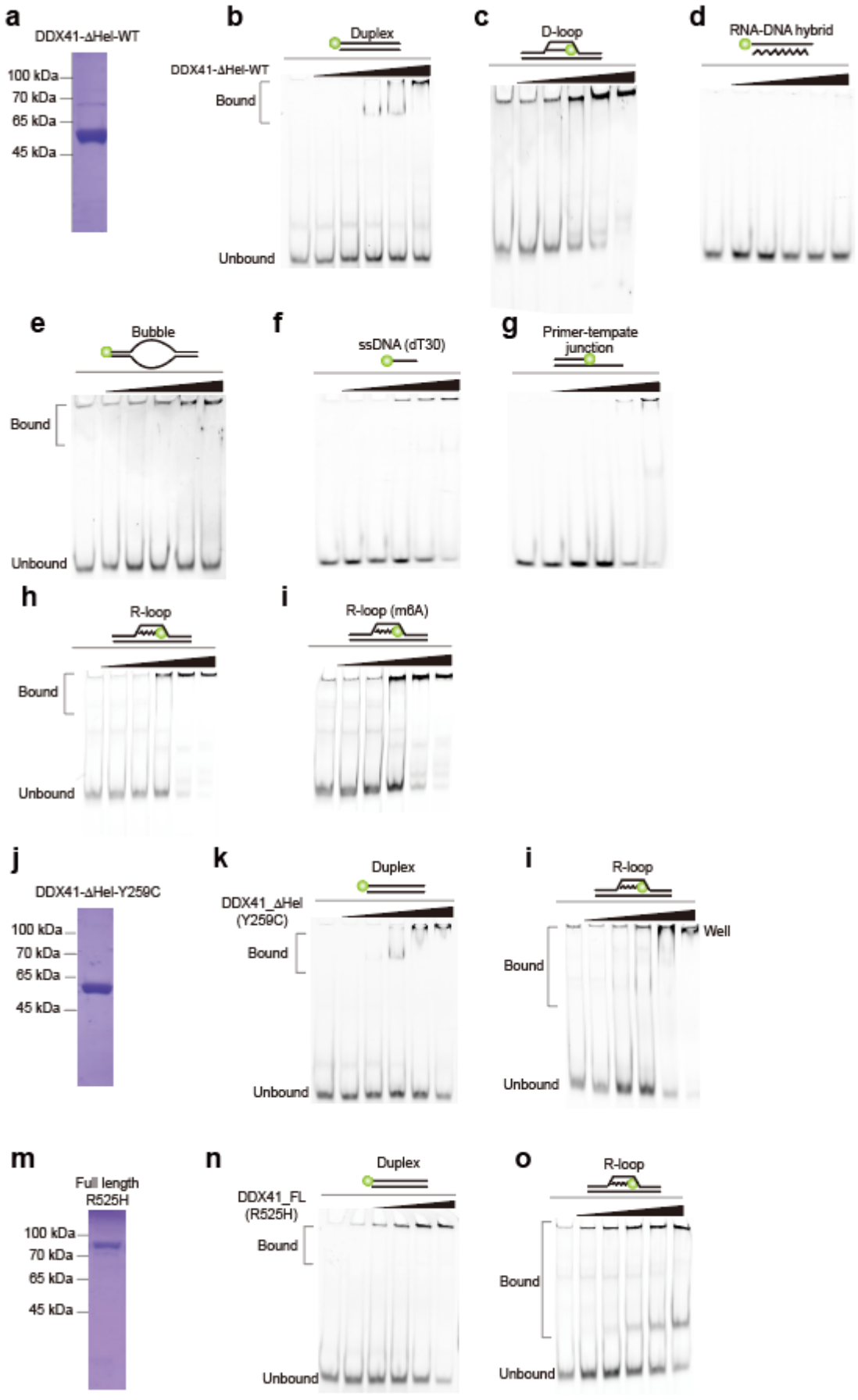
Supplementary Figure S2. DNA damage accumulated through methylated-R-loop in *DDX41* KO or KI cell lines.

(a) Human DDX41 genomic structure and guide RNAs in exon 2 and 3. Guide RNA plasmid for human DDX41 coupled with GFP vector was co-transfected to K562 cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA). 48 hr after transfection, GFP-positive K562 cells were sorted by FACS and then seeded onto 96-well plates. Each growing clones were expanded and screened for DDX41 knockout by western blotting and DNA sequencing. Red arrow: cut site, green: target sequence for guide RNA, Red (underlined): PAM sequence. **(b, c)** Human *DDX41* genomic structure and guide RNAs in exon 8 (Y259C, **b**) and 10 (R525H, **c**). **(d, e)** Viability of *DDX41* WT, or KO **(d)**, or KI **(e)** K562 cells. 5000 cells were plated and the number of viable cells was counted at indicated time points. Results are presented as the average of three independent experiments. **(f-h)** *DDX41* WT or KO **(f)** or KI **(g, h)** K562 cell lysates were immunoblotted with indicated antibodies. (**i)** DDX41 WT, R525H or Y259C expression transfected cell lysates were immunoblotted with indicated antibodies. **(j, k)** Quantification of γH2AX fluorescence intensity was determined by immunofluorescence staining with γH2AX antibodies in DDX41 WT, KO, and KI K562 cells. **(l-n)** *DDX41* WT or KO or KI K562 cells were transfected with GFP or GFP-RNase H1 expression plasmid. After 48 hr, γH2AX **(l)**, and S9.6 **(m, n)** intensity were determined by immunofluorescence. **(o and p)** γH2AX **(o)** and S9.6 **(p)** intensity in *DDX41* WT, KO, and KI K562 cells were reduced by overexpressed Myc or Myc-DDX41. After 72 hr, γH2AX and S9.6 intensity were determined by immunofluorescence. The numbers above each sample indicate the n value, which is the number of nuclei analyzed. Ordinary one-way ANOVA was used to determine the statistical significance ($^{***}P < 0.001$). Scale bar, 5 μm.


a
DDX41 DEAD
186
397
NLS
b
Live imaging (GFP-DDX41)
0min
1min
2min
5min
10min
DDX41
DDX41 DEAD (NLS)
% of cells with positive GFP on laser stripes
100
50
0
GFP-DDX41 : WT
DEAD
c
GFP-DDX41 : WT
DEAD
100
α-GFP
48
α-actin

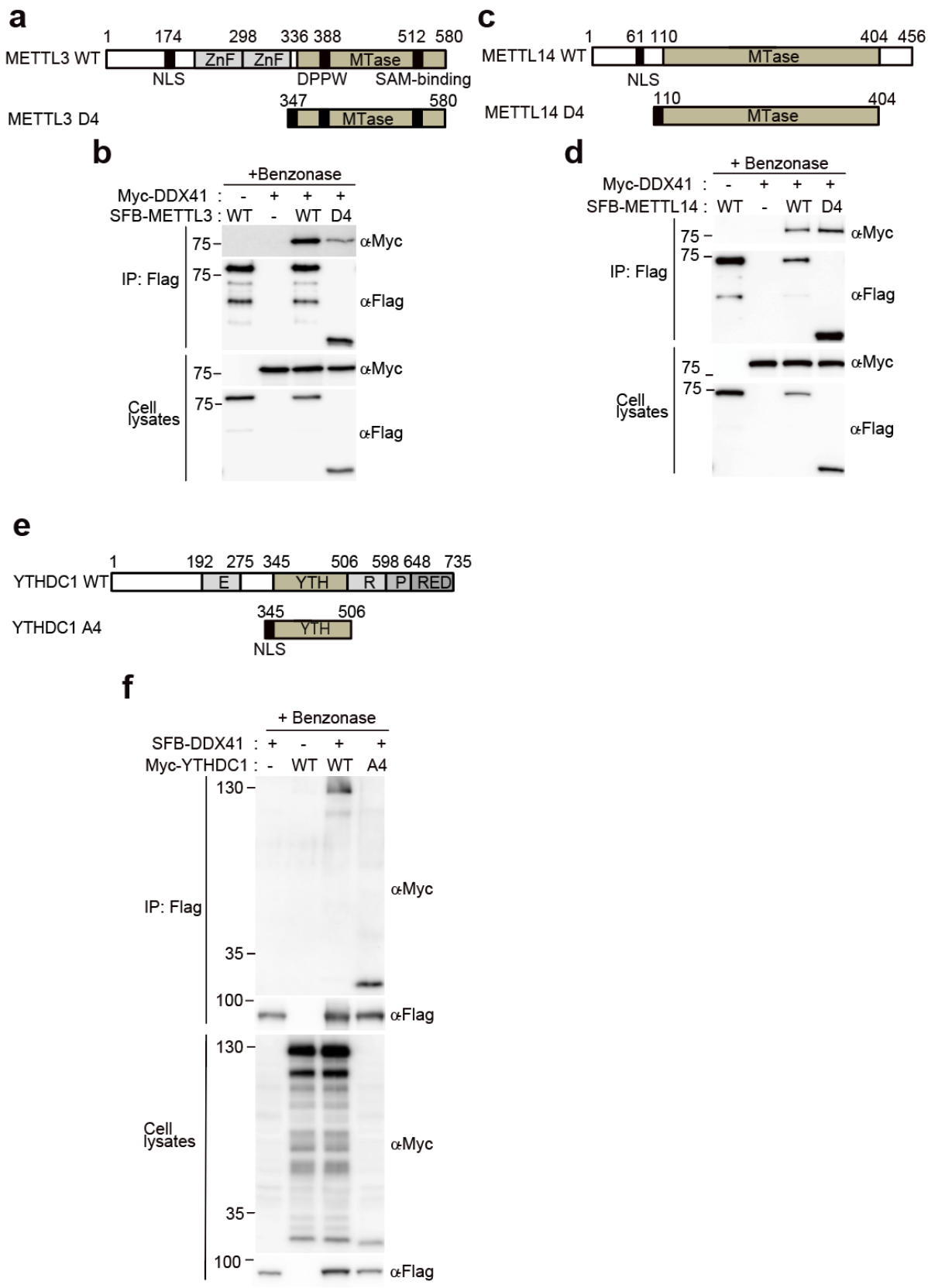
Supplementary Figure S3. DDX41 localizes to DNA damage sites via the DEAE box of DDX41.

(a) Diagram of the DDX41-DEAD mutant. The numbers indicate the amino acids residues. **(b)** GFP-DDX41-DEAD mutant translocates to the DNA damage sites. HeLa cells were transfected with GFP-tagged mutant expression plasmids, and 24 h later, the cells were treated with laser microirradiation (top panel). Scale bar, 5 μm. The cells with positive GFP expression on the laser stripes are presented in the bar graph (bottom panel). The results represent the average of two independent experiments. The error bars indicate the standard deviation for the cells transfected with each expression plasmid. **(c)** The expression validation of GFP-DDX41 WT or -DDX41-DEAD mutants by western blotting.


a
DDX41-ΔHel-WT
100 kDa
70 kDa
65 kDa
45 kDa
b
Duplex
DDX41-ΔHel-WT
Bound
Unbound
c
D-loop
d
RNA-DNA hybrid
e
Bubble
Bound
Unbound
f
ssDNA (dT30)
g
Primer-tempate junction
h
R-loop
Bound
Unbound
i
R-loop (m6A)
j
DDX41-ΔHel-Y259C
100 kDa
70 kDa
65 kDa
45 kDa
k
Duplex
DDX41_ΔHel (Y259C)
Bound
Unbound
i
R-loop
Well
Bound
Unbound
m
Full length R525H
100 kDa
70 kDa
65 kDa
45 kDa
n
Duplex
DDX41_FL (R525H)
Bound
Unbound
o
R-loop
Bound
Unbound

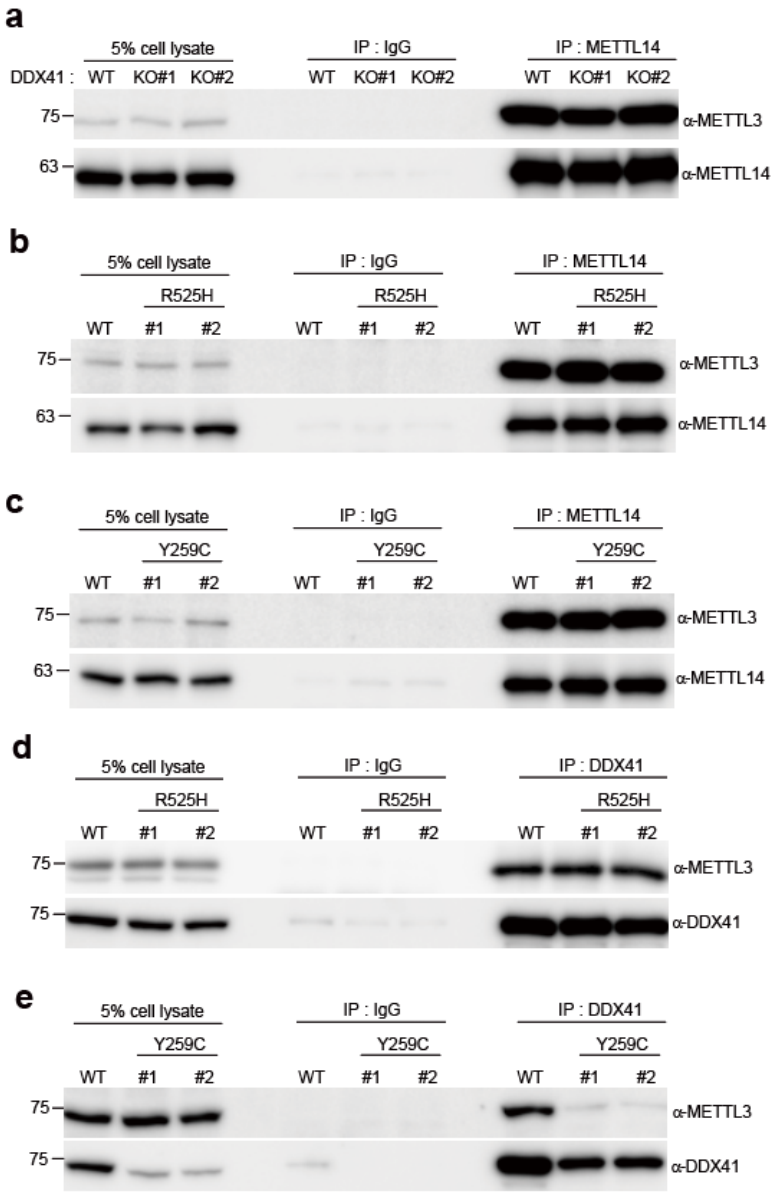
Supplementary Figure S4. Binding affinity of various construct of DDX41 to nucleotide substrates.

(a) SDS PAGE data of purified recombinant DDX41-ΔHel-WT. (**b-i)** EMSA for DDX41-ΔHel-WT and Duplex **(b)**, D-loop **(c)**, RNA-DNA hybrid **(d**), Bubble **(e)**, ssDNA(dT30) **(f)**, Primer-template junction **(g)**, R-loop **(h)**, methylated R-loop **(i)**. DDX41-ΔHel-WT (0, 80, 160, 325, 650 and 1300 nM) was titrated to 10 nM of nucleotide substrates. **(j)** SDS PAGE data of purified recombinant DDX41-ΔHel-Y259C. **(k, l)** EMSA for DDX41-ΔHel-Y259C and Duplex **(k)**, R-loop **(l)**. DDX41-ΔHel-Y259C (0, 80, 160, 325, 650 and 1300 nM) was titrated to 10 nM of nucleotide substrates. **(m)** SDS PAGE data of purified recombinant DDX41-R525H. **(n, o)** EMSA for DDX41-ΔHel-Y259C and Duplex (**N**), R-loop (**O**). DDX41-R525H (0, 80, 160, 325, 650 and 1300 nM) was titrated to 10 nM of nucleotide substrates.


a
METTL3 WT
1 174 298 336 388 512 580
ZnF ZnF MTase
NLS DPPW SAM-binding
METTL3 D4
347 580
MTase
c
METTL14 WT
1 61 110 404 456
MTase
NLS
METTL14 D4
110 404
MTase
b
+Benzonase
Myc-DDX41 : - + + +
SFB-METTL3 : WT - WT D4
IP: Flag
75
αMyc
αFlag
Cell lysates
d
+ Benzonase
Myc-DDX41 : - + + +
SFB-METTL14 : WT - WT D4
e
YTHDC1 WT
1 192 275 345 506 598 648 735
E YTH R P RED
YTHDC1 A4
345 506
YTH
NLS
f
+ Benzonase
SFB-DDX41 : + - + +
Myc-YTHDC1 : - WT WT A4
130
35
100
αMyc
αFlag
IP: Flag
Cell lysates

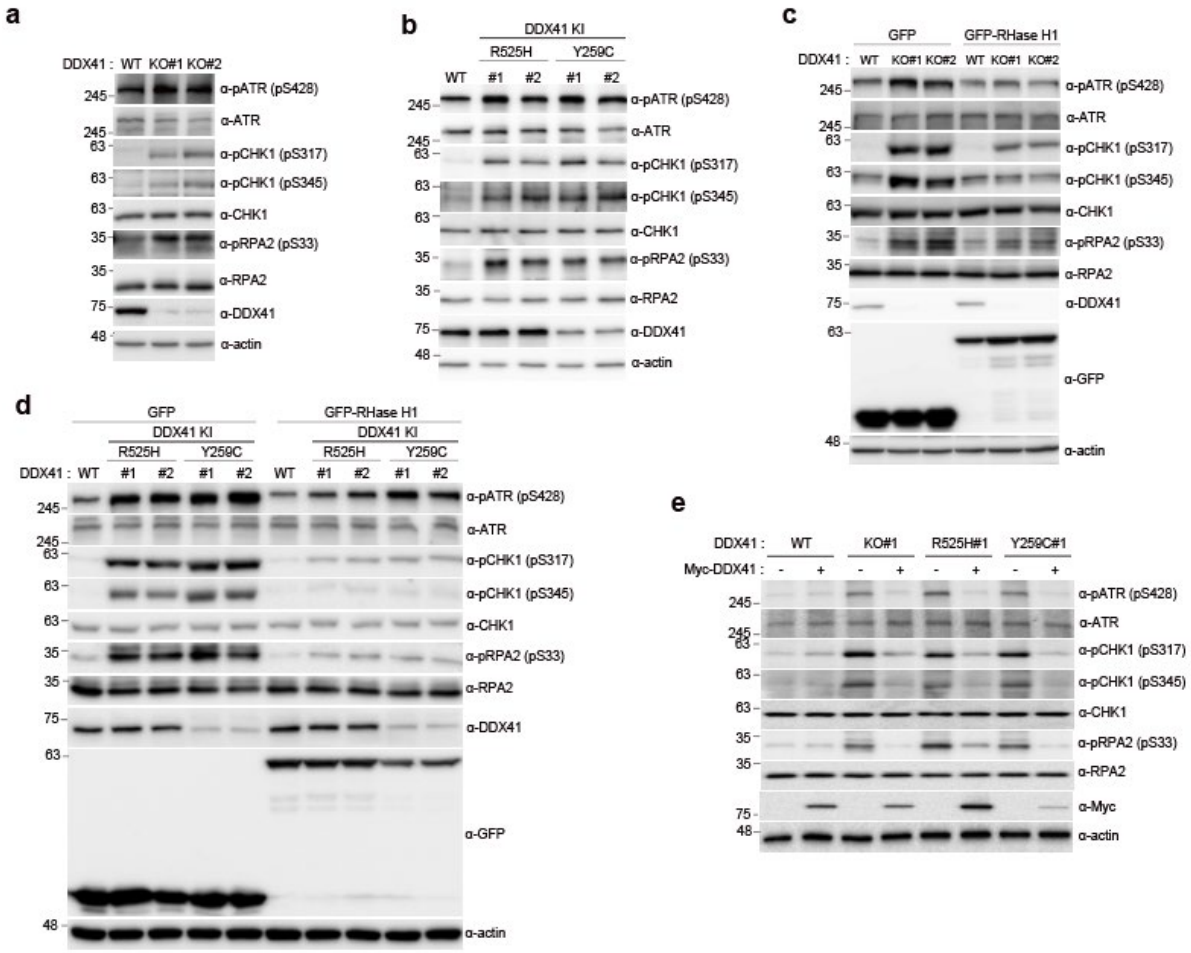
Supplementary Figure S5. DDX41 associates with METTL3, METTL14 and YTHDC1.

(a) Diagram of WT METLL3 and deletion mutants (METTL3 D4). The numbers indicate the amino acid residues. **(b)** Myc-DDX41 WT and either SFB-METTL3 or its deletion mutant were co-transfected into K562 cells. The cell lysates were immunoprecipitated with the anti-Flag antibody and then immunoblotted with the indicated antibodies. **(c)** Diagram of WT METLL14 and deletion mutants (METTL14 D4). The numbers indicate the amino acid residues. **(d)** Myc-DDX41 WT and either SFB-METTL14 or its deletion mutant were co-transfected into 293T cells. The cell lysates were immunoprecipitated with the anti-Flag antibody and then immunoblotted with the indicated antibodies. **(e)** Diagram of WT YTHDC1 and deletion mutant (YTHDC1 A4). The numbers indicate the amino acid residues. **(f)** SFB-DDX41 WT and either Myc-YTHDC1 or its deletion mutant were co-transfected into 293T cells. The cell lysates were immunoprecipitated with the anti-Flag antibody and then immunoblotted with the indicated antibodies.


a
5% cell lysate
IP : IgG
IP : METTL14
DDX41 : WT KO#1 KO#2 WT KO#1 KO#2 WT KO#1 KO#2
75
63
α-METTL3
α-METTL14
b
5% cell lysate
IP : IgG
IP : METTL14
R525H
WT #1 #2 WT #1 #2 WT #1 #2
75
63
α-METTL3
α-METTL14
c
5% cell lysate
IP : IgG
IP : METTL14
Y259C
WT #1 #2 WT #1 #2 WT #1 #2
75
63
α-METTL3
α-METTL14
d
5% cell lysate
IP : IgG
IP : DDX41
R525H
WT #1 #2 WT #1 #2 WT #1 #2
75
75
α-METTL3
α-DDX41
e
5% cell lysate
IP : IgG
IP : DDX41
Y259C
WT #1 #2 WT #1 #2 WT #1 #2
75
75
α-METTL3
α-DDX41

Supplementary Figure S6. DDX41 mediates the interaction among DDX41, METTL3 and METTL14.

(a-c) The interaction between METTL3 and METTL14 in *DDX41* KO or KI K562 cells. Immunoprecipitation reactions were performed using rabbit IgG or indicated antibodies and subjected to Western blotting analysis using the indicated antibodies. **(d, e)** The interaction between DDX41 and METTL3 in KI K562 cells. Immunoprecipitation reactions were performed using rabbit IgG or indicated antibodies and subjected to Western blotting analysis using the indicated antibodies.


a
DDX41 : WT KO#1 KO#2
245
α-pATR (pS428)
245
α-ATR
63
α-pCHK1 (pS317)
63
α-pCHK1 (pS345)
63
α-CHK1
35
α-pRPA2 (pS33)
35
α-RPA2
75
α-DDX41
48
α-actin
b
DDX41 KI
R525H
Y259C
WT #1 #2 #1 #2
245
α-pATR (pS428)
245
α-ATR
63
α-pCHK1 (pS317)
63
α-pCHK1 (pS345)
63
α-CHK1
35
α-pRPA2 (pS33)
35
α-RPA2
75
α-DDX41
48
α-actin
c
GFP
GFP-RHase H1
DDX41 : WT KO#1 KO#2 WT KO#1 KO#2
245
α-pATR (pS428)
245
α-ATR
63
α-pCHK1 (pS317)
63
α-pCHK1 (pS345)
63
α-CHK1
35
α-pRPA2 (pS33)
35
α-RPA2
75
α-DDX41
63
α-GFP
48
α-actin
d
GFP
GFP-RHase H1
DDX41 KI
R525H
Y259C
DDX41 : WT #1 #2 #1 #2 WT #1 #2 #1 #2
245
α-pATR (pS428)
245
α-ATR
63
α-pCHK1 (pS317)
63
α-pCHK1 (pS345)
63
α-CHK1
35
α-pRPA2 (pS33)
35
α-RPA2
75
α-DDX41
63
α-GFP
48
α-actin
e
DDX41 : WT KO#1 R525H#1 Y259C#1
Myc-DDX41 : - + - + - + - +
245
α-pATR (pS428)
245
α-ATR
63
α-pCHK1 (pS317)
63
α-pCHK1 (pS345)
63
α-CHK1
35
α-pRPA2 (pS33)
35
α-RPA2
75
α-Myc
48
α-actin

Supplementary Figure S7. The RNase H1 reduces replication stress response in *DDX41* defect cell lines.

(a and b) *DDX41*WT and KO or DDX41 KI K562 cell lysates were immunoblotted with indicated antibodies. **(c and d)** *DDX41* WT and KO or KI K562 cells were transfected with GFP or GFP-RNase H1 expression plasmid. After 48 hr, cell lysates were immunoblotted with indicated antibodies. **(e)** *DDX41* WT and KO or KI K562 cells were transfected with Myc or Myc-DDX41 expression plasmid. After 72 hr, cell lysates were immunoblotted with indicated antibodies. Results represent the average of two or three independent experiments.

Supplementary Table S1. Baseline characteristics of MDS patients according to presence of DDX41 mutation

	*DDX41*mutation (-)*	*DDX41*mutation (+)*	*P*
Number of cases	293	39	
Age, year, median (range)	58 (19-83)	63 (42-79)	0.001
Male sex, number (%)	184 (62.8)	32 (82.1)	0.018
Diagnosis, number (%)			0.045
SLD/MLD/MDS-U/del (5q)	182 (62.1)	20 (51.3)	
EB1/EB2	85 (29.0)	19 (48.7)	
MDS/MPN	26 (8.9)	0 (0.0)	
Chromosome, number (%)			<0.001
Normal karyotype	137 (46.8)	30 (76.9)	
Abnormal karyotype	156 (53.2)	9 (23.1)	
WBC (10^6/L), median (range)	3090 (640-126120)	1760 (800-7660)	<0.001
Hb (g/dL), median (range)	8.8 (2.8-16.8)	9.6 (4.4-13.2)	0.525
ANC (10^6/L), median (range)	1060 (0-78190)	460 (80-6430)	<0.001
PLT (10^9/L), median (range)	88 (5-654)	84 (7-260)	0.233
BM blast, %, median (range)	3.0 (0.0-18.0)	4.2 (0.7-18.0)	0.006
IPSS-R			
Score, median (range)	3.5 (0-10)	4 (1-8.5)	0.835
Risk by core, number (%)			0.567
< 4	148 (51.0)	18 (46.2)	
≥ 4	142 (49.0)	21 (53.8)	
Co-mutated genes, number (range)	1 (0-8)	1 (0-6)	0.948
HMT, number (%)			0.265
No	199 (67.9)	23 (59.0)	
Yes	94 (32.1)	16 (41.0)	
Response to HMT, number (%)			0.026
No	53/92 (57.6)	4/15 (26.7)	
Yes	39/92 (42.4)	11/15 (73.3)	
SCT, number (%)			0.500
No	156 (53.2)	23 (59.0)	
Yes	137 (46.8)	16 (41.0)	
Median survival, months	108.3	74.6	0.892

Abbreviations; SLD, single lineage dysplasia; MLD, multi-lineage dysplasia; MDS-U, myelodysplastic syndrome, unclassifiable; del(5q), deletion 5q; EB, excess blasts; MDS/MPN, Myelodysplastic syndrome/myeloproliferative neoplasm; WBC, white blood cell; Hb, hemoglobin; ANC, absolute neutrophil count; PLT, platelet; BM, bone marrow; IPSS-R, revised international prognostic scoring system; HMT, hypomethylating treatment; SCT, stem cell transplantation.*Cases with *DDX41* variant of unknown significances (n=4) were not included in either group.

Supplementary Table S2. Clinical and genomic data of MDS patients

Cases	Age	Sex	WHO diagnosis	BM blast (%)	Karyotypes	*DDX41* mutation (VAF, %)		*U2AF1* mutation (VAF, %)	Other mutation
						Germline	Somatic		
D#1	63	M	MDS-MLD	3	45,X,-Y[5]/46,XY[15]	V152G (48.7)	R525H (8.8)		None
D#2	71	M	MDS-MLD	2	46,XY[20]	V152G (50.9)	R525H (31.3)		None
D#3	65	F	MDS-MLD	3	46,XX,del(20)(q11.2)[20]/46,XX[10]	Y259C (47.9)	R525H (8.2)		None
D#4	63	M	MDS-EB-2	15	46,XY[10]	c.935+4A>T (51.0)	R525H (9.5)		None
D#5	66	M	MDS-EB-2	10.9	46,XY[10]	V152G (49.2)			None
D#6	61	M	MDS-MLD	3.4	46,XY,t(1;17)(p10;p10)c[20][a]	Y259C (49.2)	T377A (7.9)		None
D#7	79	M	MDS-EB-2	17	46,XY[20]		R525H (8.2)		None
D#8	61	M	MDS-MLD	4.8	46,XY[20]	V152G (49.3)	R525H (13.6)	Q157R (16.8)	None
D#9	55	M	MDS-EB-2	18	45,XY,t(13;14)(q34;q13),-17,-20,+mar[cp7]/46,XY[13]		G530D (9.3)		*TP53, DNMT3A*
D#10	61	M	MDS-MLD	2	46,XY[20]	Y259C (50.8)	A376V (11.6)		*DNMT3A*
D#11	74	M	MDS-U	4	46,XY[20]	Y259C (48.3)			None
D#12	42	F	MDS-MLD	4.2	46,XX,del(5)(q15q31)[7]/46,XX[13]	Y259C (48.4)	T227M (15.4)		None
D#13	51	M	MDS-EB-1	4	46,XY[20]	c.299-3C>T (49.0)			None
D#14	69	M	MDS-EB-1	6.4	46,XY[10]	Y259C (51.7)	T227M (7.8)		*DNMT3A*
D#15	69	M	MDS-MLD	2	46,XY[20]	Y259C (50.9)	T227M (7.6)		*RAD50*
D#16	75	M	MDS-SLD	1	46,XY[20]	R293H (49.7)			None
D#17	69	F	MDS-EB-1	8	46,XX[5]	Y259C (48.5)			*ATM, EZH2*
D#18	70	M	MDS-MLD	1	47,XY,+8[2]/47,idem,del(11)(q13q23)[3]/46,XY[15]	V152G (48.2)	R525H (29.8)		*ASXL1*
D#19	60	M	MDS-EB-1	6.7	46,XY[20]	Y259C (45.5)	R525H (9.4)		None
D#20	53	M	MDS-EB-2	15	46,XY,del(20)(q13.1)[3]/46,XY[17]	c.935+4A>T (48.2)			None
D#21	51	M	MDS-MLD	2	46,XY[20]	V152G (50.0)	K494E (31.6)		None
D#22	75	F	MDS-MLD	3	46,XX[20]	Y259C (51.7)			None
D#23	71	M	MDS-EB-2	15	46,XY[20]	A500Cfs (47.4)	G530D (8.7)		*ASXL1*
D#24	72	M	MDS-EB-2	15	46,XY[20]	Y259C (49.9)	R525H (6.0)		*STAG2*
D#25	71	F	MDS-EB-1	7	46,XX[20]	V152G (50.3)	P379L (26.1)		None
D#26	66	F	MDS-RS-MLD	1	46,XX[20]	V152G (49.5)	R525H (30.3)		*GNB1*
D#27	51	F	MDS-EB-1	6	46,XX[20]	M509I (49.0)			*SETBP1, STAG2, ASXL1, DNMT3A, KDM6A*
D#28	44	M	MDS-MLD	1	46,XY	T214Pfs (45.6)	P321L (30.8)		*SRSF2*
D#29	62	M	MDS-EB-1	9	46,XY,inc[4]	Y259C (51.5)	R525H (2.1)		*SRSF2*
D#30	54	M	MDS-MLD	2	46,XY[20]	Y259C (52.9)	P321L (14.7)		None
D#31	66	M	MDS-MLD	0.7	46,XY[20]	V152G (47.5)	G530D (27.1)		*ZRSR2*
D#32	58	M	MDS-EB-1	9	46,XY[20]	V152G (44.9)	E345D (15.2)		None
D#33	60	M	MDS-SLD	4	46,XY[20]	S217Ifs (50.0)	L390H (35.2)		None
D#34	64	M	MDS-MLD	1.1	47,XY,+8[7]/46,XY[13]	Y259C (49.7)			None
D#35	61	M	MDS-EB-1	7	47,XY,+8[6]/46,XY[14]	A500Cfs (47.0)	R525H (5.5)		*PHF6*
D#36	66	M	MDS-EB-2	17	46,XY[20]	A500Cfs (48.5)	G530D (4.0)		None
D#37	69	M	MDS-EB-1	7	46,XY[20]	c.935+4A>T (52.9)	G530S (5.2)		*ZRSR2*

D#38	55	M	MDS-EB-1	5	46,XY[1]	c.935+4A>T (48.3)			None
D#39	60	M	MDS-SLD	1	46,XY,inv(9)(p12q13)[20][a]	Y259C (50.2)	T227M (5.7)		None
SF#1	41	M	MDS-EB-1	7	46,XY[20]	None	None	S34F (42.6)	*ASXL1, EZH2*
SF#2	73	M	MDS-EB-1	4	47,XY,+8[20]	None	None	S34F (42.6)	*DNMT3A, TERT, BCOR, STAG2*
SF#3	50	M	MDS-EB-1	5	47,XY,+8[19]/46,XY[1]	None	None	S34Y (44.3)	*ASXL1, SF3A1, RUNX1, TET2*
SF#4	61	M	MDS-EB-1	5	47,XY,+8[20]	None	None	S34F (42.6)	*CBL, IRF1*
Non-D/SF#1	63	F	MDS-EB-1	8	46,XX,del(7)(q22q32)[18]/46,XX[2]	None	None	None	None
Non-D/SF#2	54	M	MDS-EB-1	9	46,XY,t(?17;22)(p13;q13)[10]/46,XY,del(20)(q11.2)[3]/46,idem,del(20)[3]/46,XY[4]	None	None	None	None
Non-D/SF#3	69	F	MDS-EB-1	9	46,XX[20]	None	None	None	None
Non-D/SF#4	67	M	MDS-EB-1	6.4	46,XY[20]	None	None	None	None
Non-D/SF#5	49	M	MDS-EB-1	6	46,XY,t(1;20)(p36.1;q13.3)[20]	None	None	None	*BCOR*
Non-D/SF#6	59	M	MDS-EB-1	9	44,XY,-5,del(7)(q22q35),add(12)(q24.2),-16,der(19)t(1;19)(p22;p13.3)[8]/46,XY[12]	None	None	None	*TP53*
Non-D/SF#7	60	M	MDS-EB-1	9	46,XY[20]	None	None	None	*IDH2, BCOR, DNMT3A, ASXL1*

Abbreviations; WBC, white blood cell; ANC, absolute neutrophil count; Hb, hemoglobin; PLT, platelet; BM, bone marrow; HMA, hypomethylating agent; SLD, single lineage dysplasia; MLD, multi-lineage dysplasia; MDS-U, myelodysplastic syndrome, unclassifiable; del(5q), deletion 5q; EB, excess blasts; MDS/MPN, Myelodysplastic syndrome/myeloproliferative neoplasm; IPSS-R, revised international prognostic scoring system; VAF, variant allele frequency

[a] Confirmed constitutional abnormality was categorized as normal karyotype.

Supplementary Table S3: The oligonucleotides used in this study.

Name	Sequence
DDX41 siRNA #1	5’-CUAAGAGUGCCCUUGUAAAUU-3’
DDX41 siRNA #2	5’-CCAUCUUCUCCUACUUCAAUU-3’
Control siRNA	5’-UUCAAUAAAUUCUUGAGGUUU-3’
DDX41 R525H ssDNA	5’-CTGTCCTTCTCTCTGCAGTACACCGGATTGGCCGCACCGGTCACTCGGGAAACACAGGCATCGCCACTACCTTCATCAACAAA-3’
DDX41_R525 gRNA	5’-GAGCGCCCGGTGCGGCCAAT-3’
DDX41 Y259C ssDNA	5’-AACAAGAGAAGAGGTTACCCTTCTCAAAGCGCGAGGGGCCaTgcGGACTCATCATCTGCCCCTCGGTAAGATAGGCTGGCCTGGA-3’
DDX41_Y259C gRNA	5’-GGGGCAGATGATGAGTCCATAGG-3’
hDDX41 KO#10 gRNA#1 F	5'-CACCGATGTGCCGTTACGGCAGCGC-3'
hDDX41 KO#10 gRNA#1 R	5'-AAACGCGCTGCCGTAACGGCACATC-3'
hDDX41 KO#10 gRNA#2 F	5’-CACCGCTGCTGCAGCGAAGACGCAA-3’
hDDX41 KO#10 gRNA#2 R	5'-AAACTTGCGTCTTCGCTGCAGCAGC-3’
hDDX41 KO#32 gRNA#1 F	5'-CACCGTTACCCTTCTCAAAGCGCG-3'
hDDX41 KO#32 gRNA#1 R	5'-AAACCGCGCTTTGAGAAGGGTAAC-3'
hDDX41 KO#32 gRNA#2 F	5'-CACCGGCCCCTCGCGCTTTGAGAA-3'
hDDX41 KO#32 gRNA#2 R	5'-AAACTTCTCAAAGCGCGAGGGGCC-3'
hDDX41 KO sequencing F	5’-TTGGGGGGCAGATGATGAGTCCATGTTTAAGAGC-3’
hDDX41 KO sequencing R	5’-TTAGCTCTTAAACATGGACTCATCATCTGCCCCCCAACAAG-3’
hDDX41 Myc, SFB, GFB, mCherry N1 vector F	5’-GCGCGCGAATTCATGGAGGAGTCGGAACCCGAA-3’
hDDX41 Myc, SFB vector R	5’-GCGCGCGGATCCTCAGAAGTCCATGGAGCTGTG-3’
hDDX41 GFP, mCherry N1 vector R	5’-GCGCGCGGATCCGCGAAGTCCATGGAGCTGTGGGC-3’
hDDX41 D1 GFP F	5’-AGCCGCTCCGAGGCGGAAATGAAGTTTCCTGCAGCC-3’
hDDX41 D1 GFP R	5’-GGCTGCAGGAAACTTCATTTCCGCCTCGGAGCGGCT-3’
hDDX41 D2 GFP F	5’-ATCAAGAGCTTCAAGGAAATCCAGGAGGTAGAATAT-3’
hDDX41 D2 GFP R	5’-ATATTCTACCTCCTGGATTTCCTTGAAGCTCTTGAT-3’
hDDX41 D3 GFP F	5’-GCTGCCAGCCTGGATGTCACCTTCATCAACAAAGCG-3’
hDDX41 D3 GFP R	5’-CGCTTTGTTGATGAAGGTGACATCCAGGCTGGCAGC-3’
hDDX41 D4 GFP F	5’-GCGCGCGAATTCATGGAGGAGTCGGAACCCGAA-3’
hDDX41 D4 GFP R	5’-GCGCGCGGATCCGCAGTGGCGATGCCTGTGTT-3’

hDDX41 N term (1-408 a.a) Myc vector F	5’-GCGCGCGAATTCATGGAGGAGTCGGAACCCGAA-3’
hDDX41 N term (1-408 a.a) Myc vector R	5’-GCGCGC GGATCCTCAGACATCCAGGCTGGCAGC-3’
hDDX41 C term (409-622 a.a) Myc vector F	5’-GCGCGCGAATTCATGCCCGAACGGAAGCGGGCTCGCACCGACATCCAGGAGGTAGAATAT-3’
hDDX41 C term (409-622 a.a) Myc vector R	5’-GCGCGCGGATCCTCAGAAGTCCATGGAGCTGTG-3’
hDDX41 A1 Myc vector F	5’-GCGCGCGAATTCATGGAGGAGTCGGAACCCGAA-3’
hDDX41 A1 Myc vector R	5’-GCGCGCGGATCCTCATTCCTTGAAGCTCTTGAT-3’
hDDX41 A2 (DEAD domain) Myc, GFP vector F	5’-GCGCGCGAATTCATGCCCGAACGGAAGCGGGCTCGCACCGACATGAAGTTTCCTGCAGCC-3’
hDDX41 A2 (DEAD domain) Myc vector R	5’-GCGCGCGGATCCTCAGACATCCAGGCTGGCAGC-3’
hDDX41 DEAD domain GFP vector R	5’-GCGCGCGGATCCGCGACATCCAGGCTGGCAGC-3’
hDDX41 A3 Myc vector F	5’-GCGCGCGAATTCATGCCCGAACGGAAGCGGGCTCGCACCGACATCCAGGAGGTAGAATAT-3’
hDDX41 A3 Myc vector R	5’-GCGCGCGGATCCTCAAGTGGCGATGCCTGTGTT-3’
hDDX41 A4 Myc vector F	5’-GCGCGCGAATTCATGCCCGAACGGAAGCGGGCTCGCACCGACACCTTCATCAACAAAGCG-3’
hDDX41 A4 Myc vector R	5’-GCGCGCGGATCCTCAGAAGTCCATGGAGCTGTG-3’
hDDX41 V152G (c.455T>G) GFP vector F	5’-TGGACTCCACCCCGTTATGGTCTGAGCATGTCTGAAGAG-3’
hDDX41 V152G (c.455T>G) GFP vector R	5’-CTCTTCAGACATGCTCAGACCATAACGGGGTGGAGTCCA-3’
hDDX41 T227M (c.680C>T) GFP vector F	5’-ATGATAGGCATCGCTTTCATGGGTTCAGGCAAGACACTG-3’
hDDX41 T227M (c.680C>T) GFP vector R	5’-CAGTGTCTTGCCTGAACCCATGAAAGCGATGCCTATCAT-3’
hDDX41 Y259C (c.766A>G) GFP vector F	5’-TCAAAGCGCGAGGGGCCCTGTGGACTCATCATCTGCCCC-3’
hDDX41 Y259C (c.766A>G) GFP vector R	5’-GGGGCAGATGATGAGTCCACAGGGCCCCTCGCGCTTTGA-3’
hDDX41 R293H (c.878G>A) GFP vector F	5’-GACAGCTCACCACTCCTGCACTGCGCCCTCTGCATTGGG-3’

hDDX41 R293H (c.878G>A) GFP vector R	5’-CCCAATGCAGAGGGCGCAGTGCAGGAGTGGTGAGCTGTC-3’
hDDX41 P321L (c.962C>T) GFP vector F	5’-CACATGATGGTGGCCACCCTGGGGCGCCTCATGGATTTG-3’
hDDX41 P321L (c.962C>T) GFP vector R	5’-CAAATCCATGAGGCGCCCCAGGGTGGCCACCATCATGTG-3’
hDDX41 E345D (c.1035G>C) GFP vector F	5’-CGCTACCTGGCCCTGGACGACGCTGACCGCATGATCGAC-3’
hDDX41 E345D (c.1035G>C) GFP vector R	5’-GTCGATCATGCGGTCAGCGTCGTCCAGGGCCAGGTAGCG-3’
hDDX41 A376V (c.1127C>T) GFP vector F	5’-CAGACCCTGCTCTTCAGTGTCACCATGCCGAAGAAGATT-3’
hDDX41 A376V (c.1127C>T) GFP vector R	5’-AATCTTCTTCGGCATGGTGACACTGAAGAGCAGGGTCTG-3’
hDDX41 P379L (c.1136C>T) GFP vector F	5’-CTCTTCAGTGCCACCATGCTGAAGAAGATTCAGAACTTT-3’
hDDX41 P379L (c.1136C>T) GFP vector R	5’-AAAGTTCTGAATCTTCTTCAGCATGGTGGCACTGAAGAG-3’
hDDX41 L390H (c.1169T>A) GFP vector F	5’-CAGAACTTTGCTAAGAGTGCCCATGTAAAGCCTGTGACCATCAAT-3
hDDX41 L390H (c.1169T>A) GFP vector R	5’-ATTGATGGTCACAGGCTTTACATGGGCACTCTTAGCAAAGTTCTG-3’
hDDX41 K494E (c.1480A>G) GFP vector F	5’-GCCACAGACGTTGCCTCCGAGGGCCTGGACTTCCCTGCC-3’
hDDX41 K494E (c.1480A>G) GFP vector R	5’-GGCAGGGAAGTCCAGGCCCTCGGAGGCAACGTCTGTGGC-3’
hDDX41 R525H (c.1574G>A) GFP vector F	5’-CGGATTGGCCGCACCGGGCACTCGGGAAACACAGGCATC-3’
hDDX41 R525H (c.1574G>A) GFP vector R	5’-GATGCCTGTGTTTCCCGAGTGCCCGGTGCGGCCAATCCG-3’
hDDX41 G530D (c.1589G>A) GFP vector F	5’-GGGCGCTCGGGAAACACAGACATCGCCACTACCTTCATC-3’
hDDX41 G530D (c.1589G>A) GFP vector R	5’-GATGAAGGTAGTGGCGATGTCTGTGTTTCCCGAGCGCCC-3’
hMettl3 SFB vector F	5’-GCGCGCGTCGACGCATGTCGGACACGTGGAGC-3’
hMettl3 SFB vector R	5’-GCGCGCCCGCGGCTATAAATTCTTAGGTTT-3’
hMettl3 D1 SFB vector F	5’-ATGTCGGACACGTGGAGCTCTCCAAAAAAAAAAAGAAAAGTTTCCATTGTTGAAAAATTTCGC-3’
hMettl3 D1 SFB vector R	5’-GCGAAATTTTTCAACAATGGAAACTTTTCTTTTTTTTTTTGGAGAGCTCCACGTGTCCGACAT-3’

hMettl3 D2 SFB vector F	5’-ACAACAGCCAAGGAACAACACACGCCAAGCCAGGAG-3’
hMettl3 D2 SFB vector R	5’-CTCCTGGCTTGGCGTGTGTTGTTCCTTGGCTGTTGT-3’
hMettl3 D3 SFB vector F	5’-GCCCCTGGCAGCAAAGACAAACCTAAGAATTTATAG-3’
hMettl3 D3 SFB vector R	5’-CTATAAATTCTTAGGTTTGTCTTTGCTGCCAGGGGC-3’
hMettl3 D4 SFB vector F	5’-GCGCGCGAATTCATGCCAAAAAAAAAAAGAAAAGTTCACACGCCAAGCCAG GAG-3’
hMettl3 D4 SFB vector R	5’-GCGCGCGTCGACCTATAAATTCTTAGGTTT-3’
hMettl14 SFB vector F	5’-GCGCGCGAATTCATGGATAGCCGCTTGCAG-3’
hMettl14 SFB vector R	5’-GCGCGCGGATCCTTATCGAGGTGGAAAGCC-3’
hMettl14 D1 SFB vector F	5’-GCGCGCGAATTCATGCCAAAAAAAAAAAGAAAAGTTACACAGAGCTTAAATCCC-3’
hMettl14 D1 SFB vector R	5’-GCGCGCGGATCCTTATCGAGGTGGAAAGCC-3’
hMettl14 D2 SFB vector F	5’-AGTACTTTTCTTAAGGGAAAATCTGACCGAGGAGGT-3’
hMettl14 D2 SFB vector R	5’-ACCTCCTCGGTCAGATTTTCCCTTAAGAAAAGTACT-3’
hMettl14 D3 SFB vector F	5’-GCGCGCGAATTCATGGATAGCCGCTTGCAG-3’
hMettl14 D3 SFB vector R	5’-GCGCGCGGATCCTTAAGATTTGGGAGGAGGCGA-3’
hMettl14 D4 SFB vector F	5’-GCGCGCGAATTCATGCCAAAAAAAAAAAGAAAAGTTACACAGAGCTTAAATCCC-3’
hMettl14 D4 SFB vector R	5’-GCGCGCGGATCCTTAAGATTTGGGAGGAGGCGA-3’
hYTHDC1 SFB, Myc, GFP vector F	5’-GCGCGCGAATTCATGGCGGCTGACAGTCGGG-3’
hYTHDC1 SFB vector R	5’-GCGCGCCCGCGGTTATCTTCTATATCGACC-3’
hYTHDC1 Myc vector R	5’-GCGCGCGGTACCTTATCTTCTATATCGACC-3’
hYTHDC1 GFP vector R	5’-GCGCGCGGTACCCCTCTTCTATATCGACCTCTCTCCCC-3’
hYTHDC1 D1 SFB vector F	5’-ATGGCGGCTGACAGTCGG GATGAGCAAGGGAACAAC-3’
hYTHDC1 D1 SFB vector R	5’-GTTGTTCCCTTGCTCATCCCGACTGTCAGCCGCCAT-3’
hYTHDC1 D2 SFB vector F	5’-GGCAGCAGTGGTTCTTCTTCTGAATCTGTTTCCTTC-3’
hYTHDC1 D2 SFB vector R	5’-GAAGGAAACAGATTCAGAAGAAGAACCACTGCTGCC-3’
hYTHDC1 D3 SFB vector F	5’-GAGGCCAGTGACTCTGGTACCAGTAAACTCAAATAT-3’
hYTHDC1 D3 SFB vector R	5’-ATATTTGAGTTTACTGGTACCAGAGTCACTGGCCTC-3’
hYTHDC1 D4 SFB vector F	5’-GCTGTCCGAAAAGATCAAAAAATGCGTCACAAGAGA-3’
hYTHDC1 D4 SFB vector R	5’-TCTCTTGTGACGCATTTTTTGATCTTTTCGGACAGC-3’
hYTHDC1 D5 SFB vector F	5’-TTGTATCAGGTCATTCATAATGATTATGTGAGGGAA-3’

hYTHDC1 D5 SFB vector R	5’-TTCCCTCACATAATCATTATGAATGACCTGATACAA-3’
hYTHDC1 D6 SFB vector F	5’-TTTTTAAATGGGTCCTACCATCCAGTACCACATGAA-3’
hYTHDC1 D6 SFB vector R	5’-TTCATGTGGTACTGGATGGTAGGACCCATTTAAAAA-3’
hYTHDC1 D7 SFB vector F	5’-CCCCCTTACTCAGGACATGGTCGATATAGAAGATAA-3’
hYTHDC1 D7 SFB vector R	5’-TTATCTTCTATATCGACCATGTCCTGAGTAAGGGGG-3’
hYTHDC1 A4 Myc vector F	5’-GCGCGCGAATTCATGCCAAAAAAAAAAAGAAAAGTTACCAGTAAACTCAAATAT-3’
hYTHDC1 A4 Myc vector R	5’-GCGCGCGGTACCTTAATGAATGACCTGATACAA-3’
hYTHDC1-DDX41 flag vector F	5’-GCGCGCACCGGTGCCACCATGGACTACAAGGATGACGACGACAAG GATTACAAAGATGACGACGATAAGGACTACAAGGATGACGACGACAAG ATGGCGGCTGACAGTCGGGAG-3’
hYTHDC1-DDX41 flag vector R	5’-GCGCGCGAATTCCGATCCGCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTCTTCTATATCGACCTCTCTC-3’
hYTHDC1-METTL3 flag vector F	5’-GCGCGCACCGGTGCCACCATGGACTACAAGGATGACGACGACAAGGATTACAAAGATGACGACGATAAGGACTACAAGGATGACGACGACAAG ATGGCGGCTGACAGTCGGGAG-3’
hYTHDC1-METTL3 flag vector R	5’-GCGCGCAAGCTTGCCGATCCGCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTCTTCTATATCGACCTCTCTC-3’

Supplementary Table S4. List of synthesized oligomers

Name	Sequence
R-loop oligo1*	5’-[Cy5]-GCC AGG GAC GAG GTG AAC CTG CAG GTG GGC **GGC TAC TAC TTA GAT GTC ATC CGA GGC TTA T**TG GTA GAA TTC GGC AGC GTC ATG C GA CGG C-3’
R-loop oligo2*	5’-GCC GTC GCA TGA CGC TGC CGA ATT CTA CCA **CGC GAT TCA TAC CTG TCG TGC CAG CTG CTT T**GC CCA CCT GCA GGT TCA CCT CGT C CC TGG C-3’
R-loop RNA	5’-[Cy3]-GCA GCU GGC ACG ACA GGU AUG AAU C-3’
m6A RNA**	5’-[Cy3]-GC***A*** GCU GGC ***A***CG ACA GGU AUG A***A***U C-3’
D-loop DNA	5’-GCA GCT GGC ACG ACA GGT ATG AAT C-3’
Homoduplex	5’-[Cy5]-GCC AGG GAC GAG GTG AAC CTG CAG GTG GGC AAA GCA GCT GGC ACG ACA GGT ATG AAT CGC GTG GTA GAA TTC GGC AGC GTC AT G CGA CGG C-3’
Hybrid DNA	5’-CCC ATA CCG TAT AAC CAT TTG GCT GTC CAA GCT CCG GGT-3’
Hybrid RNA	5’-[Cy5]-ACC CGG AGC UUG GAC AGC CAA AUG GUU AUA CGG UAU GG G-3’
dT_{30}	5’-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-3’
PTJ 1	5’-[Cy5]GCCTCGCTGCCGTCGCCA[Biotin]-3’
PTJ 2	5’-TGGCGACGGCAGCGAGGCTTTTTTTTTTTTTTTTT[Cy3]-3’

* Bold represents bubble for R-loop.

** Bold italic A represents N6 methylated A.

Supplementary Table S5. Annealing for diverse DNA or RNA substrates

	Components and concentration	Buffer
dsDNA	R-loop oligo 1: 6 uM Homoduplex: 5 uM	25 mM Tris-HCl [8.0] 100 mM NaCl
R-loop	R-loop oligo 1: 6 uM R-loop oligo 2: 5 uM R-loop RNA: 5 uM	
R-loop(m6A)	R-loop oligo 1: 6 uM R-loop oligo 2: 5 uM m6A RNA: 5 uM	
D-loop	R-loop oligo 1: 6 uM R-loop oligo 2: 5 uM D-loop DNA: 5 uM	
Bubble	R-loop oligo 1: 6 uM R-loop oligo 2: 5 uM	
Hybrid	Hybrid DNA: 5 uM Hybrid RNA: 5 uM	
ssDNA	dT_{30}: 5 uM	
Primer-template junction	PTJ 1: 6 uM PTJ 2: 5 uM	