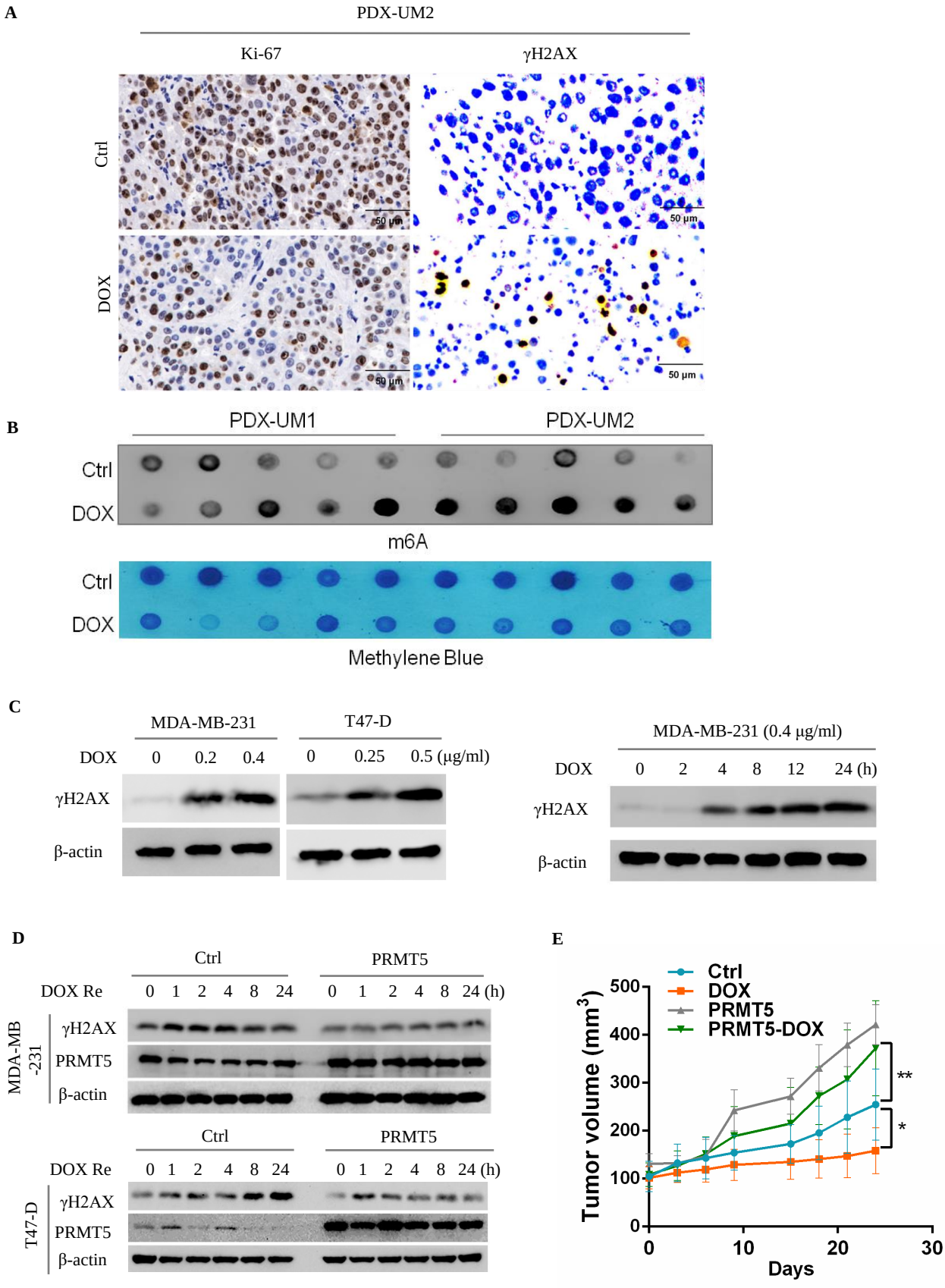
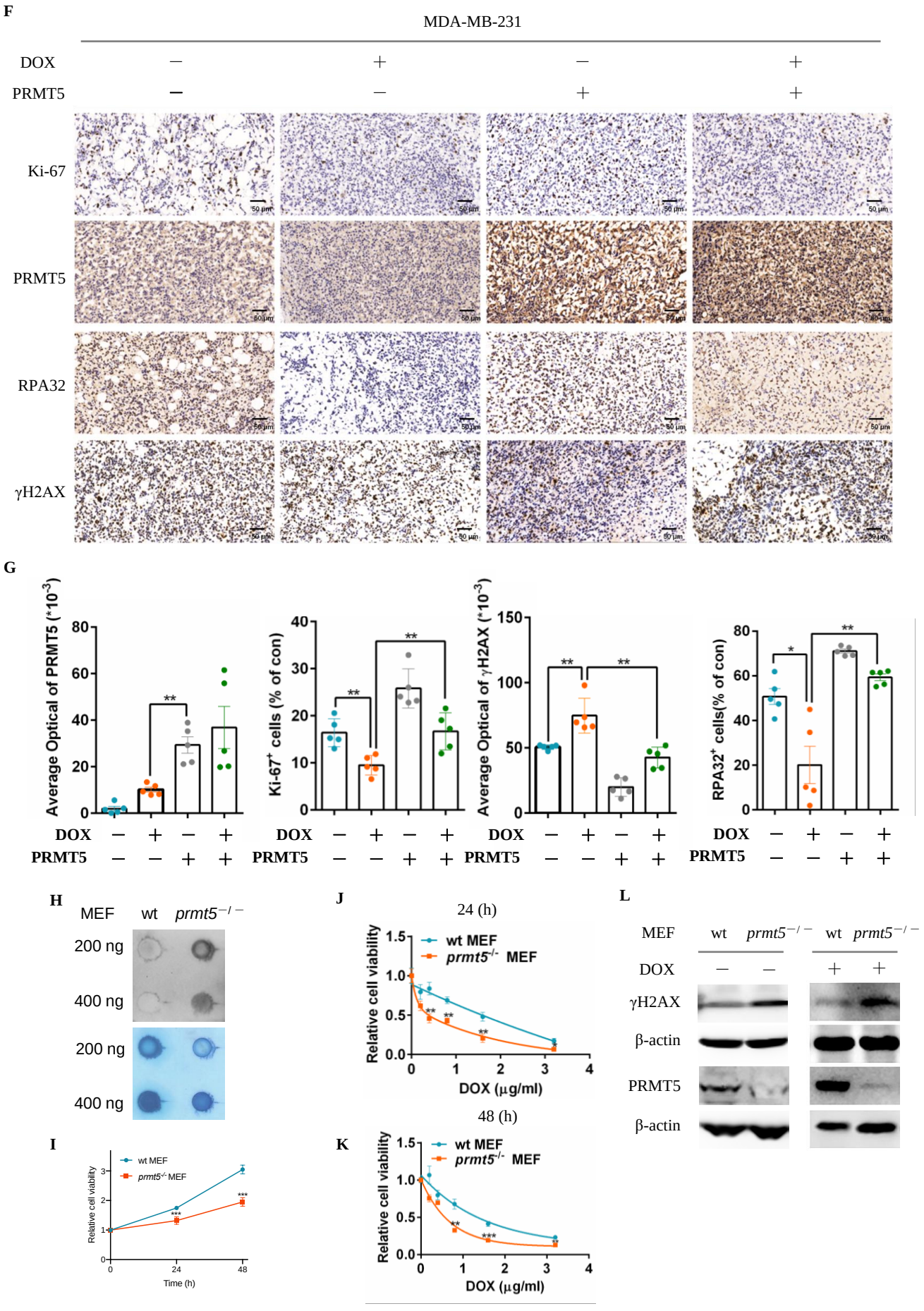

A
PDX-UM2
Ki-67
γH2AX
Ctrl
DOX
50 μm
B
PDX-UM1
PDX-UM2
Ctrl
DOX
m6A
Methylene Blue
C
MDA-MB-231
T47-D
DOX
0
0.2
0.4
0
0.25
0.5 (μg/ml)
γH2AX
β-actin
MDA-MB-231 (0.4 μg/ml)
0
2
4
8
12
24 (h)
D
Ctrl
PRMT5
DOX Re
0 1 2 4 8 24 0 1 2 4 8 24 (h)
MDA-MB-231
γH2AX
PRMT5
β-actin
T47-D
E
Ctrl
DOX
PRMT5
PRMT5-DOX
Tumor volume (mm3)
Days
**
*


F
MDA-MB-231
DOX
PRMT5
Ki-67
PRMT5
RPA32
γH2AX
G
Average Optical of PRMT5 (*10-3)
Ki-67+ cells (% of con)
Average Optical of γH2AX (*10-3)
RPA32+ cells(% of con)
DOX
PRMT5
H
MEF
wt
prmt5−/−
200 ng
400 ng
200 ng
400 ng
I
wt MEF
prmt5-/- MEF
Relative cell viability
Time (h)
J
24 (h)
wt MEF
prmt5-/- MEF
Relative cell viability
DOX (μg/ml)
K
48 (h)
wt MEF
prmt5-/- MEF
Relative cell viability
DOX (μg/ml)
L
MEF
wt
prmt5−/−
wt
prmt5−/−
DOX
γH2AX
β-actin
PRMT5
β-actin

Supplementary Figure 1. PRMT5 Decreases the Doxorubicin Sensitivity of Breast Cancer Cells

(A) Representative immunohistochemical (IHC) staining for γH2AX and Ki-67 in breast cancer tissue samples from a PDX mouse model.

(B) Dot-blot assay of RNA m6A methylation in breast cancer tissue samples from a PDX mouse model treated with or without doxorubicin.

(C) Western blot analysis of γH2AX in MDA-MB-231 and T47-D breast cancer cells treated with doxorubicin (DOX) for the duration and dose indicated.

(D) MDA-MB-231 and T47-D breast cancer cells with or without PRMT5 overexpression were treated with the indicated dose of DOX for 1 hour. The cells were then washed with PBS twice and cultured in fresh medium to recover (Re) for the indicated time, and γH2AX expression was detected by Western blotting. MDA-MB-231: 0.4 μg/ml; T47-D: 0.5 μg/ml.

(E) MDA-MB-231 cells with or without PRMT5 overexpression were orthotopically implanted subcutaneously into female athymic nude mice. The mice were treated with DOX once a week via intravenous tail vein injection at 2 mg/kg·body weight after the tumor volume reached 100-150 mm^3. Tumor volume was measured every 3 days, and the mice were sacrificed 27 days after injection. The tumor growth curves and harvested tumors are displayed.

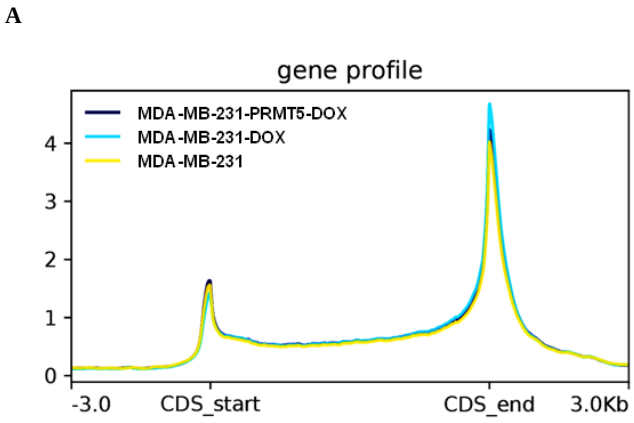
(F) (G) Immunostaining for and relative quantification of Ki-67, PRMT5, RPA32 and γH2AX in **(E)** were performed.

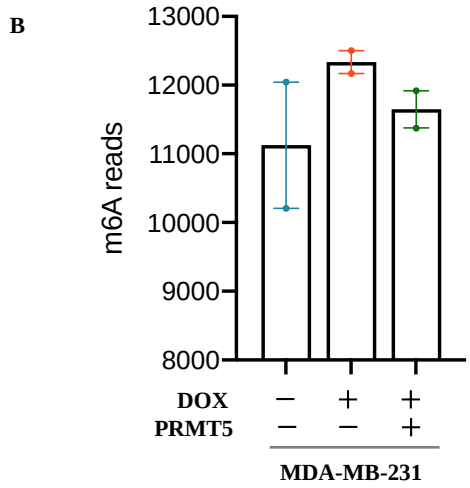
*p < 0.05, **p < 0.01, and ***p < 0.001.

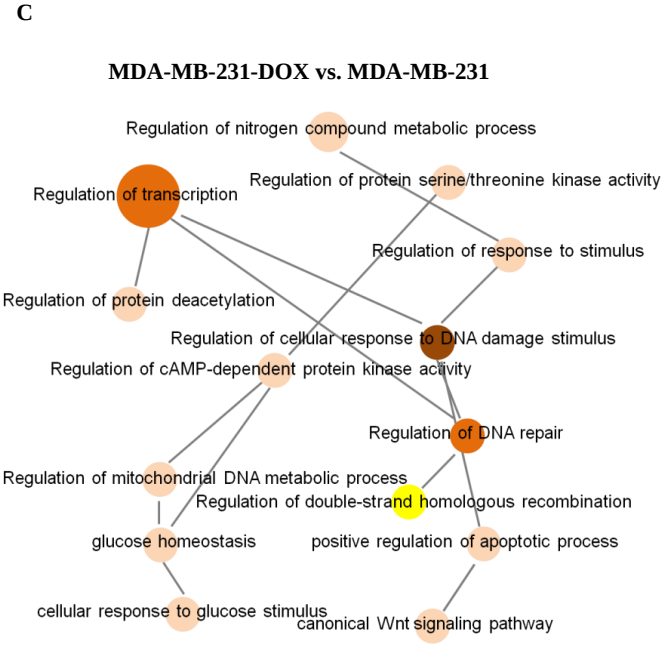
(H) Dot-blot assay of RNA m6A methylation comparing wild-type and *prmt*5-knockout mouse embryonic fibroblasts (MEFs) without doxorubicin.

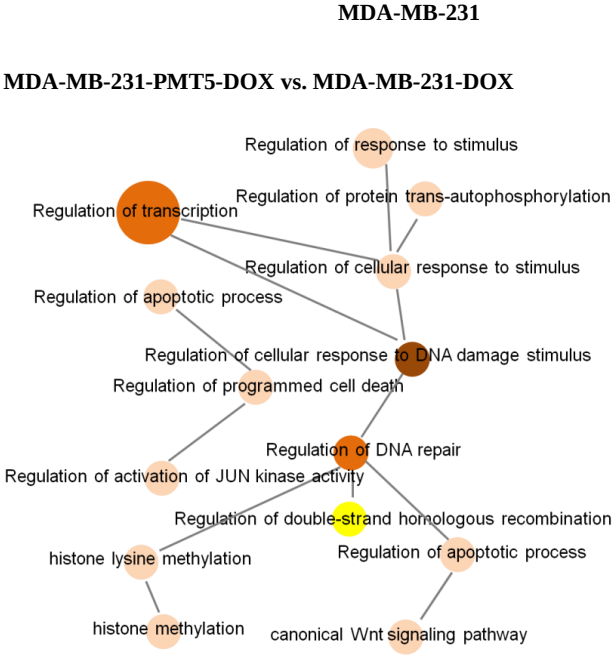
(I) (J) (K) Proliferation assay (MTT) comparing wild-type and *prmt*5-knockout mouse embryonic fibroblasts (MEFs) for 24 and 48 hours and comparing doxorubicin-treated with different doses for 24 or 48 hours, as indicated.

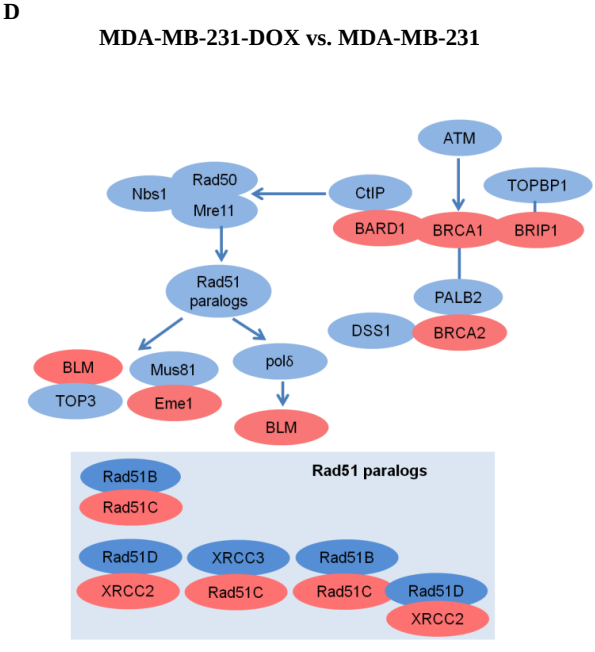
(L) Western blot analysis of γH2AX and PRMT5 in wild-type and *prmt*5-knockout MEFs treated and untreated with 0.4 μg/ml doxorubicin for 24 hours.

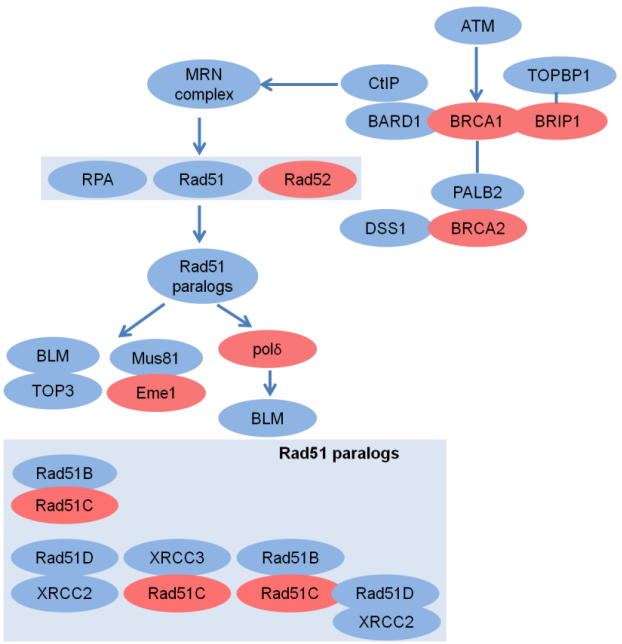

A
gene profile
MDA-MB-231-PRMT5-DOX
MDA-MB-231-DOX
MDA-MB-231
4
3
2
1
0
-3.0
CDS_start
CDS_end
3.0Kb


B
m6A reads
13000
12000
11000
10000
9000
8000
DOX
PRMT5
MDA-MB-231

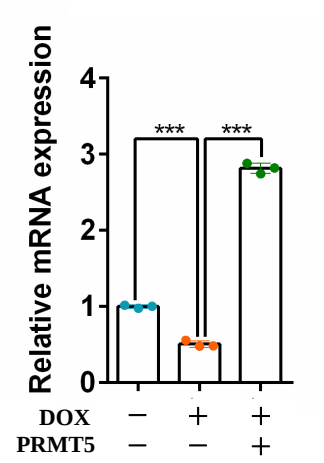

C
MDA-MB-231-DOX vs. MDA-MB-231
Regulation of nitrogen compound metabolic process
Regulation of transcription
Regulation of protein serine/threonine kinase activity
Regulation of response to stimulus
Regulation of protein deacetylation
Regulation of cellular response to DNA damage stimulus
Regulation of cAMP-dependent protein kinase activity
Regulation of DNA repair
Regulation of mitochondrial DNA metabolic process
Regulation of double-strand homologous recombination
glucose homeostasis
positive regulation of apoptotic process
cellular response to glucose stimulus
canonical Wnt signaling pathway


MDA-MB-231-PMT5-DOX vs. MDA-MB-231-DOX
Regulation of response to stimulus
Regulation of transcription
Regulation of protein trans-autophosphorylation
Regulation of cellular response to stimulus
Regulation of apoptotic process
Regulation of cellular response to DNA damage stimulus
Regulation of programmed cell death
Regulation of DNA repair
Regulation of activation of JUN kinase activity
Regulation of double-strand homologous recombination
histone lysine methylation
Regulation of apoptotic process
histone methylation
canonical Wnt signaling pathway

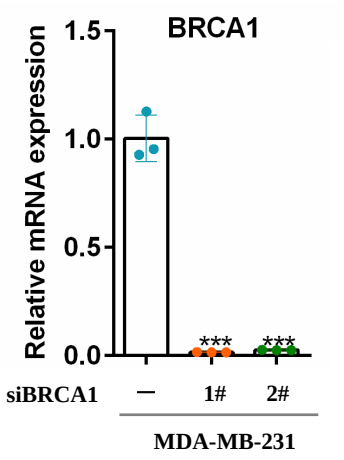

D
MDA-MB-231-DOX vs. MDA-MB-231
ATM
Nbs1
Rad50
Mre11
CtIP
TOPBP1
BARD1
BRCA1
BRIP1
Rad51 paralogs
PALB2
DSS1
BRCA2
BLM
Mus81
polδ
TOP3
Eme1
BLM
Rad51 paralogs
Rad51B
Rad51C
Rad51D
XRCC3
Rad51B
XRCC2
Rad51C
Rad51C
Rad51D
XRCC2


MDA-MB-231-PMT5-DOX vs. MDA-MB-231-DOX
ATM
MRN complex
CtIP
TOPBP1
BARD1
BRCA1
BRIP1
RPA
Rad51
Rad52
PALB2
DSS1
BRCA2
Rad51 paralogs
BLM
Mus81
polδ
TOP3
Eme1
BLM
Rad51 paralogs
Rad51B
Rad51C
Rad51D
XRCC3
Rad51B
XRCC2
Rad51C
Rad51C
Rad51D
XRCC2

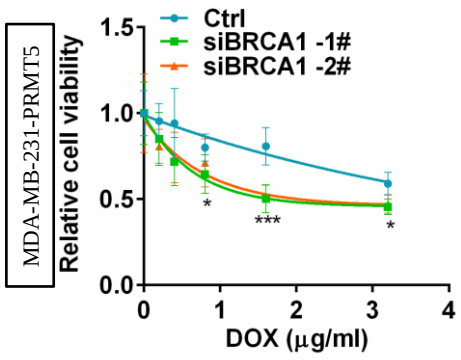
E



F



G



H

#	BRCA1 mRNA sequence of 3'UTR	Score	Decision
	149		
	UAUGUACAUCAGCCUGAAAAGGACUUCUGGCUAUGCAAGGGUCC		
1	C	0.621	m6A site (High confidence)
	1268		
	ACUGUUUAUAGCUGUUGGAAGGACUAGGUCUUCCCUAGCCCCCC		
2	C	0.567	m6A site (Moderate confidence)

Supplementary Figure 2. The GO Terms and KEGG Annotation Analysis of MeRIP-Seq Data and Doxorubicin Sensitivity Assay with BRCA1 Knockdown.

(A) Gene profile of MeRIP sequencing data for the different indicated MDA-MB-231 cells. m6A peak enrichment was analyzed in each group as indicated, with enrichment found mainly near start and stop codons.

(B) Quantification of the total m6A reads in each group.

(C) GO term enrichment analysis of biological processes with a significant difference in the m6A levels of overall RNA transcripts between MDA-MB-231 cells treated with or without 0.4 μg/ml doxorubicin for 24 hours (left) or between MDA-MB-231-PRMT5 and MDA-MB-231 cells treated with 0.4 μg/ml doxorubicin for 24 hours (right). The circle size represents the number of molecules and depth of color shows the relationship.

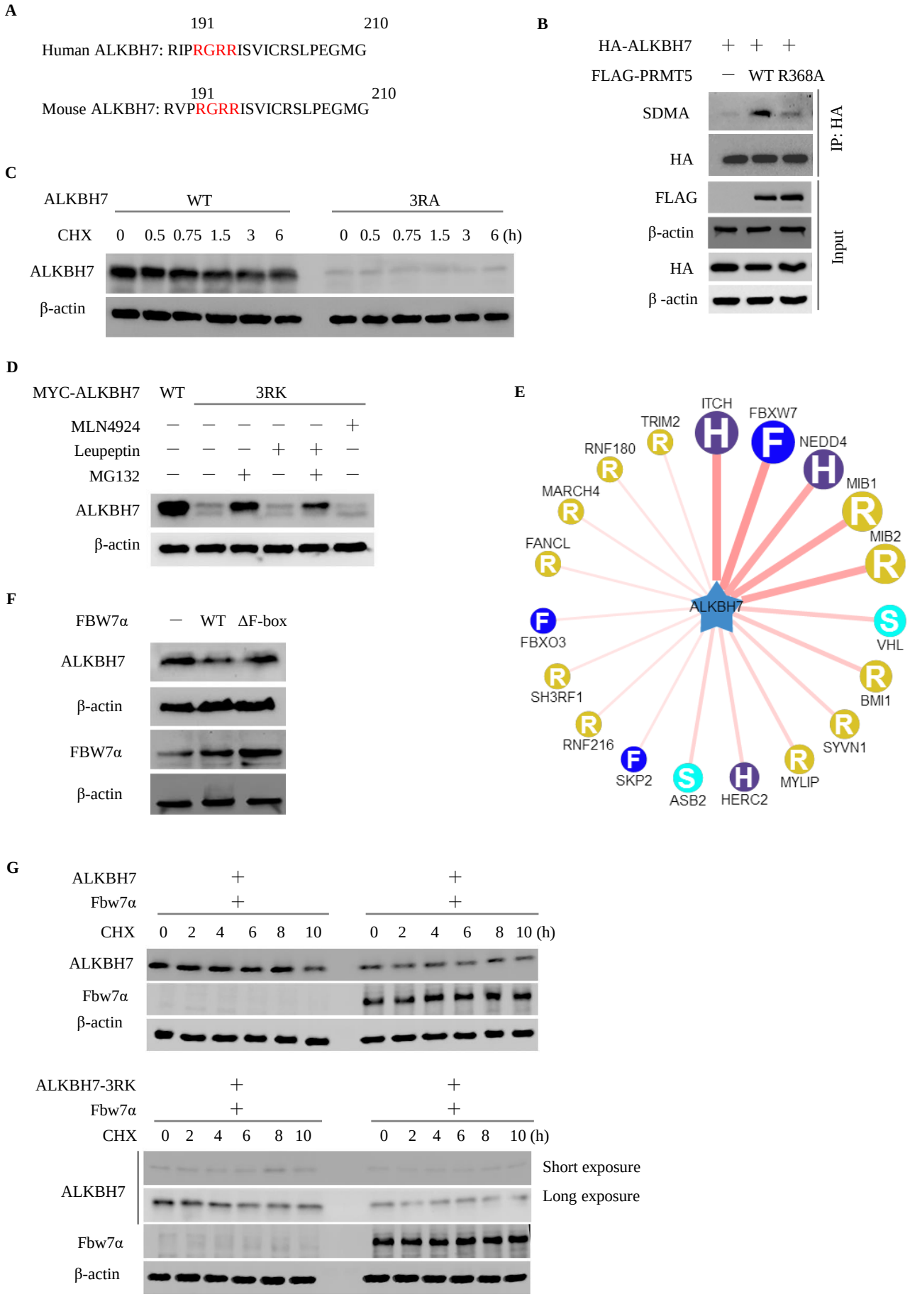
(D) Diagram of the homologous repair-related genes affected by m6A. The genes with differential expression between MDA-MB-231 cells treated with or without 0.4 μg/ml doxorubicin for 24 hours (left) or between MDA-MB-231-PRMT5 and MDA-MB-231 cells treated with 0.4 μg/ml doxorubicin for 24 hours (right) are marked in red. The diagram is based on KEGG annotations.

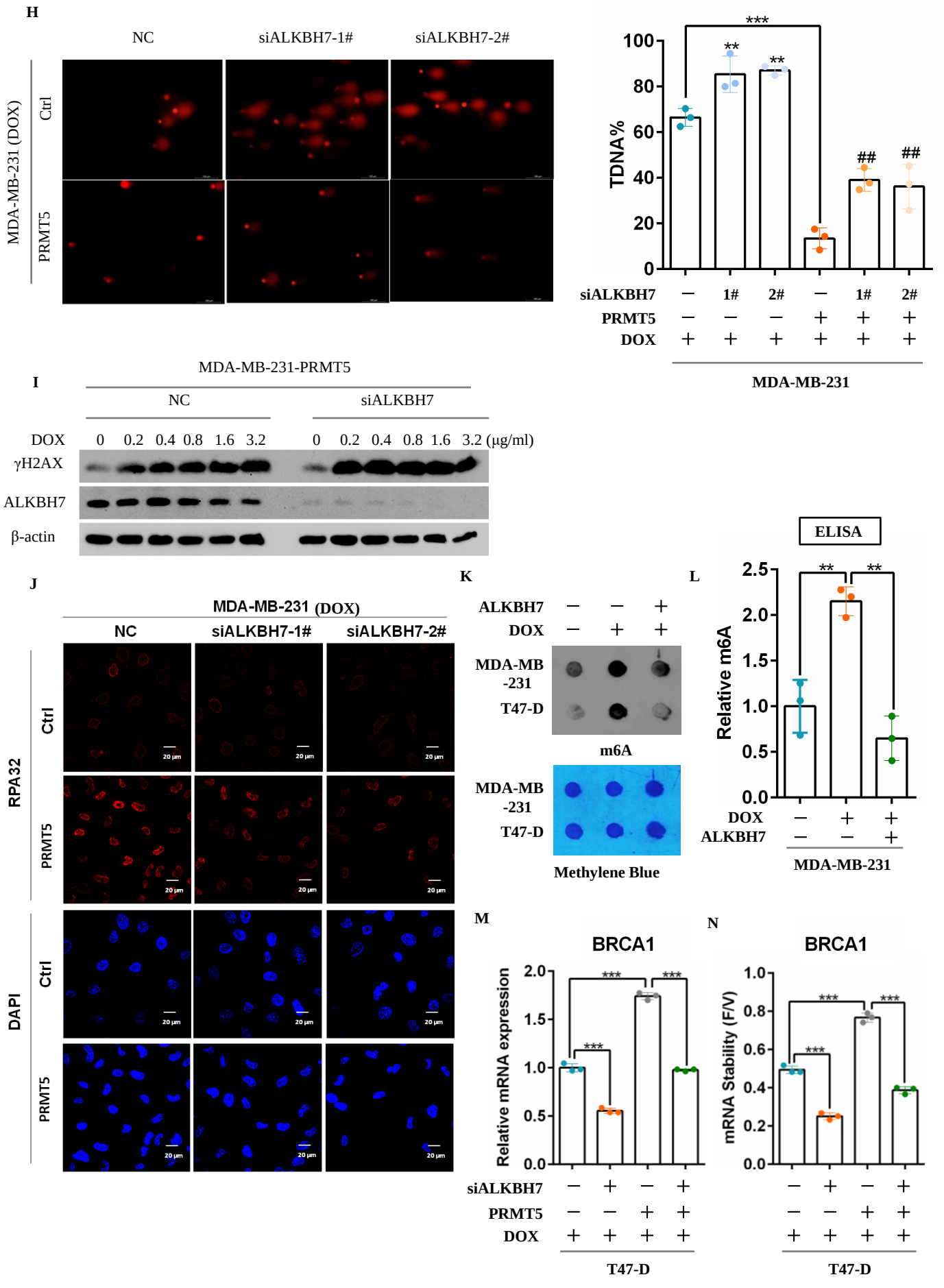
(E) BRCA1 mRNA expression in different groups determined by qPCR analysis.

(F) BRCA1 mRNA expression determined by qPCR analysis after transfection with BRCA1-specific siRNAs (siBRCA1). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

(G) MTT assay of MDA-MB-231 cells with PRMT5 overexpression and BRCA1-specific siRNA transfection treated with different doses of doxorubicin for 24 hours. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

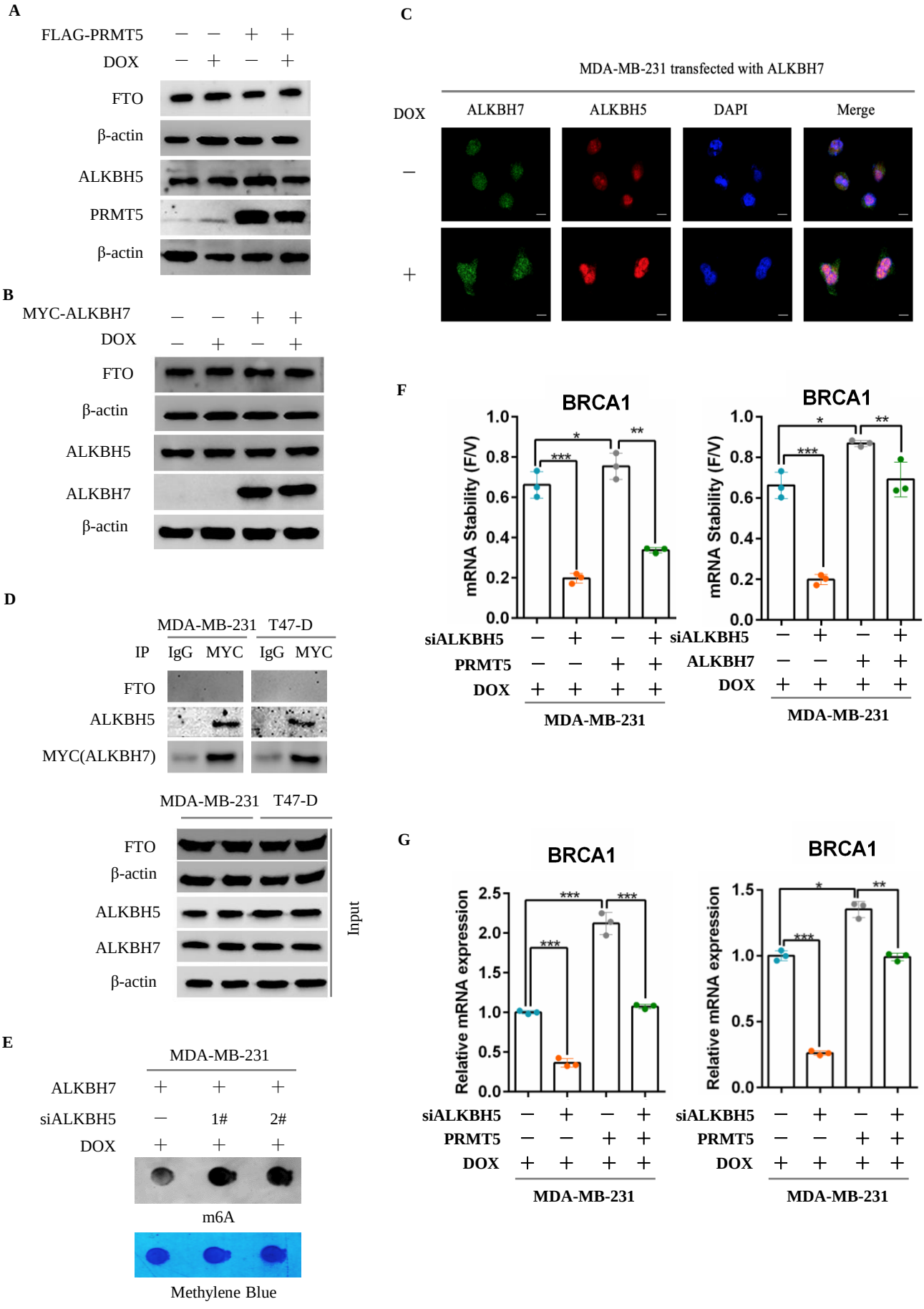
(H) Schematic analysis of the potential m6A methylation sites in the BRCA1 mRNA 3'UTR.


A
191
210
Human ALKBH7: RIPRGRRISVICRSLPEGMG
191
210
Mouse ALKBH7: RVPRGRRISVICRSLPEGMG
B
HA-ALKBH7 + + +
FLAG-PRMT5 − WT R368A
SDMA
HA
IP: HA
FLAG
β-actin
HA
β -actin
Input
C
ALKBH7
WT
3RA
CHX 0 0.5 0.75 1.5 3 6 0 0.5 0.75 1.5 3 6 (h)
ALKBH7
β-actin
D
MYC-ALKBH7 WT 3RK
MLN4924 − − − − − +
Leupeptin − − − + + −
MG132 − − + − + −
ALKBH7
β-actin
E
ITCH
FBXW7
TRIM2
NEDD4
RNF180
MIB1
MARCH4
MIB2
FANCL
ALKBH7
FBXO3
VHL
SH3RF1
BMI1
RNF216
SYVN1
SKP2
MYLIP
ASB2
HERC2
F
FBW7α − WT ΔF-box
ALKBH7
β-actin
FBW7α
β-actin
G
ALKBH7 + +
Fbw7α + +
CHX 0 2 4 6 8 10 0 2 4 6 8 10 (h)
ALKBH7
Fbw7α
β-actin
ALKBH7-3RK + +
Fbw7α + +
CHX 0 2 4 6 8 10 0 2 4 6 8 10 (h)
ALKBH7
Short exposure
Long exposure
Fbw7α
β-actin


H
NC
siALKBH7-1#
siALKBH7-2#
MDA-MB-231 (DOX)
Ctrl
PRMT5
TDNA%
siALKBH7
PRMT5
DOX
MDA-MB-231
I
MDA-MB-231-PRMT5
NC
siALKBH7
DOX
0 0.2 0.4 0.8 1.6 3.2 0 0.2 0.4 0.8 1.6 3.2 (μg/ml)
γH2AX
ALKBH7
β-actin
J
MDA-MB-231 (DOX)
NC
siALKBH7-1#
siALKBH7-2#
RPA32
DAPI
Ctrl
PRMT5
20 μm
K
ALKBH7
DOX
MDA-MB-231
T47-D
m6A
Methylene Blue
L
ELISA
Relative m6A
DOX
ALKBH7
MDA-MB-231
M
BRCA1
Relative mRNA expression
siALKBH7
PRMT5
DOX
T47-D
N
BRCA1
mRNA Stability (F/V)
T47-D

Supplementary Figure 3. PRMT5 Regulates DNA Repair and RNA m6A Methylation Dependent on the Arginine Methylation of ALKBH7.

(A) Potential arginine methylation sites of the human and mouse ALKBH7 proteins, with arginine-rich motifs indicated in red.
(B) HEK293 cells transfected with HA-ALKBH7, FLAG-PRMT5 and FLAG-PRMT5^{R368A} (catalytically dead PRMT5) as indicated. HEK293 cell lysates were immunoprecipitated with an anti-HA antibody, and Western blot analysis was performed to evaluate the arginine methylation of ALKBH7 by PRMT5 detected with an anti-symmetric dimethylarginine (SDMA) antibody.
(C) Protein stability analysis by Western blotting of MDA-MB-231 cells stably expressing wild-type ALKBH7 or ALKBH7^{3RA} (three arginine site mutations to alanine) treated with 100 μg/ml cycloheximide (CHX) for the indicated times.
(D) Western blot analysis of ALKBH7 expression in MDA-MB-231 cells stably expressing wild-type ALKBH7 or ALKBH7^{3RK} treated with MG132 (100 μM), leupeptin (20 μM) or MLN4924 (20 μM) for 6 hours as indicated.
(E) Bioinformatic analysis of the E3 ligase responsible for ALKBH7 degradation performed at http://ubibrowser.ncpsb.org/ubibrowser/.
(F) ALKBH7 expression detected by Western blotting of HEK293 cells transfected with FBW7α, FBW7α/ΔF-box and ALKBH7 for 48 hours as indicated.
(G) HEK293 cells transfected with FBW7α, ALKBH7 and ALKBH7^{3RK} as indicated and treated with 100 μg/ml CHX before harvest at the indicated times. ALKBH7 expression was detected by Western blotting.
(H) A comet assay was performed with MDA-MB-231 cells with or without PRMT5 overexpression and ALKBH7-specific siRNA knockdown treated with 0.4 μg/ml doxorubicin for 24 hours. Right panel, the quantification of the comet tail that represents broken DNA strands (TDNA%). $^{\#\#}p < 0.01$ vs. MDA-MB-231-PRMT5-siNC, $^{**}p < 0.01$ vs. MDA-MB-231-siNC, and $^{***}p < 0.001$ vs. MDA-MB-231-siNC.
(I) Western blotting was performed to analyze γH2AX expression in MDA-MB-231 cells with or without PRMT5 overexpression and ALKBH7-specific siRNA knockdown treated with doxorubicin as indicated for 24 hours.
(J) Immunofluorescence (IF) analysis of RPA foci in MDA-MB-231 cells was performed with cells treated as in **(H)**.
(K) A dot-blot assay of RNA m6A methylation in MDA-MB-231 and T47-D cells with ALKBH7 overexpression treated with doxorubicin for 24 hours was performed. The doxorubicin concentrations used to treat the MDA-MB-231 and T47-D cells were 0.4 μg/ml and 0.5 μg/ml, respectively.
(**L**) ELISA was performed to analyze the RNA m6A methylation levels in MDA-MB-231 cells overexpressing ALKBH7 treated with 0.4 μg/ml doxorubicin for 24 hours.
(**M**) RT-qPCR analysis of BRCA1 mRNA in T47-D cells overexpressing PRMT5 with ALKBH7-specific siRNA transfection treated with 0.5 μg/ml doxorubicin for 24 hours was performed.
(**N**) T47-D cells overexpressing PRMT5 with ALKBH7-specific siRNA transfection were treated with 0.5 μg/ml doxorubicin for 24 hours and then treated with vehicle or 3.2 μM flavopiridol for 6 hours. BRCA1 mRNA levels were measured by RT-qPCR, and the F/V ratio of BRCA1 mRNA was determined (mean ± SEM; n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.


A
FLAG-PRMT5 − − + +
DOX − + − +
FTO
β-actin
ALKBH5
PRMT5
β-actin
B
MYC-ALKBH7 − − + +
DOX − + − +
FTO
β-actin
ALKBH5
ALKBH7
β-actin
C
MDA-MB-231 transfected with ALKBH7
DOX
ALKBH7
ALKBH5
DAPI
Merge
−
+
D
MDA-MB-231 T47-D
IP IgG MYC IgG MYC
FTO
ALKBH5
MYC(ALKBH7)
MDA-MB-231 T47-D
FTO
β-actin
ALKBH5
ALKBH7
β-actin
Input
E
MDA-MB-231
ALKBH7 + + +
siALKBH5 − 1# 2#
DOX + + +
m6A
Methylene Blue
F
BRCA1
mRNA Stability (F/V)
siALKBH5 − + − +
PRMT5 − − + +
DOX + + + +
MDA-MB-231
BRCA1
mRNA Stability (F/V)
siALKBH5 − + − +
ALKBH7 − − + +
DOX + + + +
MDA-MB-231
G
BRCA1
Relative mRNA expression
siALKBH5 − + − +
PRMT5 − − + +
DOX + + + +
MDA-MB-231
BRCA1
Relative mRNA expression
siALKBH5 − + − +
PRMT5 − − + +
DOX + + + +
MDA-MB-231

Supplementary Figure 4. PRMT5 and ALKBH7 Regulate RNA m6A Methylation Mediated by ALKBH5.

(A) Western blot analysis of ALKBH5 and FTO in PRMT5-overexpressing MDA-MB-231 cells treated with or without 0.4 μg/ml doxorubicin for 24 hours.

(B) Western blot analysis of ALKBH5 and FTO in ALKBH7-overexpressing MDA-MB-231 cells treated with or without 0.4 μg/ml doxorubicin for 24 hours.

(C) Immunofluorescence assay of ALKBH5 and ALKBH7 in MDA-MB-231 cells overexpressing ALKBH7 treated with or without 0.4 μg/ml doxorubicin for 24 hours as indicated. The bars in each images represent for 10 μm.

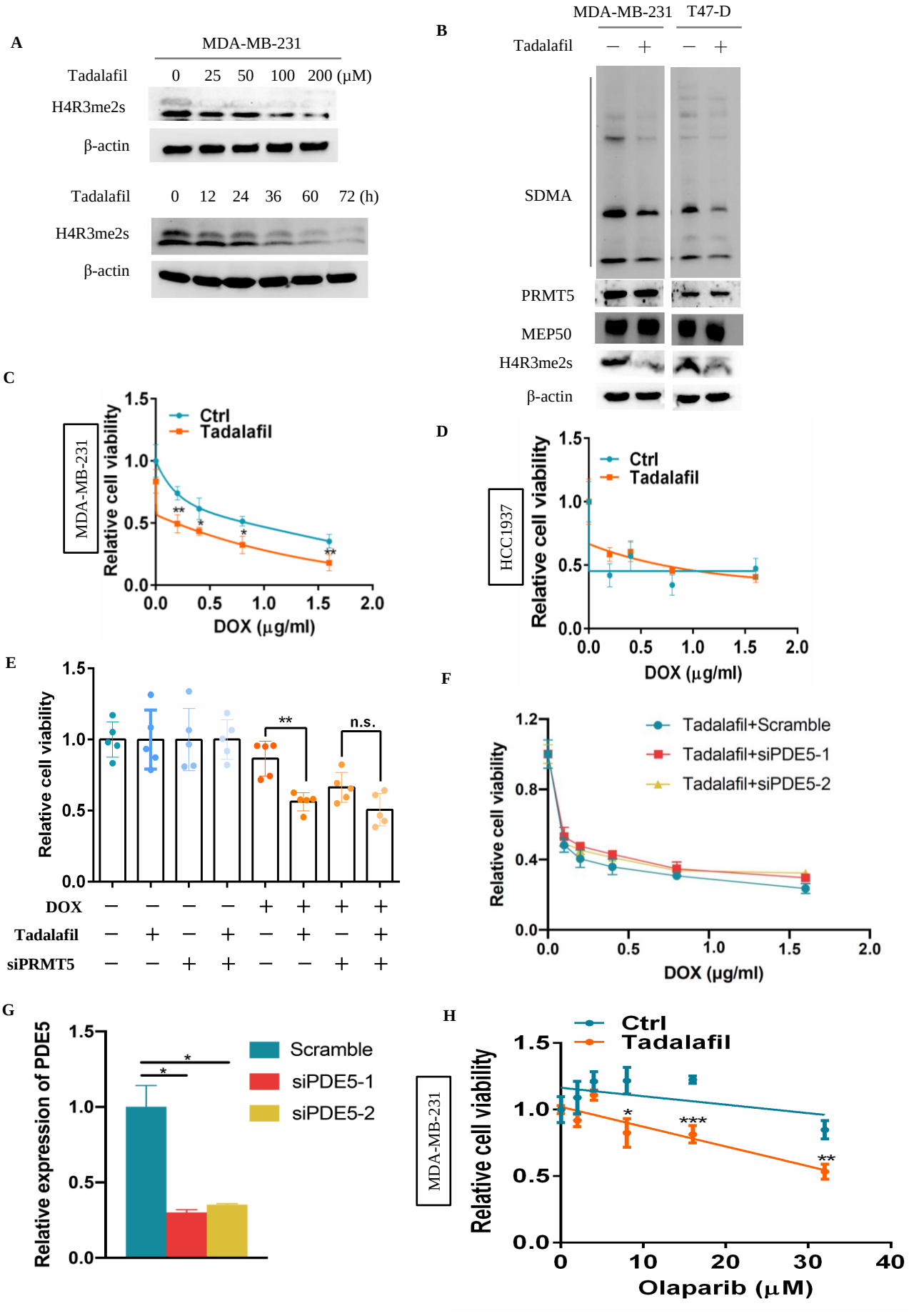
(D) Immunoprecipitation assay for MYC-ALKBH7 and ALKBH5 in MDA-MB-231 and T47-D cells. Cell lysates were immunoprecipitated with an anti-MYC tag antibody (MYC-ALKBH7), and endogenous ALKBH5 was detected with an anti-ALKBH5 antibody.

(E) Dot-blot assay with MDA-MB-231 cells overexpressing ALKBH7 with ALKBH5-specific siRNA transfection treated with 0.4 μg/ml doxorubicin for 24 hours and then subjected to poly(a)+ RNA extraction. An anti-m6A antibody was used for the dot-blot assay with 200 ng of mRNA, and methylene blue staining was used as the loading control.

(F) BRCA1 mRNA levels measured by RT-qPCR and the F/V ratio of BRCA1 mRNA. MDA-MB-231 cells overexpressing PRMT5 or ALKBH7 with ALKBH5-specific siRNA transfection were treated with 0.4 μg/ml doxorubicin for 24 hours and then treated with vehicle or 0.8 μM flavopiridol for 3 hours (mean ± SEM; n = 3; **P < 0.01 vs. Ctrl).

(G) RT-qPCR analysis of BRCA1 mRNA in MDA-MB-231 cells overexpressing PRMT5 or ALKBH7 with ALKBH5-specific siRNA transfection treated with 0.4 μg/ml doxorubicin for 24 hours. *p < 0.05, **p < 0.01, and ***p < 0.001.

Supplementary Fig. 5



Supplementary Figure 5. Tadalafil is a Novel PRMT5 Inhibitor that Increases the Doxorubicin Sensitivity of Breast Cancer Cells.

(A) (B) Western blotting of two downstream targets of PRMT5, H4R3me2s and symmetric dimethylarginine (SDMA), in MDA-MB-231 and T47-D breast cancer cells treated with 100 μM tadalafil for 24 hours as indicated.

(C) Proliferation analysis of MDA-MB-231 cells treated with different doses of doxorubicin with or without 100 μM tadalafil for 24 hours as indicated.

(D) Proliferation analysis of HCC1937 cells treated with different doses of doxorubicin and 0, 25, 50, or 100 μM tadalafil for 24 hours as indicated. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

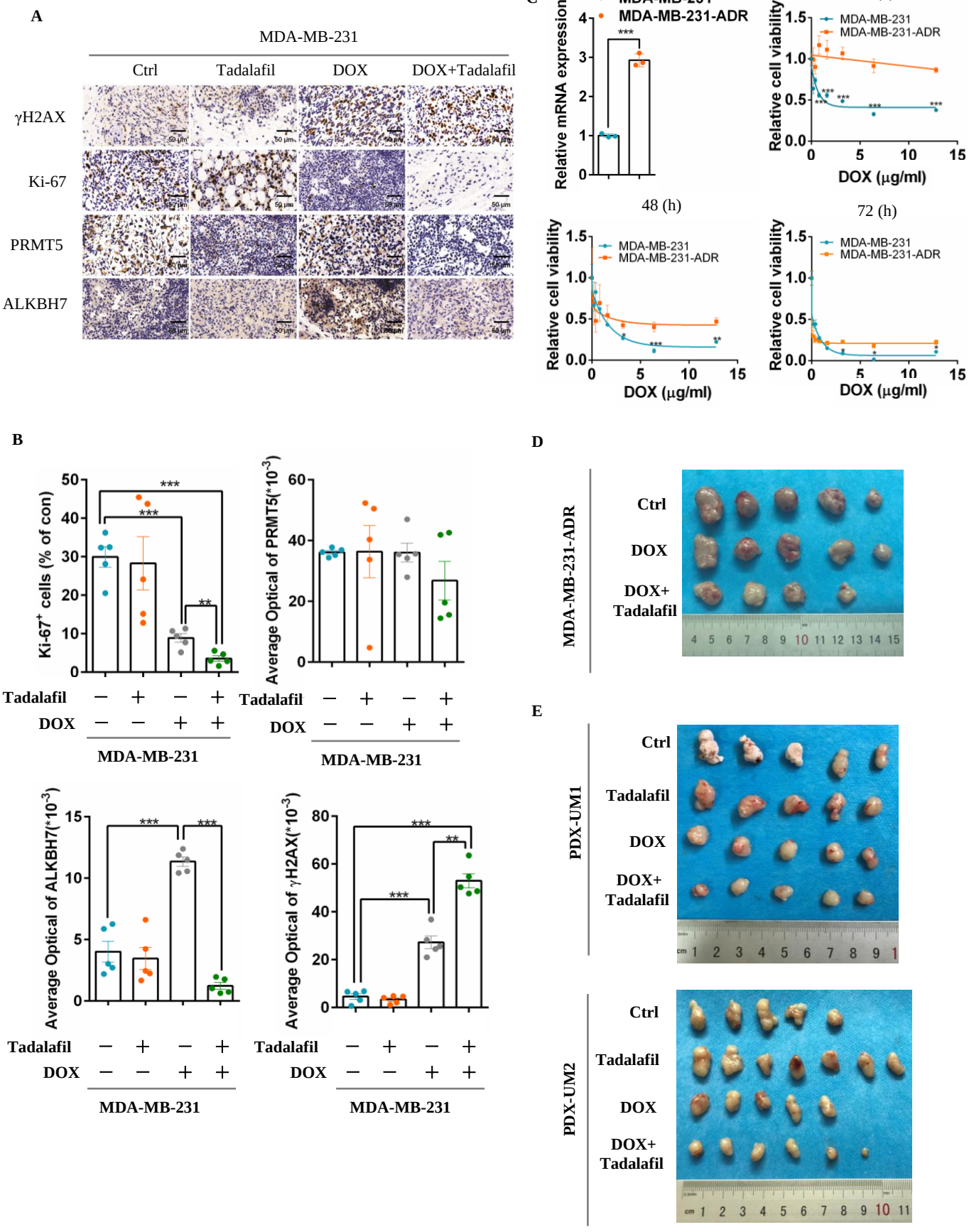
(E) Proliferation analysis (MTT) of MDA-MB-231 cells transfected with scramble or PRMT5-specific siRNA and then treated with 100 μM tadalafil and 0.4 μg/ml doxorubicin for 24 hours as indicated.

(F) Proliferation analysis (MTT) of MDA-MB-231 cells transfected with scramble or PDE5-specific siRNA and then treated with 100 μM tadalafil and different concentration of doxorubicin for 24 hours as indicated.

(G) Realtime RT-PCR analysis of PDE5 after siRNA transfection for 24 hours.

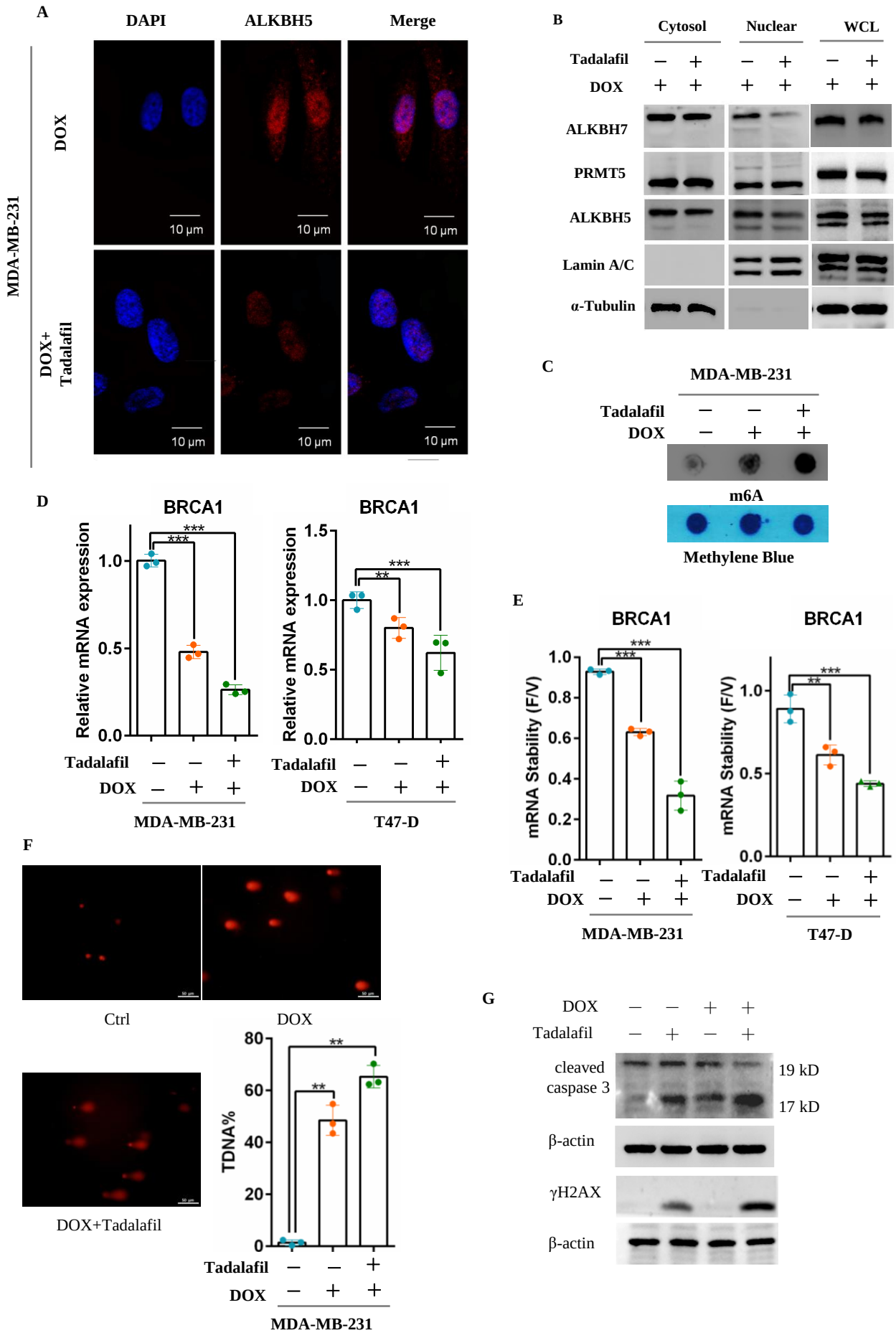
(H) Proliferation analysis of MDA-MB-231 cells treated with different doses of olaparib with or without 100 μM tadalafil for 24 hours as indicated.

Supplementary Fig. 6



Supplementary Figure 6. Tadalafil Increases Doxorubicin Sensitivity in Breast Cancer Cells and a PDX Mouse Model.

(A) Immunohistochemical analysis (top panel) and quantification (bottom panel) of γH2AX, Ki-67, PRMT5, and ALKBH7 in athymic nude mice with orthotopically implanted MDA-MB-231 cells treated with tadalafil and doxorubicin as indicated.
(B) Quantification (bottom panel) of the γH2AX, Ki-67, PRMT5, and ALKBH7 staining shown in **(A)**.
(C) Proliferation analysis of MDA-MB-231 cells with doxorubicin resistance (MDA-MB-231-ADR) treated with 100 μM tadalafil and different doses of doxorubicin for 24, 48, or 72 hours. qPCR analysis of PRMT5 expression is shown in the top left corner.
(D) Tumor tissue display of MDA-MB-231-ADR breast cancer cells treated with tadalafil or doxorubicin as indicated (n=4-5/group).
(E) Tumor tissue display of patient-derived xenograft (PDX) models of breast cancer treated with tadalafil and doxorubicin as indicated (n=5-7/group). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.


A
DAPI
ALKBH5
Merge
MDA-MB-231
DOX
DOX+ Tadalafil
10 μm
B
Cytosol
Nuclear
WCL
Tadalafil
DOX
ALKBH7
PRMT5
ALKBH5
Lamin A/C
α-Tubulin
C
MDA-MB-231
Tadalafil
DOX
m6A
Methylene Blue
D
BRCA1
Relative mRNA expression
MDA-MB-231
T47-D
E
BRCA1
mRNA Stability (F/V)
MDA-MB-231
T47-D
F
Ctrl
DOX
DOX+Tadalafil
TDNA%
MDA-MB-231
G
DOX
Tadalafil
cleaved caspase 3
19 kD
17 kD
β-actin
γH2AX
β-actin

Supplementary Figure 7. Tadalafil Increases RNA m6A Methylation by Reducing the Nuclear Translocation of ALKBH5.

(A) Immunofluorescence assay for ALKBH5 in MDA-MB-231 cells treated with tadalafil (100 μM) and 0.4 μg/ml doxorubicin for 24 hours as indicated.

(B) Nuclear, cytosolic and whole cell lysate (WCL) expression of ALKBH5 and ALKBH7 in MDA-MB-231 cells treated with 0.4 μg/ml doxorubicin with or without combination with tadalafil as indicated.

(C) Dot-blot assay of RNA m6A methylation in MDA-MB-231 cells treated with 0.4 μg/ml doxorubicin with or without tadalafil (100 μM) for 24 hours. An anti-m6A antibody was used for the dot-blot assay with 200 ng of mRNA, and methylene blue staining was used as the loading control.

(D) qPCR analysis of BRCA1 mRNA in MDA-MB-231 (left panel) and T47-D (right panel) cells treated with 0.4 μg/ml and 0.5 μg/ml doxorubicin, respectively, with or without 100 μM tadalafil for 24 hours.

(E) BRCA1 mRNA levels measured by RT-qPCR and the F/V ratio of BRCA1 mRNA. MDA-MB-231 (left panel) and T47-D (right panel) cells were treated with doxorubicin and tadalafil as in **(D)** and treated with vehicle or 0.8 μM flavopiridol for 3 hours (MDA-MB-231 cells) or vehicle or 3.2 μM flavopiridol for 6 hours (T47-D cells) (mean ± SEM; n = 3).

(F) Comet assay with MDA-MB-231 cells treated with doxorubicin and tadalafil as in **(D)**. The right bottom column shows the quantification of the comet tail that represents broken DNA strands (TDNA%). **$p < 0.01$ and ***$p < 0.001$.

(G) Western blot analysis of γH2AX and cleaved Caspase-3 expression in MDA-MB-231 cells treated with 100 μM Tadalafil and 0.4 μg/ml doxorubicin as indicated for 24 hours.

Table S1 Summarization of tumor-stage and degrade information of breast cancer patients

Neoadjuvant therapy		Yes	No
Stage	IIA	6	4
	IIB	4	2
	IIIA	2	6
	IIIC	3	2
	IV	0	1
	Not available	0	3
TNM grade	T1	0	1
	T2	15	13
	T3	0	1
	Not available	0	3

Table S2 Summarization of clinic-pathologic information of breast cancer patients

Gender	**Female**	**n=174**
Type	Male	n=0
	Invasive carcinoma	n=110
	Ductal carcinoma in situ	n=4
	Invasive ductal carcinoma	n=5
	Ductal carcinoma in situ with microinvasion	n=4
	Invasive lobular carcinoma	n=2
Curative effect	Complete response	n=2
	Partial response	n=124
	Stable disease	n=40
	Progressive disease	n=8