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Supplementary Materials for

SMYD2 inhibition–mediated hypomethylation of Ku70 contributes to impaired nonhomologous end joining repair and antitumor immunity

Ming Tang *et al.*

Corresponding author: Wei-Guo Zhu, zhuweiguo@szu.edu.cn; Wen Lu, luwen198102@126.com; Yongzhong Liu, liuyongzhong0801@126.com; Ming Tang, tangming@bjmu.edu.cn

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Figs S1 to S6

Supplementary Figures

Fig. S1

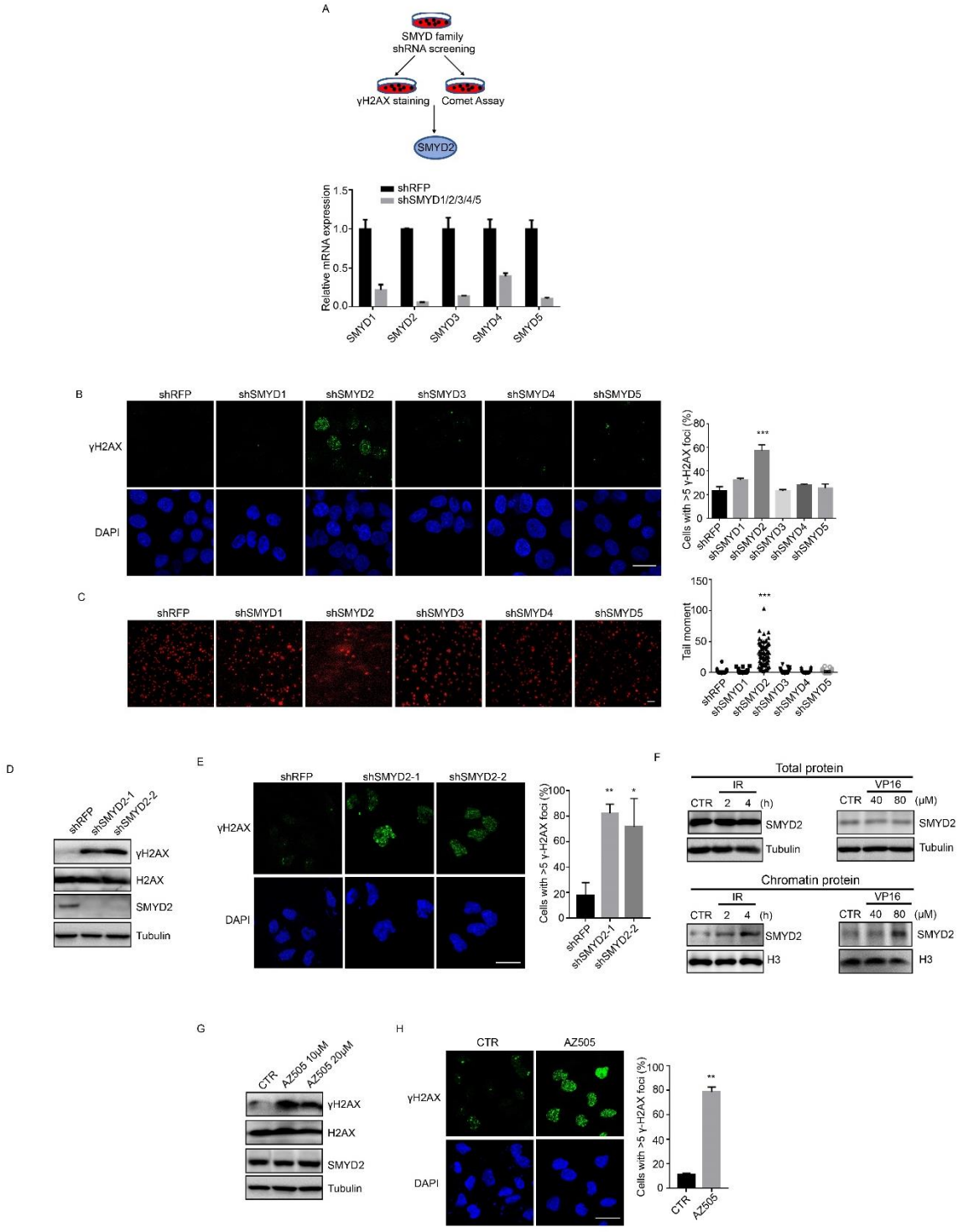

Fig. S1. Screening of SMYD family identifies the role of SMYD2 in DNA damage repair

A. Schedule of the screen of SMYD family members in DNA damage repair. Lower graph: relative mRNA expression of SMYD1-5 knockdown efficiency. **B**. Representative fluorescence images and quantification of γ-H2AX immunostaining in cells with shRNAs targeting SMYD1-5. Scale bars, 25 μm; green, γ-H2AX; blue, DAPI. **C**. Representative fluorescence images and quantification of tail moments in cells with shRNAs targeting SMYD1-5 as determined by a comet assay, scale bars, 100 μm. **D**. Immunoblot of the expression levels of γ-H2AX in SMYD2 depleted CT26 cells. **E**. Representative fluorescence images and quantification of γ-H2AX immunostaining in cells with and without SMYD2, scale bars, 25 μm; green, γ-H2AX; blue, DAPI. **F**. Immunoblot of endogenous SMYD2 in total proteins treated with IR at 10 Gy and released for 2 or 4 hr, etoposide (VP16) at 40 or 80 μM for 4 hr of CT26 cells (upper panels). Immunoblot of endogenous SMYD2 in chromatin proteins treated with IR at 10 Gy and released for 2 or 4 hr, etoposide (VP16) at 40 or 80 μM for 4 hr of CT26 cells (lower panels). **G**. Immunoblot of the expression levels of γ-H2AX in AZ505 treated CT26 cells. **H**. Representative fluorescence images and quantification of γ-H2AX immunostaining in cells with and without AZ505, scale bars, 25 μm; green, γ-H2AX; blue, DAPI.

Fig. S2

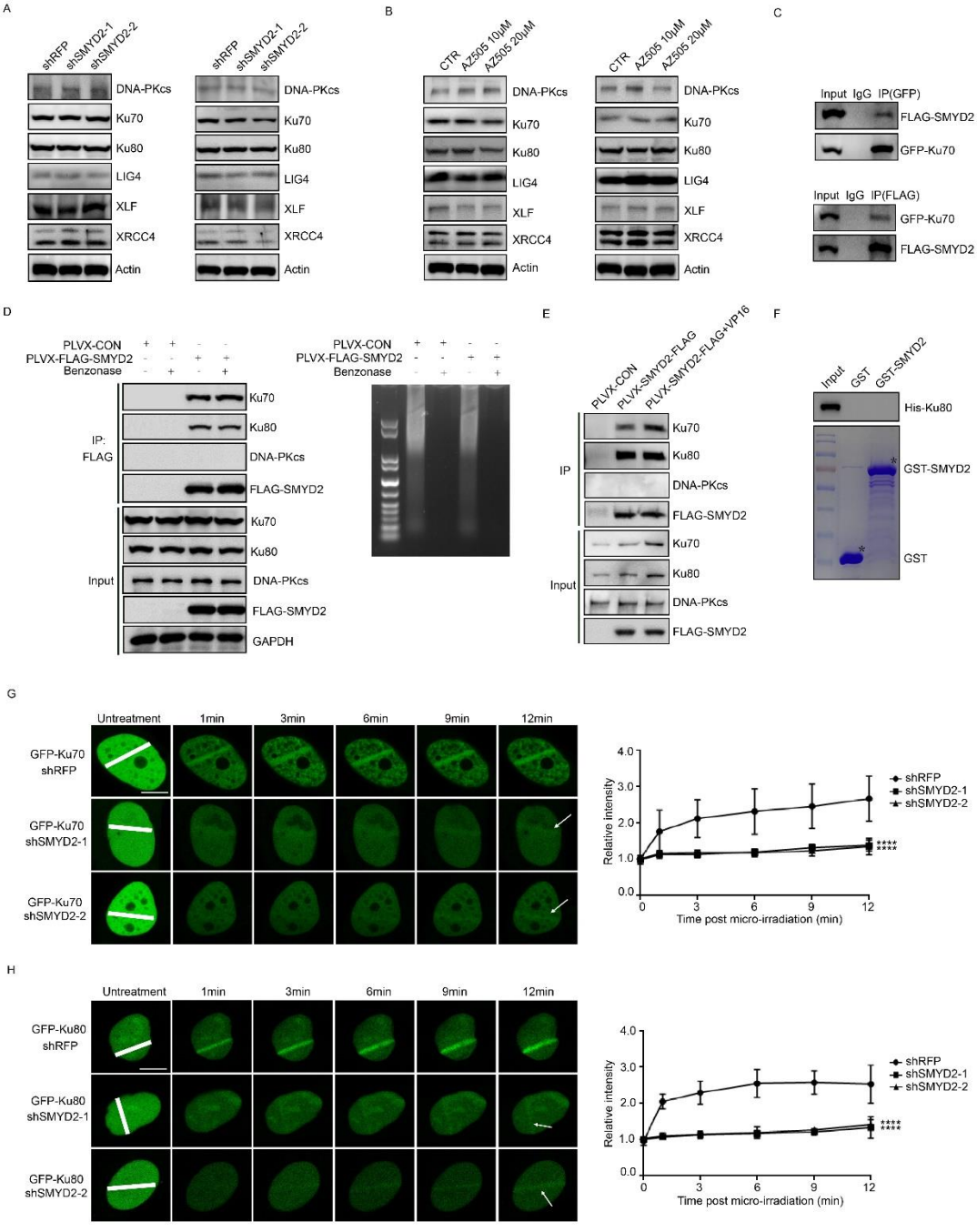
A
shRFP
shSMYD2-1
shSMYD2-2
DNA-PKcs
Ku70
Ku80
LIG4
XLF
XRCC4
Actin
B
CTR
AZ505 10μM
AZ505 20μM
C
Input IgG IP(GFP)
FLAG-SMYD2
GFP-Ku70
Input IgG IP(FLAG)
D
PLVX-CON
PLVX-FLAG-SMYD2
Benzonase
IP: FLAG
Input
GAPDH
E
PLVX-SMYD2-FLAG
PLVX-SMYD2-FLAG+VP16
IP
Input
F
Input
GST
GST-SMYD2
His-Ku80
GST
G
Untreatment
1min
3min
6min
9min
12min
GFP-Ku70 shRFP
GFP-Ku70 shSMYD2-1
GFP-Ku70 shSMYD2-2
Relative intensity
Time post micro-irradiation (min)
H
GFP-Ku80 shRFP
GFP-Ku80 shSMYD2-1
GFP-Ku80 shSMYD2-2

Fig. S2. SMYD2 interacts with Ku70 without affecting the levels of NHEJ-related proteins

A. Immunoblot of the expression levels of NHEJ-related proteins in SMYD2 depleted HCT116 or CT26 cells. **B**. Immunoblot of the expression levels of NHEJ-related proteins in AZ505 treated HCT116 or CT26 cells. **C**. Immunoblot of FLAG-SMYD2 or GFP-Ku70 protein that was immunoprecipitated with anti-GFP or anti-FLAG antibodies for exogenous interaction. **D**. Immunoblot of interactions between SMYD2 and Ku70 or Ku80 that was immunoprecipitated with anti-FLAG antibodies, lysates treated with Benzonase (Benzo) for detecting the DNA independency of the interaction. **E**. Immunoblot of indicated proteins in the chromatin lysates or anti-FLAG immunoprecipitates of HEK293FT cells transfected with FLAG-SMYD2 after treatment with VP16 at 40 μM for 2 hr. **F**. *In vitro* GST pulldown assay of his-Ku80 combined with GST or GST-SMYD2. **G**. Left graph: Accumulation of exogenous GFP-Ku70 in BrdU-sensitized U2OS cells stably expressing shSMYD2 or shRFP with GFP-Ku70 from 1 min to 12 min. Right graph: relative intensity of GFP-Ku70 at micro-irradiated sites in the experiments described in the left graph. Scale bars, 10 μm. **H**. Left graph: Accumulation of exogenous GFP-Ku80 in BrdU-sensitized U2OS cells stably expressing shSMYD2 or shRFP with GFP-Ku80 from 1 min to 12 min. Right graph: relative intensity of GFP-Ku80 at micro-irradiated sites in the experiments described in the left graph. Scale bars, 10 μm.

Fig. S3

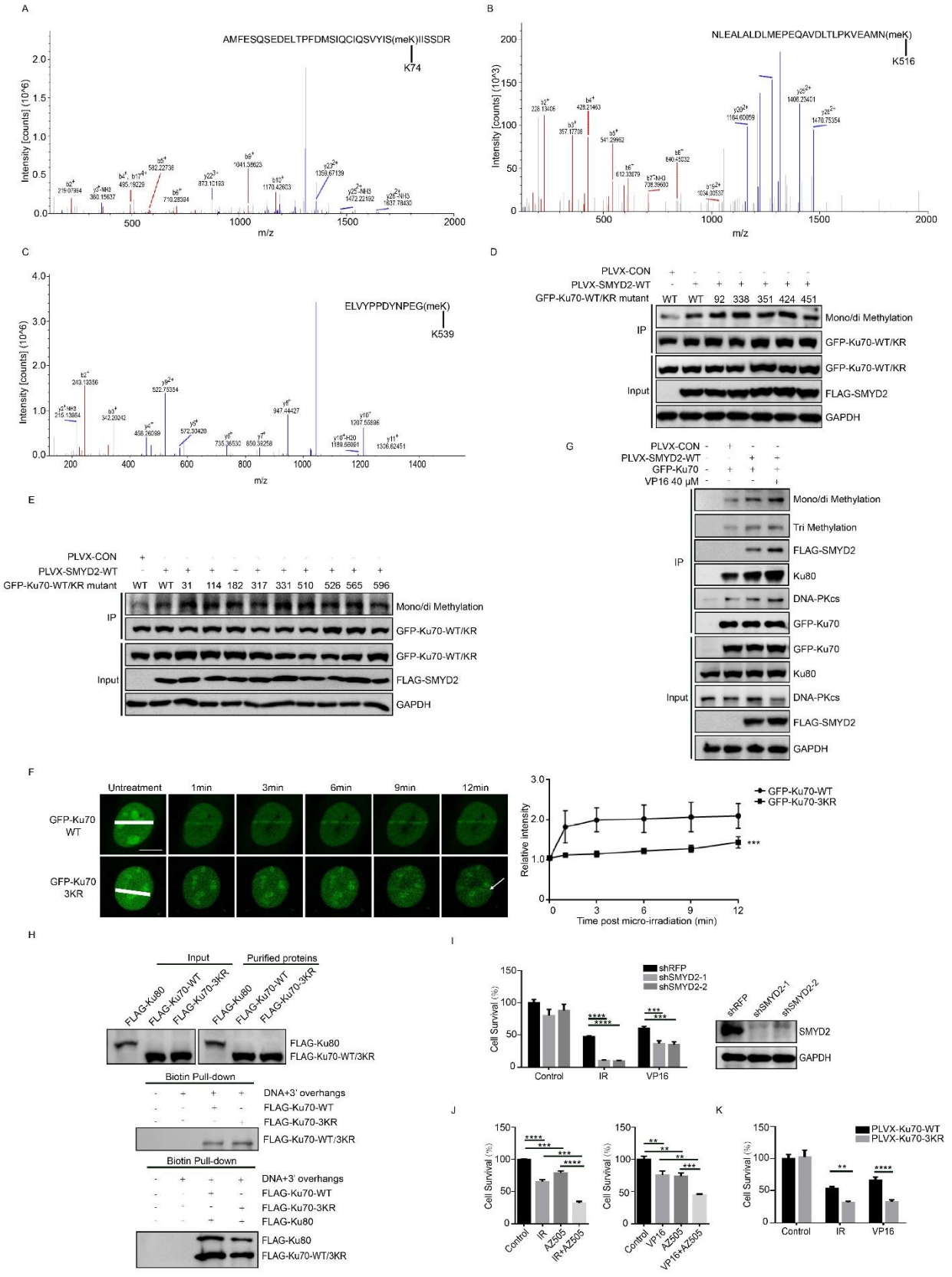
A
AMFESQSEDELTPFDMSIQCIQSVYIS(meK)IISSDR
K74
Intensity [counts] (10^6)
m/z
B
NLEALALDLMEPEQAVDLTLPKVEAMN(meK)
K516
Intensity [counts] (10^3)
m/z
C
ELVYPPDYNPEG(meK)
K539
Intensity [counts] (10^6)
m/z
D
PLVX-CON
PLVX-SMYD2-WT
GFP-Ku70-WT/KR mutant
WT WT 92 338 351 424 451
IP
Input
Mono/di Methylation
GFP-Ku70-WT/KR
GFP-Ku70-WT/KR
FLAG-SMYD2
GAPDH
E
PLVX-CON
PLVX-SMYD2-WT
GFP-Ku70-WT/KR mutant
WT WT 31 114 182 317 331 510 526 565 596
IP
Input
Mono/di Methylation
GFP-Ku70-WT/KR
GFP-Ku70-WT/KR
FLAG-SMYD2
GAPDH
G
PLVX-CON
PLVX-SMYD2-WT
GFP-Ku70
VP16 40 μM
IP
Input
Mono/di Methylation
Tri Methylation
FLAG-SMYD2
Ku80
DNA-PKcs
GFP-Ku70
GFP-Ku70
Ku80
DNA-PKcs
FLAG-SMYD2
GAPDH
F
Untreatment
1min
3min
6min
9min
12min
GFP-Ku70 WT
GFP-Ku70 3KR
Relative intensity
Time post micro-irradiation (min)
GFP-Ku70-WT
GFP-Ku70-3KR
***
H
Input
Purified proteins
FLAG-Ku80
FLAG-Ku70-WT
FLAG-Ku70-3KR
FLAG-Ku80
FLAG-Ku70-WT/3KR
Biotin Pull-down
DNA+3' overhangs
FLAG-Ku70-WT
FLAG-Ku70-3KR
FLAG-Ku70-WT/3KR
Biotin Pull-down
DNA+3' overhangs
FLAG-Ku70-WT
FLAG-Ku70-3KR
FLAG-Ku80
FLAG-Ku80
FLAG-Ku70-WT/3KR
I
shRFP
shSMYD2-1
shSMYD2-2
Cell Survival (%)
Control
IR
VP16
SMYD2
GAPDH
J
Cell Survival (%)
Control
IR
AZ505
IR+AZ505
Cell Survival (%)
Control
VP16
AZ505
VP16+AZ505
K
PLVX-Ku70-WT
PLVX-Ku70-3KR
Cell Survival (%)
Control
IR
VP16

Fig. S3. Mass spectrometry and confirmation of SMYD2 mediated Ku70 methylation sites

A-C. Mass spectrometry spectrum of digested Ku70 fragments (methylated K74, K516, K539). **D**. Immunoblot analysis of Mono/di-methylation of Ku70-WT/Mutants in anti-GFP immunoprecipitates HEK293FT cells co-transfected with GFP-Ku70-WT or K92R/K338R/K351R/K424R/K451R mutants and FLAG-SMYD2-WT. **E**. Immunoblot analysis of Mono/di-methylation of Ku70-WT/Mutants in anti-GFP immunoprecipitates HEK293FT cells co-transfected with GFP-Ku70 WT or K31R/K114R/K182R/K317R/K331R/K510R/K526R/K565R/K596R mutants and FLAG-SMYD2-WT. **F.** Left graph: Accumulation of exogenous GFP-Ku70-WT/3KR mutant in BrdU-sensitized U2OS cells from 1 min to 12 min. Right graph: relative intensity of GFP-Ku70-WT/3KR at micro-irradiated sites in the experiments described in the left graph. Scale bars, 10 μm. **G**. Immunoblot analysis of Mono/di methylation, Tri-methylation of Ku70 and the interaction between Ku70 and Ku80 or DNA-PKcs in anti-GFP immunoprecipitates. Plasmids encoding GFP-Ku70 and FLAG-SMYD2 were co-transfected in HEK293FT for 72 hr, and treated with VP16 at 40 μM for 4 hr. **H.** DNA pull-down assay of biotinylated DNA with 3'-overhangs in the presence of purified FLAG-Ku70-WT/3KR alone or in combination with FLAG-Ku80 were performed as indicated. **I.** HCT116 shRFP or shSMYD2-1 or shSMYD2-2 stable cells were subjected to colony formation assay. Cells were treated with IR (1 Gy) or etoposide (2 μM) for 2 hr and washed with fresh medium and allowed to grow for 2 weeks. **J**. HCT116 cells were subjected to colony formation assay. Cells were treated with IR (1 Gy) or etoposide (2 μM) for 2 hr combined with or without AZ505 (20 μM) for three days. After the treatment, cells were washed with fresh medium and allowed to grow for 2 weeks. **K**. HCT116 Ku70-WT or Ku70-3KR stable cells were subjected to colony formation assay. Cells were treated with IR (1 Gy) or etoposide (2 μM) for 2 hr and washed with fresh medium and allowed to grow for 2 weeks.

Fig. S4

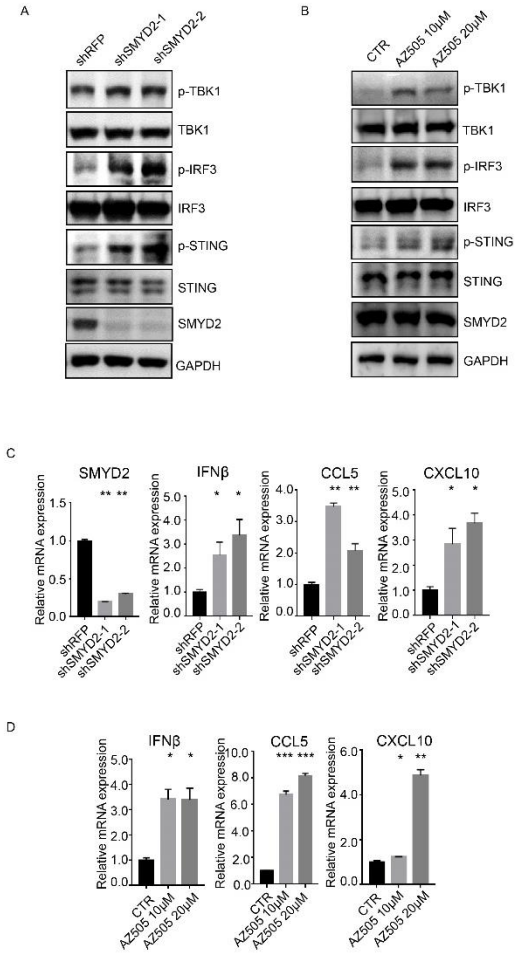

Fig. S4. Inhibition of SMYD2 activates cGAS-STING pathway

A. Immunoblot of markers in the STING pathway including total and phospho (p) TBK1 (S172), total and phospho (p) IRF3 (S396), total and phospho (p) STING (S365), in lysates collected from cells with and without SMYD2 in CT26 cells. **B**. Immunoblot of markers in the STING pathway including total and phospho (p) TBK1 (S172), total and phospho (p) IRF3 (S396), total and phospho (p) STING (S365), in lysates collected from CT26 cells with and without SMYD2 inhibitor AZ505 at 10 μM, 20 μM for 7 days. **C**. The expression of SMYD2, IFNβ, CCL5 and CXCL10 were measured via qPCR in cells with and without SMYD2 in CT26 cells. **D**. The expression of IFNβ, CCL5 and CXCL10 were measured via qPCR in cells with and without SMYD2 inhibitor AZ505 at 10 μM, 20 μM in CT26 cells.

Fig. S5

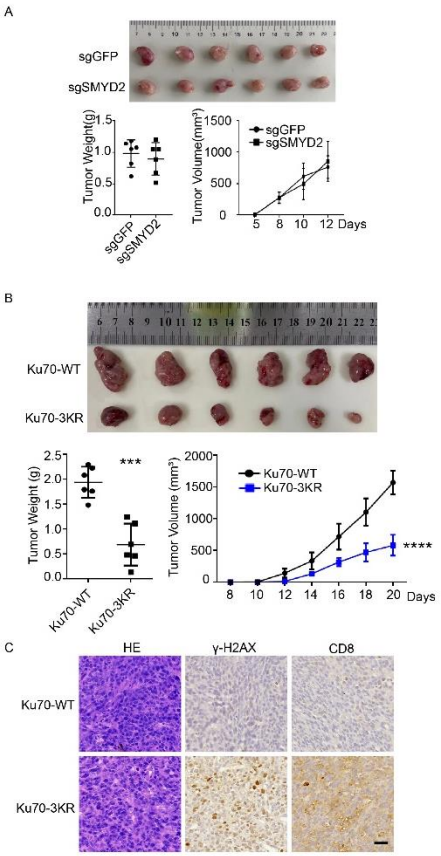

Fig. S5. SMYD2 affects tumor growth through mediating Ku70 methylation and NHEJ repair

A. Tumor weight and tumor volume of NRAS-Myc tumors with or without SMYD2 in nude mice. **B**. Tumor weight and tumor volume of CT26 tumors with Ku70-WT or Ku70-3KR stable cells in BALB/c mice. **C**. Representative images of H&E and immunohistochemistry (IHC) staining of γ-H2AX and CD8 in tissues from CT26 tumors with Ku70-WT or Ku70-3KR cells. Scale bars, 30 μm.

Fig. S6

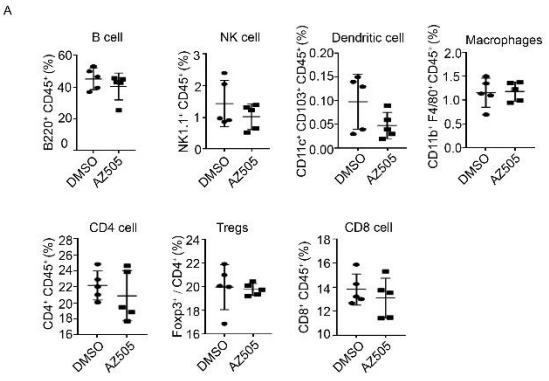

Fig. S6. SMYD2 inhibitor AZ505 does not affect the immune cells in spleen

A. Flow cytometry analysis of immune cells of the spleen tissue in BALB/c mice bearing CT26 tumors treated with or without AZ505.