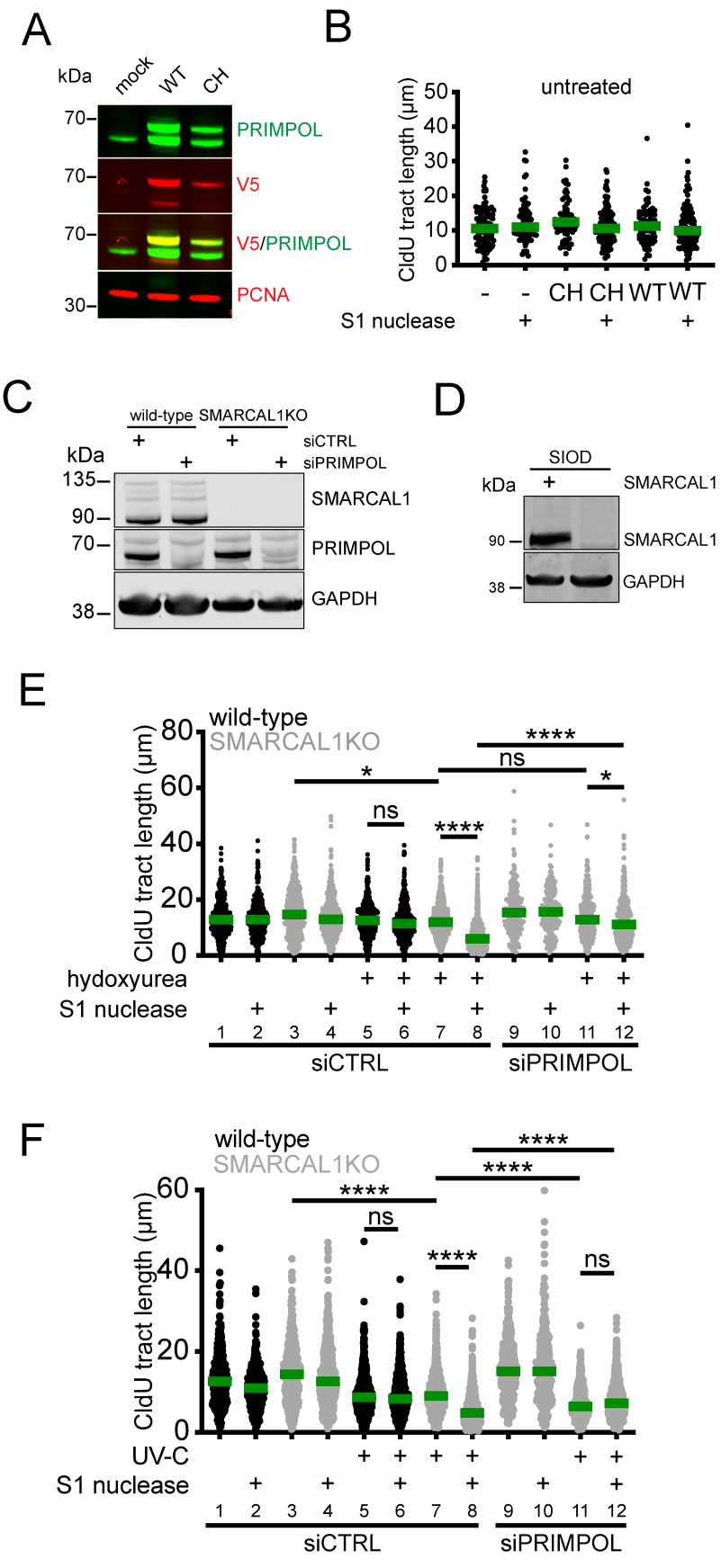
A
kDa
mock
WT
CH
70
PRIMPOL
70
V5
70
V5/PRIMPOL
30
PCNA
B
untreated
CldU tract length (µm)
50
40
30
20
10
0
- - CH CH WT WT
S1 nuclease + + +
C
wild-type SMARCAL1KO
siCTRL
siPRIMPOL
kDa
135
90
70
38
SMARCAL1
PRIMPOL
GAPDH
D
SIOD
kDa
SMARCAL1
90
SMARCAL1
38
GAPDH
E
wild-type
SMARCAL1KO
CldU tract length (µm)
80
60
40
20
0
****
ns
*
ns
****
*
hydoxyurea
S1 nuclease
1 2 3 4 5 6 7 8 9 10 11 12
siCTRL
siPRIMPOL
F
wild-type
SMARCAL1KO
CldU tract length (µm)
60
40
20
0
****
****
****
ns
****
ns
UV-C
S1 nuclease
1 2 3 4 5 6 7 8 9 10 11 12
siCTRL
siPRIMPOL

**Figure S1. Suppression of fork reversal leads to PRIMPOL-dependent ssDNA gaps in response to hydroxyurea and UV-C (related to Figure 1).** (A) PRIMPOL expression in U2OS cells after transfection with wild-type (WT) or primase-dead (CH) V5-tagged PRIMPOL constructs. A representative Western blot from three independent experiments is shown. (B) Untreated samples from DNA fiber data shown in Figure 1A. (C) Expression of PRIMPOL and SMARCAL1 after siRNA (siCTRL or siPRIMPOL) knockdown in wild-type and SMARCAL1KO U2OS cells. (D) Expression of SMARCAL1 in SIOD cells or SIOD cells complemented with SMARCAL1. (E) Dot plot and median of CldU tract lengths in wild-type or SMARCAL1KO U2OS cells ± 50 μM hydroxyurea ± siCTRL or siPRIMPOL ± S1 nuclease (*n*=3). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, *p < 0.05, ****p < 0.0001. (F) Dot plot and median of CldU tract lengths in wild-type or SMARCAL1KO U2OS cells ± 15 J/m$^2$ UV-C ± siCTRL or siPRIMPOL ± S1 nuclease (*n*=3). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, ****p < 0.0001.

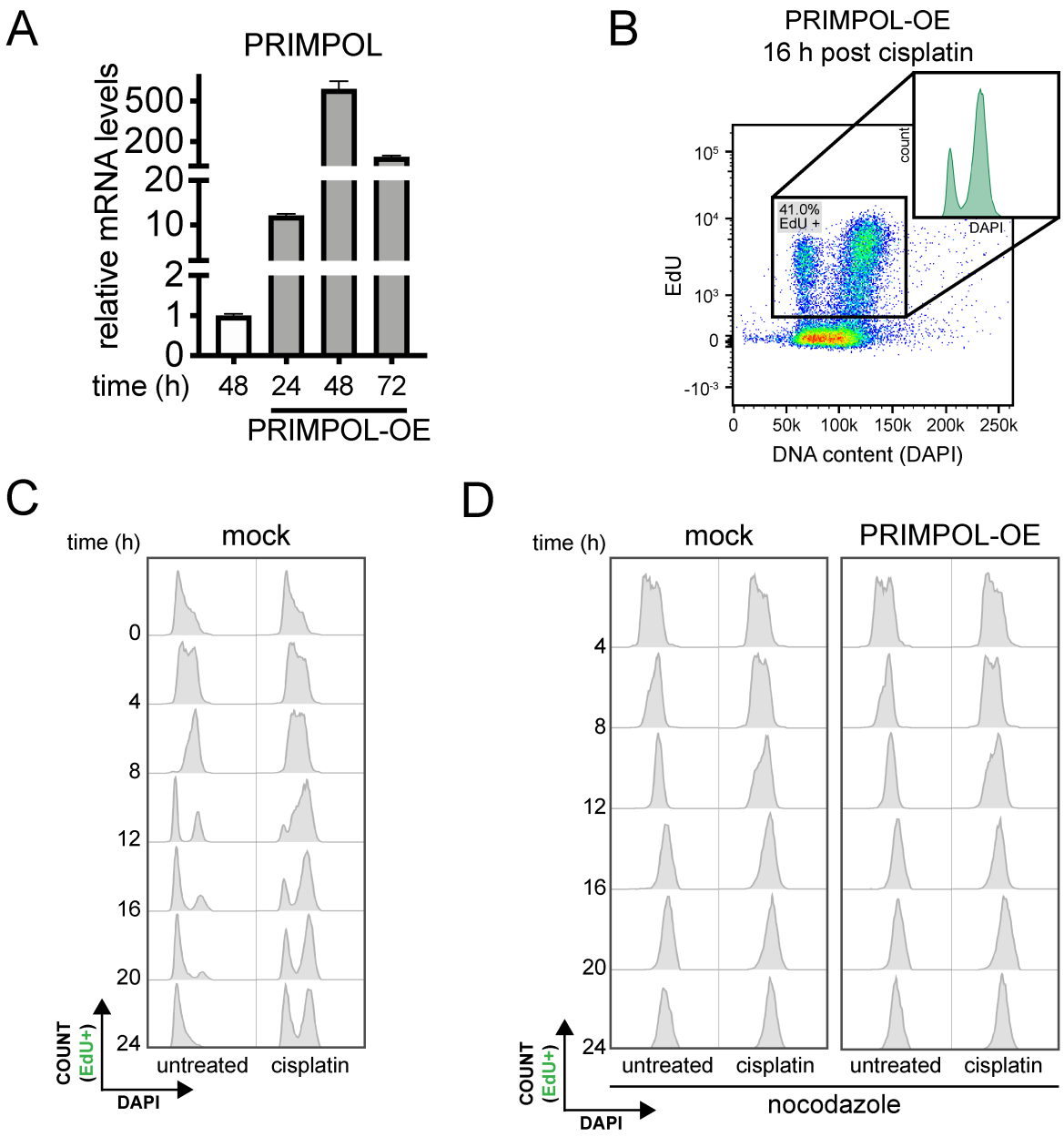


**Figure S2. Impact of cisplatin treatment on cell cycle progression (related to Figure 2).** (A) mRNA expression of PRIMPOL after transfection with PRIMPOL in mock-transfected or PRIMPOL-OE U2OS cells at indicated times. (B) PRIMPOL-OE U2OS cells were pulse labeled with 10 μM EdU + 150 μM cisplatin, then fixed 16 hours post-cisplatin. Cells were first gated for EdU positivity, then cell cycle of EdU-positive cells was assessed via DAPI content (top right). (C) Cell cycle histograms (stained with DAPI to assess DNA content) of EdU-positive mock-transfected U2OS cells + 10 μM EdU ± 150 μM cisplatin, collected at indicated times. (D) Cell cycle histograms (stained with DAPI to assess DNA content) of EdU-positive wild-type (mock) or

PRIMPOL-OE U2OS cells + 10 μM EdU ± 150 μM cisplatin + 200 ng/mL nocodazole, collected at indicated times.

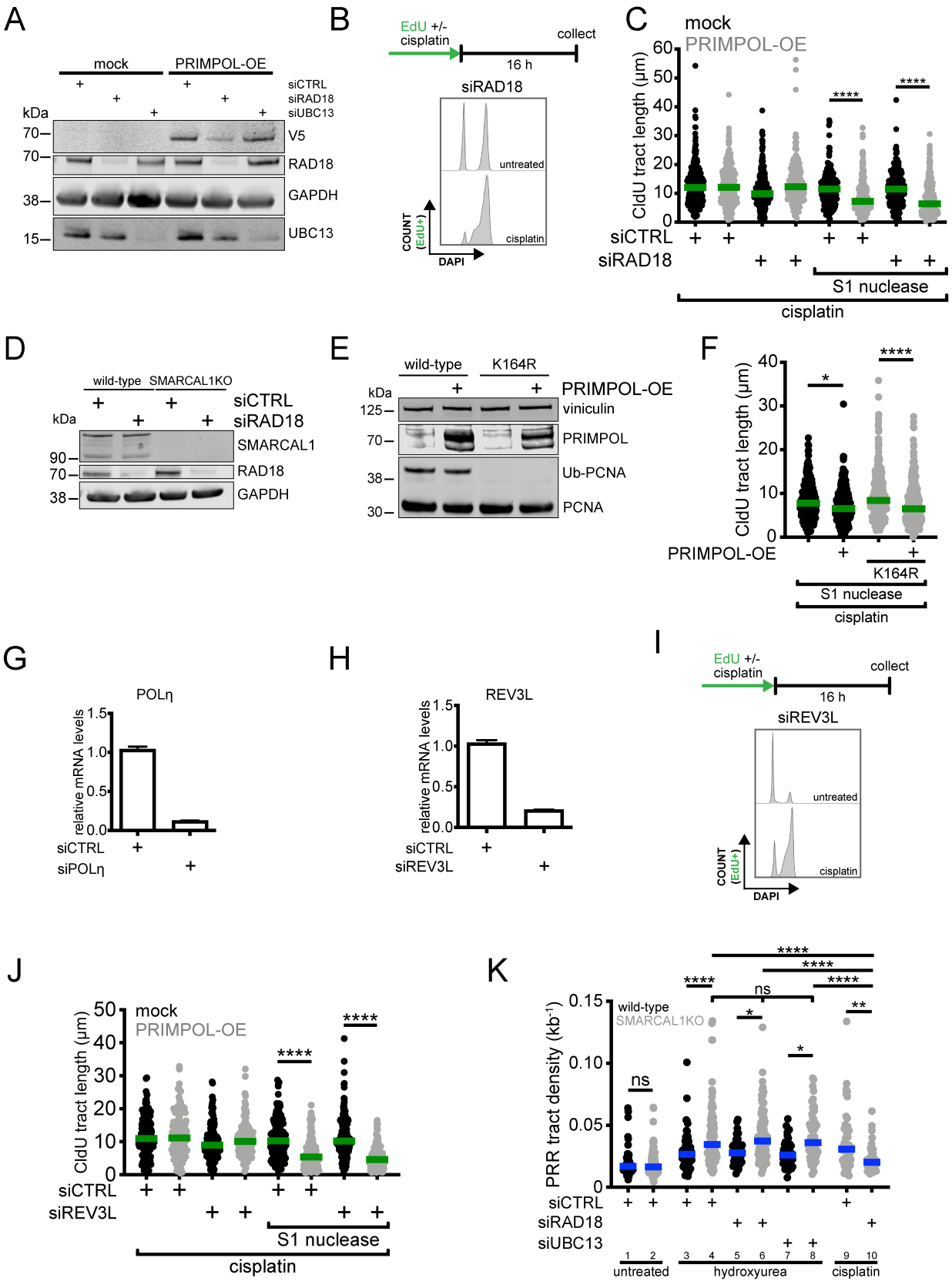
A
mock
PRIMPOL-OE
siCTRL
siRAD18
siUBC13
kDa
70–
70–
38–
15–
V5
RAD18
GAPDH
UBC13
B
EdU +/-
cisplatin
collect
16 h
siRAD18
untreated
cisplatin
COUNT (EdU+)
DAPI
C
mock
PRIMPOL-OE
CldU tract length (µm)
siCTRL
siRAD18
S1 nuclease
cisplatin
****
D
wild-type
SMARCAL1KO
siCTRL
siRAD18
kDa
90–
70–
38–
SMARCAL1
RAD18
GAPDH
E
wild-type
K164R
PRIMPOL-OE
kDa
125–
70–
38–
30–
viniculin
PRIMPOL
Ub-PCNA
PCNA
F
CldU tract length (µm)
PRIMPOL-OE
K164R
S1 nuclease
cisplatin
*
****
G
POLη
relative mRNA levels
siCTRL
siPOLη
H
REV3L
relative mRNA levels
siCTRL
siREV3L
I
EdU +/-
cisplatin
collect
16 h
siREV3L
untreated
cisplatin
COUNT (EdU+)
DAPI
J
mock
PRIMPOL-OE
CldU tract length (µm)
siCTRL
siREV3L
S1 nuclease
cisplatin
****
K
PRR tract density (kb⁻¹)
wild-type
SMARCAL1KO
ns
*
**
****
siCTRL
siRAD18
siUBC13
untreated
hydroxyurea
cisplatin

**Figure S3. Depletion of RAD18 or REV3L does not lead to cell cycle defects or prevent generation of ssDNA gaps (related to Figure 3).** (A) Expression of RAD18, UBC13, and V5-tagged PRIMPOL after siRNA (siCTRL or siRAD18 or siUBC13) knockdown in wild-type or PRIMPOL-OE U2OS cells. (B) Cell cycle histograms (stained with DAPI to assess DNA content) of EdU-positive SMARCAL1KO RAD18-depleted U2OS cells + 10 μM EdU ± 150 μM cisplatin, collected at 16 hours post cisplatin. (C) Dot plot and median of CldU tract lengths in mock-transfected or PRIMPOL-OE U2OS cells + 150 μM cisplatin ± siCTRL or siRAD18 ± S1 nuclease (*n*=2). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; ****p < 0.0001. (D) Expression of RAD18 and SMARCAL1 after siRNA (siCTRL or siRAD18) knockdown in wild-type or SMARCAL1KO U2OS cells. (E) Expression of PRIMPOL, PCNA, and ubiquitinated PCNA (Ub-PCNA) in wild-type or K164R HEK293T cells ± PRIMPOL overexpression. (F) Dot plot and median of CldU tract lengths in wild-type or K164R HEK293T cells ± PRIMPOL-OE + 150 μM cisplatin + S1 nuclease (*n*=2). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *p < 0.05, ****p < 0.0001. (G) mRNA expression of POLη after siRNA (siCTRL or siPOLη) knockdown in PRIMPOL-OE U2OS cells. (H) mRNA expression of REV3L after siRNA (siCTRL or siREV3L) knockdown in PRIMPOL-OE U2OS cells. (I) Cell cycle histograms (stained with DAPI to assess DNA content) of EdU-positive SMARCAL1KO REV3L-depleted U2OS cells + 10 μM EdU ± 150 μM cisplatin, collected at 16 hours post-cisplatin. (J) Dot plot and median of CldU tract lengths in mock-transfected or PRIMPOL-OE U2OS cells + 150 μM cisplatin ± siCTRL or siREV3L ± S1 nuclease (*n*=2). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; ****p < 0.0001. (K) Dot plot and median of PRR densities in wild-type or SMARCAL1KO U2OS cells ± 50 μM hydroxyurea ± 150 μM cisplatin ± siCTRL or siRAD18 or siUBC13 (*n*=3). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, *p < 0.05, **p < 0.01, ****p < 0.0001.

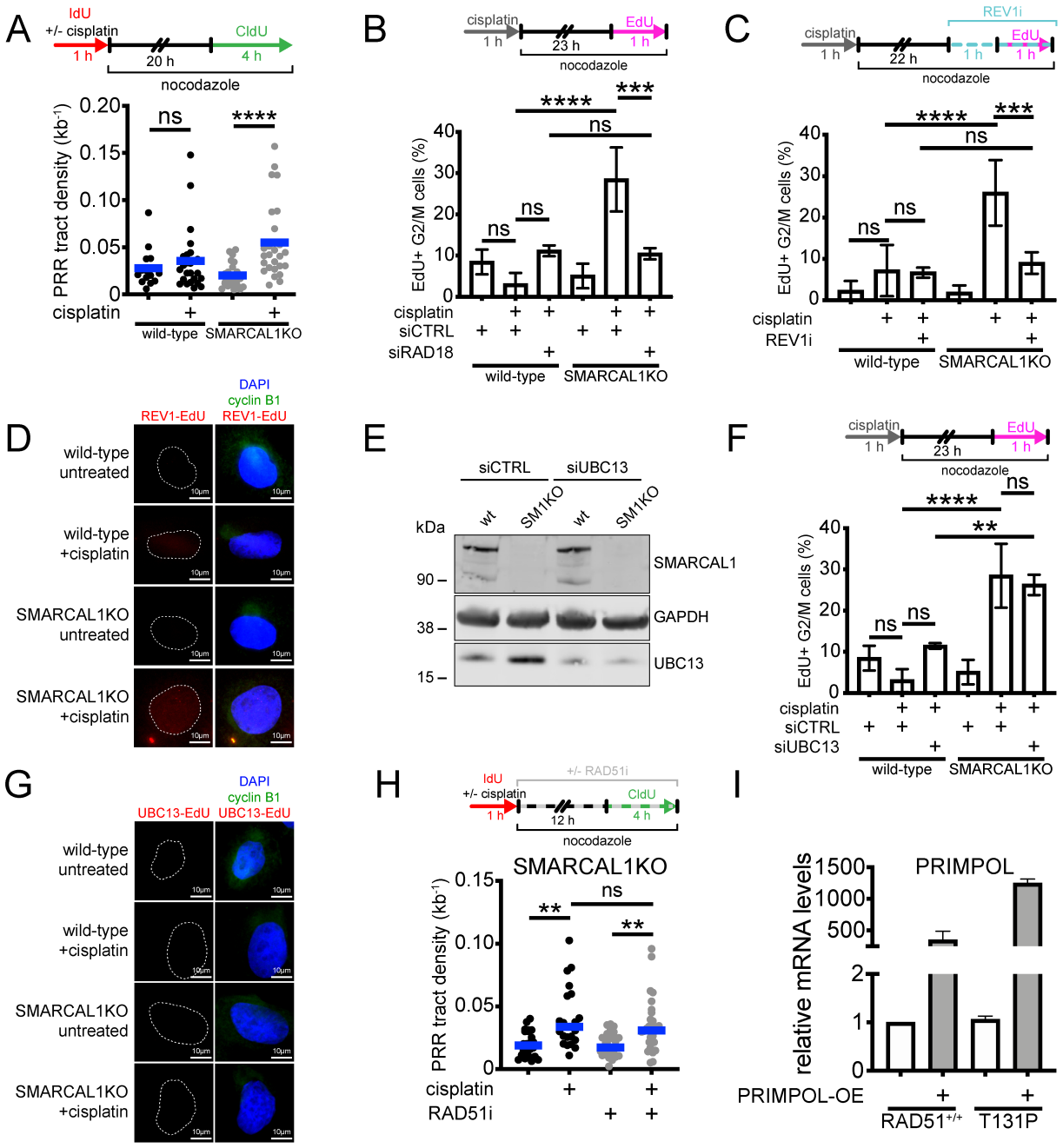


**Figure S4. RAD18 and REV1 but not UBC13 are required for EdU incorporation during gap filling in G2 (related to Figure 4).** (A) Top, schematic for PRR assay at 24 hours. Bottom, dot plot and median of PRR densities in wild-type or SMARCAL1KO U2OS cells ± 150 μM cisplatin at 24 hours (*n*=3). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, ****p < 0.0001. (B) Top, schematic for gap filling detection in G2 *via* EdU incorporation at 24 hours. Bottom, percentage of cells in G2 (cyclin B1+) with EdU foci ± 150 μM cisplatin ± siCTRL or siRAD18 in wild-type and SMARCAL1KO U2OS cells (mean ± SEM) (*n*=3).

Statistics: one-way ANOVA followed by Tukey's multiple comparisons test; *ns*, non-significant, ***p < 0.001, ****p < 0.0001. (C) Top, schematic for gap filling detection in G2 *via* EdU incorporation ± REV1i at 24 hours. Bottom, percentage of cells in G2 (cyclin B1+) with EdU foci ± 150 μM cisplatin ± 2 μM REV1i in wild-type and SMARCAL1KO U2OS cells (mean ± SEM) (*n*=3). Statistics: one-way ANOVA followed by Tukey's multiple comparisons test; *ns*, non-significant, ***p < 0.001, ****p < 0.0001. (D) Representative images of REV1-EdU PLA foci (red) in wild-type and SMARCAL1KO U2OS cells ± 150 μM cisplatin. Cyclin B1 is used a marker for G2. White dashes outline nuclear contours as detected by DAPI staining. (E) Expression of UBC13 and SMARCAL1 after siRNA (siCTRL or siUBC13) knockdown in wild-type (wt) or SMARCAL1KO (SM1KO) U2OS cells. (F) Top, schematic for gap filling detection in G2 *via* EdU incorporation at 24 hours. Bottom, percentage of cells in G2 (cyclin B1+) with EdU foci ± 150 μM cisplatin ± siCTRL or siUBC13 in wild-type and SMARCAL1KO U2OS cells (mean ± SEM) (*n*=3). Statistics: one-way ANOVA followed by Tukey's multiple comparisons test; *ns*, non-significant, **p < 0.01, ****p < 0.0001. (G) Representative images of UBC13-EdU PLA foci (red) in wild-type and SMARCAL1KO U2OS cells ± 150 μM cisplatin. Cyclin B1 is used a marker for late S/G2. White dashes outline nuclear contours as detected by DAPI staining. (H) Top, schematic for PRR assay ± RAD51i. Bottom, dot plot and median of PRR densities in SMARCAL1KO U2OS cells ± 150 μM cisplatin ± 27 μM RAD51 inhibitor (RAD51i, B02) (*n*=2). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, **p < 0.01. (I) mRNA expression of PRIMPOL following transfection of PRIMPOL in RAD51$^{+/+}$ or T131P-RAD51 patient fibroblasts.

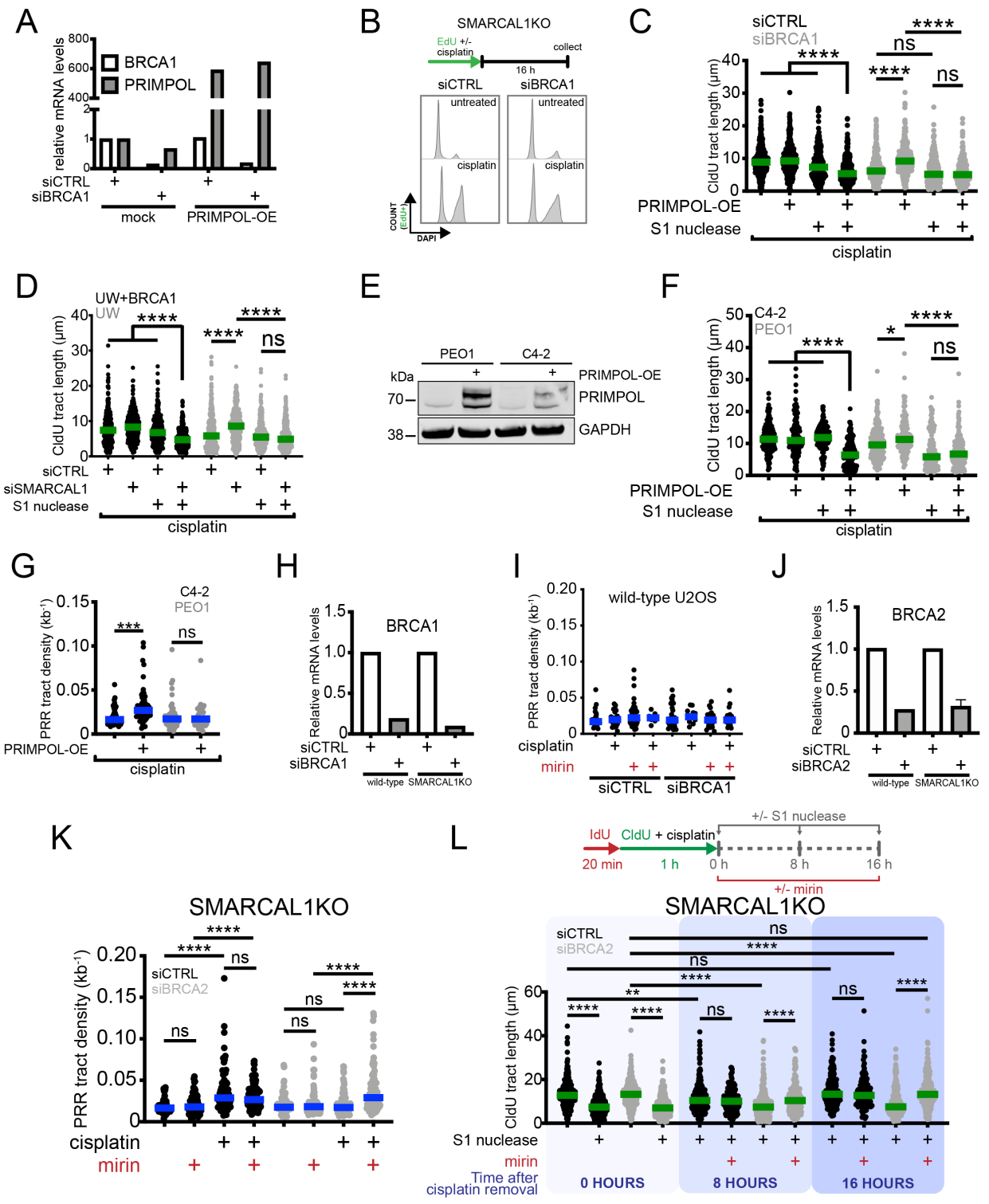


**Figure S5. BRCA2 promotes gap filling by limiting MRE11 activity in S and G2 phase (related to Figure 5).** (A) mRNA expression of PRIMPOL and BRCA after siRNA (siCTRL or siBRCA1) knockdown in wild-type or PRIMPOL-OE U2OS cells. (B) Cell cycle histograms

(stained with DAPI to assess DNA content) of EdU-positive SMARCAL1KO U2OS cells ± siCTRL or siBRCA1 + 10 μM EdU ± 150 μM cisplatin, collected at 16 hours post cisplatin. (C) Dot plot and median of CldU tract lengths in mock-transfected or PRIMPOL-OE U2OS cells + 150 μM cisplatin ± siCTRL or siBRCA1 ± S1 nuclease (*n*=2). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, ****p < 0.0001. See also Quinet et al. for more details on the effect of PRIMPOL overexpression on nascent tract length in BRCA-deficient cells (Quinet et al., Mol. Cell 2020). (D) Dot plot and median of CldU tract lengths in UW or UW+BRCA1 cells + 150 μM cisplatin ± siCTRL or siSMARCAL1 ± S1 nuclease (*n*=3). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, ****p < 0.0001. (E) Expression of PRIMPOL after mock transfection or transfection with V5-tagged PRIMPOL in PEO1 or C4-2 cells. (F) Dot plot and median of CldU tract lengths in C4-2 or PEO1 cells + 150 μM cisplatin ± PRIMPOL-OE ± S1 nuclease (*n*=2). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; ns, non-significant, *p < 0.05, ****p < 0.0001. (G) Dot plot and median of PRR densities in C4-2 or PEO1 cells ± 150 μM cisplatin ± PRIMPOL-OE (*n*=3). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; ns, non-significant, ***p < 0.001. (H) mRNA expression of BRCA1 after siRNA (siCTRL or siBRCA1) knockdown in wild-type and SMARCAL1KO U2OS cells. (I) Dot plot and median of PRR densities in wild-type U2OS cells ± siCTRL or siBRCA1 ± 150 μM cisplatin ± 50 μM mirin (*n*=3). (J) mRNA expression of BRCA2 after siRNA (siCTRL or siBRCA2) knockdown in wild-type and SMARCAL1KO U2OS cells. (K) Dot plot and median of PRR densities in SMARCAL1KO U2OS cells ± 150 μM cisplatin ± siCTRL or siBRCA2 ± 50 μM mirin (*n*=3). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, ****p < 0.0001. (L) Top, schematic of DNA fiber assay with S1 nuclease and mirin. Bottom, dot plot and median of CldU tract lengths in SMARCAL1KO cells ± siCTRL or siBRCA2 ± 50 μM mirin ± S1 nuclease at 0 or + S1 at 8 and 16 hours post a 1 hour treatment with 150 μM cisplatin. Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; *ns*, non-significant, **p < 0.01, ****p < 0.0001.

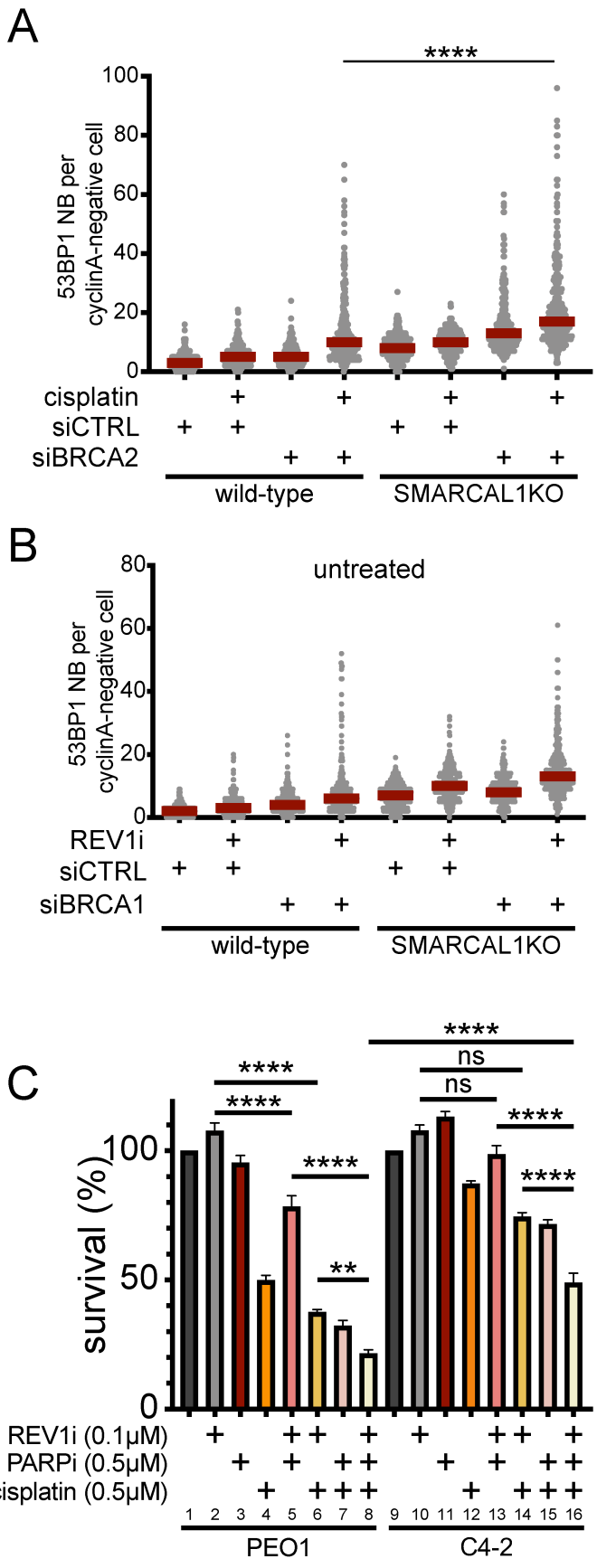
A
****
100
80
60
40
20
0
53BP1 NB per cyclinA-negative cell
cisplatin
siCTRL
siBRCA2
wild-type
SMARCAL1KO
B
untreated
80
60
40
20
0
53BP1 NB per cyclinA-negative cell
REV1i
siCTRL
siBRCA1
wild-type
SMARCAL1KO
C
****
ns
ns
****
****
****
****
**
100
50
0
survival (%)
REV1i (0.1μM)
PARPi (0.5μM)
cisplatin (0.5μM)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
PEO1
C4-2

**Figure S6. BRCA2 loss leads to G1-specific 53BP1 nuclease bodies and increases cell sensitivity in response to cisplatin (related to Figure 6).** (A) Dot plot and median of G1-specific 53BP1 bodies in wild-type and SMARCAL1KO U2OS cells ± siCTRL or siBRCA2 ± cisplatin (*n*=3). Statistics: Kruskal-Wallis followed by Dunn's multiple comparisons test; ****p < 0.0001. (B) Dot plot and median of G1-specific 53BP1 bodies in wild-type and SMARCAL1KO U2OS cells ± siCTRL or siBRCA1 ± 2 μM REV1i (*n*=2). (C) Cell survival of PEO1 and C4-2 cells ± indicated chronic doses of PARPi (Olaparib), cisplatin, and REV1i (JH-RE-06) (*n*=3). Statistics: one-way ANOVA followed by Tukey's multiple comparison test; *ns*, non-significant, **p < 0.01, ****p < 0.0001.

A

| % Reversed forks (total # molecules analyzed) | | | | |
|---|---|---|---|---|
| | untreated | + cisplatin | SMARCAL1KO + cisplatin | PARPi + cisplatin |
| Exp 1 | 11(90) | 23 (91) | 12 (82) | 4 (70) |
| Exp 2 | 4 (84) | 24 (83) | 14 (78) | 9 (78) |
| Exp 3 | 8 (91) | 24 (75) | 7 (89) | 10 (91) |

B

| % Forks with 1, 2, ≥ 3 internal gaps (total # molecules analyzed) | | | | |
|---|---|---|---|---|
| | untreated | + cisplatin | SMARCAL1KO + cisplatin | PARPi + cisplatin |
| Exp 1 | 4 (90) | 2 (91) | 16 (82) | 11 (70) |
| Exp 2 | 7 (84) | 8 (83) | 15 (78) | 14 (78) |
| Exp 3 | 7 (91) | 7 (75) | 16 (89) | 13 (91) |

**Table S1. Electron microscopy (EM) data (related to Figure 1).** (A) Percentage of reversed replication forks observed in three independent EM experiments for samples in Figure 1E. (B) Percentage of replication forks with 1, 2, or ≥ 3 ssDNA gaps behind forks observed in three independent EM experiments for samples in Figure 1F. Number of total molecules analyzed per sample is indicated in parentheses.