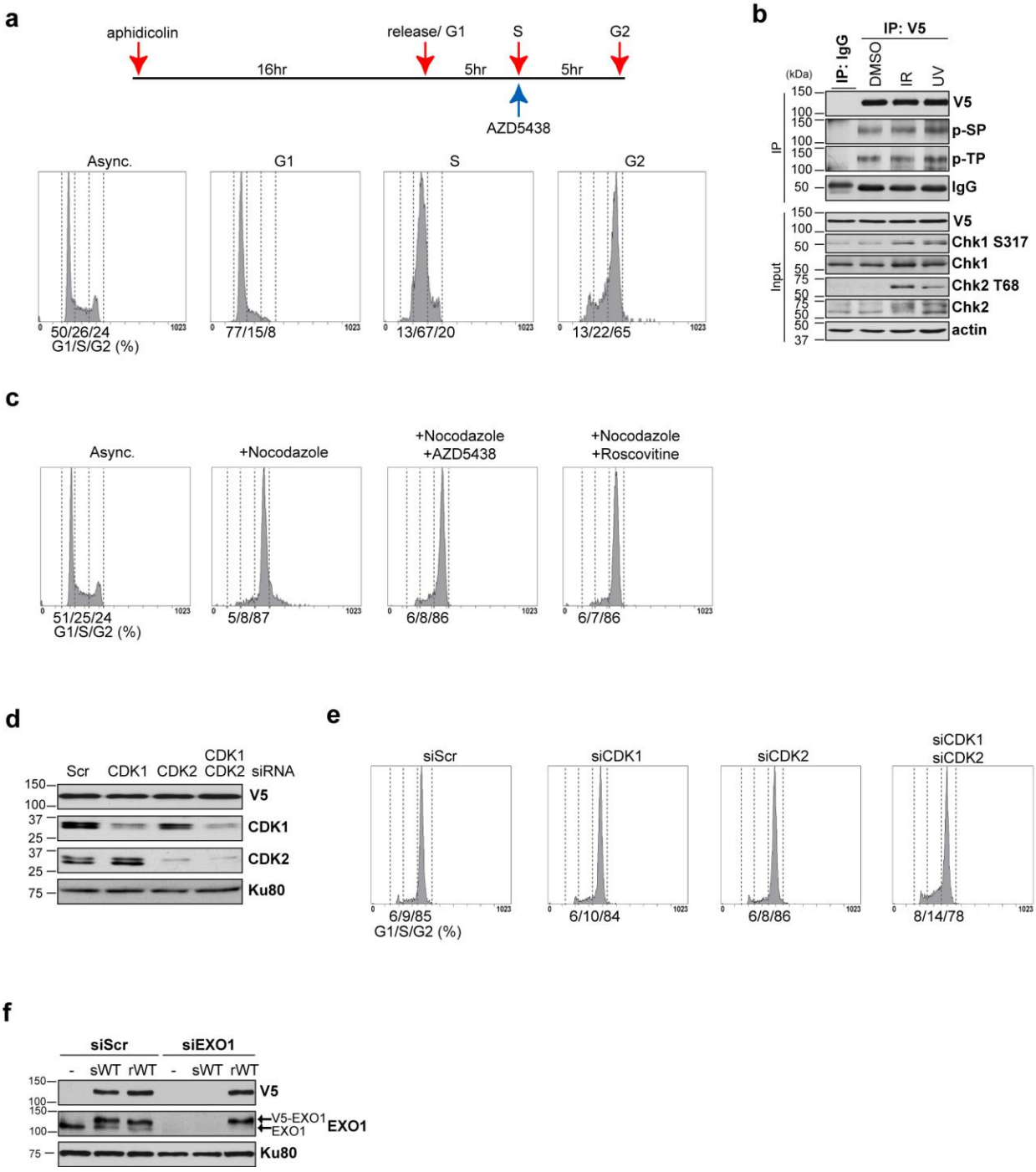
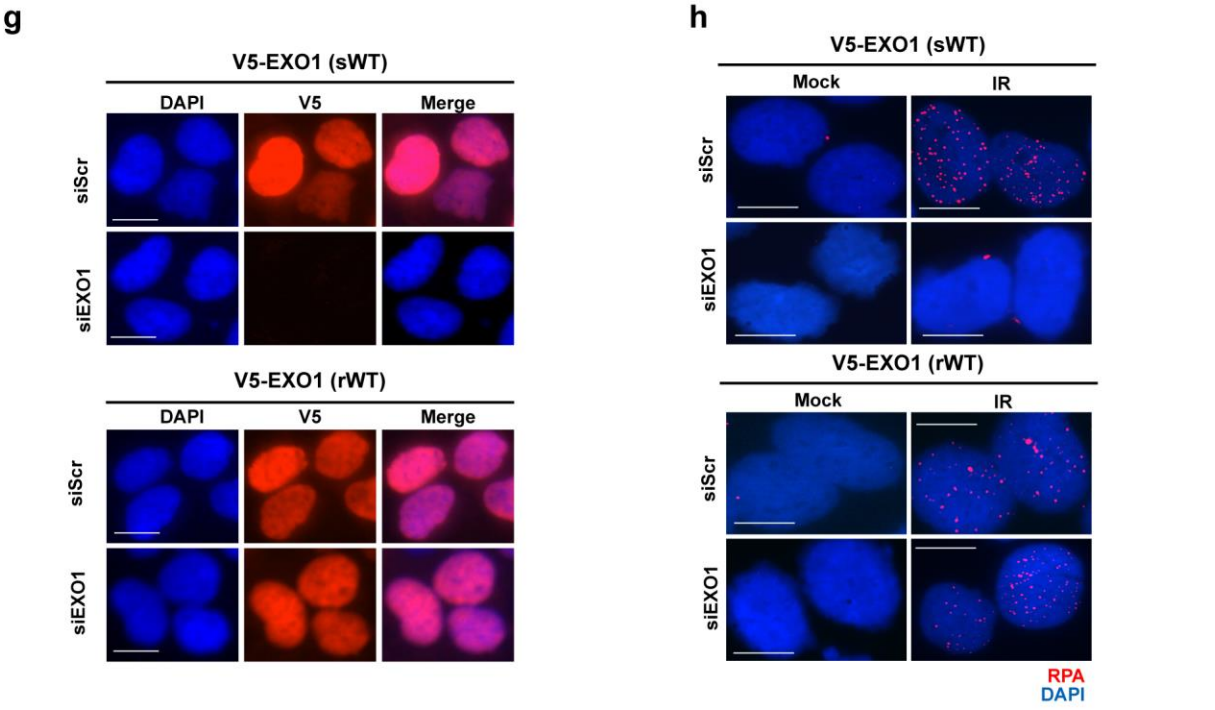
Supplementary Figure 1



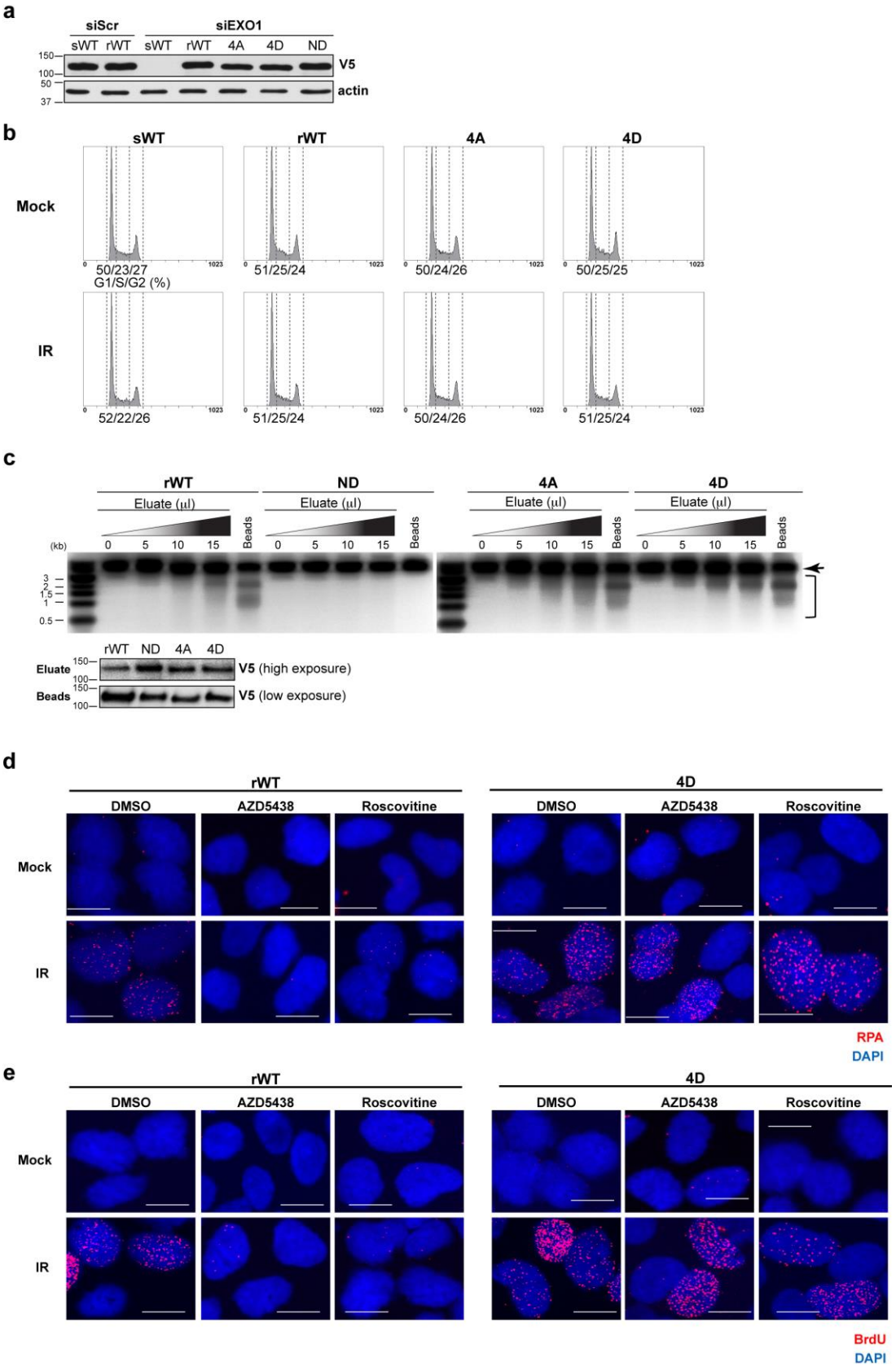
Supplementary Figure 1



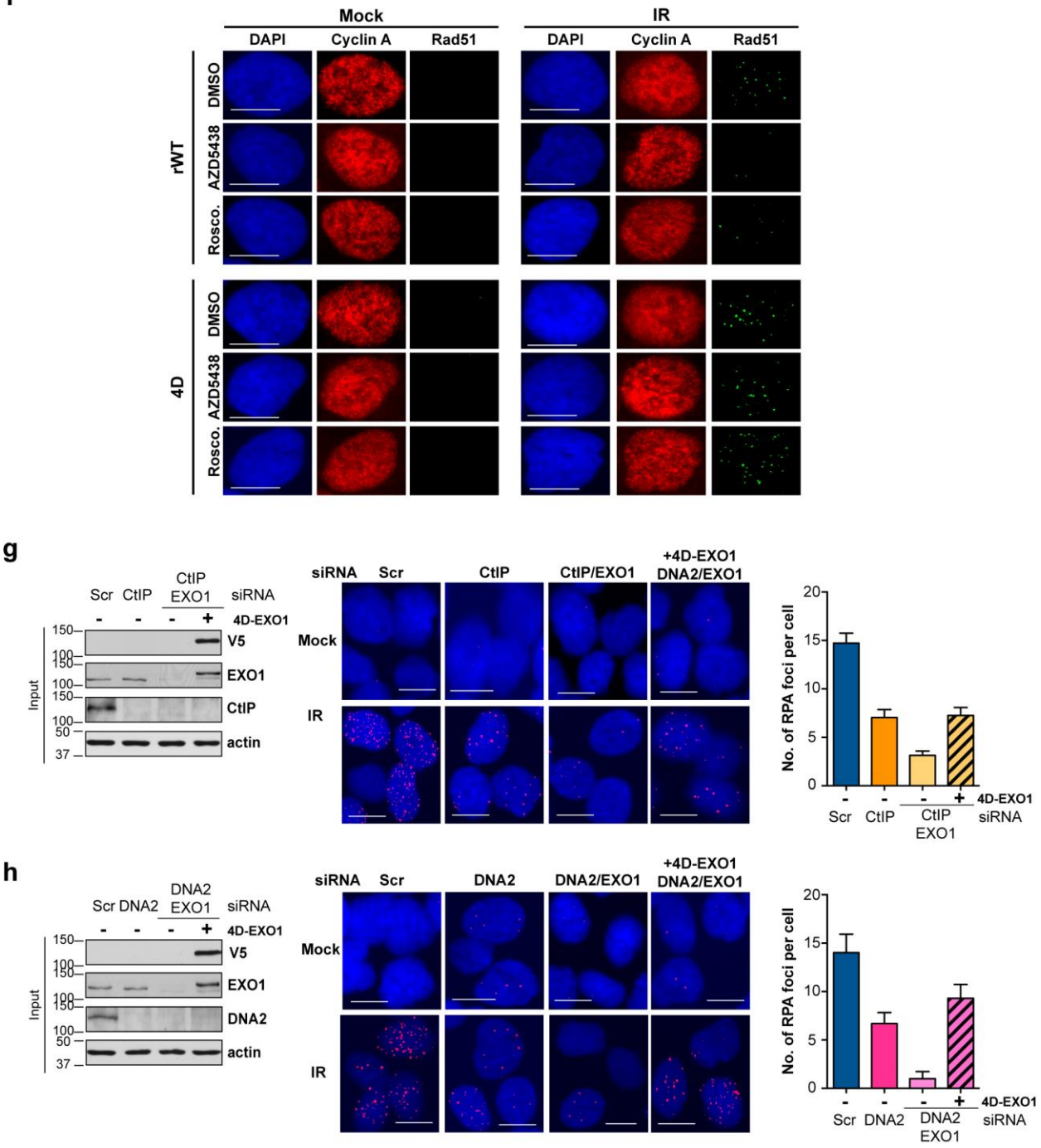
Supplementary Figure 1. (a) HEK-293 cells ectopically expressing V5-tagged EXO1 were synchronized in G1, S, or G2 phases of the cell cycle by aphidicolin treatment for 16 hours followed by release into fresh media for the time periods indicated. Synchronization was confirmed by single parameter flow cytometry after propidium iodide staining for DNA content (*x* axis). The G1, S, and G2 compartments are demarcated and the percentage of cells in each compartment is shown. For CDK inhibition studies, AZD5438 was added to media 5 hours before cells were harvested. **(b)** HEK-293 cells expressing V5-EXO1 were irradiated with X-rays (10 Gy) or with UV-C (10 J/m^2) and harvested after 1 hour. EXO1 was immunoprecipitated (IP) with anti-V5 antibody and Western blotted with anti-phospho-Ser (p-SP) or anti-phospho-Thr (p-TP) CDK substrate antibodies, as indicated. Please note phosphorylation of CHK1 and CHK2 kinases in response to DNA damage but no increase in phosphorylation of EXO1. **(c)** HEK-293 cells expressing V5- EXO1 were synchronized in late G2 by nocodazole treatment for 16 hours and G2 accumulation confirmed by single parameter flow cytometry. For CDK inhibition studies, AZD5438 or Roscovitine was added to media 5 hours before cells were harvested. The G1, S, and G2 compartments are demarcated and the percentage of cells in each compartment is shown. **(d)** Depletion of CDK1 and/or CDK2 by siRNA in HEK-293 cells expressing V5-EXO1 was confirmed by Western blotting with the indicated antibodies. The following siRNAs (Invitrogen) were used –
CDK1, CCUAGUACUGCAAUUCGGGAAAUUU, and
CDK2, CCUAUUCCCUGGAGAUUCUGAGAUU. **(e)** Depletion of CDK1 and/or CDK2 does not affect cell synchronization as confirmed by single parameter flow cytometry using propidium iodide staining. The G1, S, and G2 compartments are demarcated and the percentage of cells in each compartment is shown. Please note total percentage of cells in S and G2 is similar for control and siRNA-treated cells. **(f)** To screen for resection-relevant S/TP sites on EXO1, U2OS cells were depleted of endogenous EXO1 (bottom arrowhead) using siRNA and complemented with siRNA-resistant, V5-tagged EXO1 (top arrowhead). Cells were transfected with scrambled siRNA (siScr) as control. Efficacy of knockdown and comparable levels of protein expression were verified for every experiment by Western blotting with the indicated antibodies. Please note that both endogenous EXO1 and siRNA-sensitive EXO1 (sWT) were depleted by siRNA, while siRNA-resistant EXO1 (rWT) was unaffected. The following siRNA (Invitrogen) was used to target endogenous EXO1 – UGCCUUUGCUAAUCCAAUCCCACGC. Ectopically-expressed wild type and mutant EXO1were rendered resistant to this siRNA by changing three nucleotides in the EXO1 siRNA-targeting region (A702T, T703C, and A705T), which did not result in any changes in the amino acid sequence. **(g)** Immunofluorescence staining of U2OS cells with anti-V5 antibody was carried out to ensure that ectopically-expressed EXO1 (both wild type and mutant) was nuclear and that more than 90% of transfected cells exhibited robust protein expression. **(h)** Representative images of U2OS cells immunostained with anti-RPA antibody (red) 3 hours after irradiation (IR). Nuclei were stained with DAPI (blue). Please note attenuation of RPA foci formation after depletion of endogenous EXO1 (sWT panel) and restoration upon expression of siRNA-resistant WT EXO1 (rWT panel). Cells expressing mutant EXO1 were similarly stained and average numbers of RPA foci were plotted in Fig. 1e. **(i)** HEK-293 cells expressing either WT-EXO1, EXO1 with all four C-terminal S/TP sites mutated to Ala (4A-EXO1), or EXO1 with individual mutations at the identified S/TP sites (S639A, T732A, S815A, or T824A) were synchronized in G2, and EXO1 phosphorylation status determined by IP-

Western with anti-phospho-Ser (p-SP) or anti-phospho-Thr (p-TP) CDK substrate antibodies, as indicated. **(j)** A rabbit polyclonal phospho-specific antibody against p-T732 of EXO1 was generated using the phospho-peptide NH_2-Cys-FSKKD(pT)PLRNK-COOH, where pT denotes phospho-Thr. The antibody was affinity purified and tested by immunoblotting synthetic peptides with or without phosphorylation at T732. **(k)** To confirm that the anti-phospho-T732 antibody recognized phosphorylated EXO1, V5-EXO1 was immunoprecipitated (IP) from HEK-293 cells synchronized in G2. Immunoprecipitated EXO1 was treated with 200 Units of lambda phosphatase (Millipore) for 30 minutes at 30°C in the presence or absence of a phosphatase inhibitor cocktail (Sigma), as indicated, prior to Western blotting. Please note loss of p-T732 signal in the Western blot upon treatment with lambda phosphatase that is rescued by the presence of phosphatase inhibitors. **(l)** Endogenous EXO1 was immunoprecipitated from HEK-293 cells synchronized in G1 or G2, as indicated, and Western blotted with anti-phospho-Ser (p-SP) or anti-phospho-Thr (p-TP) CDK substrate antibodies, or with the anti-phospho-EXO1 (p-T732) antibody. "+AZD" indicates cells pre-treated with the CDK inhibitor AZD5438 prior to analysis.
Scale bars denote 10 μm for all images. All experiments were replicated three times.

Supplementary Figure 2



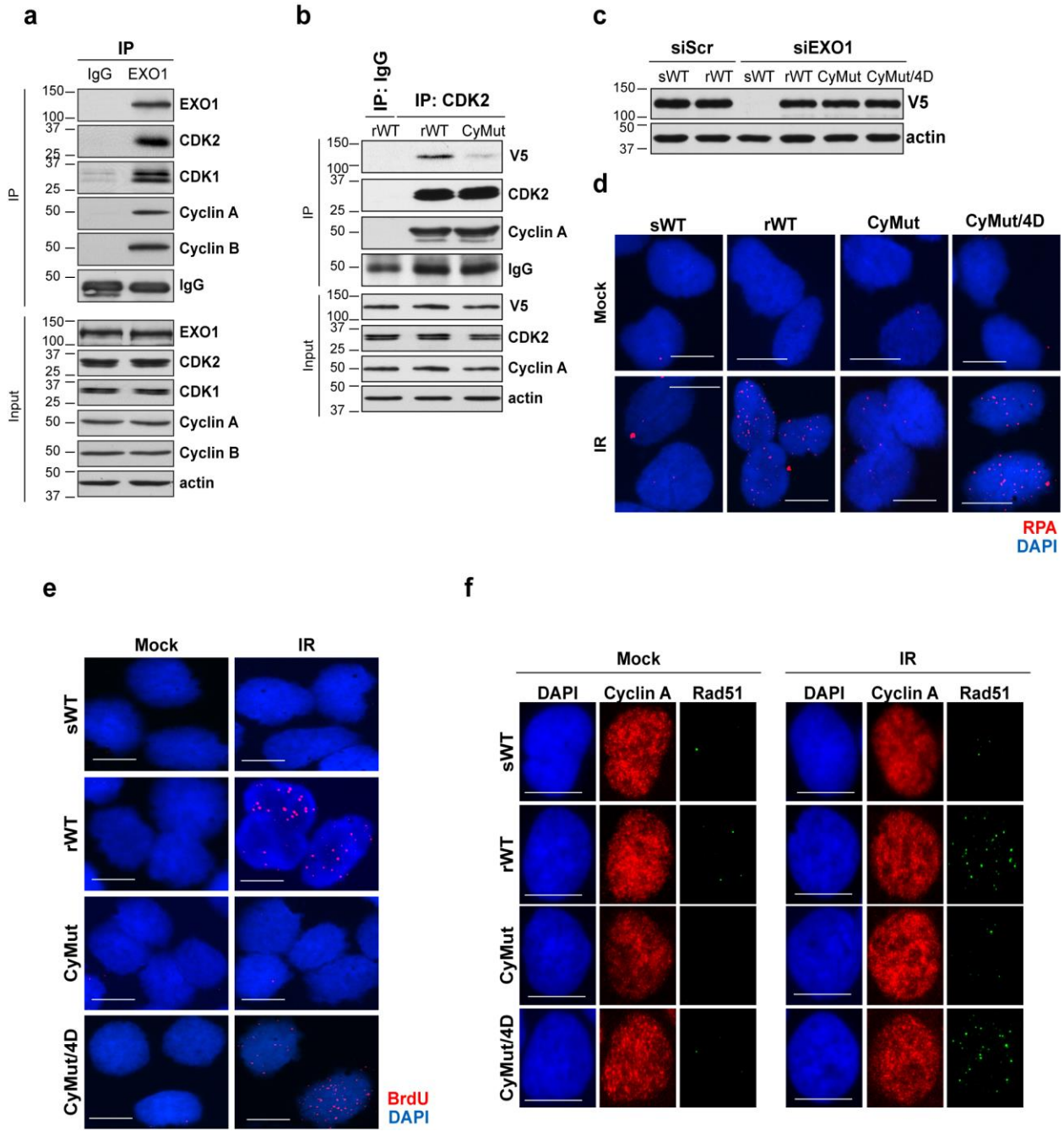
Supplementary Figure 2



Supplementary Figure 2. (a) Expression of siRNA-resistant, V5-tagged wild type and mutant EXO1 in U2OS cells was verified by Western blotting with the indicated antibodies. Efficacy of siRNA knockdown is evident from depletion of sWT-EXO1. **(b)** Cells expressing siRNA-sensitive wild type EXO1 (sWT) or siRNA-resistant wild type or mutant EXO1 (rWT, 4A, or 4D) were mock-irradiated or irradiated as in Figure 2b and cell cycle profiles were determined by single parameter flow cytometry after PI staining for DNA content (*x* axis). The G1, S, and G2 compartments are demarcated and the percentage of cells in each compartment is shown. Please note that expression of the different forms of EXO1 does not significantly alter cell cycle profiles and the percentage of cells in S/G2. **(c)** Mutation of all four S/TP sites on EXO1 does not affect its intrinsic nuclease activity as assessed by in vitro nuclease assay. V5-tagged WT-, ND-, 4A-, or 4D-EXO1 were immunoprecipitated (IP) from HEK-293 cells using Dynabeads as described in Methods, except that IP was carried out for only two hours. Nuclease assays were performed either with V5-EXO1 bound to beads or with the eluate after elution with V5 peptide (APExBIO). Briefly, beads were washed extensively after IP and then incubated in nuclease assay buffer (20 mM HEPES pH7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.05% Triton X-100, 0.1 mg/ml BSA). For V5-EXO1 elution, 10 μL of V5 peptide (5mg/ml in water) was added to 40 μl of beads in nuclease assay buffer and incubated overnight. Nuclease activity was tested by examining the degradation of a linearized 6.2 kb plasmid. Nuclease assays were performed in a final volume of 16 μl with 200 ng of plasmid DNA per reaction and 0, 5, 10, or 15 μl of eluates or 15 μl of beads, as indicated. After incubation for 60 minutes at 37°C, samples were directly run on a 0.8% agarose gel, transferred to a hybond XL membrane (Amersham) and hybridized using the ^{32}P-labeled plasmid as a probe. 5 μl of the eluates and beads were analyzed separately by Western blotting with anti-V5 antibody (only a small fraction of V5-EXO1 could be eluted from beads, necessitating higher exposures for detection). Nuclease activity is detectable as a smear (*bracket*) beneath the plasmid band (*arrowhead*) and is concentration dependent. Note that the nuclease activity detected is specific to EXO1 as no activity is seen with nuclease dead (ND)-EXO1. Importantly, both 4A-EXO1 and 4D-EXO1 show nuclease activity, indicating that amino acid substitutions at the four C-terminal S/TP sites do not directly compromise nuclease functions of EXO1. **(d)** Representative images of mock-irradiated or irradiated U2OS cells immunostained with anti-RPA antibody. Nuclei were stained with DAPI (blue). Cells were depleted of endogenous EXO1, and siRNA-resistant WT- or 4D-EXO1 was expressed. Cells were treated with AZD5438 or Roscovitine, as indicated or with DMSO as a control prior to irradiation. **(e)** Representative images of IR-induced BrdU/ssDNA foci (red) in U2OS cells expressing WT- or 4D-EXO1, with or without drug treatment. **(f)** Representative images of U2OS cells co-immunostained with anti-Cyclin A (red) and anti-Rad51 (green) antibodies after irradiation (IR). Please note reduction in RPA, BrdU/ssDNA, and Rad51 foci in WT-EXO1 cells treated with CDK inhibitors but not so in 4D-EXO1 cells. **(g)** U2OS cells were depleted of CtIP or both CtIP and EXO1 using siRNA and complemented with siRNA-resistant phospho-mimic EXO1 with all four C-terminal S/TP sites replaced with Asp (4D), as indicated. Cells were irradiated and scored for RPA foci numbers. Average numbers of RPA foci per cell are plotted after subtracting background (average numbers of foci in mock-irradiated cells). Efficacy of knockdown and expression of V5-EXO1 were verified by Western blotting with the indicated antibodies. The following siRNA (Invitrogen) was used to target endogenous CtIP – GGGUCUGAAGUG AACAGAUAUUA. **(h)** U2OS cells were depleted of DNA2 or both DNA2 and EXO1 using siRNA and complemented with siRNA-resistant 4D-EXO1, as indicated. Cells were irradiated and RPA foci numbers were scored and plotted. Efficacy of

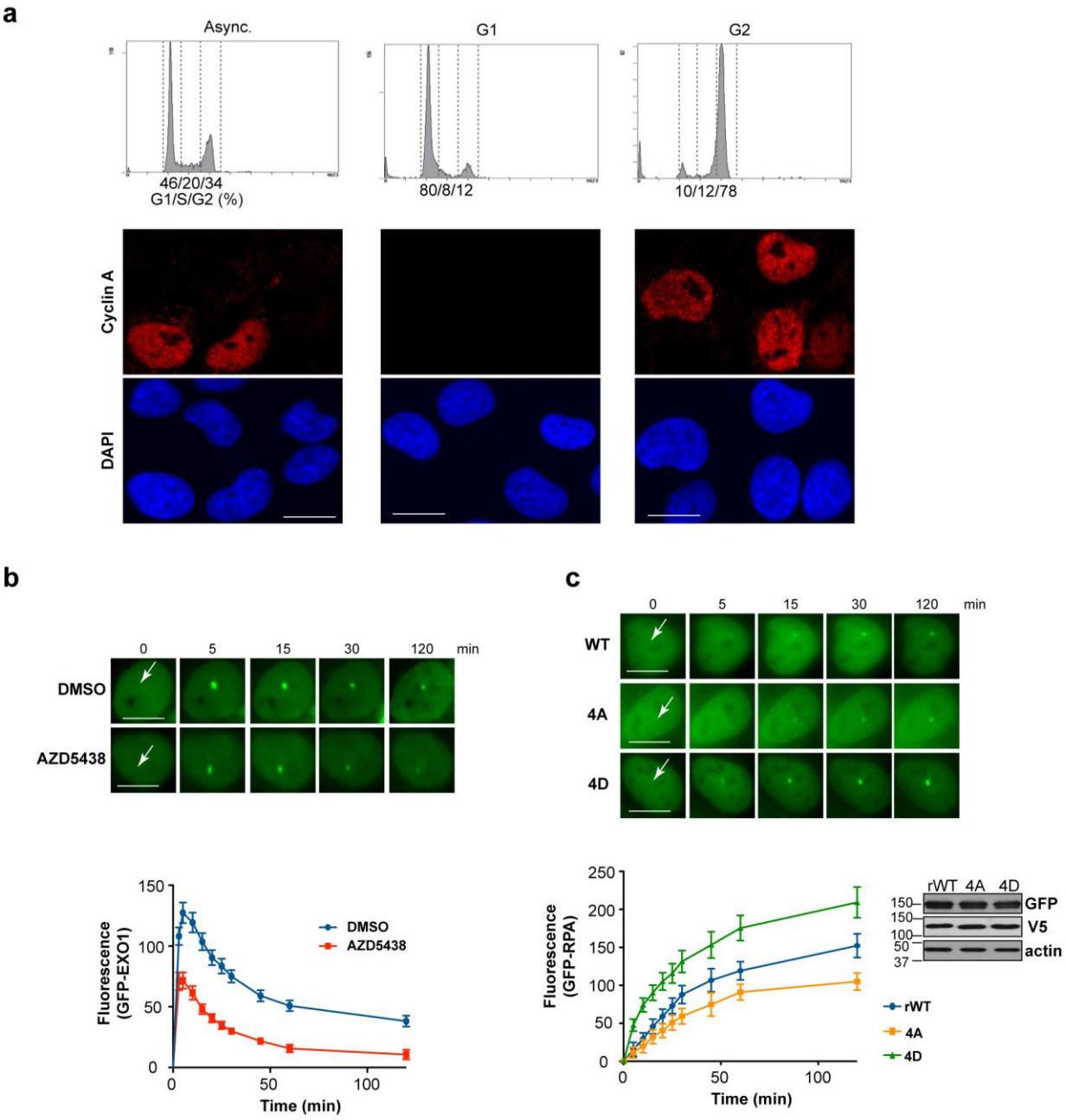
knockdown and expression of V5-EXO1 were verified by Western blotting. The following siRNA (Invitrogen) was used to target endogenous DNA2 - GCCUGCAUUCUAAAGGGUUUGAAUA.
Scale bars denote 10 μm for all images. All experiments were replicated three times. Error bars indicate S.E.M. for all plots.

Supplementary Figure 3

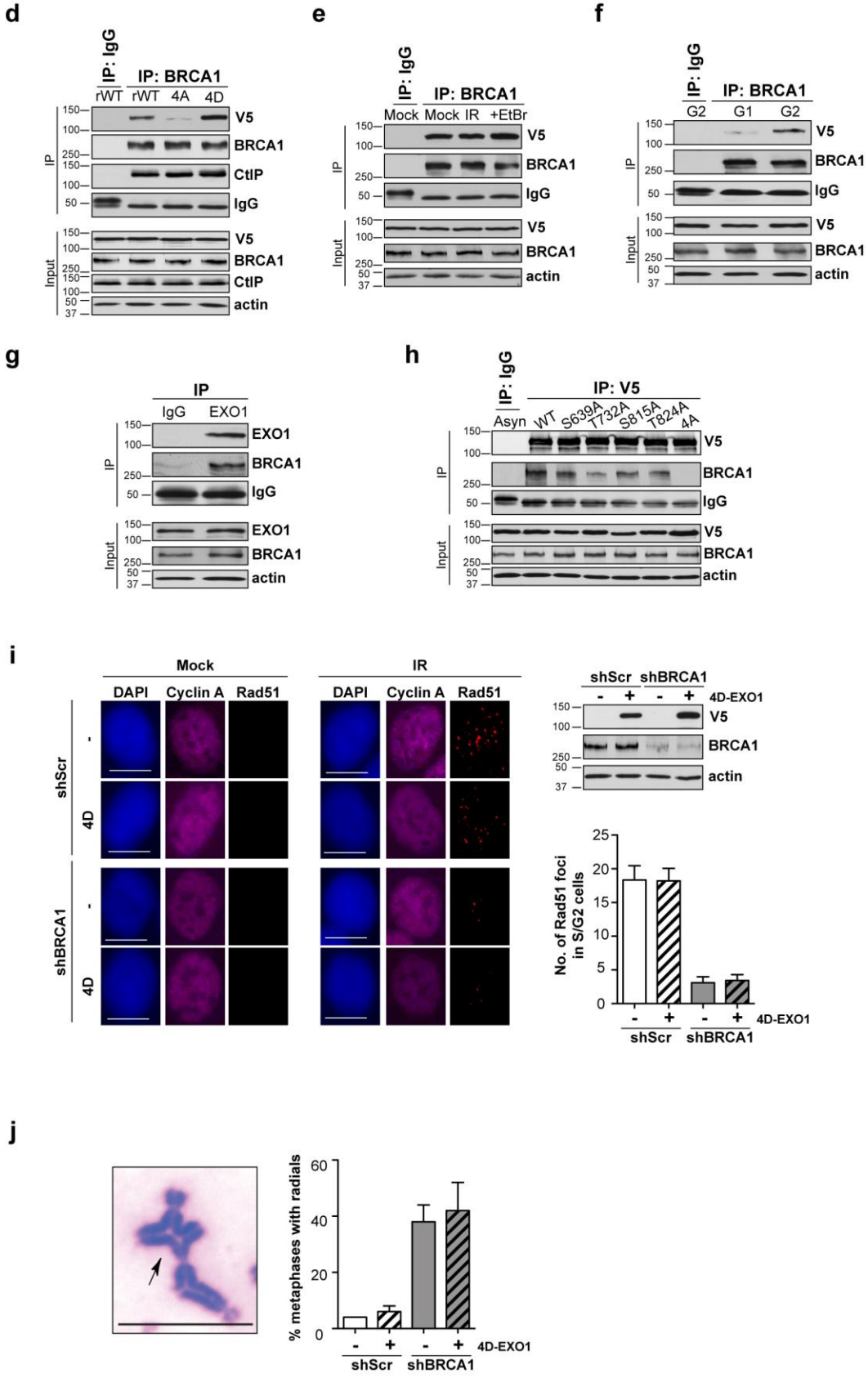


Supplementary Figure 3. (a) HEK-293 cells were synchronized in G2 as described in Supplementary Fig. 1a. Whole cell extracts were immunoprecipitated with anti-EXO1 antibody or with IgG as control, and immunoprecipitates were probed by Western blotting with the indicated antibodies. **(b)** HEK-293 cells expressing rWT- or CyMut-EXO1 were synchronized in G2. Whole cell extracts were immunoprecipitated with anti-CDK2 antibody or with IgG as control, and immunoprecipitates were probed by Western blotting with the indicated antibodies. **(c)** Expression of siRNA-resistant, V5-tagged WT-, CyMut-, CyMut/4D-EXO1 in U2OS cells with depletion of endogenous EXO1 was verified by Western blotting with the indicated antibodies. Efficacy of siRNA knockdown is evident from depletion of sWT-EXO1. **(d)** Representative images of mock-irradiated or irradiated U2OS cells immunostained with anti-RPA antibody (red). Cells were depleted of endogenous EXO1, and siRNA-resistant wild type or mutant EXO1 was expressed. Nuclei were stained with DAPI (blue). **(e)** Representative images of U2OS cells showing IR-induced BrdU/ssDNA foci (red). **(f)** Representative images of U2OS cells co-immunostained with anti-Cyclin A (red) and anti-Rad51 (green) antibodies after irradiation (IR). Please note defects in RPA, BrdU/ssDNA, and Rad51 foci in cells expressing EXO1 with mutated Cy sites (CyMut) and rescue of these defects by EXO1 with additional phospho-mimic substitutions (CyMut/4D).
Scale bars denote 10 μm for all images. All experiments were replicated three times.

Supplementary Figure 4



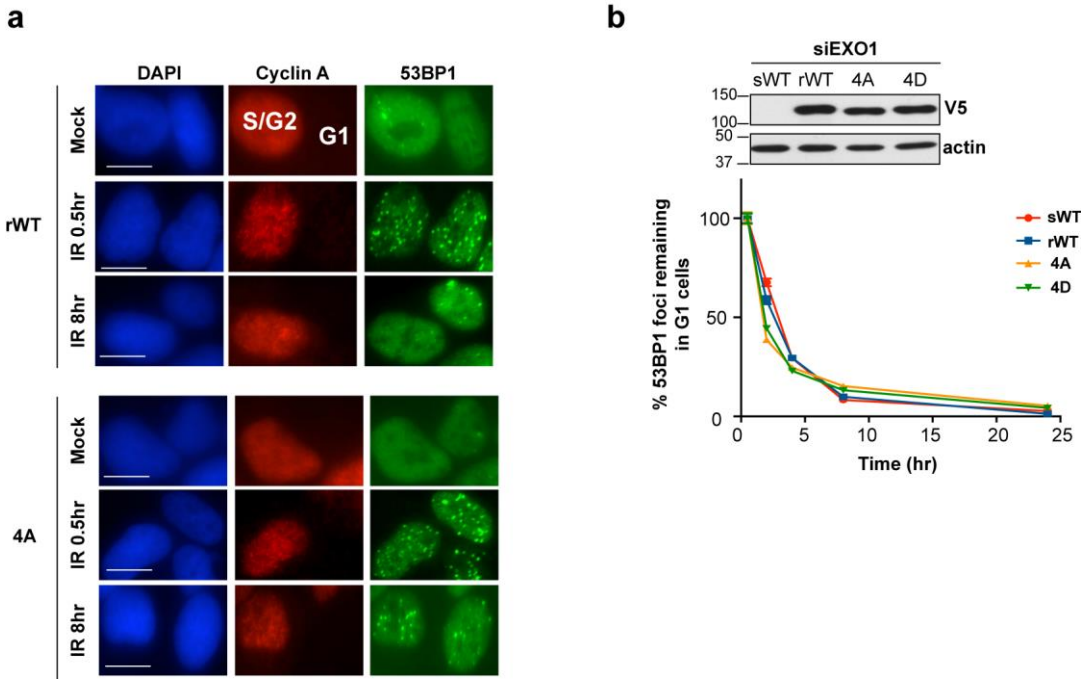
Supplementary Figure 4



Supplementary Figure 4. (a) To rule out cell cycle effects while quantifying GFP-EXO1 or DsRed-EXO1 recruitment to micro-laser-induced DSBs, U2OS cells were first synchronized in G2 as described in Supplementary Fig. 1a. Synchronization was confirmed by single parameter flow cytometry. The G1, S, and G2 compartments are demarcated and the percentage of cells in each compartment is shown. Synchronization was re-confirmed by staining synchronized cells with anti-Cyclin A antibody (red) to ensure that most cells were positive for Cyclin A at time of laser micro-irradiation. Nuclei were stained with DAPI (blue). **(b)** GFP-EXO1-expressing U2OS cells synchronized in G2 were pre-treated with AZD5438 or with DMSO before laser micro-irradiation. Time-lapse images of accumulation of GFP-EXO1 (green) at DSBs induced by laser micro-irradiation (*arrows*) are shown. Plot shows recruitment of GFP-EXO1 in cells with or without AZD5438 treatment. **(c)** Time-lapse images of accumulation of GFP-RPA (green) at DSBs induced by laser micro-irradiation (*arrows*) of U2OS cells. Cells were depleted of endogenous EXO1 and siRNA-resistant WT-, 4A-, or 4D-EXO1 was expressed, along with GFP-RPA. The kinetics of recruitment of GFP-RPA is plotted. Inset shows equal levels of expression of GFP-RPA as determined by Western blotting. **(d)** HEK-293 cells expressing rWT-, 4A-, or 4D-EXO1 were synchronized in G2. Whole cell extracts were immunoprecipitated with anti-BRCA1 antibody or with IgG as control and immunoprecipitates were probed by Western blotting with the indicated antibodies. **(e)** HEK-293 cells expressing rWT-EXO1 were synchronized in G2, mock-irradiated or irradiated (IR) with 10 Gy, and harvested after 1 hr. Whole cell extracts were immunoprecipitated with anti-BRCA1 antibody or with IgG as control and immunoprecipitates were probed by Western blotting with the indicated antibodies. Additionally, immunoprecipitation was carried out in the presence of 50 μg/ml ethidium bromide to negate any DNA-mediated interactions. **(f)** HEK-293 cells expressing rWT-EXO1 were synchronized in G1 or G2. Whole cell extracts were immunoprecipitated with anti-BRCA1 antibody or with IgG as control and immunoprecipitates were probed by Western blotting with the indicated antibodies. **(g)** HEK-293 cells were synchronized in G2. Whole cell extracts were immunoprecipitated with anti-EXO1 antibody or with IgG as control and immunoprecipitates were probed by Western blotting with the indicated antibodies. **(h)** HEK-293 cells expressing WT-EXO1, EXO1 with individual mutations at the identified S/TP sites (S639A, T732A, S815A, or T824A), or EXO1 with all four C-terminal S/TP sites mutated to Ala (4A-EXO1), were synchronized in G2. Whole cell extracts were immunoprecipitated with anti-V5 antibody or with IgG as control, and immunoprecipitates were probed by Western blotting with the indicated antibodies. **(i)** U2OS cells were stably transfected with a shRNA vector targeting Brca1 (shBrca1) or with a scrambled shRNA vector (shScr) as control and BRCA1 knockdown verified by Western blotting. Cells were additionally transfected with a vector expressing 4D-EXO1 as indicated. Cells were irradiated (IR) and co-immunostained with anti-Rad51 (red) and anti-Cyclin A (purple) antibodies after 3 hours. Average numbers of Rad51 foci in Cyclin A positive cells are plotted. **(j)** U2OS cells (shScr or shBRCA1), expressing 4D-EXO1, as indicated, were irradiated and metaphase chromosome spreads scored for tri- and quadri-radial chromosomes (inset). Plot shows percent metaphases with one or more radials. Please note reduced Rad51 foci

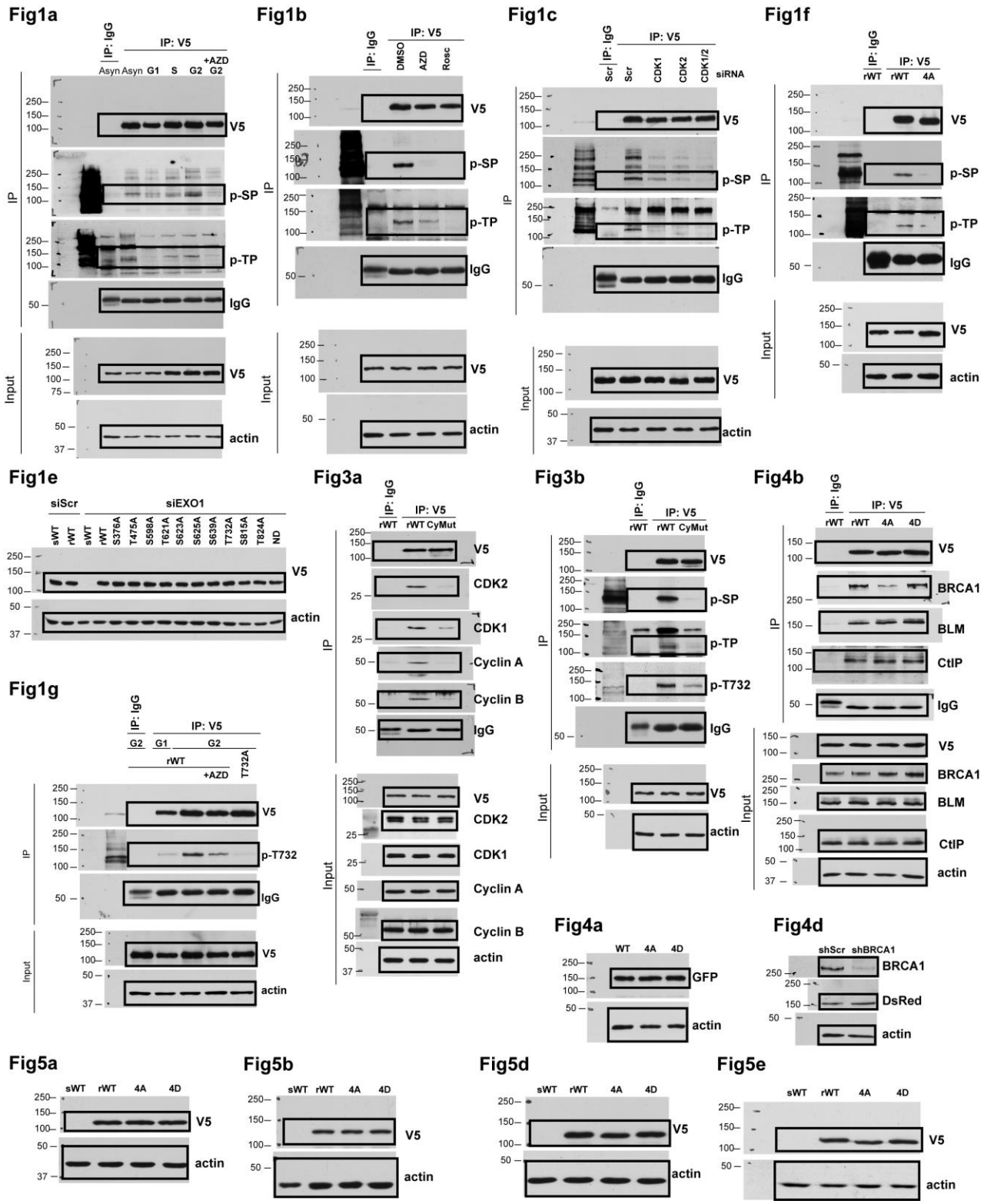
and increased radials in cells with BRCA1 knockdown that are not rescued by expression of 4D-EXO1.
Scale bars denote 10 μm for all images. All experiments were replicated three times. Error bars indicate S.E.M. for all plots.

Supplementary Figure 5



(a) U2OS cells were irradiated (IR), fixed at the indicated times post-IR, and co-immunostained for Cyclin A (red) and 53BP1 foci (green). Cyclin A staining was used to distinguish between cells in G1 (Cyclin A negative) versus cells in S/G2 (Cyclin A positive). Nuclei were stained with DAPI (blue). Cells were depleted of endogenous EXO1 using siRNA and complemented with siRNA-sensitive EXO1 (sWT) or siRNA-resistant WT-, 4A-, or 4D-EXO1. Representative pictures are shown for U2OS cells expressing rWT- or 4A-EXO1. **(b)** 53BP1 foci were scored in Cyclin A-negative (G1) nuclei, and after subtracting background (number of foci in mock-irradiated nuclei), percent foci remaining was plotted against the indicated times post-IR. Inset shows levels of EXO1 protein expression as determined by Western blotting. Please note absence of major differences in DSB repair kinetics in G1 cells, in contrast to the repair defect seen in 4A-EXO1-expressing cells in G2 (compare this figure with Figure 5c).
Scale bars denote 10 μm for all images. All experiments were replicated three times. Error bars indicate S.E.M.

Supplementary Figure 6 (Uncropped Western blot scans)



Supplementary Table 1: Primers for site-directed mutagenesis of EXO1

rWT	TCA CTG CGT GGG ATT GGT CTT GCA AAG GCA TGC AAA GTC C
S376A	T TGG CAT AGG AAT TAC GCT CCC AGA CCA GAG TCG GGT ACT GTT T
T475A	C AAA AAG AGT GTA AGC GCC CCA CCT AGG ACG AGA AAT AAA TTT G
S598A	C AAA TTT ACA AGG ACC ATT GCA CCA CCC ACT TTG GGA ACA CTA A
T621A	T GGA GAT TTT TCA AGA GCG CCG AGC CCC TCT CCA AGC ACA GCA T
S623A	T GGA GAT TTT TCA AGA ACG CCG GCC CCC TCT CCA AGC ACA GCA T
S625A	AGA ACG CCG AGC CCC GCT CCA AGC ACA GCA TTG CAG
S639A	G TTC CGA AGA AAG AGC GAT GCC CCC ACC TCT TTG CCT GAG AAT A
T732A	T TTC TCA AAA AAA GAC GCA CCT CTA AGG AAC AAG GTT CCT GGG C
S815A	T TGT AAG AAA CCC CTG GCC CCA GTC AGA GAT AAC ATC CAA CTA A
T824A	C AGA GAT AAC ATC CAA CTA GCT CCA GAA GCG GAA GAG GAT ATA T
S639D	G TTC CGA AGA AAG AGC GAT GAC CCC ACC TCT TTG CCT GAG AAT A
T732D	T TTC TCA AAA AAA GAC GAC CCT CTA AGG AAC AAG GTT CCT GGG C
S815D	T TGT AAG AAA CCC CTG GAC CCA GTC AGA GAT AAC ATC CAA CTA A
T824D	C AGA GAT AAC ATC CAA CTA GAT CCA GAA GCG GAA GAG GAT ATA T
CyMut1 R204A	CAA GCT CGG CTA GGA ATG TGC GCA CAG CTT GGG GAT GTA TTC ACG GAA
CyMut1 Q205A	CAA GCT CGG CTA GGA ATG TGC GCA GCG CTT GGG GAT GTA TTC ACG GAA
CyMut1 L206A	CAA GCT CGG CTA GGA ATG TGC GCA GCG GCT GGG GAT GTA TTC ACG GAA
CyMut2 R293A	CTA GTT TTT GAT CCC ATC AAA GCG AAA CTT ATT CCT CTG AAC GCC TAT
CyMut2 K294A	CTA GTT TTT GAT CCC ATC AAA GCG GCA CTT ATT CCT CTG AAC GCC TAT
CyMut2 L295A	CTA GTT TTT GAT CCC ATC AAA GCG GCA GCT ATT CCT CTG AAC GCC TAT
ND	ATA ATT ACA GAG GAC TCG GCT CTC CTA GCT TTT GGC TG

Supplementary Table 2: siRNA sequences

EXO1	UGCCUUUGCUAAUCCAAUCCCACGC
CDK1	CCUAGUACUGCAAUUCGGGAAAUUU
CDK2	CCUAUUCCCUGGAGAUUCUGAGAUU
CtIP	GGGUCUGAAGUG AACAGAUAUUA
DNA2	GCCUGCAUUCUAAAGGGUUUGAAUA

Supplementary Table 3: Antibody sources and dilutions

Antibody	Vendor	Catalog No.	WB	IF
V5	Invitrogen	R960-25	1:5000	1:1000
Phospho-(Thr) CDK substrate	Cell Signaling	2321	1:1000	
Phospho-(Ser) CDK substrate	Cell Signaling	9477	1:1000	
Phospho-Chk1 S317	Cell Signaling	2344	1:1000	
Chk1	Cell Signaling	2345	1:1000	
Phospho-Chk2 T68	Cell Signaling	2197	1:1000	
actin	Sigma	A-2066	1:1000	
Phospho-EXO1(T732)	custom (Abmart)		1:1000	
RPA	Calbiochem	NA19L-100ug		1:500
BrdU	Becton Dickinson	347580		1:500
Rad51	Santa Cruz	sc-8349		1:500
Cyclin A	Vector Laboratory, Santa Cruz	VP-C391/sc-751	1:1000	1:500
CDK1	Santa Cruz	sc-954	1:1000	
CDK2	Santa Cruz	sc-6248	1:1000	
Cyclin B	Santa Cruz	sc-752	1:1000	
53BP1	Santacruz	sc-22760	1:1000	1:2000
BRCA1	ThermoFisher	OP92	1:1000	
BLM	Bethyl	A300-110A	1:1000	
CtIP	Bethyl	A300-488A	1:1000	
Chk2	Bethyl	A300-620A	1:1000	
DsRed	Clontech	632496	1:1000	
GFP	Abcam	ab290	1:1000	
EXO1	ThermoFisher, Bethyl	MS1534P, A302-639A	1:1000	
Ku80	kind gift from Dr. B. Chen		1:1000	
HRP-conjugated secondary antibodies	Bio-rad	170-6515, 170-6516	1:3000	
Alexa488/568-conjugated secondary antibodies	Invitrogen	A-21202, A-11008, A-10037, A-11011, A-21235		1:1000