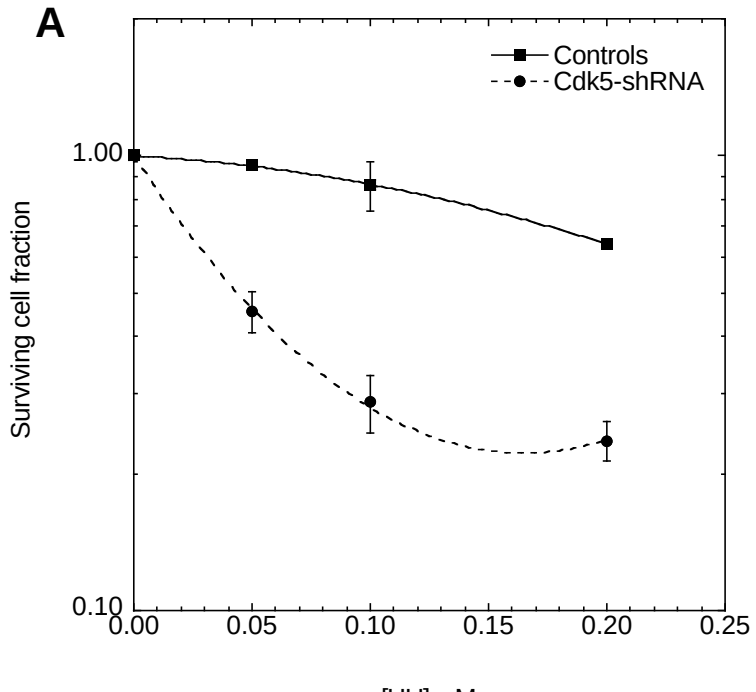
Figure S1



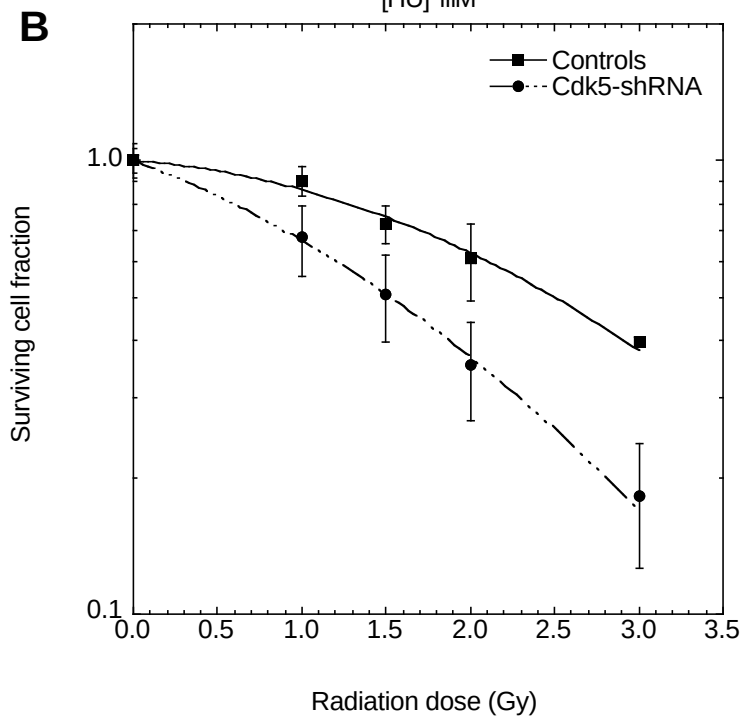


Figure S1: Clonogenic cell survival of U2OS Control and Cdk5-shRNA1499 cell lines to increasing doses of (A) Hydroxyurea, (B) ^{137}Cs gamma rays.

(A) Asynchronous cells were exposed to increasing concentrations of HU present in the culture medium until colony fixation **(B)** Asynchronous cells were irradiated and colonies were allowed to grow for 10-15 days. Data represents mean ± SD from two independent experiments in triplicate for all conditions.

Figure S2

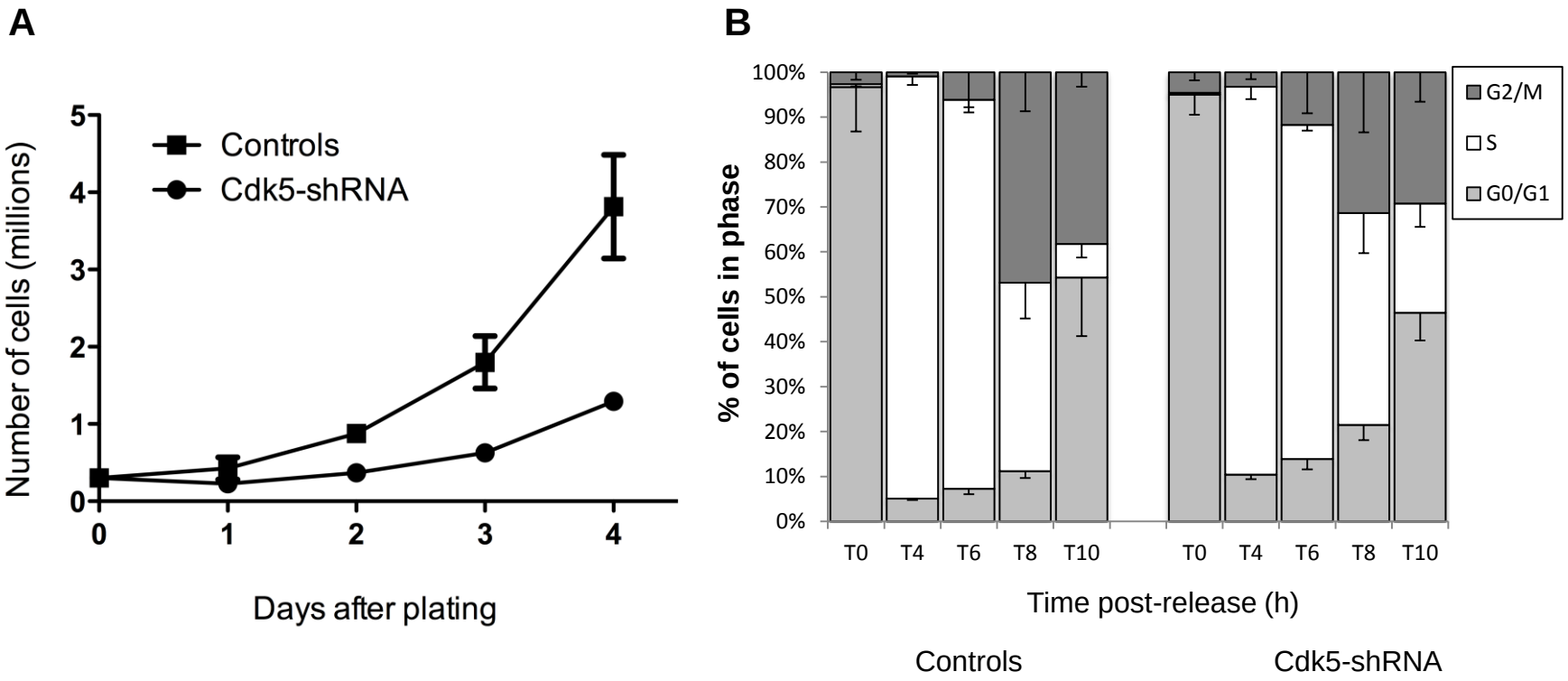
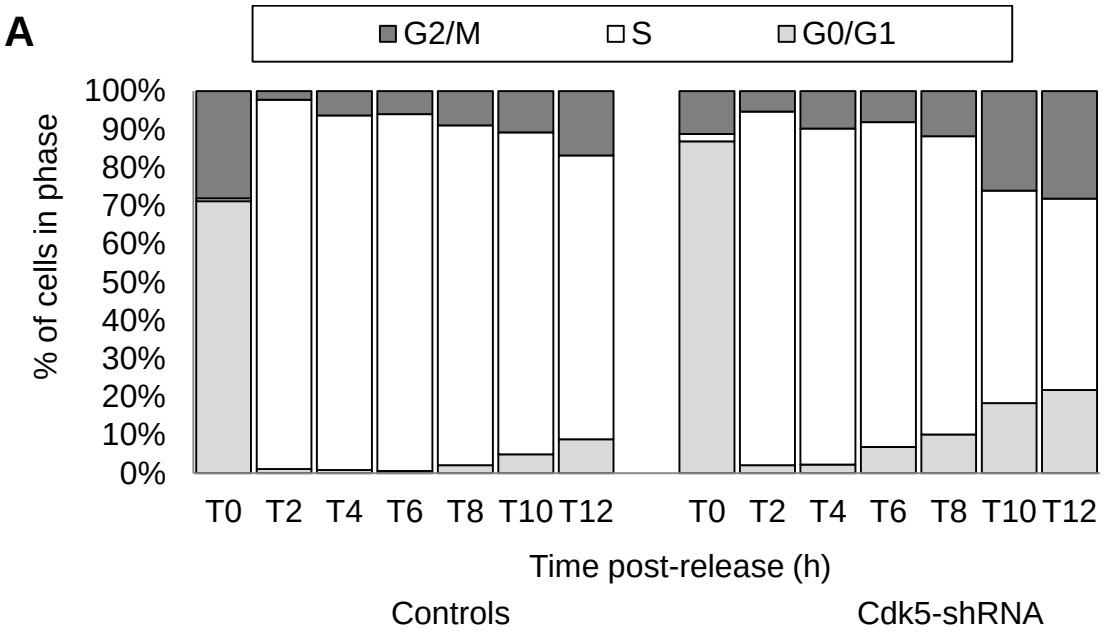


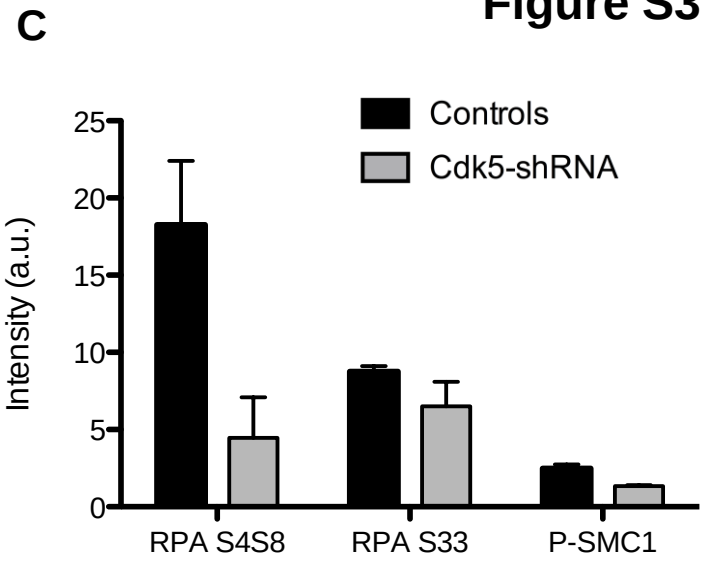
Fig S2: Reduced S-phase dynamics in the absence of the Cdk5 protein

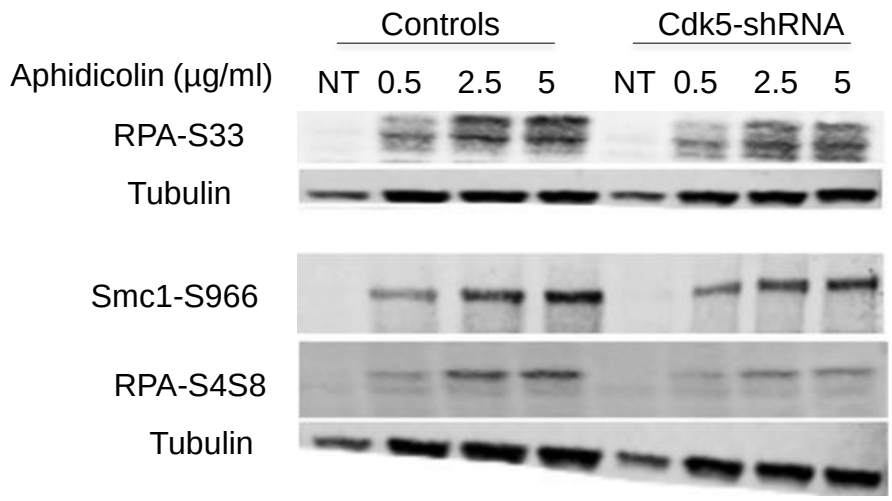
(A) Proliferation curves of Control and Cdk5-shRNA cells growing under normal growth conditions. 3.10^5 cells were plated in 25 cm^2 flasks and 3 flasks per time point were counted at the indicated times.

(B) In order to determine the cell cycle progression and the length of S phase, cells were synchronized by a double thymidine block then pulsed labeled with BrdU (10 µM/15 min) and analyzed by FACS at the indicated times. Data are mean ± SD from 3 independent experiments.

Figure S3


A
G2/M
S
G0/G1
% of cells in phase
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
T0 T2 T4 T6 T8 T10 T12
T0 T2 T4 T6 T8 T10 T12
Time post-release (h)
Controls
Cdk5-shRNA


C
Controls
Cdk5-shRNA
Intensity (a.u.)
25
20
15
10
5
0
RPA S4S8
RPA S33
P-SMC1


B
Controls
Cdk5-shRNA
Aphidicolin (µg/ml)
NT 0.5 2.5 5 NT 0.5 2.5 5
RPA-S33
Tubulin
Smc1-S966
RPA-S4S8
Tubulin

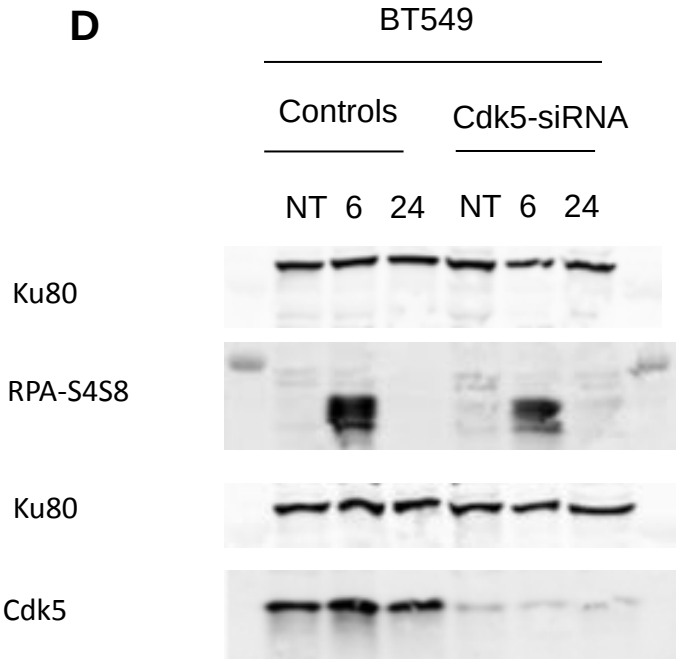

D
BT549
Controls
Cdk5-siRNA
NT 6 24 NT 6 24
Ku80
RPA-S4S8
Ku80
Cdk5

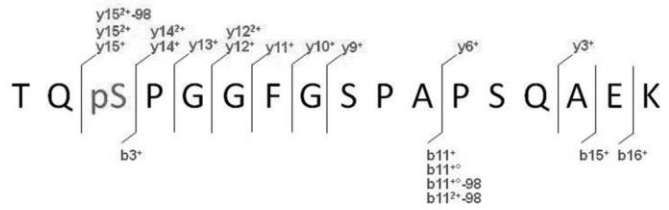
Fig S3: Cdk5-shRNA cells show a faster progression through S and G2 after exposure to Aphidicolin and reduced phosphorylation of RPA32-S33 and S4S8

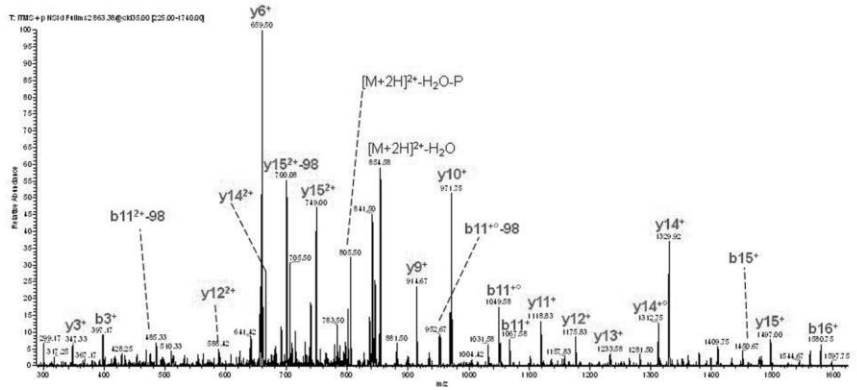
(A) Cells were treated with Aphidicolin (4µg/ml) (Sigma) for 24 h, released into fresh medium (0 h corresponds to 24 h of Aphidicolin treatment) then pulse labelled with BrdU (10 µM, 15 min) at different times post-release before collection. The percentage of Control and Cdk5-shRNA cells in G1, S and G2/M phases were determined by FACS analysis. Data presented is from one experiment. **(B)** Representative western blot analysis of phospho-SMC1 (S966), and phospho-RPA32 S4S8 and S33 in protein extracts from Control and Cdk5-shRNA cells treated for 24 h with 0.5/2.5/5 µg/ml of Aphidicolin. Total Tubulin was used as a loading control. **(C)** Quantification data are ratios of intensities from treated (24 h /2.5 µg/ml) *vs* non-treated cellular extracts, data are means ± SD from 2 independent experiments. **(D)** Representative western-blots of total cellular extracts from Control and Cdk5-siRNA BT549 cells after HU treatment, the protein extraction was done 6 or 24h after exposure to 2mM of HU for 24h.

Figure S4

A

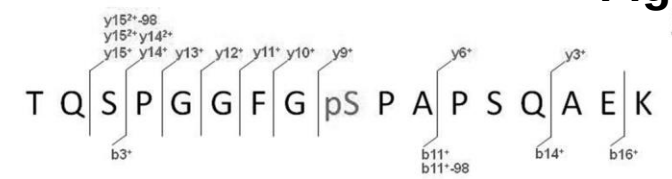
S23

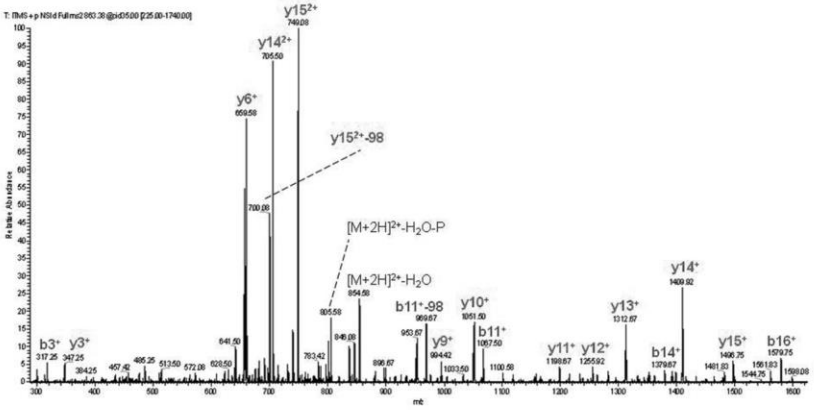




B

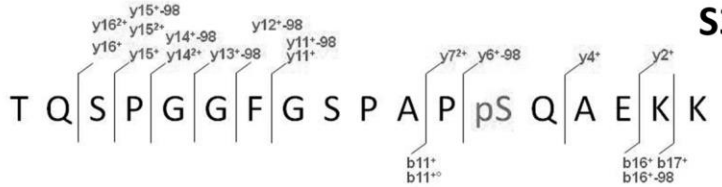
S29

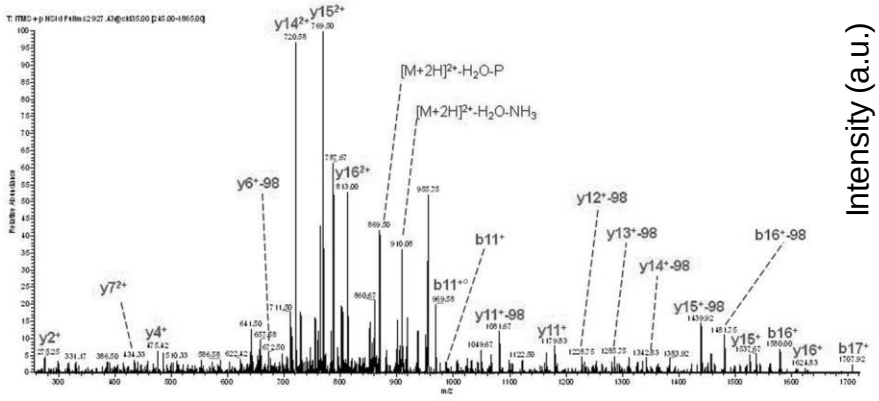




C

S33





D

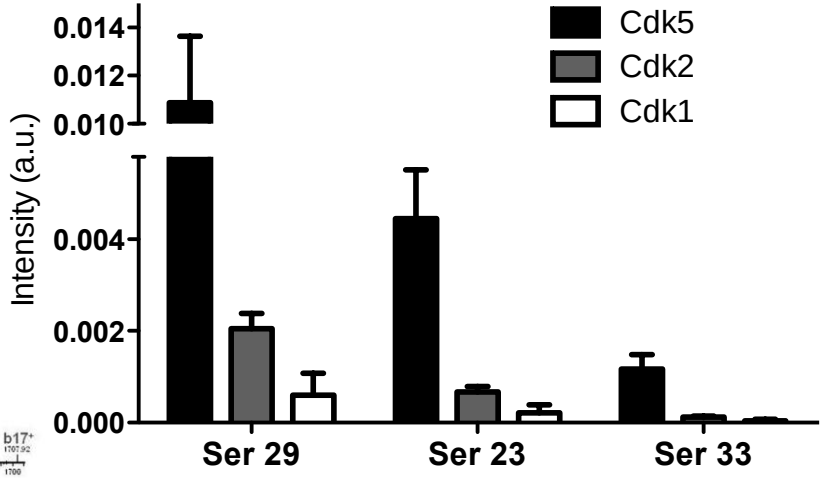
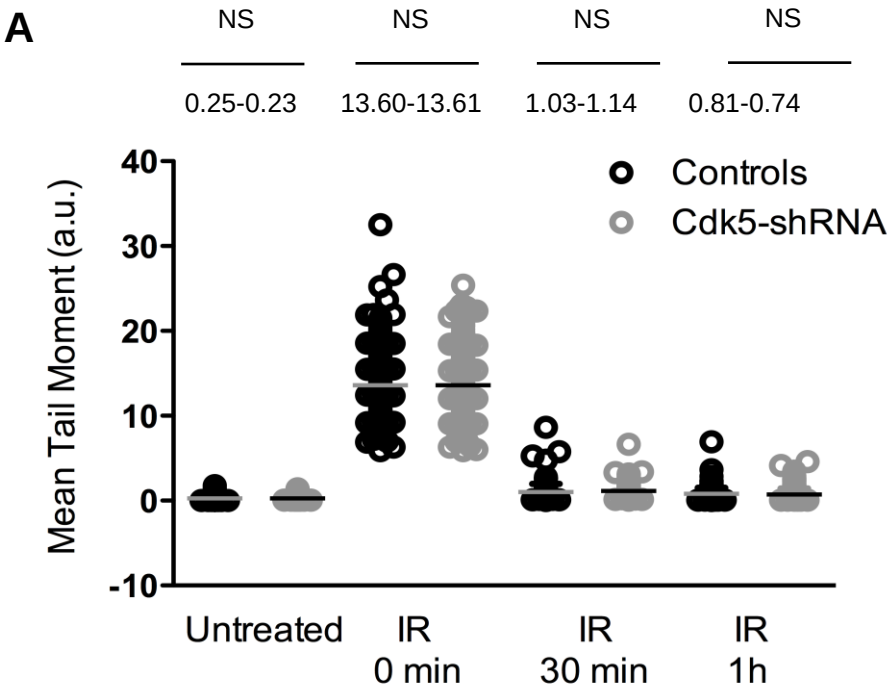


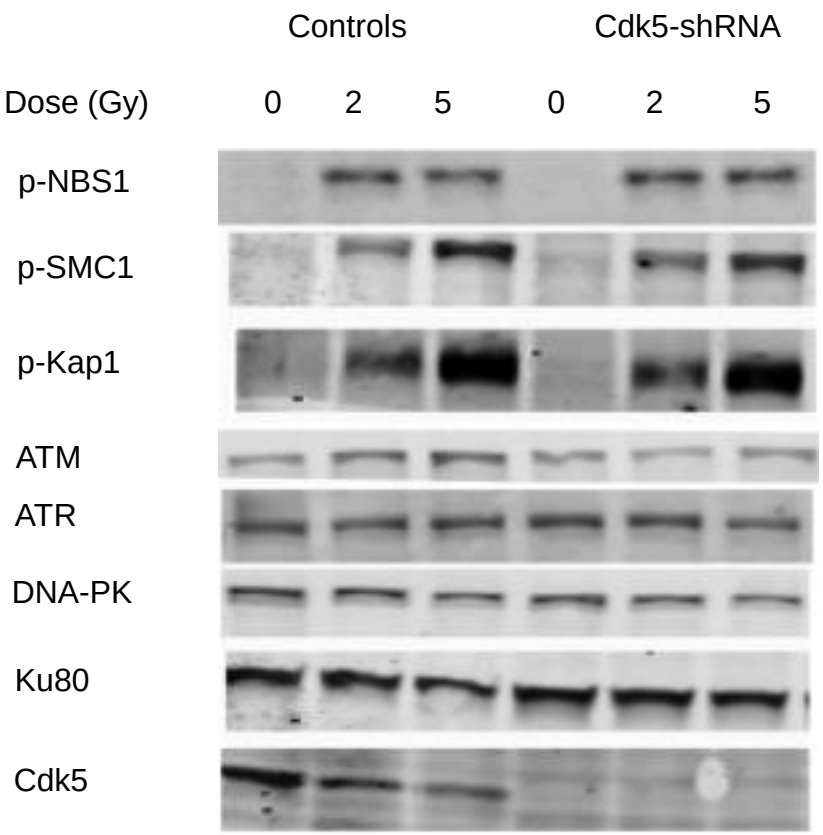
Fig S4: Identification of RPA phosphorylation sites by mass spectrometry

Liquid chromatography-MS/MS spectra (MSA enabled) of RPA32 phosphopeptides containing **(A)** Ser23 (TQpSPGGFGSPAPSQAEK: 863.38$^{(2+)}$*m/z*), **(B)** Ser29 (TQSPGGFGpSPAPSQAEK: 863.38$^{(2+)}$*m/z*) and **(C)** Ser33 (TQSPGGFGSPAPpSQAEKK: 927.43$^{(2+)}$*m/z*). The fragmentation spectra are trypsin/chymotrypsin derived peptides and show the peptide sequence based on the interpretation of the observed ions obtained. Tandem mass spectrum were labeled to show singly and doubly charged *b* and *y* ions, as well as ions corresponding to neutral losses of water (*H_2O, circles* °) and H3PO4 (*P, 98 Da*) groups; *M*: parent ion mass are shown.

(D) Quantification of the phosphorylation of RPA32 at positions Ser29, Ser23 and Ser33. The bar charts represent the relative phosphorylation intensity of RPA32 by Cdk5, Cdk2 and Cdk1. Data are means ± SD from four independent experiments.

Figure S5


A
NS
NS
NS
NS
0.25-0.23
13.60-13.61
1.03-1.14
0.81-0.74
Controls
Cdk5-shRNA
Mean Tail Moment (a.u.)
40
30
20
10
0
-10
Untreated
IR 0 min
IR 30 min
IR 1h


B
Controls
Cdk5-shRNA
Dose (Gy)
0 2 5 0 2 5
p-NBS1
p-SMC1
p-Kap1
ATM
ATR
DNA-PK
Ku80
Cdk5

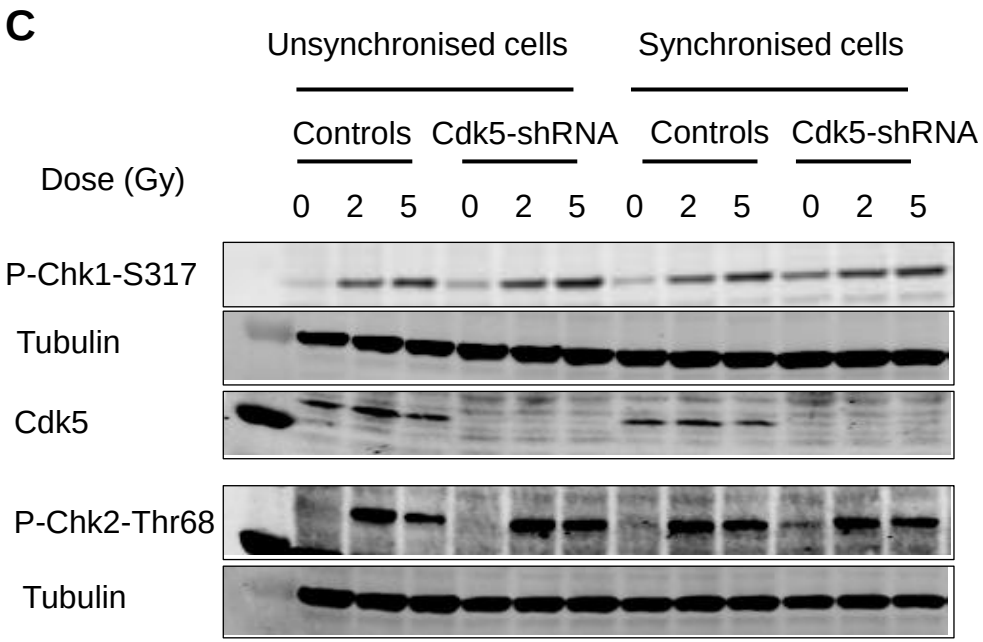

C
Unsynchronised cells
Synchronised cells
Controls
Cdk5-shRNA
Controls
Cdk5-shRNA
Dose (Gy)
0 2 5 0 2 5 0 2 5 0 2 5
P-Chk1-S317
Tubulin
Cdk5
P-Chk2-Thr68
Tubulin

Fig S5: DNA damage response after irradiation in Cdk5-shRNA *vs* Control cells

(A) DNA strand break formation and persistence measurement in the Cdk5-shRNA and Control cells using the alkaline Comet assay, immediately, 30 min and 1 h after exposure to gamma-rays (8 Gy) **(B)** Representative western-blots of total cellular extracts from Control and Cdk5-shRNA HeLa cells unsynchronised after irradiation. Protein extraction was done 1h after exposure to 2 or 5 Gy of IR. Ku80 was used as a control for protein loading. **(C)** Representative western-blots of total cellular extracts from Control and Cdk5-shRNA HeLa cells irradiated when synchronised in S-phase. Protein extraction was done 1h after exposure to 2 or 5 Gy of IR. Tubulin was used as a control for protein loading.

Figure S6

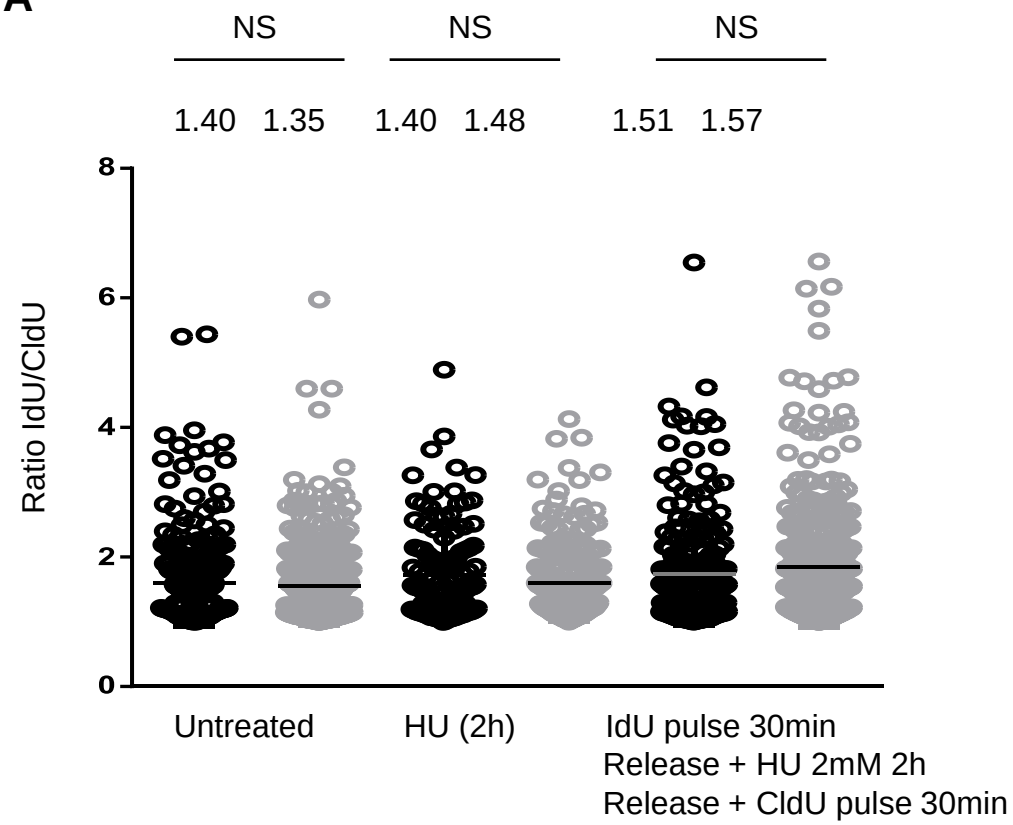


Fig S6: Distribution of asymmetric replication bubbles in Control and Cdk5-shRNA cells

The ratio of IdU/CldU was calculated for each replication fork. Distribution of ratios was analyzed as described previously[1] with no significant difference observed between the cells lines.

1. Techer H, Koundrioukoff S, Azar D, Wilhelm T, Carignon S, Brison O *et al*. Replication dynamics: biases and robustness of DNA fiber analysis. J Mol Biol 2013; 425: 4845-4855.

Supplemental Table 1: Characteristics of the 456 breast cancer relative to mRNA over-expression of Cdk5.

		Number of patients (%)		
	Total population (%)	CdK5 mRNA expression <=3.8 relative to normals	CdK5 mRNA expression >3.8 relative to normals	p^a
Total	456 (100.0)	322 (70.6)	134 (29.4)	
Age				
≤50	98 (21.5)	65 (66.3)	33 (33.7)	0.29 (NS)
>50	358 (78.5)	257 (71.8)	101 (28.2)	
SBR histological grade[b,c]				
I	58 (13)	45 (77.6)	13 (22.4)	0.21 (NS)
II	229 (51.2)	165 (72.1)	64 (27.9)	
III	160 (35.8)	106 (66.3)	54 (33.8)	
Lymph node status[d]				
0	119 (26.1)	73 (61.3)	46 (38.7)	0.024
1-3	237 (52.1)	173 (73.0)	64 (27.0)	
>3	99 (21.8)	76 (76.8)	23 (23.2)	
Macroscopic tumor size[e]				
≤25mm	223 (49.8)	167 (74.9)	56 (25.1)	0.070 (NS)
>25mm	225 (50.2)	151 (67.1)	74 (32.9)	
*ERα status**				
Negative	118 (25.9)	79 (66.9)	39 (33.1)	0.31 (NS)
Positive	338 (74.1)	243 (71.9)	95 (28.1)	
*PR status**				
Negative	194 (42.5)	130 (67.0)	64 (33.0)	0.15 (NS)
Positive	262 (57.5)	192 (73.3)	70 (26.7)	
*ERBB2 status**				
Negative	357 (78.3)	261 (73.1)	96 (26.9)	0.026
Positive	99 (21.7)	61 (61.6)	38 (38.4)	
Molecular subtypes				
RH- ERBB2-	68 (14.9)	44 (64.7)	24 (35.3)	0.032
RH- ERBB2+	45 (9.9)	30 (66.7)	15 (33.3)	
RH+ ERBB2-	289 (63.4)	217 (75.1)	72 (24.9)	
RH+ ERBB2+	54 (11.8)	31 (57.4)	23 (42.6)	
KI67 mRNA expression				
median	12.5 (0.80-117)	11.5 (0.85-77.8)	17.2 (0.80-117)	0.00000011 [f]
Relapse				
No	287 (62.9)	213 (74.2)	74 (25.8)	0.028
Yes	169 (37.1)	109 (64.5)	60 (35.5)	

a χ^2Test, b Scarff Bloom Richardson classification, c Information available for 447 patients, d Information available for 455 patients, e Information available for 448 patients,f Kruskall Wallis's H test, NS: non-significant, * Estrogen receptor (ER), progesterone receptor (PR) and ERBB2 status was determined at the protein level by biochemical methods (dextran-coated charcoal method, enzymatic immuno-assay or immunohistochemistry) at the time of surgery and confirmed by *ERα, PR* and *ERBB2* real-time qRT-PCR assays[1,2].

Supplemental Table 2: Primers and RT-qPCR conditions and Cdk5 siRNA

Sequences for Cdk5 siRNA
5’ CAACAUCCCUGGUGAACGUTT 3’
3’ TTGUUGUAGGGACCACUUGCA 5’

Sequences for primers RTqPCR
Cdk5 - U: 5’-CAAGCTGCCAGACTATAAGCCCTA-3’
Cdk5 - L: 5’-TGCAGCAGATCCCTCCCTGT-3’

TBP - U:5’-TGCACAGGAGCCAAGAGTGAA-3’
TBP – L:5’CACATCACAGCTCCCCACCA-3’

PCR conditions
95°C 10 min ; (95°C 15 sec 65°C 1 min) 50 cycles.

Supplemental Table 3 : Details of antibodies

Antibody	Supplier
Cdk5 (Py15)	Epitomics (1947-S)
RPA 34 (9H8)	Pierce (MA1-26418)
p.RPA S33	Bethyl (A300-246A)
p.RPA S4S8	Bethyl (A300-245A)
p.Chk1 317	Bethyl (A300-163A)
p.Chk2 T68	Cell Signaling (2661)
Rad51 (H-92)	Santa Cruz (SC-8349)
Phospho-p95/NBS1 (Ser343)	Cell Signaling (3001)
p.KAP1 S824	Abcam (ab70369)
ATR Antibody (N-19)	Santa Cruz (sc-1887)
ATM	Novus (NB100-104)
DNA-PK	BioLegend (612701)
KU80	Abcam (ab87860)
H2AX (Ser139) FITC conjugated	Euromedex (16-202A)
p.SMC1 S966	Bethyl (A300-050A)
ssDNA	Milipore (MAB3034)
BrdU (Monoclonal Antibody) Rat Clone: BU1/75	Ab Serotec (OBT0030)
Anti-BrdU FITC (clone B44)	BD Bioscience (347583)

Supplementary References

1 Bieche I, Onody P, Laurendeau I, Olivi M, Vidaud D, Lidereau R *et al*. Real-time reverse transcription-PCR assay for future management of ERBB2-based clinical applications. Clinical chemistry 1999; 45: 1148-1156.

2 Bieche I, Parfait B, Le Doussal V, Olivi M, Rio MC, Lidereau R *et al*. Identification of CGA as a novel estrogen receptor-responsive gene in breast cancer: an outstanding candidate marker to predict the response to endocrine therapy. Cancer Res 2001; 61: 1652-1658.

Supplementary Experimental Procedures

Mass Spectrometry Analyses

After incubation of RPA32 with Cdk5, proteins were separated on SDS–PAGE gels stained with LabSafe GEL Blue Stain (G-Biosciences) and the RPA bands excised. Gel slices were washed and proteins reduced with 10 mM dithiothreitol prior to alkylation with 55 mM iodoacetamide. After washing and shrinking the gel pieces with 100% acetonitrile (MeCN), an in-gel digestion was performed using trypsin (Promega) and adding Chymotrypsin (Sequencing Grade, Promega) after 1 h in 25 mM NH_4HCO_3 at 37°C and continuing the digest for another 4 additional hours. The extracted peptides were analysed by nano-liquid chromatography tandem mass spectrometry using an Ultimate 3000 system (Dionex S.A.) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Samples were loaded onto a C18 precolumn (300 µm inner diameter x 5 mm; Dionex) at 20 µl/min in 2% MeCN, 0.1% trifluoroacetic acid. After 3 min of desalting, the precolumn was switched with the analytical C18 column (75 µm i.d. x 50 cm, packed with C18 PepMap™, 3 µm, 100 Å; LC Packings) equilibrated in solvent A (2% MeCN, 0.1% HCO_2H). Bound peptides were eluted using a two-step linear gradient of 157 min (from 0 to 30%) and 20 min (from 30 to 50%) of solvent B (80% MeCN, 0.085% HCO_2H) at a 150 nl/min flow rate and an oven temperature of 40°C. A LTQ-Orbitrap mass spectrometer was used in the positive ion mode and Survey MS scans acquired in the Orbitrap on the 350-1000 m/z range with the resolution set to a value of 100,000. We recalibrated each scan in real time by co-injecting an internal standard from ambient air into the C-trap. We selected the five most intense ions per survey scan for collision induced fragmentation and analyzed the resulting fragments in the linear trap (LTQ) and, if a neutral loss of 98 Da from the precursor ion was observed, then we enabled a multistage activation (MSA). Target ions already selected for MS/MS were dynamically excluded for 30 s. We acquired data using the Xcalibur software (v 2.2) and the resulting spectra were interrogated by MascotTM and SEQUEST® through Proteome Discoverer (v 1.4, Thermo Scientific) with the SwissProt Homo sapiens database. We set carbamidomethylation of Cys, oxidation of Met, N-terminal acetylation, phosphorylation of Ser and phosphorylation of Thr as variable modifications for all searches. We set specificity of trypsin and chymotrypsin digestion and allowed two missed cleavage sites and we set the mass tolerances in MS and MS/MS to 2 ppm and 0.5 Da, respectively. The resulting Mascot and Sequest files were further processed using myProMS[1]. We validated phosphorylated peptides by combining phosphoRS analysis and by manually inspecting the peak assignment. The same procedure was used to analyse the phosphorylation of RPA32 by Cdk1 and Cdk2.

Label-free Quantification

To quantify the phosphorylated peptides, we extracted from the MS survey of nano-LC-MS/MS raw files the extracted ion chromatogram (XIC) signal of the well characterized tryptic peptide ions using the Pinpoint^{TM} (version: 1.2, Thermo Scientific) software. We integrated XIC areas in Xcalibur under the QualBrowser interface using the ICIS algorithm. We calculated Mean values of normalized areas by using the total RPA 32 kDa signal (all RPA 32 kDa peptide ions). We calculated mean values and S.D. for four replicate measurements of independent experiments.

1. Poullet P, Carpentier S, Barillot E. myProMS, a web server for management and validation of mass spectrometry-based proteomic data. Proteomics 2007; 7: 2553-2556.