Supplemental Information

Non redundant functions of ATM and DNAPK in response to DNA Double-Strand Breaks.

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Experimental Procedures

AID-DIvA 53BP1GFP stable cell line

The pEGFP-53BP1 plasmid (kindly provided by Dr D. Jullien, ITAV, Toulouse) was transfected into AID-DIvA cells by using the Cell Line Nucleofactor kit V (Amaxa) and selection was performed with1µg/mL puromycine. Monoclonal, stable cell lines were selected based on 53BP1-GFP expression level and 4OHT response.

Cell culture

DIvA (AsiSI-ER-U20S), AID-DIvA (AID-AsiSI-ER-U20S) and AID-DIvA 53BP1-GFP cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics, 10% FCS (InVitrogen) and either 1 µg/mL puromycin (DIvA and AID-DIvA 53BP1-GFP cells) or 800 µg/mL G418 (AID-DIvA cells; AID-DIvA 53BP1-GFP) at 37°C under a humidified atmosphere with 5% CO2. Primary MEFs were isolated from embryos at day 13 p.c. and cultured at 37°*C*, 5% CO_2, 3% O_2 in Dubelcco's Modified Eagle's Medium (DMEM) supplemented with glutamine, penicillin, streptomycin, 15% FCS and non-essential aminoacids. Experiments were carried out between P1 and P5.

For AsiSI-dependent DSB induction, cells were treated with 300 nM 4OHT (Sigma; H7904) for 4h. When indicated, 4OHT treated cells were washed 3 times in pre-warmed PBS and further incubated with 500 µg/mL auxin (Sigma; I5148). KU55933 and Nu7441 (Tocris Bioscience) were respectively used at the final concentrations of 20µM and 2µM. ETP-46464 (ATRi) was a kind gift from Dr O. Fernandez Capetillo, and used at a final concentration of 5µM. Rapamycin (mTORi) and SP600125 (JNKi) were respectively used at 20nM and 50µM Inhibitors were added to the medium one hour before the addition of 4OHT and during the 4h stage of break induction (4OHT) and/or during the repair step (auxin) as indicated.

ATM and DNAPK siRNAs transfection was performed using the Amaxa® Cell Line Nucleofector® Kit V, and program X001for 48h hours followed by a 4h 4OHT treatment. The following siRNA were used: DNAPK: GGG-CGC-UAA-UCG-UAC-UGA-tt, ATM: GCC-UCC-AGG-CAG-AAA-AAG-Att

Clonogenic assays

AID-DIvA cells were seeded at a clonal density in 10 cm diameter dishes. The next day, cells were treated with 300 nM 4OHT for 4 hours and, when indicated, washed 3 times in pre-

warmed PBS and further incubated with 500 µg/mL auxin in presence of the inhibitors or not, for another 4 hours. After 3 washes in pre-warmed PBS, complete medium was added to each dish. After 7 to 10 days, cells were stained with crystal violet (Sigma) and counted. Only colonies containing more than 50 cells were scored.

Western Blot

Western blot analysis was performed using NuPAGE Tris-acetate 3-8% gels and reagents (Invitrogen) according to the manufacturer's indications. Briefly, cells were rinsed twice with ice-cold PBS and then lysed in the appropriate lysis buffer with sample reducing agent (Invitrogen). Liquid transfer of resolved proteins was performed onto PVDF membranes (Invitrogen). After 1 h block in 5% non-fat dry milk / 0.5% PBS-Tween, membranes were incubated overnight with anti-γH2AX antibody (Cell Signaling, 2577s, 1/500) and anti-α-tubulin (Sigma, DM1A, 1/50000). Validations for these antibodies are available on the manufacturers' websites. Horseradish peroxidase-coupled secondary antibodies were from Amersham Biosciences, and the chemiluminescence Lumilight reagent was from Roche Diagnostic.

Chromatin immunoprecipitation

ChIP assays were carried out according to the protocol described in (Iacovoni et al., 2010). Briefly, 200 µg of chromatin was immunoprecipitated by using 2 µg of anti-γH2AX (Epitomics, 2212-S), anti-P-ATM S1981 (10H11 Cell Signaling), anti-P-DNAPK S2056 (Abcam ab 18192) or without antibody (mock). P-ATM and P-DNAPK antibodies were validated in immunostaining and ChIP (Figure S2). Immunoprecipitated DNA and input DNA were analyzed in duplicate by RT-qPCR using the following primers DSB1 FW TGCCGGTCTCCTAGAAGTTG, REV GCGCTTGATTTCCCTGAGT; DSB2 FW GATTGGCTATGGGTGTGGAC, REV CATCCTTGCAAACCAGTCCT. IP efficiencies were calculated as the percent of input DNA immunoprecipitated.

ChIP chip and analysis

For ChIP-chip, 8ng of inputs and samples were amplified as in (Iacovoni et al., 2010), labelled and hybridized on Affymetrix tiling arrays covering human chromosomes 1 and 6. ChIP samples were normalized against the input samples using the Tiling Array analysis Software (bandwidth 300bp), converted to .WIG files using R/Bioconductor software, when necessary, and visualized using the Integrated Genome Browser (bioviz.org)

To plot data with respect to the 24 DSBs induced on chromosome 1 and 6 (Iacovoni et al., 2010), AsiSI site positions were retrieved from the human genome (hg18) and average ChIP-chip signal was computed for 200bp windows spanning 40kb (P-ATM and P-DNAPK) or 2Mb (γH2AX) surrounding each annotated AsiSI site.

For heatmap representations, average ChIP-chip signal was determined in 500 bp bins for P-ATM and P-DNAPK, and 50 kb bins for γH2AX, centered on each cleaved AsiSI site using custom R/Bioconductor scripts. The resulting matrix was represented as a heatmap using Java Treeview (http://www.jtreeview.sourceforge.net). DSBs were ordered based on the overall γH2AX level in untreated cells (Figure 3E) or the overall P-ATM levels in 4OHT treated cells (Figure 2E).

Repair kinetics and repair fidelity at AsiSI sites.

Repair kinetics at specific AsiSI induced DSBs were measured as described in (Aymard et al., 2014) by a cleavage assay permitting the capture of unrepaired DSBs, at the indicated times after auxin addition. The full procedure for the cleavage assay has been previously described (Chailleux et al., 2014). Briefly, a biotinylated double-stranded oligonucleotide, cohesive with AsiSI sites, was ligated *in vitro* to genomic DNA after break induction. T4 ligase was heat inactivated at 65°C for 10 min and DNA was fragmented by *Eco*RI digestion at 37°C for 2 h, followed by heat inactivation at 70°C for 20 min. After a preclearing step, DNA was pulled down with streptavidin beads (Sigma) at 4°C overnight, and then washed 5 times in RIPA buffer and twice in TE. Beads were resuspended in 100 µL of water and digested with *Hind*III at 37°C for 4 h. After phenol/chloroform purification and precipitation, DNA was resuspended in 100 µL of water. Precipitated DNA was quantified for each site by RT-qPCR using the following primers: NHEJ1: FW TCCCCTGTTTCTCAGCACTT, REV CTTCTGCTGTTCTGCGTCCT; NHEJ2: FW GGAAGGAGGGGCTACTAGGG, REV GAAAGCCCCATTCAGTTTGA; NHEJ3: FW ATCGGGCCAATCTCAGAGG, REV GCGACGCTAACGTTAAAGCA; HRIII: FW CCGTCCGTTACGTAGAATGC, REV GGGCGGGGATTATGTAATTT; DSB Figure S1C FW GATTGGCTATGGGTGTGGAC, REV CATCCTTGCAAACCAGTCCT.

For fidelity assays, DIvA cells were treated with 4OHT to induce DSBs for 4h (300nM) followed by an auxin treatment for 4h, in the presence or absence of ATMi (KU55933). The next day, cells were treated again with 4OHT for 4 hours. DNA was extracted and subjected to a cleavage assay as described above.

Immunofluorescence

Detailed methods for immunofluorescence against γH2AX (JBW301) in DIvA cells have already been described in (Iacovoni et al., 2010). XRCC4 staining was performed according the protocol described in (Britton et al., 2013). Briefly cells grown on coverslips were incubated twice six minutes in a CSK Buffer (10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) supplemented with TritonX100 0.7% and 0.3mg/ml Rnase A, After PBS washes, cells were fixed with 4% paraformaldehyde and subjected to immunostaining using a standard protocol (Iacovoni et al., 2010). Both γH2AX (mouse JBW301) and XRCC4 antibodies (Abcam ab145) were used at a dilution of 1:1000. All secondary antibodies were purchased from molecular probes. Image acquisition was performed using MetaMorph on a wide field microscope equipped with a with a cooled charge-coupled device camera (CoolSNAP HQ2), using either a 100X objective (for details) or a X40 objective (for quantification). Quantification were performed using plugins in Image J, measuring the percentage of each nuclei positively stained (above a defined threshold).

For immunofluorescence in MEF, cells were grown on coverslips for 7 days until confluency arrested, preincubated for 30 min with the required inhibitor, treated as indicated and fixed 10 min in ice-cold methanol. Fixed cells were then permeabilized (2 min in PBS-0.2% Triton X-100), blocked (30 min in PBS-5% BSA), incubated with the γH2AX primary antibody for 1 h in PBS-1% BSA (Millipore, 05-636), washed (3x 5 min in PBS-0.1% Tween 20), incubated for 30 min with the corresponding AlexaFluor-conjugated secondary antibody (1/1000 dilution in 1% BSA-PBS) and washed again as described above. Finally, they were counterstained with DAPI (Sigma) and mounted in Vectashield (Vector Labs). γH2AX foci were manually counted (double-blind) in 40 cells from each experimental condition.

High resolution microscopy

Fluorescent widefield microscopy was used to produce 3D image of nucleus. The acquisition have been made with a Nikon eclipse Ti E with a 100x microscope objective lenses with numerical aperture equal to 1.49. EMCCD camera Evolve from photometrics has been used (16µm pixel size). For a best respect of Nyquist criterion 2.5 magnification lens was installed after the collection tube lens. High resolution deconvolution was performed using non linear

deconvolution called Richardson lucy algorithm with total variation (TV) regularization (Dey et al., 2006). The algorithm is implemented in the image J software deconvolution lab (http://bigwww.epfl.ch/algorithms/deconvolutionlab/). The TV regularization is a constraint which suppresses unstable oscillations while preserving object edges. TV regularization parameter from each image was determined with the method described in (Laasmaa et al., 2011). The experimental Point Spread Function of the microscope (PSF) used for inversion was an average from the 30 isolated experimental PSF. The zero frequency components of the noise was removed in the final PSF.

Ripley analysis

Spot distribution analysis of γH2AX has been performed in 2D. Spots were identified as performed in (Feuerbach et al., 2002), on a selected slice of an N-SIM acquired image using the spot detector based on Decimated Wavelet Transform available as a plug-in in Icy. A spot detector scale value of 2 was used together with a threshold based on the individual experiment. The spatial analysis used Ripley's K function to robustly analyze the spatial organization of γH2AX foci. The Ripley K function allows counting the number of γH2AX points that are closer than a parameter *r*. For positive values of the Ripley's K function foci are clustered, while for negative values of the K function, foci are considered as dispersed. For a value around 0, the distribution is considered to be random, following a poisson distribution. Real cluster sizes can be directly inferred from the maximum of the Ripley function by the relation: $Rc \approx \frac{r_{max}}{1.3}$

Time lapse microscopy

Live cells analysis was performed in a 2-well chamber (Lab-Tek) in 1mL of DMEM supplemented with antibiotics, 10% FCS (InVitrogen) and 25mM Hepes (InVitrogen), at 37°C. Confocal Microscopy was performed with an Andor Revolution Nipkow-disk confocal system installed on a Olympus IX-81, featuring a CSU22 confocal spinning disk unit (Yokogawa) and an EMCCD camera (DU 888, Andor). The system was controlled using the mode "Full but not XY" of Andor Revolution IQ1 software (Andor). Images were acquired using an Olympus 100x objective (Plan APO, 1.4 NA, oil immersion). Single laser lines used for excitation were diode pumped solid state lasers (DPSSL) exciting GFP fluorescence at 488 nm (50 mW, Coherent) and Semrock bi-bandpass emission filter (Em01-R488/568-15). Pixel size was 65 nm. For 3D analysis, Z-stacks images with a 250-nm Z-step were acquired.

Exposure time was adjusted for each individual experiment, but usually set at 100 ms. Txt. files describing the used parameters (numbers of stacks, total time of acquisition, time lapse between images, exposure time, as well as the time after 4OHT treatment) are linked to each Supplemental 4D movies files are available upon request. For illustration shown Figure S5C, maximum projections using Image J were performed to generate 2D movies.

Movie S1, Related to Figure 5:

Time lapse microscopy was performed on 4OHT treated 53BP1-GFP DIvA using a spinning disk confocal microscope. Acquisition was started straight after 4OHT treatment, all z stacks were acquired every 2 minutes. Maximum projection was computed with Image J. Full 4D movies are available upon request.

Movie S2, Related to Figure 5:

Same as Movie S1

Movie S3, Related to Figure 5:

Same as Movie S1, except that acquisition was performed after 2h of 4OHT treatment and every 15 seconds. A magnification of 53BP1 foci ongoing clustering is presented. Related to Figure S5C bottom sequence.

Movie S4, Related to Figure 5:

Same as Movie S1, except that acquisition was performed after 2h of 4OHT treatment and every 3 minutes. Related to Figure S5C top sequence.

Movie S5, Related to Figure 5:

Same as Movie S1, except that acquisition was performed after 2h of 4OHT treatment and every 3 minutes.

Figure S1, Related to Figure 1

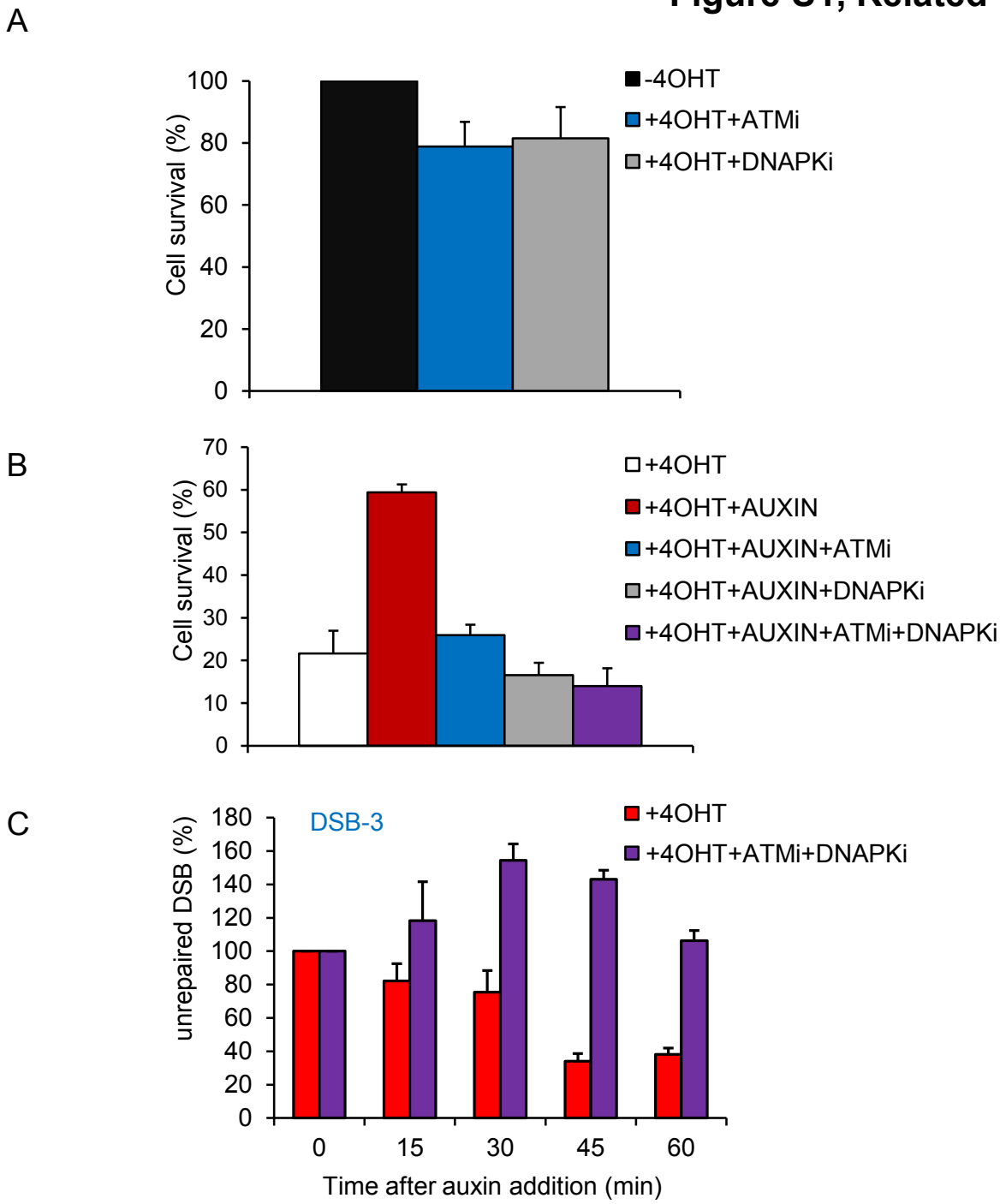

Figure S1: Effect of PI3K inhibitors on cell survival and DSB repair kinetics, related to Figure1
A. Clonogenic assays in AID-DIvA cells, in presence of ATM inhibitor (KU55933), DNAPK inhibitor (Nu7441) or without inhibitor, in the absence of damage induction. Colonies were counted ten days after treatments. Average and s.e.m of biological replicates are shown (n=3). B. Clonogenic assays in AID-DIvA cells after 4OHT treatment (4h), followed by auxin treatment (4h) in absence or presence of ATM inhibitor (KU55933), DNAPK inhibitor (Nu7441), or both ATM and DNAPK inhibitors as indicated. Colonies were counted ten days after 4OHT/auxin treatments. Average and s.e.m of biological replicates are shown (n=3). **C**. Cleavage assay in AID-DIvA cells treated with 4OHT (4h) followed by auxin (4h), in absence or presence of ATM and DNAPK inhibitors, as indicated. Immunoprecipitated DNA was analyzed close to one DSB. The percentage of sites that remain broken after the indicated time of auxin treatment is presented. Average and s.e.m. (n=3, technical replicates) of a representative experiment are shown (out of 2 independent experiments).

Figure S2, Related to Figure 2

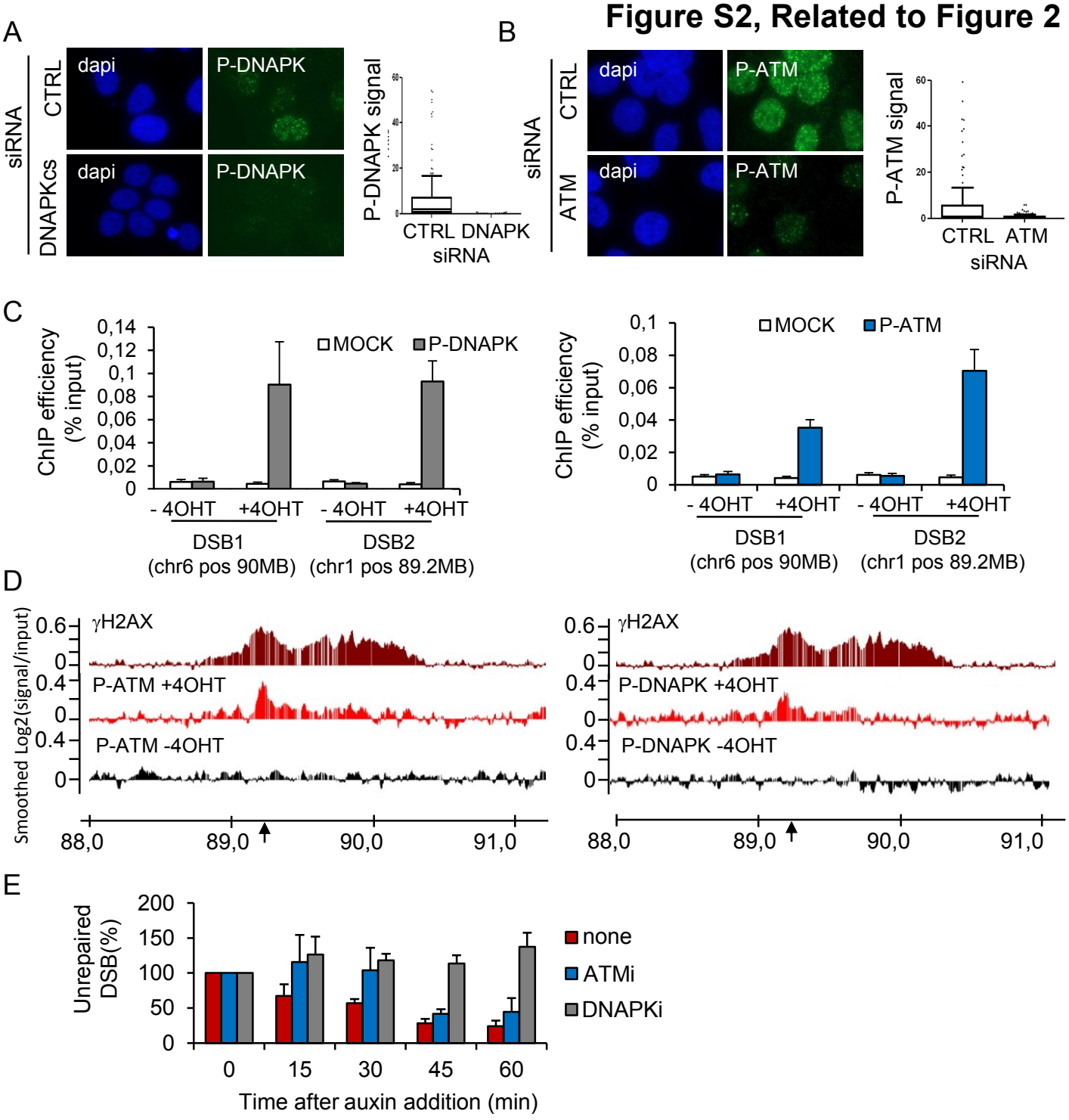

Figure S2: ChIP-chip profile of activated kinases, related to Figure2

A-B DIvA cells were transfected with a siRNA control or against DNA-PK, or ATM for 48h (as indicated), further treated for 4h with 4OHT and stained with the P-DNAPK (S2056) or P-ATM (S1981) antibodies. Quantification are shown on the right. **C.** ChIP against P-ATM (S1981) and P-DNAPK (S2056) were performed in 4OHT treated or untreated DIvA cells as indicated. Enrichment were assayed at two AsiSI induced DSB by qPCR. **.D** Profiles of both activated kinases before (in black) and after (in red) 4OHT treatment are shown at an AsiSI-induced DSB (indicated by black arrow), on the chromosome 1, where considerable spreading (on roughly 500kb) of the kinases was observed. Positions are indicated in megabases. As a comparison, the γH2AX profile is also shown (dark red, top panels). **E.** Repair kinetics was investigated at the above DSB, similarly to Figure 1B. Average and s.e.m. (n=3, technical replicates) of a representative experiment are shown (out of 3 independent experiments).

Figure S3, Related to Figure 4

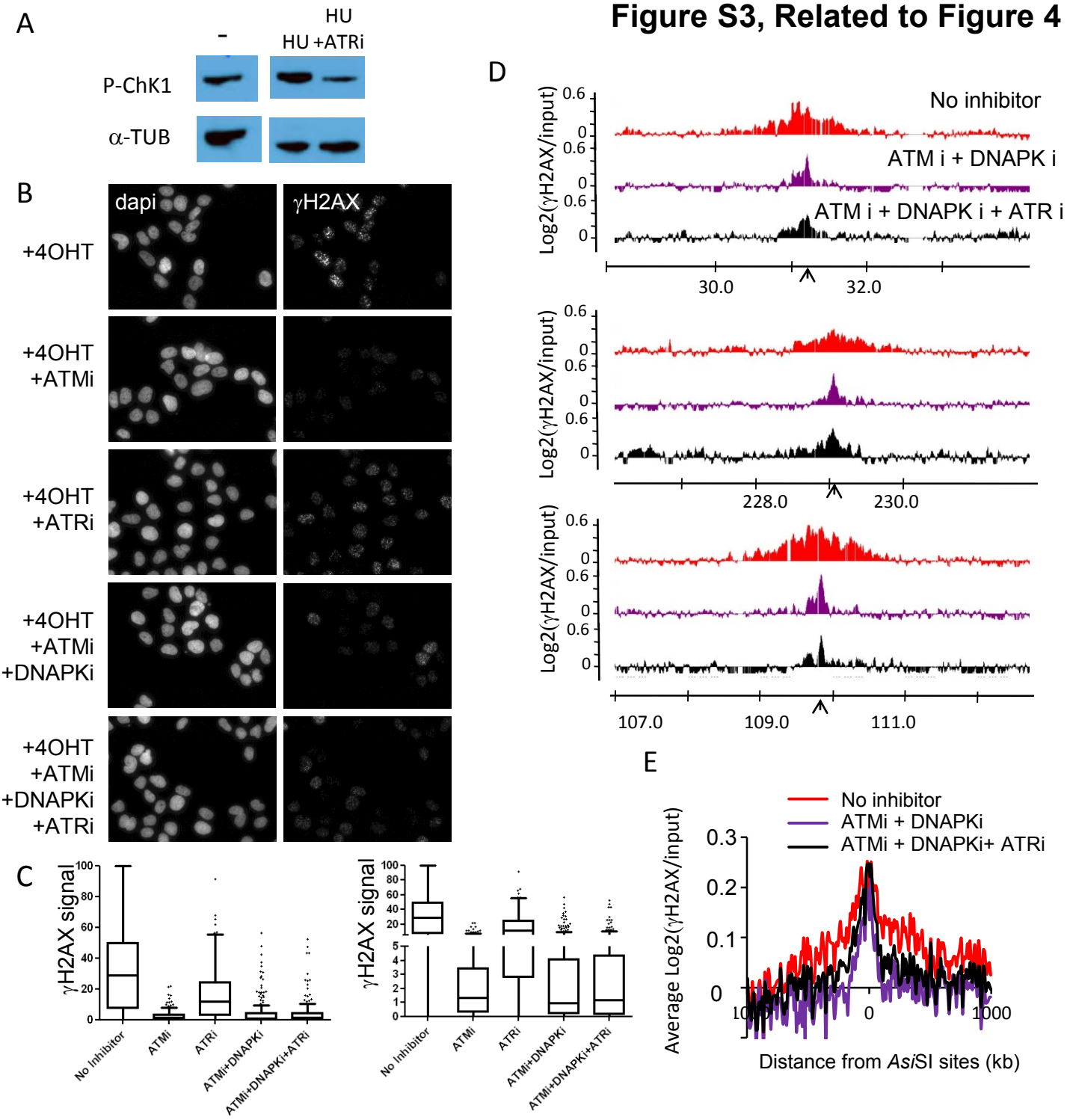

Figure S3: ATR does not mediate γH2AX in cells where ATM and DNAPK activities are impaired, related to Figure 4. A. DIvA cells were treated with HU and/or ATRi (ETP-46464) as indicated, and subjected to western blot against P-ChK1, to control ATR inhibition by the ATR inhibitor. **B.** γH2AX staining was performed in DIvA cells after 4OHT treatment (4h), in presence of ATR, ATM and DNAPKi inhibitors, as indicated. **C**. Quantification of the γH2AX staining presented Figure S3B were performed. Right panel: the same graph is presented with a modified y axis for better visualization of the ATMi, and combined inhibitors conditions. **D.** γH2AX ChIP-chip analyses in DIvA cells after 4OHT treatment (4h), in presence of ATM+DNAPK inhibitor or in presence of a combination of ATM, DNAPK and ATR inhibitors, as indicated. Profiles of γH2AX are shown at three AsiSI-induced DSBs (indicated by black arrows). **E**. Averaged γH2AX signals in 4OHT treated cells (in red) supplemented with ATM+DNAPK inhibitors (purple) or ATM+DNAPK+ATR inhibitors (black) over a two megabases region flanking cleaved AsiSI sites are shown.

Figure S4, Related to Figure 4

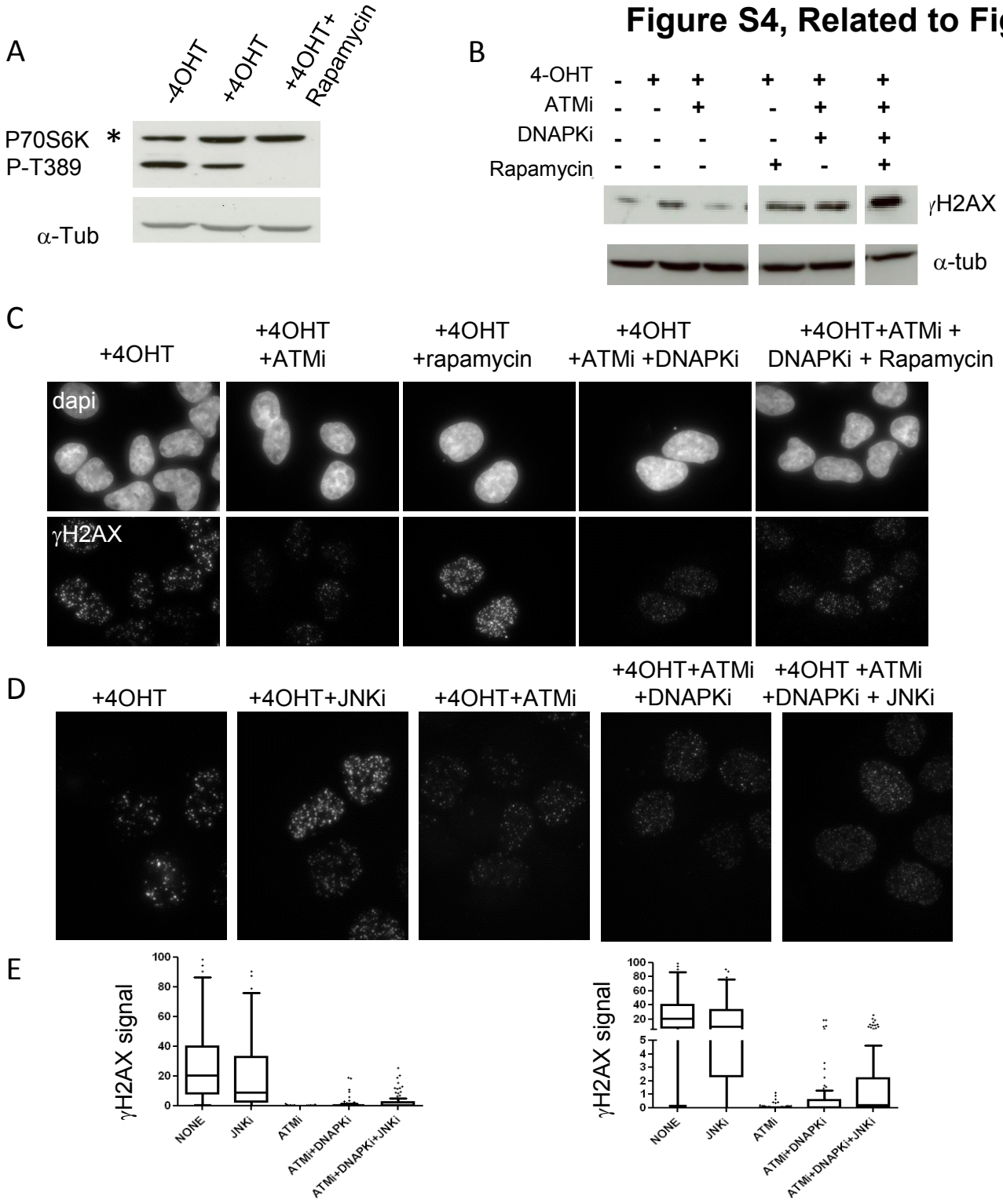

Figure S4: The mTOR pathway and c-Jun N-terminal Kinase (JNK) do not mediate γH2AX in cells where ATM and DNAPK activities are impaired, related to Figure 4.

A. DIvA cells were treated with 4OHT in presence or absence of rapamycin as indicated and subjected to western blot against P70S6K P-T389 (a known target for mTOR pathway, Cell Signalling, #9205) * non specific. **B.** DIvA cells were treated or not with 4OHT in presence of inhibitors as indicated, and subjected to western blot against γH2AX and alpha tubulin (loading control). **C**. DIvA cells were treated with 4OHT and inhibitors as indicated, and subjected to an immunostaining against γH2AX. **D.** DIvA cells were treated with 4OHT in presence or absence of the indicated inhibitors and subjected to γH2AX staining. **E.** Quantification of γH2AX signal. A representative experiment is shown. Right panel: same box plot except for the y axis, to better visualize variations between ATMi, ATMi+DNAPKi and ATMi+DNAPKi+JNKi conditions.

Figure S5, Related to Figure 5

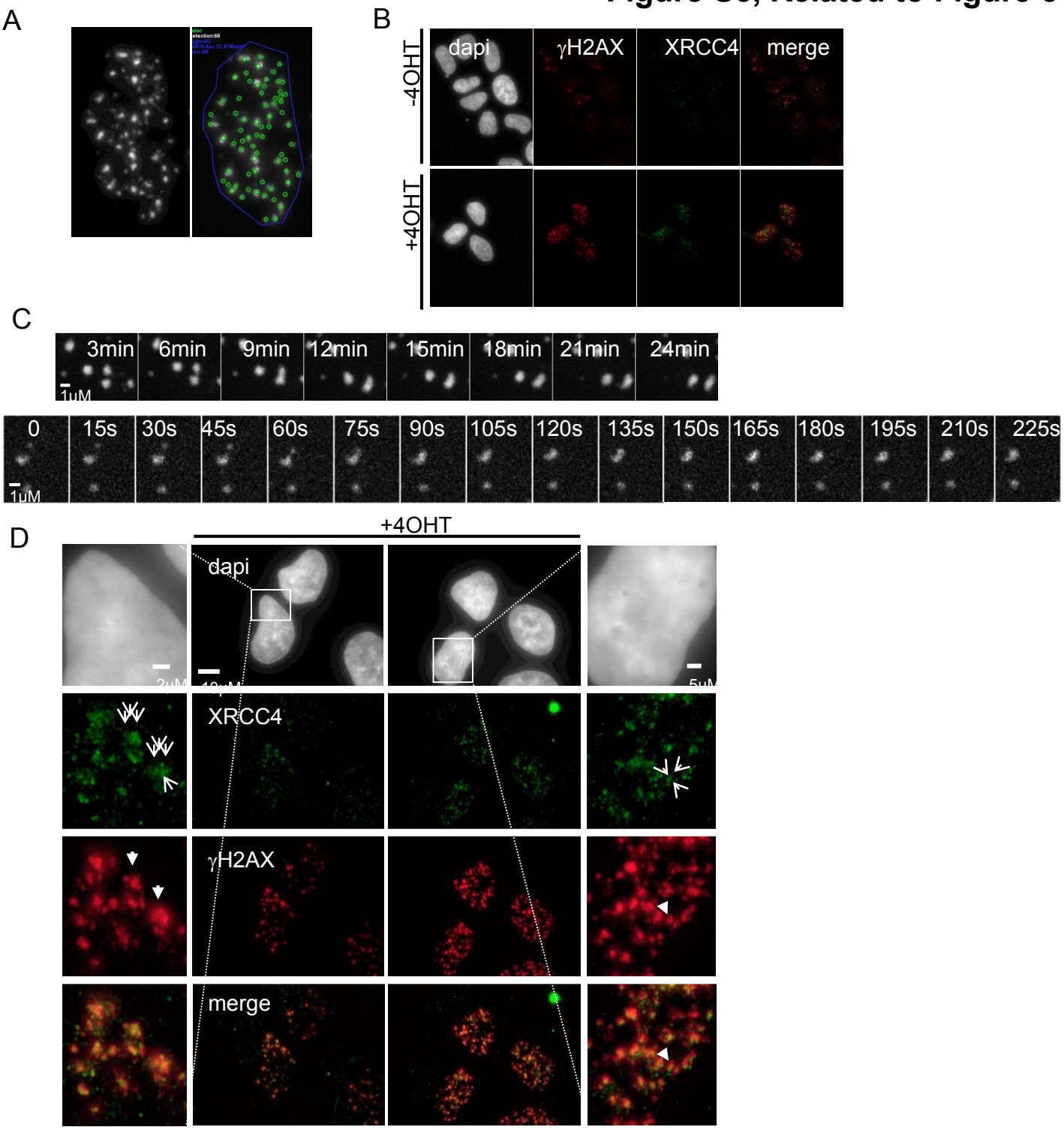

Figure S5: Analyses of AsiSI induced foci by microscopy, related to Figure 5

A. γH2AX staining in 4OHT treated DIvA cells revealed a reduced number of foci compared to the amount of γH2AX domains linearly depicted on chromosomes by ChIP-seq. Images were acquired using a wide field microscope, and foci were manually counted on more than 20 cells. **B**. γH2AX and XRCC4 staining were performed in 4OHT treated DIvA cells , untreated (left panels) or treated with 4OHT for 4 hours, according the protocol described in (Britton et al, 2013). **C**. Time lapse acquisition of 53BP1-GFP foci in 4OHT treated DIvA cells. Movies are available as Supplemental material. Legends for Movies are available in the Supplemental Experimental Procedure file. **D**. γH2AX and XRCC4 staining were performed in 4OHT treated DIvA cells, and images were acquired using a wide field microscope (X100 objective). Two magnifications are presented on left and right panels. Arrows indicates γH2AX foci that contain more than one XRCC4 spot.