Supplementary Information

for

γH2AX in the S Phase After UV Irradiation Corresponds to DNA Replication and Does Not Report on the Extent of DNA Damage

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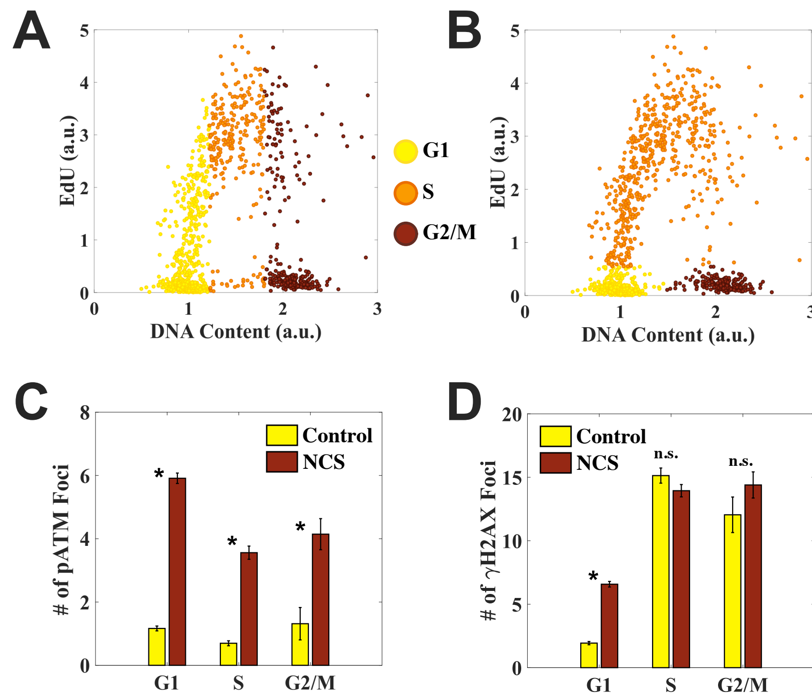
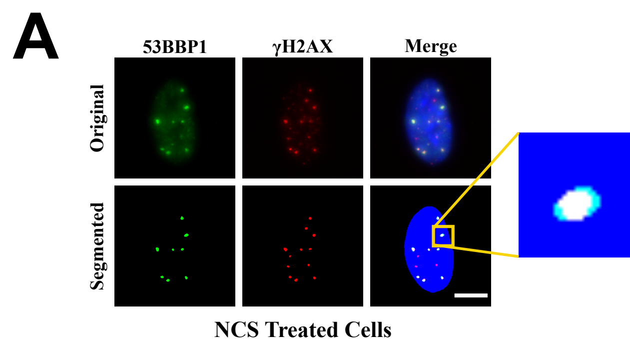
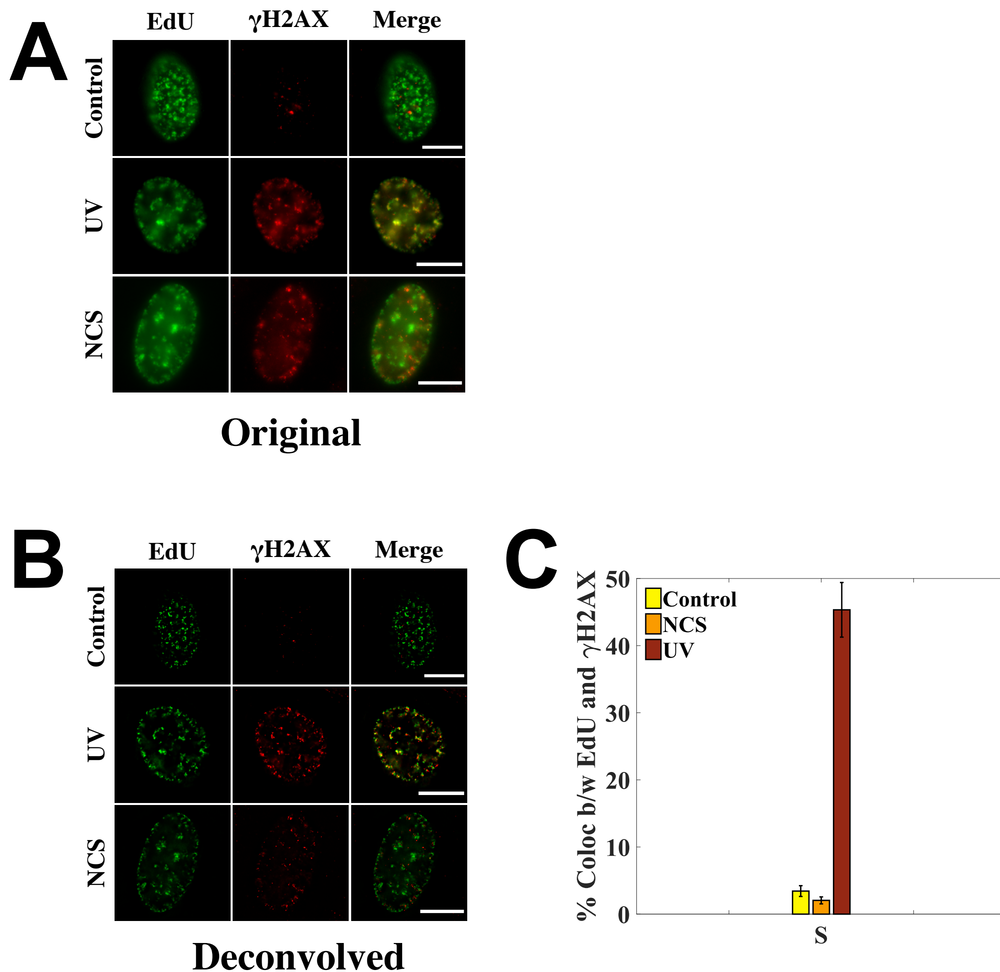
Running Title: γH2AX in the S phase post UV irradiation



Supplementary Figure S1: Cell cycle-dependent DNA damage responses (Related to Figure 1)
(A) Cell cycle staging based on just the DNA content. (B) The same cells as in (A) are staged also according to their EdU content. EdU helps identify true S phase cells. (C) Mean numbers of pATM foci in control and NCS-treated cells. (D) Mean numbers of γH2AX foci in control and NCS-treated cells. Error bars are standard errors of the mean. Foci counts depend just on the relative differences in intensities between the homogeneous background and the sites of focus and are agnostic of overall protein inductions (compare foci counts for γH2AX and pATM with their total nuclear levels in NCS treated cells). There is an increase in the diffuse background of pATM with NCS, and also while γH2AX foci counts may be comparable in specific phases, the individual foci are much brighter in NCS-treated cells. This explains the different trends observed for total intensity and foci counts for NCS treatment. Asterisks mark significant differences with $p < 0.01$ (t-test). Scale bar: 10 μm.

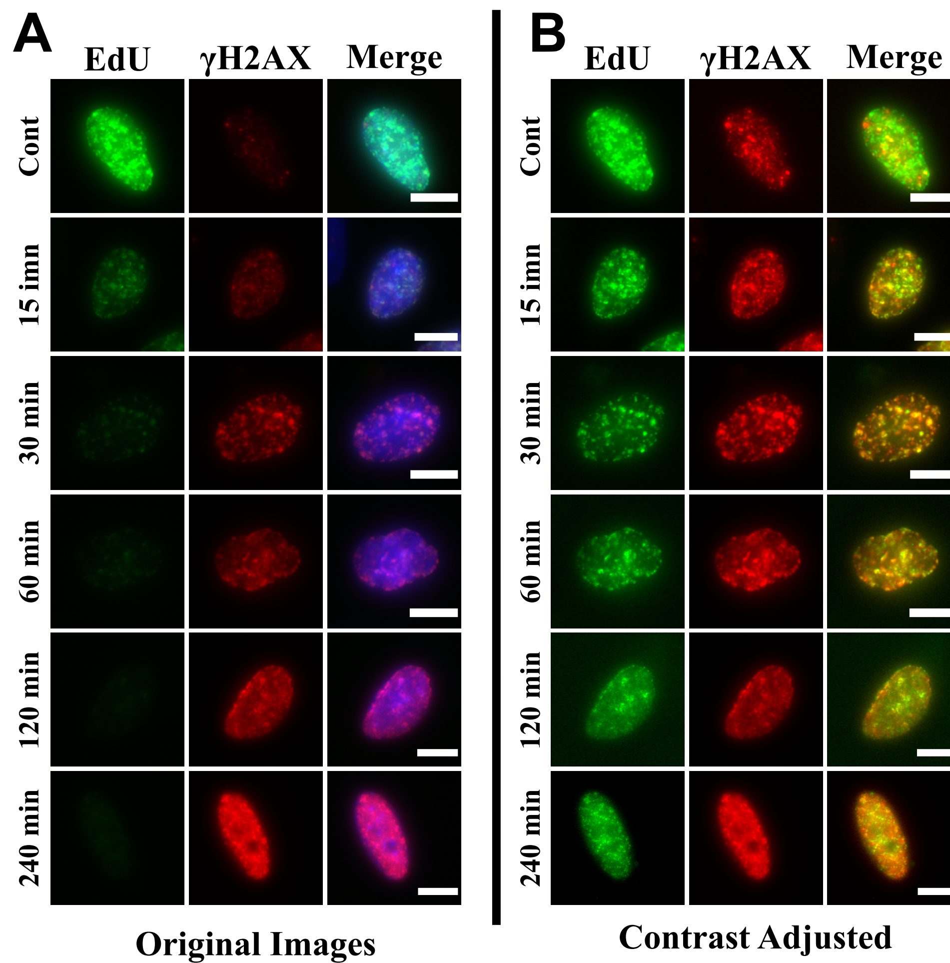


Supplementary Figure S2: Definition of the colocalization metric (Related to Figure 3)
(A) Definition of the colocalization metric: more than 50% overlap between the foci from the two channels at a position. Images are of NCS-treated cells: those 53BP1 foci colocalizing with γH2AX foci are considered as DSBs. Merge images also have DAPI channel shown here in blue. Scale bar: 10 μm.



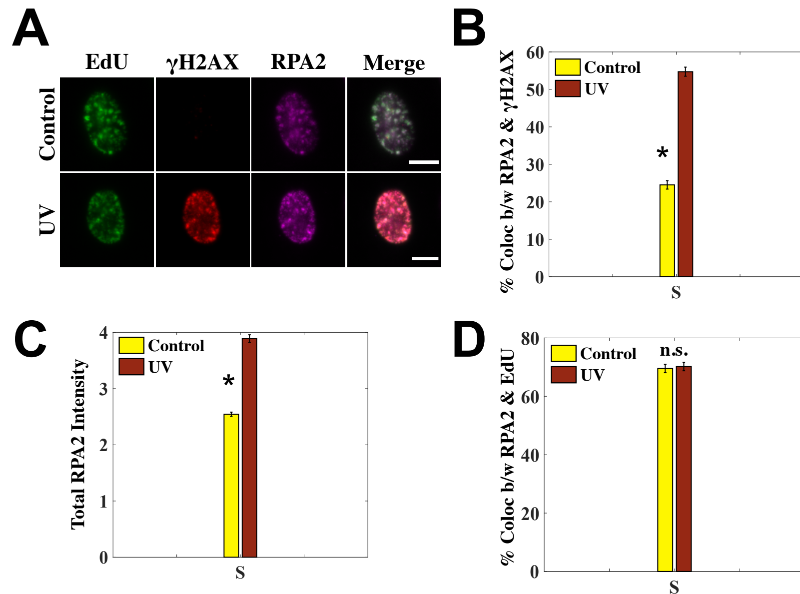
Supplementary Figure S3: Colocalization between EdU and γH2AX at high resolution (Related to Figure 4)

(A) A plane from the original 60X images showing colocalization between EdU and γH2AX foci in the S-phase cells from control, UV- and NCS-treated populations. (B) The same plane from the corresponding deconvolved images as in (A). (C) Percentage colocalization between γH2AX and EdU foci obtained from 60X images. Although smaller because of higher resolution, the colocalization metric obtained from high-resolution 60X images still captures the increased colocalization between γH2AX and EdU foci indicating that γH2AX is induced close to the sites of EdU incorporation upon UV irradiation. The overall conclusion remains the same as that obtained from a lower resolution 40X, 0.75 NA objective. Cells were irradiated with 10 J/m^2 and were let to recover for 60 minutes. Images are contrast adjusted to better visualize spots. Control cells were fixed right after EdU labelling. At least 70 cells were analysed for all the experiments. Error bars are standard errors of the mean. Scale bars: 10 μm.



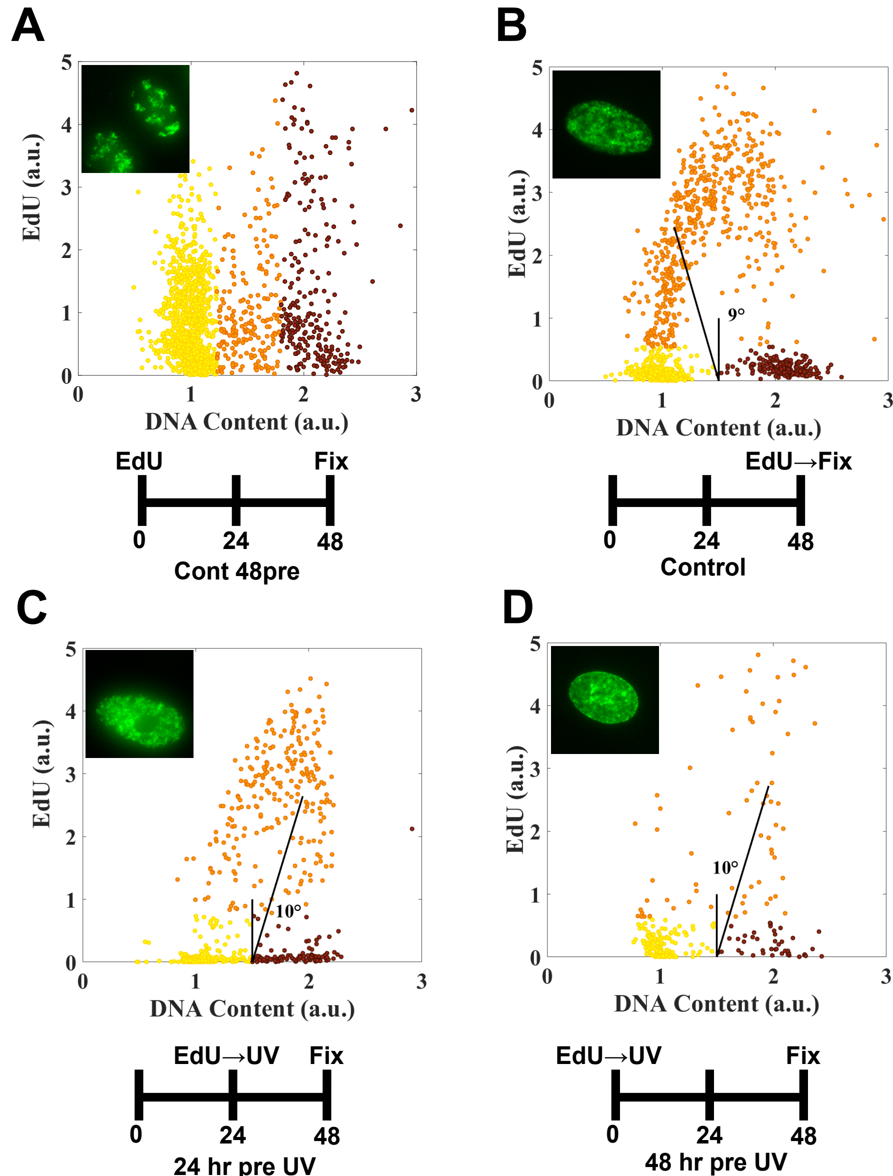
Supplementary Figure S4: γH2AX accumulation at the sites of active replication after UV does not stop replication (Related to Figure 4)

Cells were labeled with EdU just before fixation in all the populations. (A) EdU incorporation goes down with time after UV exposure. Merge images also have DAPI channel shown here in blue. (B) Colocalization between γH2AX and EdU is intact as can be seen for the same cells as in (A), but contrast adjusted to show weak signals. This colocalization implies that UV exposure slows down the replication in cells but does not stop it altogether. Cells were irradiated with 10 J/m^2. Control cells were fixed right after EdU labelling. Scale bars: 10 μm.



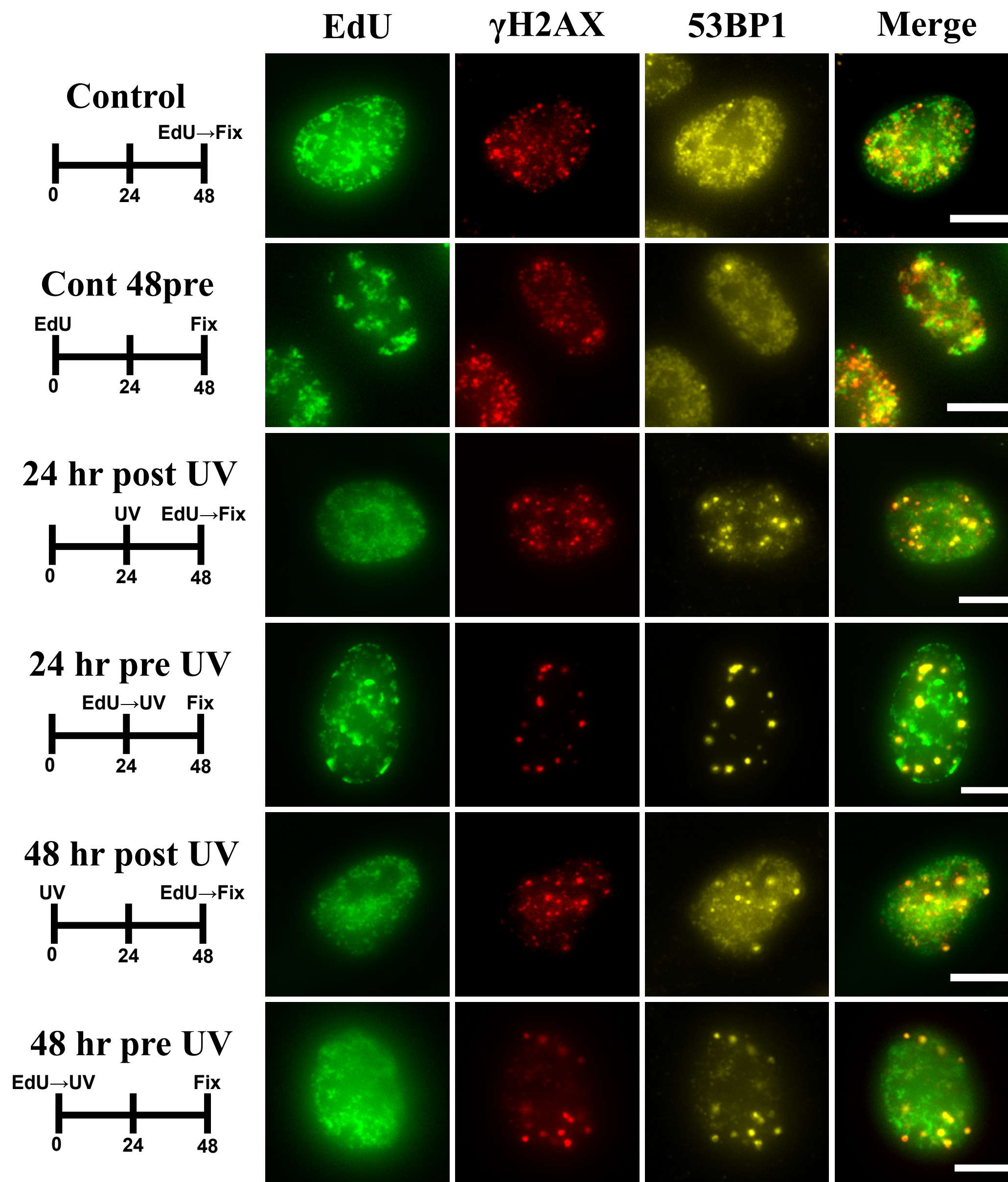
Supplementary Figure S5: Replication protein A colocalizes with γH2AX in the UV-treated S-phase cells

(A) Cells were labelled for EdU (green), γH2AX (red) and RPA2 (magenta). UV treatment causes colocalization of γH2AX and RPA2 foci in the S phase cells. Images are processed with the same contrast adjustments to aid the comparison. (B) Percentage of RPA2 foci colocalizing with γH2AX foci increases significantly in the S phase of UV-treated population. (C) Comparison of total RPA2 levels between control and UV-treated population. While total RPA2 levels go down in G1 and G2/M phases, they increased significantly in the S phase of UV-treated population. (D) Percentage of RPA2 foci colocalizing with EdU foci (replication factories) does not change between control and UV-treated population. Control cells were fixed right after EdU labelling while UV-treated cells were let to recover for 60 minutes. Control cells were fixed right after EdU labelling. Error bars are standard errors of the mean. Asterisks mark significant differences with $p < 0.01$ (t-test). Scale bars: 10 µm.



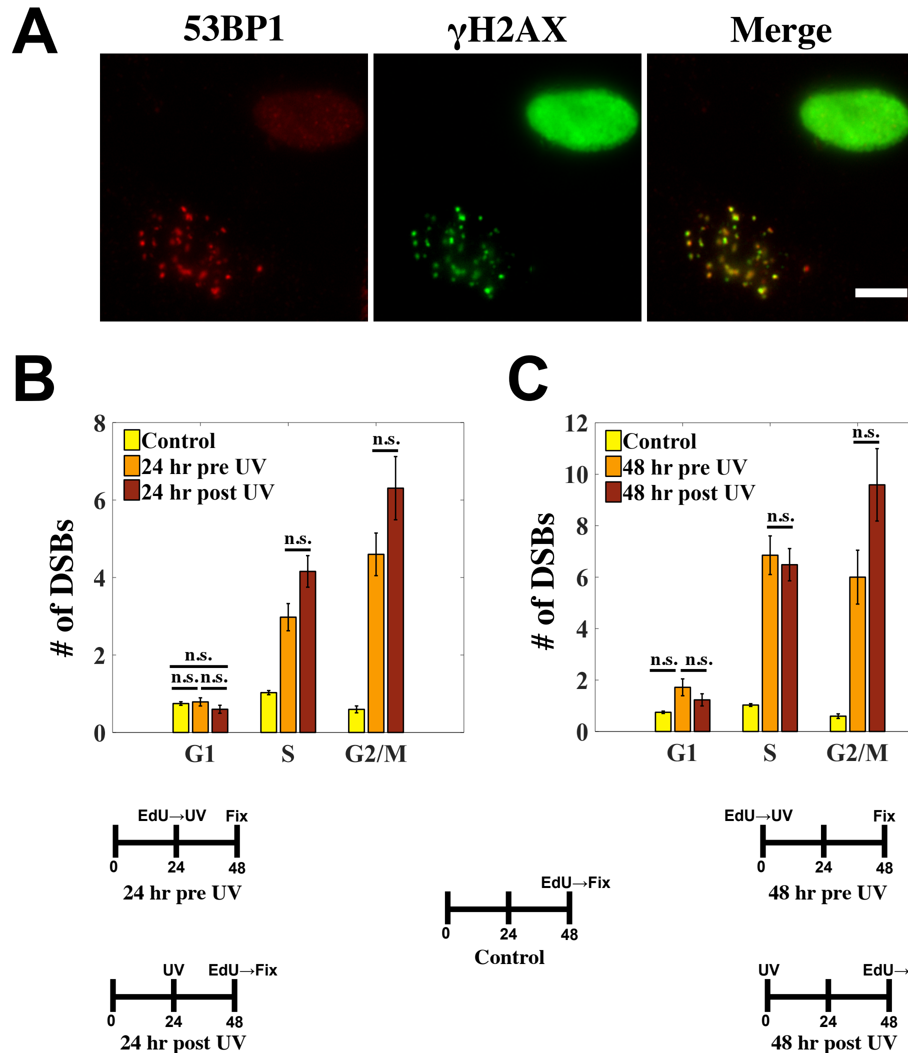
Supplementary Figure S6: S phase cells at the time of UV irradiation completes replication and arrest in the G2 phase of the cell cycle (Related to Figure 5)

(A) Cells labelled with EdU 48 hours prior to fixation. Cell cycle distribution of EdU in this population does not show an arch as seen in that for freshly-labelled EdU cells as shown in (B). Inset shows the staining pattern for these cells forming distinct patches. (B) Cell cycle distribution for EdU in freshly-labelled EdU cells. The DNA content mode of the S phase cells is closer to that of G1 phase cells as indicated by the line making a 9° angle towards left from the vertical. (C, D) Cells were labelled with EdU right before UV irradiation and let to recover for 24-48 hours. The EdU distributions and staining patterns are strikingly similar to that for freshly-labelled EdU cells. The DNA content modes of S phase cells in these population have shifted closer to that of G2 phase cells as indicated by the lines forming 10° angles towards right with the vertical in both the cases. This shows that most of the S phase cells have completed the replication and are arrested in G2 phase of the cell cycle even at 48 hours post UV. The cells for all these experiments were plated at the same time and were treated exactly the same but for their respective experimental conditions; this explains the fewer cells observed in UV-treated populations. Cells were irradiated with 10 J/m^2.



Supplementary Figure S7: Secondary DSBs induced by UV at later time points do not peak in the S phase (Related to Figure 5)

S phase cells are shown for all the different cases. Colocalization of γH2AX and 53BP1 marks DSBs. S phase cells at the time of UV have as many DSBs at later timepoints (4th and 6th rows) as the cells which were in G1 phase but now have exited G1 arrest and started replicating (3rd and 5th rows). The quantification for this observation is shown in Figure 5. The images are contrast-adjusted to aid visualization. Cells were irradiated with 10 J/m^2. Scale bar: 10 μm.



Supplementary Figure S8: Secondary DSBs induced by UV at later time points do not peak in the S phase (Related to Figure 5)

(A) Example of pre-apoptotic and non-apoptotic cells from the population of cells which were irradiated with UV followed by 24-hour recovery before they were labelled with EdU and fixed. 53BP1 here marks DSBs wherever it colocalizes with γH2AX. The cell in top-right corner with very high γH2AX levels with no DSBs and homogeneous staining is a pre-apoptotic cell. (B) Comparing numbers of DSBs in control and UV-treated cells with 24-hour recovery. The S-phase cells in '24 hr pre' population mark the cells which were in the S phase at the time of UV irradiation while those in '24 hr post' population mark those G1 cells which have exited the G1 arrest and started to replicated their genome. Both the populations show similar numbers of DSBs in the respective cell cycle phases. (C) Comparing numbers of DSBs in control and UV-treated cells with 48-hour recovery. The S-phase cells in '48 hr pre' population mark the cells which were in the S phase at the time of UV irradiation while those in '48 hr post' population mark those G1 cells which have exited the G1 arrest and started to replicated their genome. Both the populations show similar numbers of DSBs in the respective cell cycle phases. Cells were irradiated with 10 J/m^2. Error bars are standard errors of the mean. All differences with the exception of those marked n.s. (not significant) are significant with $p < 0.01$ (t-test). Scale bar: 10 µm.

Calculation of doubling time for A549 cells (Related to Figure 5):

In 24-48-hour recovery experiments, all the populations were seeded at the same time with the same cell density. Also all the populations were later fixed at the same time. We aimed to ask that if we started with the same cell density in each plate, and that UV-treated cells did arrest after irradiation with little cell death, would we get reasonable estimates of the doubling times as calculated from the observed cell number densities (CNDs). If there were cell death in addition to cell cycle arrest, in the observed time frames, the estimates would not match with doubling time expected for A549 cells under these serum conditions.

Thus, we calculate the doubling time for A549 cells starting with the assumption that UV irradiation arrests the cells in the cell cycle without causing any cell death within the time periods of observation (24-48 hours). With these assumptions, the cell number density in such UV-treated populations should stay arrested at the values observed at the time points of UV irradiation. This together with the fact that cells in all the population were seeded with the same density at the same time implies that the CNDs observed for UV-treated populations (D_{UV24} and D_{UV48}) after respective recovery periods must also be the CND of the control population observed at time points of UV irradiation as shown in the Supplementary Table T1 (see CNDs for control row).

Population \ Time point		T-24	T	T+24	T+48
Control 48-hour growth	**Action**	*Plate*	*Grow*	*Grow*	*Fix*
	CND	D_0	D_{UV48}	D_{UV24}	D_{cont}
UV-treated 24-hour recovery	**Action**	*Plate*	*Grow*	*UV*	*Fix*
	CND	D_0	D_{UV48}	D_{UV24}	D_{UV24}
UV-treated 48-hour recovery	**Action**	*Plate*	*UV*	*Recover*	*Fix*
	CND	D_0	D_{UV48}	D_{UV48}	D_{UV48}

It is assumed that UV-treated population arrest in the cell cycle and stop dividing without any cell death.
***CND: Cell number density.**

Supplementary table T1: Cell number densities at different time points in control and UV-treated populations (Related to Figure 5 and Supplementary Figures S6, S7 and S8)
Cells for all the three populations were seeded at the same time with the same cell number density (D_0). It is assumed that UV irradiation stops cells from dividing while not causing any cell death. With this assumption, one can see that the cell number density (CND) of control cells at time T will be that observed for UV-treated population let to recover for 48 hours (D_{UV48}). Similarly, CND of control cells at time T+24 will be that for UV-treated population let to recover for 24 hours (D_{UV24}). While the CND for control cells at T+48 will be the one observed for control cells fixed after 48 hours of logarithmic growth from the start of the experiment at time T.

Using the CNDs for control cells which were let to grow for 48 hours at various time points with the above-mentioned assumption, we calculated the doubling time (T_2) for A549 cells using the following equation (1):

$$T_2 = \frac{(T_{final} - T_{initial})}{log_2\left(D_{final}/D_{initial}\right)} \quad hours$$

Where D_{final} and $D_{initial}$ are the cell number densities at time points T_{final} and $T_{initial}$ respectively.

The values of cell number densities in the above table were found to be the following:

1) For control cells let to grow for 48 hours (D_{cont}): 75 cells per field averaged over 42 fields

2) For UV-treated cells let to recover for 24 hours (D_{UV24}): 29 cells per field averaged over 42 fields

3) For UV-treated cells let to recover for 48 hours (D_{UV48}): 10 cells per field averaged over 58 fields

Doubling times were calculated for the combination of three possible pairs of time points as follows:

1) Between $T_{initial}$ = T and T_{final} = T+24 (from control row in table T1)

$$T_2 = \frac{24}{log_2\left(D_{UV24}/D_{UV48}\right)} = \frac{24}{log_2(29/10)} = 15.6 \text{ hours}$$

2) Between $T_{initial}$ = T+24 and T_{final} = T+48 (from control row in table T1)

$$T_2 = \frac{24}{log_2\left(D_{cont}/D_{UV24}\right)} = \frac{24}{log_2(75/29)} = 17.5 \text{ hours}$$

And finally,

3) Between $T_{initial}$ = T and T_{final} = T+48 (from control row in table T1)

$$T_2 = \frac{48}{log_2\left(D_{cont}/D_{UV48}\right)} = \frac{48}{log_2(75/10)} = 16.5 \text{ hours}$$

Taking the average of the three values, we get T_2 = 16.3 hours which, although smaller, is very close to the previously reported value of ~18 hours for A549 cells in similar growth conditions (1, 2). However, it is interesting to note that one can also obtain the same doubling time if the cells were dying and dividing at the same time with similar rates in the populations. This possibility, though, is ruled out from our previous result where we show that the UV-treated cells arrest in the cell cycle and fail to undergo mitosis in the time periods of observation (Figure 5A and Supplementary Figure S6)). This leaves us with the only possibility with which the correct doubling time can be obtained: that UV-irradiated cells arrest in the cell cycle and fail to undergo cell division within the time periods of observation with little to no cell death.

References:

1. Akram KM, Lomas NJ, Spiteri MA, Forsyth NR. 2013. Club cells inhibit alveolar epithelial wound repair via TRAIL-dependent apoptosis. Eur Respir J 41:683–694.
2. Brower M, Carney DN, Oie HK, Gazdar AF, Minna JD. 1986. Growth of Cell Lines and Clinical Specimens of Human Non-Small Cell Lung Cancer in a Serum-free Defined Medium. Cancer Res 46.