Supplementary information for:

APOBEC3A can activate the DNA damage response and cause cell cycle arrest

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Supplementary Methods

Cell Lines

U2OS cells were purchased from the American Tissue Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/mL of penicillin and 100 μg/mL of streptomycin, and supplemented with 10% fetal bovine serum. HepaRG cells stably expressing the pLKOneo.CMV.EGFPnlsTetR plasmid were a gift from Roger Everett (MRC Virology Unit, Glasgow, Scotland) and were grown as described (Everett et al, 2009). The 293T Phoenix packaging cell line was obtained from Orbigen. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmids and Transfections

Plasmids expressing wild-type and mutant APOBEC3 cDNAs containing a carboxy-terminal HA epitope have been described previously (Chen et al, 2006). HA-tagged A3A and C106S cDNAs were inserted into a puromycin resistant lentivirus backbone (Everett et al, 2009) with the DCMV promoter and downstream TetO sites to create pLKO.DCMV.TetO.A3A and pLKO.DCMV.TetO.C106S. The retroviral pLGC-hUGI plasmid was a gift from Michael Emerman (University of Washington). pLGC-hUGI encodes a codon-optimized UGI for expression in human cells and the green fluorescent protein (GFP) (Kaiser & Emerman, 2006). Production of retroviral particles was achieved by transfecting the 293T Phoenix packaging cells. To generate UGI-expressing and control U2OS-based stable cell lines, cells were infected with retroviral particles for 48 hres and selected for GFP expression. The pLPC-UGI plasmid was generated by cloning the HindIII/Nhe1 fragment of pLGC-hUGI in the pLPC vector. Transfections for

immunofluorescence and Western blot analyses were performed using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Production of lentiviral particles was achieved by transfection of 293T cells as described (Everett et al, 2009).

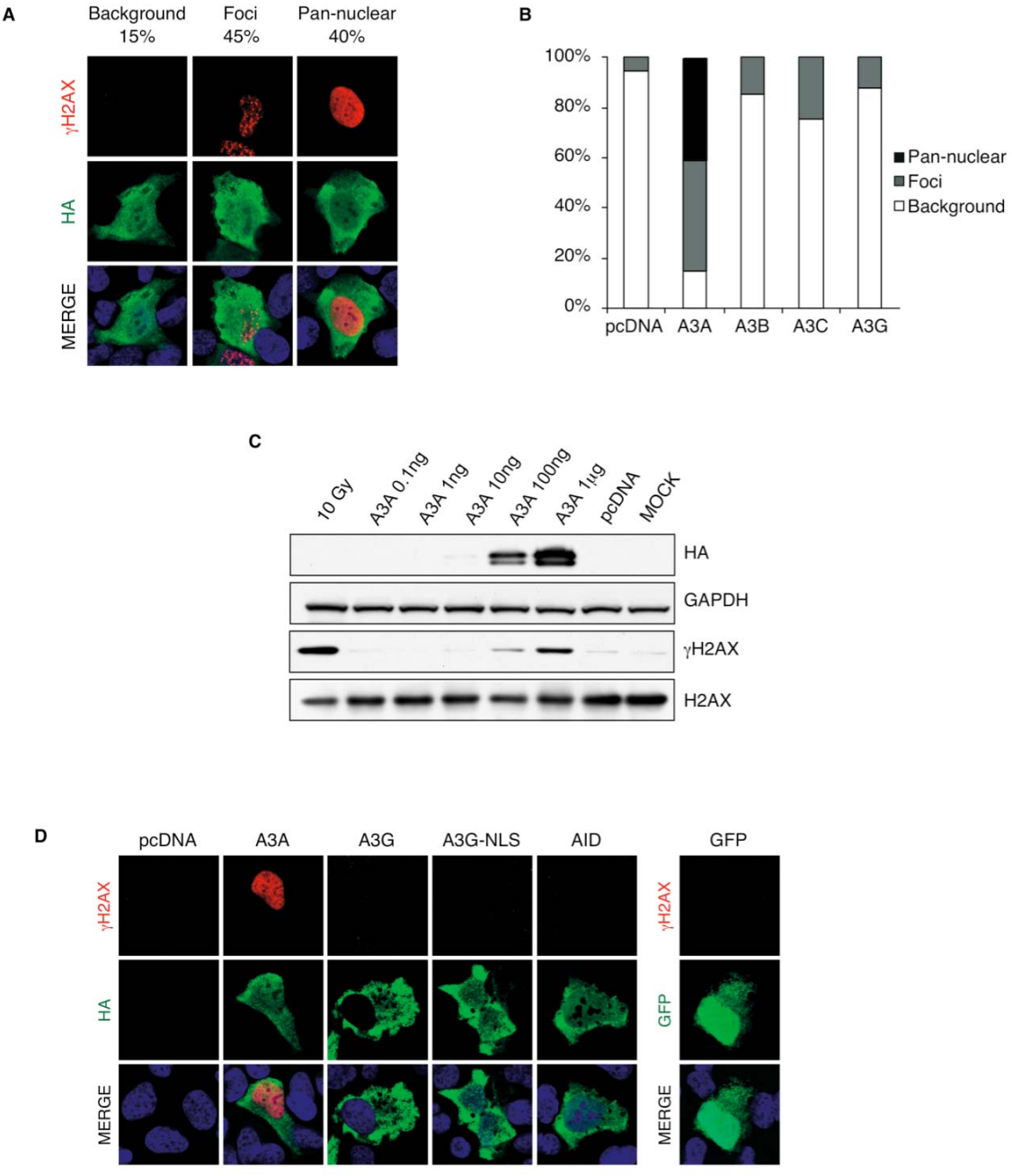
Antibodies

Commercially available antibodies used in this study were obtained from Cell signaling (H2AX, Chk2-P-T68, cleaved caspase-3 (Asp175)), Millipore (γH2AX, Chk2), Epitomics (ATM-P-S1981, ATM), Santa Cruz (Ku70, 53BP1), Fitzgerald (GAPDH), Novus (Nbs1, Nbs1-P-S343) R&D systems (γH2AX), Bethyl (RPA32-P-S4/8), and Covance (HA). The mouse monoclonal antibody against RPA32 was a gift from Tom Melendy (SUNY-Buffalo). For detection of endogenous A3A, a rabbit antiserum raised against a N-terminal peptide was used (Narvaiza et al, 2009). All secondary antibodies were from Invitrogen.

UNG Activity Assay

U2OS-UGI cells and U2OS-pLGCX control cells were seeded at 2 x 10^6 cells/well in 10 cm plates. When cells were 80-90% confluent, they were collected, washed twice with cold PBS and lysed for 30 min on ice in 500 μl of lysis buffer (50 mM Tris, pH 8.0, 40 mM KCl, 50 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 10 mM DTT). Lysates were clarified by centrifugation at 13,000 x g for 10 min. To determine UNG activity, 10 ml of whole-cell lysates and 1:2 serial dilutions were incubated with 5 pmol of a fluoresceine-labeled single strand deoxyoligonucleotide substrate in a final reaction volume of 20 ml containing 40 mM Tris, pH 8.0, 10% glycerol, 40 mM KCl, 50 mM NaCl, 5 mM EDTA, and 1 mM DTT. The reactions were incubated at 37°C for 2 hrs and stopped by addition

of TBE-Urea loading buffer (Invitrogen). Then, samples were incubated at 95°C for 5 min, 4°C for 2 min and separated by 15% TBE/urea-PAGE as previously described (Bulliard et al). Gels were directly analyzed using a FLA-5100 scanner (Fuji). The PAGE-purified ssDNA oligonucleotide (Invitrogen) used for the UNG assays contains a single uracil (Fluoresceine-5'-TATTATTATTATTATTATTUATTTATTTATTTATTTATTT-3').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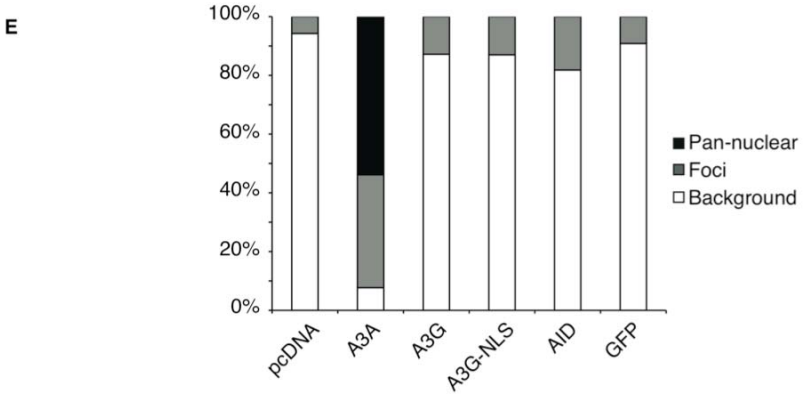
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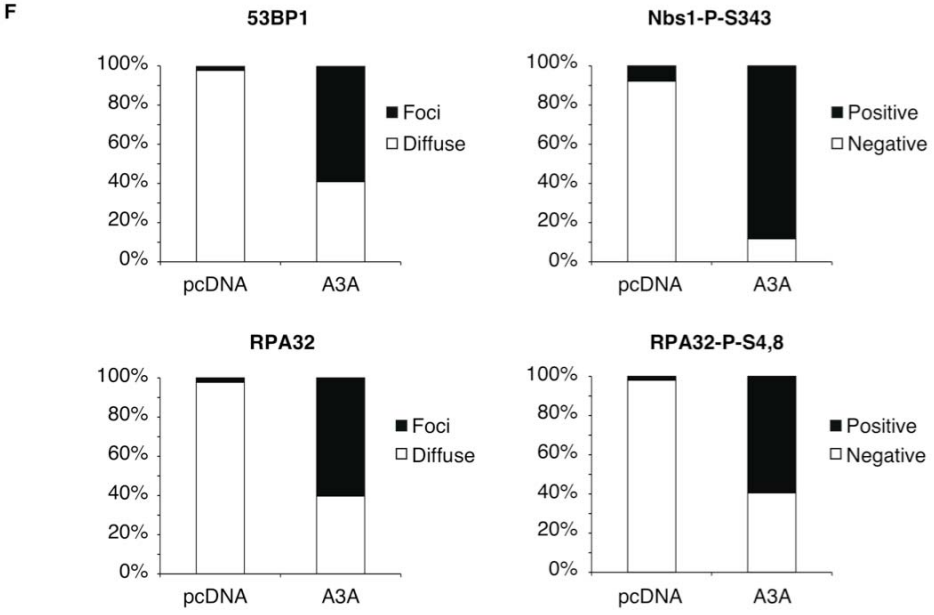
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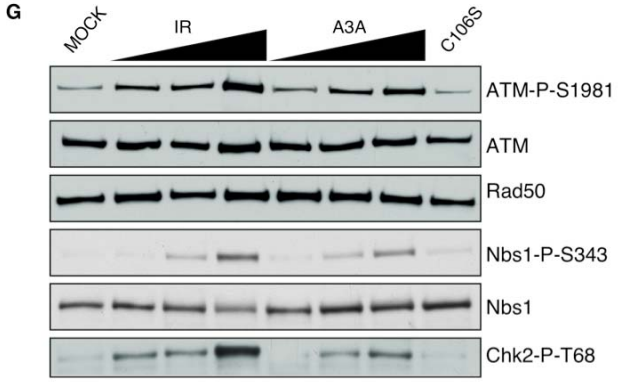
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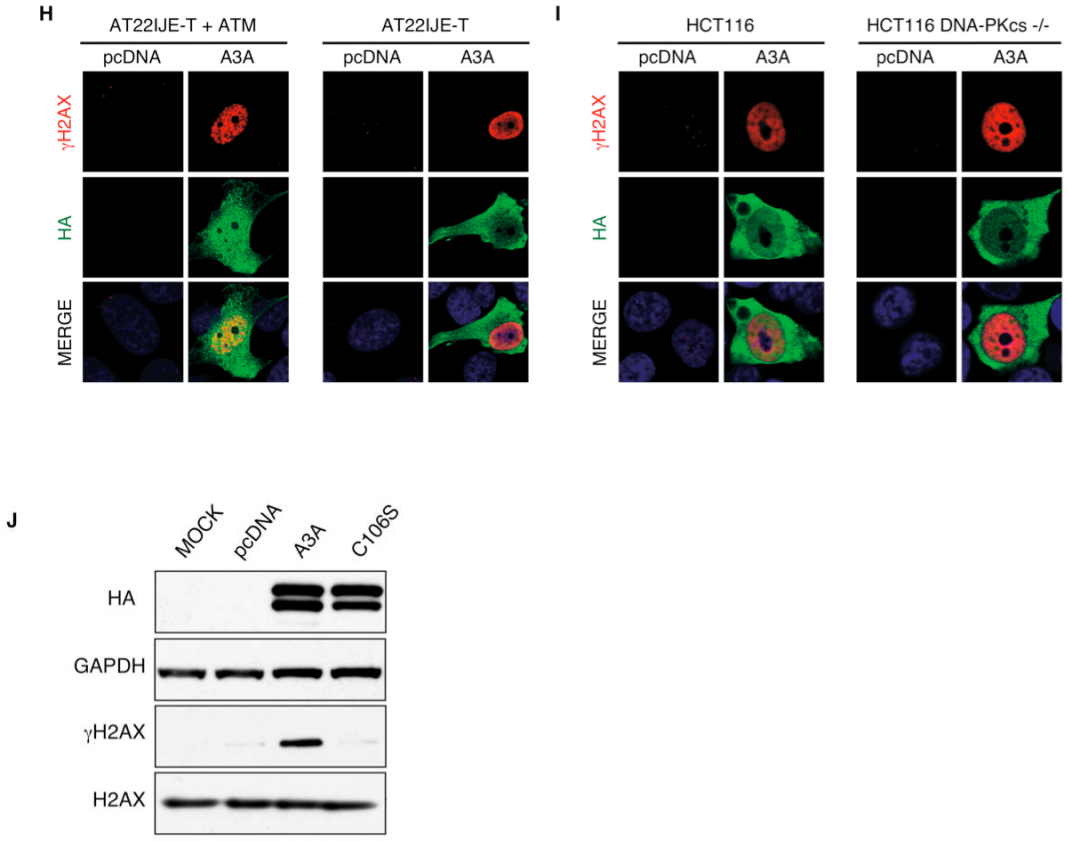


Supplementary Figure S1. A3A expression activates DNA damage signaling.

(**A**) Different γH2AX staining pattern in cells transfected with A3A. Figure shows a representative cell with pan-nuclear γH2AX staining or γH2AX foci. Approximately 15% of cells showed negative γH2AX staining which was comparable to the non-transfected control (Background).

(**B**) Analysis of γH2AX staining in cells transfected with different APOBEC3 proteins. Transfected cells were scored for γH2AX staining. Graph shows the percentage of cells with pan-nuclear γH2AX staining, γH2AX foci and background staining.

(**C**) Western blot detection of phosphorylated H2AX in cells transfected with A3A. U2OS cells were plated in 60 mm plates and transfected with varying amount of an A3A expression vector (1 μg, 100 ng, 10 ng, 1 ng and 0.1 ng) or the control pcDNA3.1 plasmid. Cells were harvested at 24 hrs post-transfection, and lysates were analyzed by Western blotting using antibodies to HA, H2AX and γH2AX. GAPDH served as a loading control.

(**D, E**) Analysis of γH2AX staining in cells transfected with A3A, A3G, A3G-NLS and AID. Transfected cells were scored for γH2AX staining. Graph shows the percentage of cells with pan-nuclear γH2AX staining, γH2AX foci and background staining. A GFP-expressing plasmid served as a transfection control.

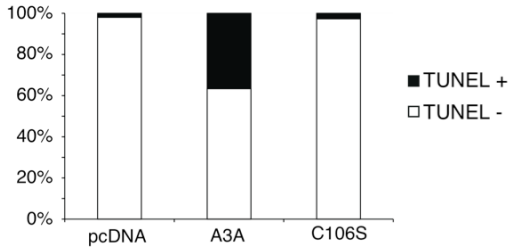
(**F**) Quantitative analysis of 53BP1, NBS-P-S343, RPA and RPA-P-S4,8 in U2OS cells transfected with A3A (see Figure 1C).

(**G**) Nbs1, ATM and Chk2 are phosphorylated in cells transfected with A3A. U2OS cells were transfected with wild-type A3A or the C106S mutant and harvested at 24 hrs post-transfection. Lysates were analyzed by Western blotting using antibodies against Nbs1, Nbs1-P-S343, ATM, ATM-P-S1981, Chk2-P-T68 and Rad50. Lysates from cells treated with ionizing radiation (IR) (0.4 Gy, 3 Gy and 10 Gy) were used as a positive control.

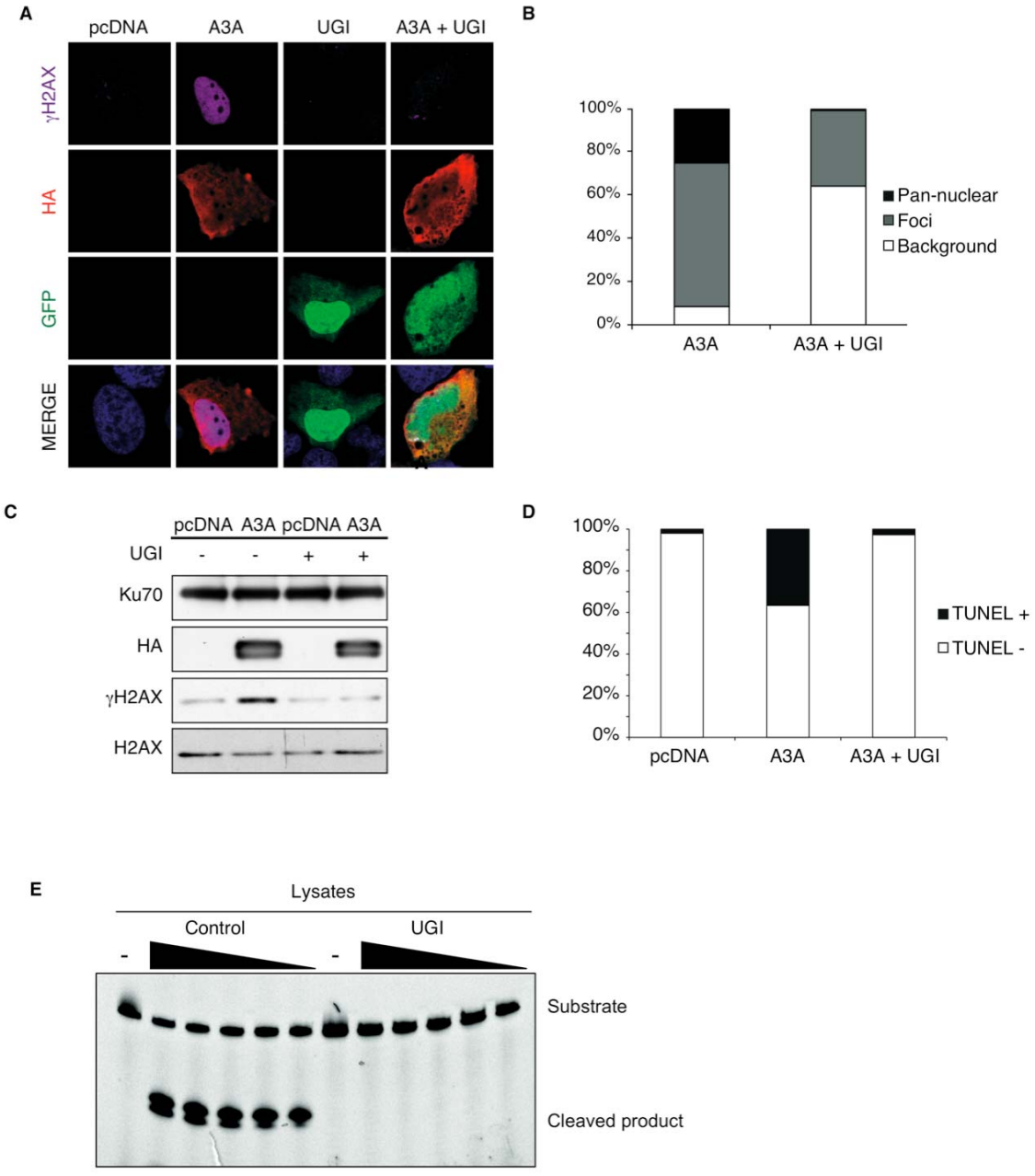
(**H**) ATM kinase is dispensable for A3A-induced H2AX phosphorylation. ATM deficient and ATM complemented AT22IJE-T cells (gift from Y. Shiloh) were transfected with an A3A expression vector or the pcDNA3.1 control plasmid. After 24 hrs, cells were fixed, stained with antibodies to HA and γH2AX and analyzed by confocal microscopy. Nuclei were stained with DAPI.

(**I**) DNA-PKcs is dispensable for A3A-induced H2AX phosphorylation. HCT116 control cells or HCT116-derived cells lacking DNA-PKcs were transfected with an A3A expression vector or the pcDNA3.1 control plasmid. After 24 hrs, cells were fixed, stained with antibodies to HA and γH2AX and analyzed by confocal microscopy.

(**J**) Western blot detection of phosphorylated H2AX in cells transfected with wild-type A3A, the C106S mutant or the pcDNA3.1 control plasmid. Transfected U2OS cells were harvested at 24 hrs. Cell lysates were analyzed by Western blotting using antibodies against HA, H2AX and γH2AX. GAPDH served as a loading control.



Supplementary Figure S2. Quantitative analysis of TUNEL staining in A3A-transfected cells. Analysis of TUNEL staining in cells transfected with A3A or the C106S mutant. Graph shows the percentage of cells with positive TUNEL staining.


A
pcDNA
A3A
UGI
A3A + UGI
γH2AX
HA
GFP
MERGE
B
100%
80%
60%
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20%
0%
A3A
A3A + UGI
Pan-nuclear
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C
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UGI - - + +
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60%
40%
20%
0%
pcDNA
A3A
A3A + UGI
TUNEL +
TUNEL -
E
Lysates
Control
UGI
-
-
Substrate
Cleaved product

Supplementary Figure S3. UNG Activity is Required for A3A-induced DNA Damage.

(**A**) The UNG inhibitor (UGI) blocks H2AX phosphorylation in cells transfected with A3A. U2OS cells were co-transfected with an A3A expression vector or the pcDNA3.1 control plasmid together with a plasmid co-expressing UGI and GFP (pLGC-hUGI). Cells were fixed after 24 hrs, stained with antibodies to HA and γH2AX, and analyzed by confocal microscopy. Nuclei were stained with DAPI.

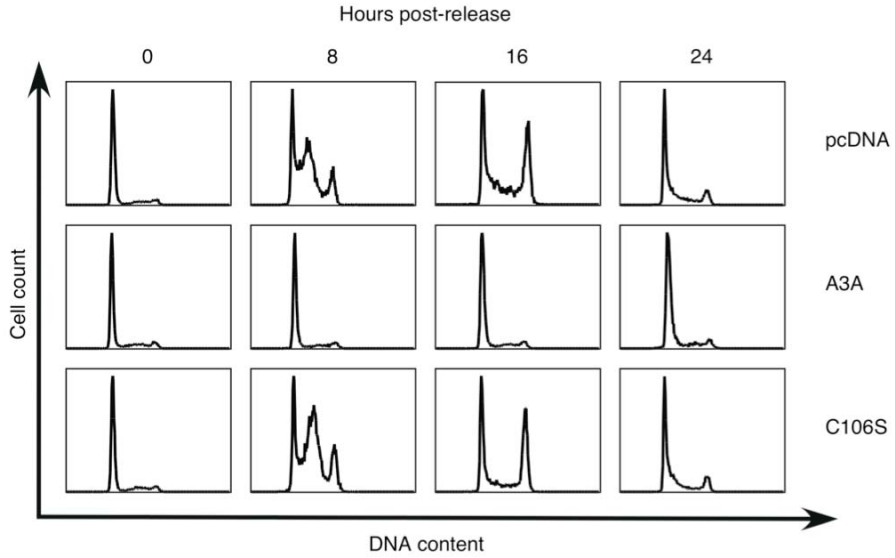
(**B**) Analysis of γH2AX staining in cells transfected with A3A and UGI. Cells showing HA staining were scored for γH2AX staining. Graph shows the percentage of cells with pan-nuclear H2AX activation, γH2AX foci and background staining.

(**C**) Western blot detection of phosphorylated H2AX in cells transfected with A3A and UGI. U2OS cells were transfected with an A3A expression vector or the pcDNA3.1 control plasmid together with pLGC-hUGI. Cells were harvested at 24 hrs and lysates were analyzed by Western blotting using antibodies to HA, H2AX and γH2AX. Ku70 served as a loading control.

(**D**) Induction of DNA breaks by A3A requires UNG activity (see figure 4D). Quantification of TUNEL staining in U2OS cells co-transfected with an A3A expression vector or the pcDNA3.1 control plasmid together with a plasmid expressing UGI (pLPC-UGI) or the empty pLPC vector.

(**E**) Stable U2OS cells expressing UGI lack detectable UNG activity. Inhibition of UNG activity by UGI was analyzed in whole-cell lysates obtained from U2OS-UGI cells and U2OS-pLGCX control cells. UNG activity was determined in an in vitro assay using a fluoresceine-labelled oligonucleotide containing a single uracil. Substrate oligonucleotide

(upper band) and the cleaved product of UNG activity (lower band) were resolved by 15% TBE/urea-PAGE.



Supplementary Figure S4. A3A expression induces a cell cycle block in U2OS cells. U2OS cells were synchronized in G1/S using a double thymidine block and release, and collected at different time points. Briefly, cells were plated in normal medium and after adherence, 2.5 μM thymidine was added for 18 hrs. Cells were washed three times in PBS, and placed in normal medium for 12 hrs and thymidine was added for an additional 18 hrs before release. U2OS cells were co-transfected with pcDNA-A3A-HA and pcDNA-GFP in a 4:1 ratio. Cells were washed three times with PBS (this point was designated t = 0). Cells were resuspended briefly in 1% paraformaldehyde for 15 min and washed in PBS before ethanol fixation. GFP-positive cells were analyzed for cell cycle progression by flow cytometry after staining with propidium iodide. Cell cycle was analyzed using Flowjo software.

Supplementary References

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