ATM Loss Confers Greater Sensitivity to ATR Inhibition than PARP Inhibition in Prostate Cancer

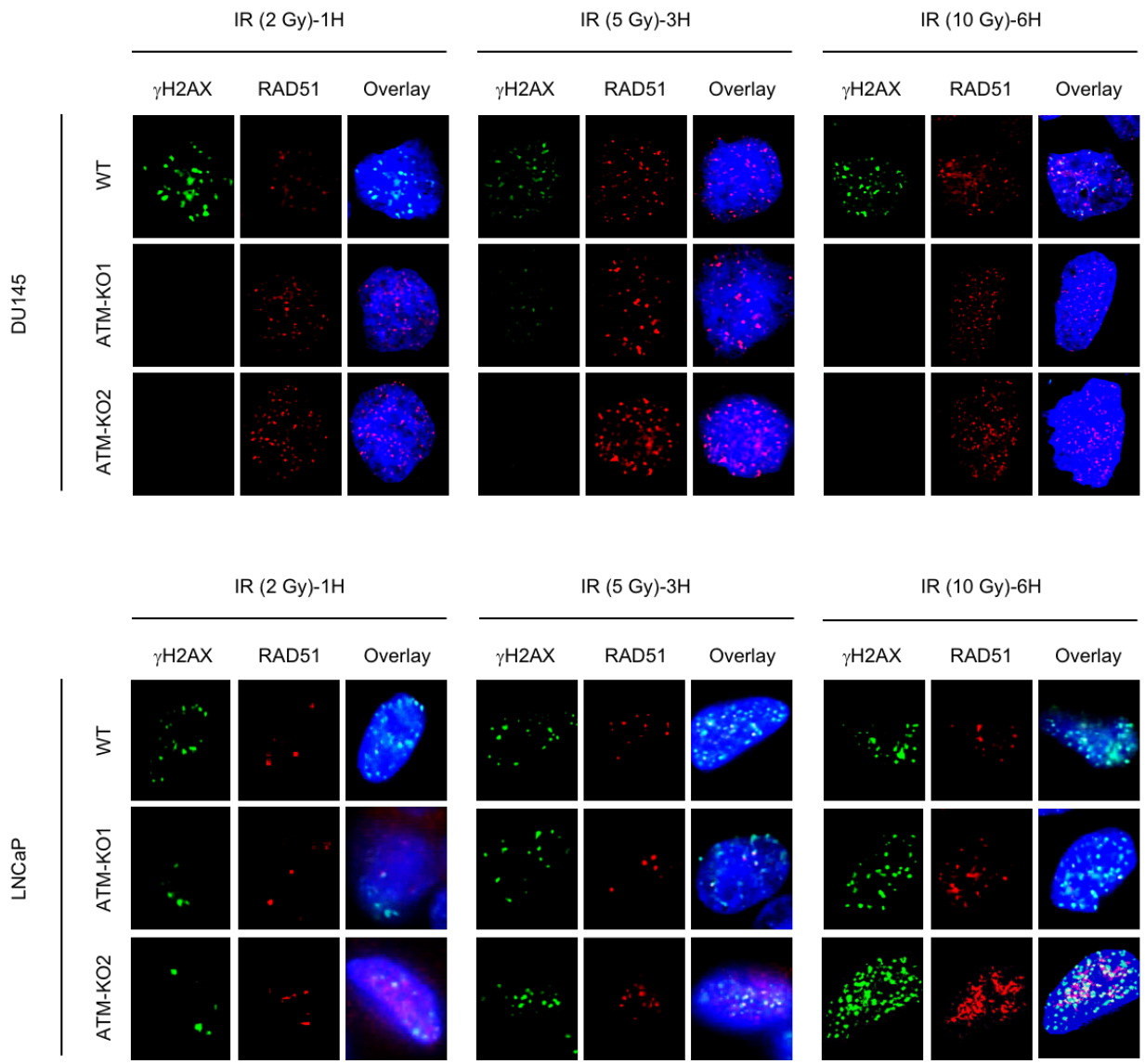
SUPPLEMENTARY INFORMATION

Supplementary Table 1 (*see separate file*)

Supplementary Figures 1-3

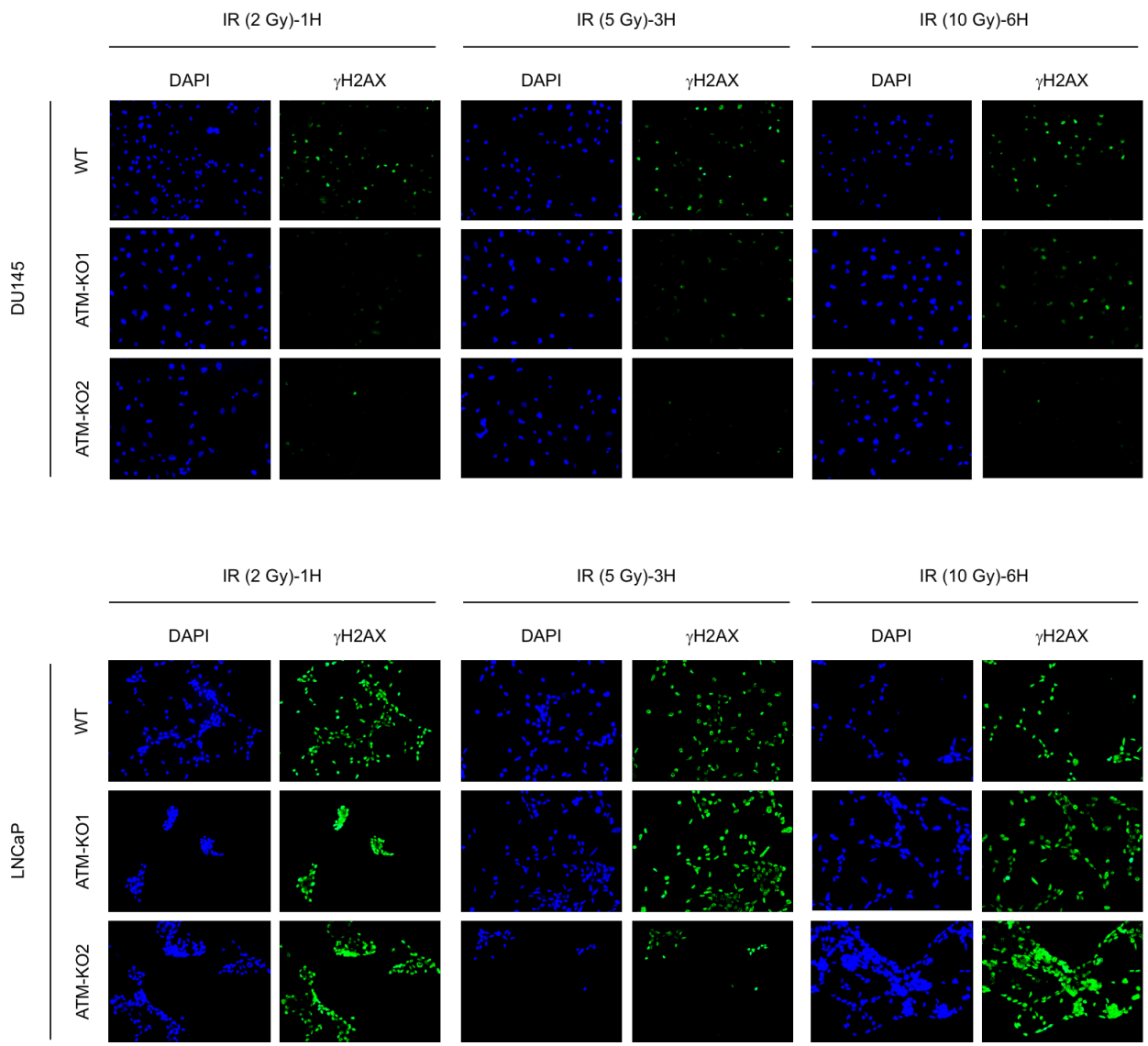
Supplementary Materials & Methods

Supplementary Figure 1



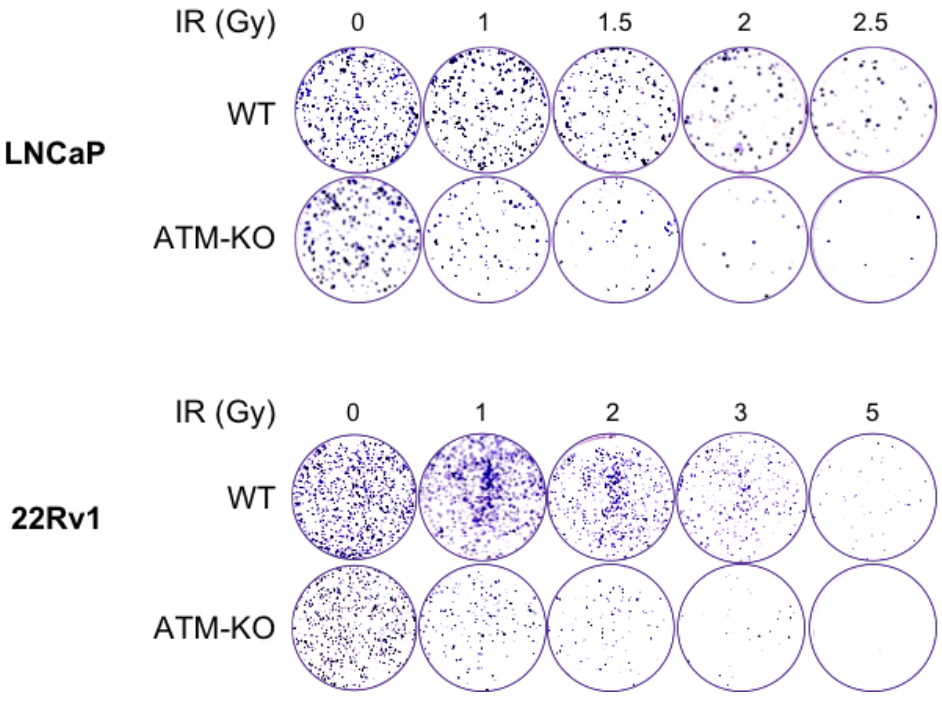
High-power (100X) images of co-stained Rad51 and γH2AX foci in DU145 (top) and LNCaP (bottom) cells following ionizing radiation (IR). ATM deleted (KO) DU145 cell lines have significantly fewer γH2AX foci than ATM wild-type (WT) cell, while there is minimal difference in γH2AX foci in LNCaP ATM–WT vs ATM–KO cells. In both DU145 and LNCaP, there was no difference in Rad51 foci formation between ATM–WT and ATM–KO cell lines. DAPI (nuclear) staining is shown in blue.

Supplementary Figure 2



Low-power (20X) images of γH2AX foci in DU145 (top) and LNCaP (bottom) cells following ionizing radiation (IR) treatment. ATM deletion (KO) results in decrease in IR-induced γH2AX foci formation in DU145 cells compared to ATM wild-type (WT) cells, particularly at lower IR doses. Conversely, there was no significant difference in γH2AX foci formation between ATM WT vs ATM–KO LNCaP cells. DAPI (nuclear) staining is shown in blue.

Supplementary Figure 3



Clonogenic survival following ionizing radiation (IR) for LNCaP and 22Rv1 ATM parental (WT) and ATM-deleted (KO) cell lines.

Supplementary Materials & Methods

Cell lines and cell culture

Human prostate cancer lines DU145, 22Rv1, and LNCaP were purchased from ATCC and were maintained in phenol red free RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Sigma). Human embryonic kidney (HEK)–293 T cells were purchased from ATCC and were maintained in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco). The U2OS DR-GFP HR reporter cell line was a kind gift from Dr. Alan D'Andrea (Dana-Farber Cancer Institute) and was maintained in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco). All cell lines were grown at 37°C in a humidified 5% CO_2 incubator.

Virus production and infection

HEK-293T cells were plated at 7.0 x 10^5 cells per 6 cm dish. The following day, cells were transfected with packaging vectors (pMD2.G envelope plasmid and psPAX2 packaging plasmid) as well as the plasmid of interest (see below). Three days later, viral supernatant was collected, passed through a 0.45 μm filter, and immediately added to existing media in dishes containing adherent prostate cancer cells. Polybrene was also added at 4 ug/ml to increase infection efficiency.

CRISPR/Cas9 gene editing

ATM-deficient prostate cancer cell lines were created using a two vector CRISPR/Cas9 system. First, Cas9 was stably expressed in the cells using the lentiCas9-Blast plasmid (Addgene 52962). Cells were selected with blasticidin and screened for Cas9 protein expression by immunoblot. sgRNA oligonucleotides targeting ATM (Table S1) were cloned into the lenti-Guide Puro plasmid (Addgene 52963). Following lentiviral production and infection, cells were selected with puromycin for 4-7 days. Surviving cells were single cell sorted into 96 well plates and clonal populations were expanded and screened for ATM loss by immunoblot. Knockout clones identified by immunoblot were confirmed by next-generation sequencing.

Drugs and antibodies

PARP inhibitors (olaparib, rucaparib) were purchased from Selleck Chemicals. The ATR inhibitor VX-970 (M6620) was supplied by Vertex Pharmaceuticals. Drugs were reconstituted in DMSO, aliquoted, and stored at -20° C. Aliquots were thawed and diluted in RPMI immediately prior to use.

ATM, ATR, KAP1, and phospho-KAP1 antibodies were purchased from Cell Signaling. Vinculin and α-Tubulin antibodies were purchased from Santa Cruz Biotechnology. The BRCA2 antibody was purchased from Millipore. All antibodies were diluted in 2% milk/BSA (1:200-1:500) prior to use.

siRNA transfection

siRNA transfections were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol (Qiagen). Briefly, cells were seeded at 40% confluence in 6-well plates and transfected with siRNA at a final concentration of 20 nM (see Table S1 for siRNA sequences) the following day. Transfected cells were incubated for 48 hours and then re-plated for the desired assay. A non-targeting siRNA (si-NEG) was used as a negative control in all siRNA assays.

Sensitivity Assays

Cells were seeded into 24-well plates (2,500-4000 cells/well) in duplicate and treated with increasing concentrations of drug the following day. Cell viability was assessed after 72-96 hours by adding CellTiter-Glo reagent (Promega) and measuring luminescence using a plate reader (BioTek). Cell survival at each drug concentration was plotted as the log-10 survival relative to drug-free media.

For radiation sensitivity assays, cells were plated in 6-well plates at a density of 500-750 cells/well. The following day, the cells were irradiated with 1-10 Gray (Gy) using a calibrated cell irradiator (Rad Source).

For clonogenic survival assays, cells were seeded in 6-well plates (500 or 4000 cells/well) in triplicate. Cells were treated with drug 16 to 18 hours after plating and were allowed to grow for 10-14 days. For radiation sensitivity assays, cells were plated in 6-well plates at a density of 500-750 cells/well. The following day, the cells were irradiated with 1-10 Gray (Gy) using a calibrated cell irradiator (Rad Source) and allowed to grow for 10-14 days. The resulting colonies

were fixed using 100% methanol for 40 min at -20^{o}C. Fixed cells were stained using crystal violet (1%) for 1 hour. Images were analyzed and quantified using ImageJ software (National Institutes of Health). Survival at each drug (or IR) concentration was calculated as the number of colonies divided by the number of colonies in the untreated wells.

Immunoblotting

For radiation-based assays, cells were plated in 10 cm plates. The following day, the cells were irradiated with 2-10 Gray (Gy) using a calibrated cell irradiator (Rad Source). Cells were allowed to recover for 0.5-5 hours following irradiation, and then harvested. Samples pre-treated with ATR inhibitor (750 nM) were irradiated with 10 Gy ionizing radiation (IR) and harvested 1 h later.

Cell were washed with PBS, scraped, and lysed in cold RIPA lysis buffer (Thermo Scientific). Phosphatase inhibitors (phosSTOP, Roche), protease inhibitors (Roche), and 1 mM PMSF (Cell Signaling Technology) were added to the RIPA lysis buffer. Total protein concentration was quantified using Protein Assay Dye Reagent Concentrate (Bio-Rad) and measured at 595 nm with a spectrophotometer. Protein extracts were resolved on 3-8% NuPAGE Bis-Tris gradient gels (Invitrogen) supplied with Tris-Acetate SDS running buffer and transferred to PVDF membrane (Thermo Scientific). Membranes were blocked with 5% milk in TBST and probed with the primary antibody solution overnight at 4°C on a rocking platform. The following day, the membrane was rinsed and then incubated in the secondary antibody solution (IRDye® 680RD) for 1 hour at room temperature. Blots were imaged using an Odyssey imaging device (Li-Cor Biosciences).

Immunohistochemistry

Cells were washed in saline, fixed in 10% neutral-buffered formalin for 10 min, washed and embedded in Histogel (Thermo Scientific). The Histogel plugs were dehydrated and embedded in paraffin using standard protocols. Sections of the formalin fixed paraffin embedded cells were stained on a Leica BOND staining platform using an antibody to ATM (Abcam; ab32420). Images of stained slides were captured on a QColor camera (Olympus) attached to a brightfield microscope at 40X magnification.

Immunofluorescence

Cells were grown on laminin-coated glass cover slips and fixed in 4% paraformaldehyde and 0.5% Triton X-100 in PBS for 40 min at room temperature. Fixed cells were washed with PBS twice and then incubated for 1 hour in permeabilization/blocking buffer. Next, the primary RAD51 (H-92, Santa Cruz Biotechnology) or phospho-H2AX (Millipore) antibody was added at a 1:200 dilution. Following a 2 h incubation of primary antibody at room temperature, coverslips were washed and incubated with the secondary antibody (488-conjugated anti-rabbit or Alexa Fluor 594-conjugated anti-mouse; Life Technologies) at 1:2000 at room temperature for 1 hour. Coverslips were then washed, mounted on glass slide with 49,6-diamidino-2-phenylindole containing Vectashield mounting medium (Vector Laboratories) and visualized by fluorescence microscopy. Slides were scanned on an Axio Imager Z2 microscope (Zeiss) equipped with a CoolCube1 camera at 20X-100X magnification. Each experiment was performed in triplicate, and at least 50 cells were counted for each replicate at each condition.

In vivo HR assays using DR-GFP plasmid

Homologous recombination (HR) efficiency was measured using the DR-GFP reporter assay as described.[11] Briefly, U2OS cells with GFP expression cassette (U251-DR-GFP) were seeded in 6-well cell plates and the following day were transfected with the indicated siRNA. At 24 hours post-transfection, cells were infected with the I-Sce adenovirus, and the GFP-positive population was analyzed 32 hours after infection. The percentage of viable GFP-positive cells was determined for each condition using CytoFLEX flow cytometer platform (Beckman-Coulter), and normalized to the non-targeting condition (siCTRL). FACS data were analyzed using FlowJo 7.6.5.

Statistical Analysis

All cell line experiments were performed in triplicate and data is plotted as the mean $\pm$ standard deviation of multiple independent experiments. Prism 7 (GraphPad) was used to display and quantify data. Student's t-tests were used to compare difference between means, and p-values <0.05 were considered to be statistically significant.