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Authors

Maranto, Cristina

Udhane, Vindhya

Hoang, David

et al.

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STAT5A/B BLOCKADE SENSITIZES PROSTATE CANCER TO RADIATION THROUGH INHIBITION OF RAD51 AND DNA REPAIR

Cristina Maranto[*,1,2], **Vindhya Udhane**[*,1,2], **David T. Hoang**[3], **Lei Gu**[3], **Vitali Alexeev**[4], **Kareem Malas**[1,2], **Karmel Cardenas**[1,2], **Jonathan R Brody**[5], **Ulrich Rodeck**[4], **Carmen Bergom**[6], **Ken A Iczkowski**[1], **Ken Jacobsohn**[7], **William See**[7], **Sara M. Schmitt**[1,2], and **Marja T Nevalainen**[1,2]

[1]Department of Pathology, Prostate Cancer Center of Excellence at Medical College of Wisconsin Cancer Center, Medical College of Wisconsin, Milwaukee, WI

[2]Department of Pharmacology & Toxicology, Prostate Cancer Center of Excellence at Medical College of Wisconsin Cancer Center, Medical College of Wisconsin, Milwaukee, WI

[3]Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

[4]Department of Dermatology, Thomas Jefferson University Medical College, Philadelphia, PA

[5]Department of Surgery, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

[6]Department of Radiation Oncology, Medical College of Wisconsin, Milwaukee, WI

[7]Department of Urology, Prostate Cancer Center of Excellence at Medical College of Wisconsin, Milwaukee, WI

Abstract

Purpose—The standard treatment for organ-confined prostate cancer (PC) is surgery or radiation, and locally advanced PC is typically treated with radiotherapy alone or in combination with androgen deprivation therapy. Here, we investigated whether Stat5a/b participates in regulation of double strand DNA break repair in PC, and whether Stat5 inhibition may provide a novel strategy to sensitize PC to radiation therapy.

Experimental Design—Stat5a/b regulation of DNA repair in PC was evaluated by comet and clonogenic survival assays, followed by assays specific to Homologous Recombination (HR) DNA repair and Non-Homologous End-Joining (NHEJ) DNA repair. For HR DNA repair, Stat5a/b regulation of Rad51 and the mechanisms underlying the regulation were investigated in PC cells, xenograft tumors and patient-derived PCs *ex vivo* in 3D explant cultures. Stat5a/b induction of Rad51 and HR DNA repair and responsiveness to radiation were evaluated *in vivo* in mice bearing PC xenograft tumors.

Address correspondence to: Marja T. Nevalainen, MD, PhD, Depts. of Pathology, Pharmacology/Toxicology, Prostate Cancer Center of Excellence at Medical College of Wisconsin Cancer Center, Medical College of Wisconsin, 8701 Watertown Plank Rd. TBRC C4960, Milwaukee, WI 53226, Tel: 414-955-2103, Fax: 414-955-6059, mnevalainen@mcw.edu.

*These authors contributed equally.

Conflicts of Interest: None

Results—Stat5a/b is critical for Rad51 expression in PC via Jak2-dependent mechanisms by inducing Rad51 mRNA levels. Consistent with this, genetic knockdown of Stat5a/b suppressed HR DNA repair while not affecting NHEJ DNA repair. Pharmacological Stat5a/b inhibition potently sensitized PC cell lines and PC tumors to radiation, while not inducing radiation sensitivity in the neighboring tissues.

Conclusion—This work introduces a novel concept of a pivotal role of Jak2-Stat5a/b signaling for Rad51 expression and HR DNA repair in PC. Inhibition of Jak2-Stat5a/b signaling sensitizes PC to radiation and, therefore, may provide an adjuvant therapy for radiation to reduce radiation-induced damage to the neighboring tissues.

Keywords

Stat5a/b; Rad51; prostate cancer; ionizing radiation

INTRODUCTION

Organ-confined prostate cancer (PC) is typically treated with surgery or radiation therapy (1–4), while radiation alone or in combination with androgen deprivation therapy is a key treatment option for locally advanced PC (1–4). In addition, radiation therapy is elementary for post-prostatectomy PC patients who have high risk features such as extracapsular extensions, positive surgical margins or persistent prostate specific antigen (PSA) levels (1–4).

Irradiation, even with intensity modulated conformal radiotherapy, is associated with significant toxicities to the surrounding tissues, which can cause debilitating side effects (5,6). Identification of effective strategies to sensitize PC cells to radiation would allow the use of lower radiation doses by reducing radiation-associated side effects while enhancing PC cell death. Radiation induces double-strand breaks (DSBs), which are the primary cause of cell death following radiation due to decreased DNA repair capacity of cancer cells (7–9). The major repair mechanisms of DSBs are homologous recombination repair (HR) and non-homologous end-joining (NHEJ) repair (7–9). HR repair occurs during late S or G_2 phases of the cell cycle and uses sister chromatids as templates for DNA repair, which allows for nearly error-free repair of the damaged DNA (7–10). NHEJ, in contrast, occurs rapidly throughout the cell cycle (11,12), and is error-prone due to the nature of the repair by simple joining of the broken DNA ends (7–9,13). Malignant tissues have an increased ratio of cells in S and G_2 phase, leading them to favor HR repair over NHEJ repair (14).

HR DNA repair occurs through a highly regulated series of events, where the key protein controlling the DNA repair process is Rad51 recombinase (7–9,15). The DNA breaks are initially recognized by the MRN (Mre11, Rad50, NbS1) protein complex, which together with phosphorylated CtIP (C-terminal binding protein interacting protein) and BRCA1 (Breast Cancer Susceptibility 1), generate the necessary 3’ single-stranded DNA (ssDNA) overhangs for HR repair (7–9,15,16). BRCA1 promotes this resection by dephosphorylating the inhibitory 53BP1 protein (7–9,15–17). Following end-resection, the MRN complex recruits and activates ATM (ataxia telangiectasia mutated) kinase, which phosphorylates histone H2AX on regions around DNA DSBs (18). Importantly, loading of Rad51

recombinase onto exposed ssDNA repair sites is carried out by BRCA1 and requires assistance of BRCA2 (16,19). Rad51 is a requisite for HR DNA repair as it catalyzes DNA strand invasion and exchange (16,20). Rad51 paralogues including Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3 have only a supporting role in this process by assisting Rad51 in the initiation and execution of HR DNA repair (21). BRCA1 and BRCA2 have a central role in assisting Rad51, and BRCA1/2-deficient cells have defective HR DNA repair (termed BRCAness), and consequently increased sensitivity to radiation, platinum chemotherapy and PARP-inhibitors (22). In contrast, Rad51 is indispensable for HR DNA repair (7–9,15).

Stat5a/b is both a signaling protein and a transcription factor that mediates diverse cellular responses to cytokines and growth factors (23), and has been linked to DNA repair in chronic myeloid leukemia (CML)(24,25). Stat5 is comprised of two highly homologous isoforms, Stat5a (94kDa) and Stat5b (92kDa), which are activated through phosphorylation of a conserved tyrosine residue, typically by Jak2, leading to formation of Stat5a/b dimers and subsequent translocation to the nucleus and binding to DNA (23). Stat5a/b is overexpressed in PC compared to normal prostate epithelium (26), and Stat5a/b levels positively correlate with Gleason grades of PC (26–29). Moreover, the Stat5a/b gene locus undergoes amplification during PC progression to castrate-resistant metastatic disease (28). Stat5a/b is critical for viability of PC cells *in vitro* and for PC xenograft tumor growth *in vivo* through both AR-dependent and AR-independent mechanisms (30–34). Stat5a/b inhibition suppresses growth of castrate-resistant PC following surgical castration (35), and Stat5a/b induces epithelial-to-mesenchymal transition (EMT), stem-like cancer cell properties and distant metastases formation of PC *in vivo* (36). The significance of Stat5a/b in PC growth and progression, demonstrated in pre-clinical PC models, is corroborated by significant predictive value of active Stat5a/b for early disease recurrence of organ-confined PC in patients after intent-to-cure radical prostatectomy (29). Recently, we developed a family of novel small-molecule Stat5a/b inhibitors (IST5), with high efficacy of blocking Stat5a/b action in PC and Bcr-Abl driven leukemias *in vitro* and *in vivo* (37).

The promoter region of Rad51 contains Stat5a/b response elements (24,25), which led us to explore the possibility that Stat5a/b may regulate HR DNA repair and radiation response in PC. Here, we demonstrate, for the first time, that Stat5a/b is required for Rad51 expression in PC, and Stat5a/b inhibition sensitizes PC to radiation *in vitro* and *in vivo* through suppression of HR DNA repair. Stat5a/b activation increased, while genetic or pharmacological Stat5a/b inhibition decreased Rad51 expression in PC cells and in clinical PCs. Moreover, Stat5a/b inhibition suppressed HR DNA repair in PC as indicated by decreased Rad51 foci formation, increased DNA damage and decreased repair of I-SceI-generated DSBs. Importantly, genetic or pharmacological Stat5a/b inhibition sensitized both PC cells and PC xenograft tumors to radiation, while not affecting the radiation sensitivity of the surrounding tissues. In summary, this work introduces the novel concept of a critical role of Jak2-Stat5a/b signaling for HR DNA repair in PC through induction of Rad51 expression. Conceptually, inhibition of Jak2-Stat5a/b signaling represents a novel strategy to introduce transient BRCA-ness in PC to sensitize PC to radiation by impairing HR DNA repair and, therefore, may provide an adjuvant therapy for radiation treatment of PC.

MATERIALS AND METHODS

Cell Lines and Reagents

CWR22Rv1, PC-3, DU145, LNCaP (all from ATCC, between 2003 and 2006), LAPC-4 (provided by Dr. Charles Sawyers) and CWR22Pc (38). PC cells were cultured in RPMI-1640 medium (Mediatech) containing 10% fetal bovine serum (FBS; Gemini), 2 mM L-glutamine (Mediatech) and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively; Mediatech). LNCaP and CWR22Pc cells were cultured in the presence of 0.5 nM and 0.8 nM dihydrotestosterone (DHT), respectively. All cell lines included in this study have been authenticated on a regular basis in the users' laboratory. The testing has been conducted by, DNA fingerprinting, isozyme collection, observation of characteristic cell morphology, hormone/growth factor responsiveness and the expression of cell lines specific markers such as PSA, hormone receptors, Stat3/Stat5, Erk1/2Protein, and Akt. All cell lines were tested for mycoplasma contamination (PCR Mycoplasma Detection Set; Takara Bio Inc.,) every 3 months.IST5-002 (37) (Fox Chase Chemical Diversity), AZD1480 (35) and ruxolitinib (MedChem Express), and MG132 and Cyclohexamide (Calbiochem) were dissolved according to the manufacturers' instructions.

Generation of Adenoviral and Lentiviral Expression Vectors

Detailed information is provided in Supplementary Materials and Methods.

Single-cell Gel Electrophoresis (Neutral Comet Assay)

CWR22Rv1 cells expressing wild-type (WT) Stat5b, DNStat5a/b or LacZ (adenoviral gene transfer at MOI=5) were irradiated with a single dose of 10 Gy, mixed with 0.7% low melting-point agarose (Sigma-Aldrich), spread on CometSlide™ microscope slides (Trevigen), followed by cell lysis. After electrophoresis, slides were stained with ethidium bromide and comets were scored (100 cells per treatment) under a fluorescence microscope (HistoRx Inc.) followed by analysis using CometScore 15 software (TriTek Corp.). The comet parameter (olive tail moment) reflects the amount of unrepaired DNA released from the cells (39).

SiRNA and Stat5a/b Antisense Transfections

Detailed information is provided in Supplementary Materials and Methods.

Pulsed Field Gel Electrophoresis (PFGE)

Stat5a/b was depleted by lentiviral expression of Stat5a/b shRNA (shStat5a/b) vs. non-target shRNA in CWR22Rv1 cells for 72 h followed by irradiation with a single dose of 10 Gy. Cells were harvested 10, 20, 30 or 60 min after irradiation, mixed with CleanCut agarose (0.75%) and transferred to agarose embedded DNA plug molds (CHEF genomic DNA Plug Kit; BioRad). For 0 min incubation, cells were irradiated on ice. DNA was lysed and digested with Proteinase K, incubated overnight (50°C) and run in 0.5% Pulsed Field Certified Agarose using CHEF Mapper® XA Pulsed Field Electrophoresis System (BioRad) in 0.5% TBE at the following conditions: 0.9 V/cm of voltage, 60° angle time for 75 min, 120° angle time for 65 hours and 25 mA at 14°C with linear ramping factors. Quantitative

analysis of the intensity of stained and released broken DNA in the gel was performed by Scion 2 software (Meyer Instruments, Inc.).

Immunofluorescence Staining

Detailed information is provided in Supplementary Materials and Methods.

Immunoprecipitation and Immunoblotting

Detailed information is provided in Supplementary Materials and Methods.

Rad51 Foci Formation Assay

Stat5a/b shRNA or control shRNA were expressed using lentivirus in CWR22Rv1 cells, irradiated with a single dose of10 Gy after 48 hours, and immunostained with primary anti-Rad51 pAb (Santa Cruz Biotechnology), fluorescein-conjugated goat anti-rabbit secondary antibody (Vector Labs), followed by analysis using a Zeiss LSM 510 laser-scanning microscope (Zeiss). For quantification, 200 nuclei were analyzed per each treatment group and cells with more than five Rad51 positive foci were counted as Rad51 foci positive cells.

Immunohistochemistry and Scoring of Cell Viability and Immunostainings

Detailed information is provided in Supplementary Materials and Methods.

Homologous Recombination DNA Repair (HRR) Assay

CWR22Rv1 cells stably expressing pDR-GFP reporter were established (CWR22Rv1/pDR-GFP). LacZ and CAStat5a/b were introduced to CWR22Rv1/pDR-GFP cells using adenoviral vector at MOI=5, and genetic knockdown was achieved by lentiviral expression of Stat5a/b shRNA (shStat5a/b) vs. control shRNA (shCtrl). After 48 h, cells were transiently transfected using X-fect (Clontech) with 10 μg pCbA-Sce, which contains I-SceI cDNA to generate DSBs in Sce-GFP cDNA (40,41). After 72 h, the amount of functional GFP reflecting homologous recombination DNA repair was analyzed by fluorescence-activated cell sorting (FACS). Ethanol-fixed cells were stained with propidium-iodide and treated with RNase (Sigma Aldrich) followed by analysis by BD LSR II flow cytometry using the FL-2 channel.

Quantitative real-time RT-PCR

Detailed information is provided in Supplementary Materials and Methods.

Clonogenic Survival Assay

CWR22Rv1, CWR22Pc and DU145 cells were irradiated with 0–8 Gy and seeded at various densities. After 21 days, colonies were stained for 30 minutes with 0.25% crystal violet (Sigma-Aldrich) and counted (minimum 50 cells/colony). Plating efficiency (colonies counted/cells seeded × 100) and survival fraction [colonies counted/cells seeded × (plating efficiency/100)] were calculated for each group.

Ex vivo 3D Tumor Explant Cultures of Patient-Derived PCs

Detailed information is provided in Supplementary Materials and Methods.

Human PC Xenograft Tumor Growth Studies

Castrated athymic nude male mice (Taconic), cared for according to institutional guidelines, were implanted with sustained-release DHT pellets (90 d release, 1 pellet/mouse; Innovative Research of America, Sarasota) 7 days before PC cell inoculation. CWR22Rv1 cells (20×10^6) in 0.2 ml Matrigel (BD Biosciences) were injected subcutaneously (s.c.) into the right flank of each mouse ($n = 10$/group, one tumor per mouse), and the treatments were initiated when tumors reached 100 mm^3. Daily intraperitoneal (i.p.) injections of IST5-002 (37) (10 mg/kg or 20 mg/kg) were initiated 3 days prior to irradiation, which was administered at a dose of 2 Gy daily for 3 consecutive days to more closely mimic fractionated radiation. Radiation was performed on anesthetized mice (75 mg/kg ketamine/0.35 mg/kg acepromazine) using an X-ray machine (Gulmay Medical, Bethel) operating at 250 kV, 10 mA, with 2-mm aluminum filtration and effective photon energy of ≈ 90 keV. Tumor volumes were measured 3 times weekly and calculated using the formula $V= (\pi/6) \times d_1 \times (d_2)^2$, with d_1 and d_2 being two perpendicular tumor diameters.

In Vivo Pelvic Irradiation Studies

The lower abdomens of athymic nude male mice were irradiated at 2 Gy/day for 3 consecutive days (250 kVp, approximate dose rate 1.4 Gy/min, 50 cm distance, 2 mm copper filter; Pantak). Half of the mice were injected i.p. with vehicle or IST5-002 (20 mg/kg) for 3 days before irradiation and for 3 day additional days during the irradiation, while control mice did not receive irradiation. On day 7, the animals were euthanized. Cryosections (7 μm) of small intestines were immunostained for Ki-67 (mAb; Abcam), CD45 (pAb; R&D Systems), and TUNEL (Apo-Green Detection Kit; Biotool). For quantitation of apoptotic and proliferating cells in the crypts, the total number of crypt-associated cells was assessed on 3 independent microscopic (20×) fields for each assay based on DAPI staining, followed by counting of either TUNEL-, CD45 or Ki-67-positive crypt-associated cells on each image.

Statistical Analyses

Detailed information is provided in Supplementary Materials and Methods.

RESULTS

Stat5a/b is critical for Rad51 expression in prostate cancer

To investigate whether Rad51 expression is regulated by Jak2-Stat5a/b signaling in PC, we first evaluated basal expression levels of Rad51, BRCA1 and BRCA2 in a panel of PC cell lines, as shown in Figure 1A. Stat5a/b was inhibited by genetic knockdown of Stat5a/b by lentiviral expression of Stat5a/b shRNA or transfection with Stat5a/b siRNA, which down-regulated Rad51 levels in DU145 and CWR22Rv1 cells (Fig. 1B). Similarly, Stat5a/b inhibition by antisense oligodeoxynucleotides (ODN) for 72 h decreased Rad51 protein levels markedly in CWR22Rv1 and LNCaP cells compared with mismatch ODN (Fig. 1B). Conversely, adenoviral expression of constitutively active Stat5a/b (CAStat5a/b) in CWR22Pc and CWR22Rv1 cells resulted in a robust increase in Rad51 expression (Fig. 1B). Importantly, direct inhibition of Stat5a/b by a specific small molecule Stat5a/b

inhibitor, IST5-002 (37), caused a dose-dependent reduction in Rad51 levels in CWR22Rv1, CWR22Pc, LAPC-4, DU145 and LNCaP cells at 72 h (Fig. 1C). Collectively, these results indicate that Stat5a/b is critical for Rad51 protein expression in PC cells.

To evaluate if Stat5a/b induction of Rad51 occurs via Jak2 signaling, Jak2 was inhibited in CWR22Pc and CWR22Rv1 cells by genetic or pharmacological knockdown. Lentiviral expression of Jak2 shRNA suppressed Rad51 in both cell lines (Fig. 1D). Similarly, inhibition of Jak2 by pharmacological Jak2-inhibitors AZD1480 (0.8 μM) or ruxolitinib (0.4 μM) resulted in a robust decrease in Rad51 protein expression in both cell lines at 72 h (Fig. 1D), indicating that Stat5a/b up-regulation of Rad51 occurs through Jak2-dependent processes. To determine if Stat5a/b is critical for Rad51 expression in patient-derived PCs, we utilized 3D tumor explant culture system of patient-derived PCs *ex vivo*, which we have rigorously described previously (26,35–37,42). All tissue components of PC, including epithelium and stroma, are retained in this culture system, thus offering a more physiological model of PC growth than PC cell lines. PCs from four patients (Table 1) were cultured *ex vivo* in 3D explant cultures and treated with IST5-002 (25 μM) or AZD1480 (25 μM) for 7 days (37). As shown in Figure 1E, inhibition of Jak2-Stat5a/b signaling by IST5-002 or AZD1480 potently reduced Rad51 expression in the clinical PCs tested. Collectively, these data indicate that Jak2-Stat5a/b signaling is an inducer of Rad51 expression not only in human PC cell lines but also in clinical PCs. Moreover, these data show that Rad51 levels in PC can be effectively reduced by pharmacological inhibitors of Jak2-Stat5a/b signaling pathway.

We next sought to determine whether Stat5a/b regulates Rad51 at the mRNA or protein level in PC. First, to assess if loss of Rad51 protein after Stat5a/b knockdown was due to increased flux of Rad51 through the proteasome, we genetically inhibited Stat5a/b for 72 h followed by treatment with proteasome inhibitor MG132 (10 μM) for 6 h (Fig. 2A). Suppression of proteasomal degradation did not reverse down-regulation of Rad51 protein induced by genetic depletion of Stat5a/b by shRNA (Fig. 2A). To further evaluate if Rad51 is down-regulated by Stat5a/b knockdown at the protein level, new protein synthesis was restricted by cycloheximide (CHX) (35 μM) for 6 h, which lowered the Rad51 levels as expected. However, Rad51 levels were not rescued by inhibition of the proteasome with MG132, indicating that Stat5a/b does not protect Rad51 from proteasomal degradation (Fig. 2A, right panel). At the same time, inhibition of Stat5a/b by shRNA resulted in a marked decrease in Rad51 mRNA expression in both CWR22Rv1 ($P<0.001$) and DU145 ($P<0.01$) cells compared to cells expressing control shRNA at 72 h after genetic Stat5a/b depletion, as indicated by quantitative RT-PCR using cells with genetic Rad51 knockdown as a positive control for the assay (Fig. 2B). Effective Stat5 knockdown was verified by immunoblotting (Fig. 2B). In summary, these data indicate that Stat5a/b regulates Rad51 expression in PC cells at the mRNA level.

Stat5a/b promotes radiation-induced DNA repair in prostate cancer

Since Stat5a/b regulates Rad51 expression in PC, we investigated whether Stat5a/b is involved in radiation-induced DNA repair in PC. We first analyzed radiation-induced DNA damage repair using the neutral comet assay. In this assay, damaged DNA is released from

the cell, generating a “tail” (termed the olive tail moment), the length and density of which reflects unrepaired DNA (43). Stat5a/b signaling was inhibited in CWR22Rv1 cells by adenoviral expression of DNStat5a/b, with AdLacZ, AdWTStat5b or mock-infected cells as controls for 48 h. As shown in Figure 3A, CWR22Rv1 cells displayed similar amounts of DNA damage regardless of the status of Stat5a/b signaling activity one hour after irradiation at a dose of 10 Gy. Most DNA damage was repaired at 24 hours when Stat5a/b signaling was intact (Fig. 3A). In contrast, Stat5a/b inhibition by AdDNStat5a/b resulted in a 13-fold increase in the olive tail moment 24 h after irradiation when compared to the AdLacZ control group ($P<0.001$) (Fig. 3A). In conclusion, Stat5a/b inhibition resulted in decreased repair of radiation-induced DNA damage, indicating that Stat5a/b regulates radiation response of PC cells.

Stat5a/b induces Homologous Recombination DNA repair in prostate cancer cells through up-regulation of Rad51

Given that Stat5a/b up-regulates Rad51 expression in PC cells (Fig. 1), we next investigated if Stat5a/b regulates loading of Rad51 onto dsDNA breaks and formation of Rad51 repair foci (7–9,15) in PC cells following radiation. Stat5a/b signaling was inhibited by RNA interference in CWR22Rv1 cells for 48 h. Following irradiation with 10 Gy, inhibition of Stat5a/b suppressed Rad51 foci formation by 75% compared to non-target control shRNA (shCtrl) ($p<0.001$) (Fig, 3B), indicating that Stat5a/b induces DNA repair through a Rad51-dependent mechanism.

To directly assess whether Stat5a/b upregulates radiation-induced DNA repair through promotion of the HR DNA repair pathway, we utilized the Sce-GFP assay which is based on a recombination reporter pDR-GFP plasmid containing an inactive GFP gene (Sce-GFP) and a fragment of the GFP gene as a donor for homologous repair (40,41). The Sce-GFP cassette has an inactivating insertion, which consists of two STOP codons and a restriction site for the rare cutting endonuclease, I-SceI. When I-SceI is expressed in pDR-GFP expressing cells, it inflicts DSBs within the Sce-GFP fragment and provides a signal for HR DNA repair. This is followed by reconstruction of functional GFP, which is readily detectable by fluorescence microscopy or FACS (40,41). Stat5a/b signaling was increased by adenoviral expression of constitutively active (CA) Stat5a/b (AdLacZ as control) in CWR22Rv1 cells stably expressing pDR-GFP. In parallel experiments, Stat5a/b was inhibited in CWR22Rv1/pDR-GFP cells by lentiviral expression of Stat5a/b shRNA. After 48 hours, DNA DSBs were introduced by transfection of the cells with pCbA-SceI for 72 h. Compared to LacZ control, Stat5a/b activation resulted in an approximately 40% increase in HR DNA repair ($P<0.01$) (Fig. 3C; left panel). At the same time, Stat5a/b inhibition reduced HR DNA repair by approximately 30% compared to cells expressing non-target shRNA ($P<0.01$) (shCtrl; Fig. 3C; right panel). To mechanistically test the linkage of Stat5a/b with Rad51 expression and HR DNA repair in PC cells, introduction of CAStat5a/b using adenovirus (AdLacZ as control) lead to 52% increase ($P<0.001$) in HR DNA repair in CWR22Rv1 cells (Fig. 3D). Genetic knockdown of Rad51 by lentiviral expression of Rad51 shRNA reversed ($P<0.001$) CAStat5a/b induction of HR DNA repair. As control, depletion of Rad51 by lentiviral Rad51 shRNA decreased HR DNA repair below the basal levels in control groups (AdLacZ +

shCtrl, shCtrl) (P<0.001) (Fig. 3D) Together, these data support the concept that Stat5a/b up-regulates HR repair through induction of Rad51 expression in PC cells.

To evaluate if Stat5a/b regulates NHEJ DNA repair in PC cells, we next measured DNA fragmentation following irradiation by pulsed field gel electrophoresis (PFGE). This assay quantifies the amount of dsDNA damage based on the pattern of migratory DNA. The repair kinetics within a window of 0–60 min after irradiation reflects NHEJ repair of dsDNA breaks (11,12). Inhibition of Stat5a/b expression by lentiviral expression of Stat5a/b shRNA (48 h) had no effect (P=0.88) on dsDNA break repair in CWR22Rv1 cells irradiated with 20 Gy for the indicated times (Fig. 3E). Specifically, the DNA fragmentation pattern of cells depleted of Stat5a/b by shRNA was similar to that of non-irradiated controls 60 min after the irradiation (Fig. 3E). These results demonstrate that Stat5a/b does not influence dsDNA break repair through the NHEJ DNA repair pathway.

Inhibition of Stat5a/b sensitizes prostate cancer cells to radiation-induced cell death

Having established that Stat5a/b up-regulates HR DNA repair in PC cells, we investigated whether Stat5a/b inhibition sensitizes PC cells to radiation-induced cell death. Stat5a/b signaling was inhibited by IST5-002 (37) in CWR22Rv1, CWR22Pc, and DU145 cells for 48 h followed by irradiation with 0 or 3 Gy, and immunostaining for the dsDNA break marker γH2AX 4 h after irradiation. Treatment of CWR22Rv1, CWR22Pc, and DU145 cells with IST5-002 resulted in a marked increase in γH2AX levels in irradiated cells in a dose-dependent manner (Fig. 4A), indicating that accumulation of dsDNA breaks is associated with Stat5a/b inhibition. To further evaluate Stat5a/b inhibition as a strategy to sensitize PC cells to radiation, Stat5a/b was disrupted by expression of AdDNStat5a/b in CWR22Rv1 cells for 48 h followed by irradiation (0, 1, 2, 3, 4, 5, 6, 8 Gy) with assessment of clonogenic survival by crystal violet staining after 21 days. Stat5a/b inhibition by genetic depletion potently reduced clonogenic survival of PC cells compared with controls (P<0.001) (Fig. 4B). To evaluate whether pharmacological inhibition of Stat5a/b also sensitized PC cells to radiation, Stat5a/b was inhibited in CWR22Rv1 cells by IST5-002 (48 h), followed by irradiation with 0, 1, 2, 3, 3.5 or 4 Gy. Inhibition of Stat5a/b by IST5-002 robustly reduced the fraction of surviving cell clones from approximately 0.25 to 0.1, 0.08 to 0.02, and 0.02 to <0.01 at irradiation doses of 2, 3, and 4 Gy, respectively (P<0.01) (Fig. 4B). Similarly, CWR22Pc and DU145 cells demonstrated significantly decreased clonogenic survival after treatment with IST5-002 and irradiation with 0, 0.5, 1.5, 2.5, or 3 Gy (P<0.001) (Fig. 4C). Collectively, these data show that Stat5a/b inhibition effectively sensitizes PC cells to radiation-induced.

Pharmacologic inhibition of Stat5a/b sensitizes prostate cancer to radiation, induces accumulation of dsDNA breaks and suppresses Rad51 levels *in vivo*, while not affecting viability of gastro-intestinal mucosal epithelium

To evaluate whether Stat5a/b inhibition sensitizes PC to radiation *in vivo*, CWR22Rv1 PC cells were inoculated subcutaneously (s.c.) into castrated male athymic nude mice supplemented with sustained-release DHT pellets to normalize circulating androgen levels. Once the tumors reached 5–6 mm in diameter, Stat5a/b was inhibited by IST5-002 at two different doses (10 mg/kg, 20 mg/kg), which by themselves are insufficient to significantly

suppress tumor growth if administered without radiation (Fig. 5A). Three days after Stat5a/b inhibitor treatment was started, the tumors were irradiated for three consecutive days at 0 or 2 Gy. Tumor growth was suppressed by approximately 40–45% on day 29 in mice treated with 10 mg/kg IST5-002 combined with radiation compared to mice receiving radiation alone (P=0.07) (Fig. 5A). At the same time, treatment with 20 mg/kg IST5-002 combined with radiation suppressed growth of the tumors by 60% compared to mice treated with radiation only (P<0.01) or IST5-002 (20 mg/kg) without radiation (P<0.01) (Figure 5A). A significant loss (80%; P<0.001) in cell viability was associated with irradiation combined with 20 mg/kg IST5-002 compared to vehicle control, and 50% decrease (P<0.01) compared to radiation alone (Fig. 5B, left panel). Immunohistochemical analysis of tumor sections showed significant decrease in nuclear active Stat5a/b levels in all tumors treated with IST5-002 compared to vehicle or irradiation (P<0.001), with the greatest decrease evident in tumors of mice treated with IST5-002 (20 mg/kg; Fig. 5B, right panel). As expected, radiation alone did not affect nuclear active Stat5a/b levels. At the same time, radiation increased both γH2AX (p<0.01) and Rad51 (P<0.001) levels in CWR22Rv1 tumors. The γH2AX levels in irradiated tumors, indicating DNA breaks, were further increased by IST5-002 compared to either vehicle or irradiation-only groups (P<0.001), while Rad51 levels were decreased in the tumors treated by IST5-002 compared to vehicle group (P<0.01) (Fig. 5B). In summary, these results demonstrate that inhibition of Stat5a/b by IST5-002 sensitizes PC xenograft tumors to radiation, while preventing nuclear localization of Stat5a/b, leading to accumulation of dsDNA breaks, and suppression of Rad51 expression.

Intestinal epithelial cells undergo apoptosis if exposed to critical amounts of radiation during pelvic radiation, which leads to radiation-induced acute side effects including diarrhea and potentially serious late toxicities such a bowel obstruction and/or perforation of the intestinal wall (5,6). To evaluate if IST5-002 combined with radiation is associated with increased damage to the intestinal epithelium, mice were treated with vehicle or IST5-002 while receiving lower abdominal irradiation with 2 Gy (no irradiation as control). Overall, the structure and morphology of the intestinal crypts harboring intestinal stem cells were similar in mice receiving radiation alone or IST-002 with radiation (Fig. 5C). Inhibition of Stat5a/b by IST5-002 did not lead to sensitization of the cells in the intestinal crypts to radiation, as shown by lack of increase in TUNEL positivity indicative of apoptotic cells (Fig. 5C). Moreover, proliferating cells within the crypts were not decreased by IST5-002 combined with radiation compared to radiation only (Fig. 5C). Collectively, these data demonstrate that Stat5a/b inhibition sensitizes PC to radiation *in vitro* and *in vivo* without increased damage to the gastro-intestinal mucosa.

DISCUSSION

Radiation therapy is the mainstay of the treatment for locally advanced PC (1,3), and one of the key treatment options for organ-confined PC (1). Radiation therapy can be associated with debilitating side effects due to unintentional radiation delivered to neighboring organs especially the bowel and rectum (5,6). Therefore, identification of new strategies to selectively sensitize PC to radiation would allow the use of lower radiation doses resulting in less damage to the normal tissues surrounding prostate and thus fewer side effects. In this

study, we show, for the first time, that Jak2-Stat5a/b signaling in PC induces HR DNA repair through up-regulation of Rad51. Inhibition of Stat5a/b depletes Rad51 in PC cells, disrupts HR DNA repair and sensitizes PC to radiation and, therefore, may provide an adjuvant therapy for radiation, improve outcomes and reduce radiation-induced side effects.

One of the key findings of the work presented here is the concept that Stat5a/b is a critical inducer of Rad51 and HR DNA repair in PC. We demonstrate that Stat5a/b up-regulates Rad51 expression in a panel of PC cell lines, in PC xenograft tumors *in vivo* and in clinical patient-derived PCs *ex vivo* in 3D explant cultures. Stat5a/b-regulation of Rad51 was independent of BRCA1/2 status of PC, demonstrating that Stat5a/b has a primary role in controlling Rad51 expression in PC. We further show that Rad51 induction by Stat5a/b occurs at the mRNA rather than the protein level, consistent with the previous findings of the presence of Stat5a/b response element in the regulatory regions of the Rad51 gene (24). Genetic or pharmacological knock-down of Jak2 by small-molecule Jak2 inhibitors significantly suppressed Rad51 levels in PC cells indicating that Stat5a/b-promotion of Rad51 expression occurs through Jak2-dependent mechanisms. In addition, a specific small-molecule inhibitor of Stat5a/b, IST5-002 (37), effectively inhibited Rad51 expression both *in vitro and in vivo* in PC xenograft tumors. Most importantly, IST5-002 and AZD1480, both, robustly decreased Rad51 levels in patient-derived PCs *ex vivo* in 3D explant cultures indicating that Stat5 regulation of Rad51 extends to clinical PCs. Collectively, this work introduces a novel concept of Jak2-Stat5a/b signaling pathway as a critical regulator of Rad51 expression in PC.

Inhibition of Stat5a/b suppressed HR DNA repair and sensitized PC cells and xenograft tumors to radiation through down-regulation of Rad51 expression. Mechanistic association of Rad51 to Stat5a/b regulation of HR DNA repair was demonstrated in a functional assay of HR DNA repair, where Stat5a/b-induced increase in HR-DNA repair was abolished by genetic knockdown of Rad51. At the same time, Stat5a/b inhibition sensitized PC cells and prostate xenograft tumors to radiation. In these experiments, IST5-002 was administered at low doses, which were sufficient to reduce Stat5a/b action but were insufficient to significantly block growth of PC cells and xenograft tumors alone without radiation. Concomitant with Stat5a/b suppression, Rad51 expression was decreased, while gamma-H2AX immunostaining marking dsDNA breaks was increased, indicating involvement of Rad51 suppression in sensitization of PC to radiation *in vivo*. Importantly, IST5-002 did not sensitize epithelial cells in the crypts of small intestines of mice to radiation implying specificity of Stat5a/b-regulation of Rad51 to PC tissues, which may be due to both elevated Rad51 and Stat5a/b levels in PC (26–29,44). A recent study reported reduction of radiation-induced intestinal injury induced by Stat5a/b (45). However, both the experimental systems as well as the designs were different between our study and the work by Gilbert et al. (45) where Gilbert et al. used single doses ranging from 8.5 – 20 Gy, while we used a cumulative dose of 6 Gy (3 × 2 Gy). Of note, only moderate effects on crypt numbers and morphology were observed at the lowest dose (8.5 Gy), consistent with the results presented here. Furthermore, transient pharmacological inhibition of Stat5a/b activity is distinct from genetic knock-out of Stat5 in gastrointestinal stem cells in that, absent radiation, the knockout mice exhibited altered expression of differentiation makers potentially predisposing these cells to more pronounced radiation damage. In conclusion, our results

support the concept that Stat5a/b inhibition may provide a strategy to sensitize PC to radiation while sparing the surrounding normal tissues.

Establishment of a pharmacologic strategy to suppress Rad51 expression and HR DNA repair by targeting Jak2-Stat5a/b signaling in PC has high translational significance. This is because active Stat5a/b and Rad51, both, are highly expressed in malignant, but not in normal prostate epithelium (26–29). Moreover, potent inhibitors targeting Jak2-Stat5a/b signaling are in clinical development and can be readily used to suppress HR DNA repair in PC. Conceptually, depletion of Rad51 by Stat5a/b inhibition induces transient BRCA-ness in PC cells to impair HR DNA repair of radiation-induced dsDNA breaks (14,19,46). The term BRCA-ness refers to dysfunctional BRCA1/2 which leads to defects in HR DNA repair through compromising Rad51 function, and it is known to sensitize cancer cells to therapies inducing dsDNA breaks such as radiation and platinum chemotherapy (46). Moreover, BRCA1/2 defective cancer cells have increased sensitivity to PARP inhibitors (22,47), which suppress nucleotide excision repair leading to accumulation of ssDNA breaks that convert to dsDNA breaks during DNA replication (48) making the cancer cells increasingly dependent on HR DNA repair (49). Future studies need to address whether Stat5a/b inhibitor-induced BRCA-ness will also sensitize PC cells to PARP-inhibitors and platinum-based chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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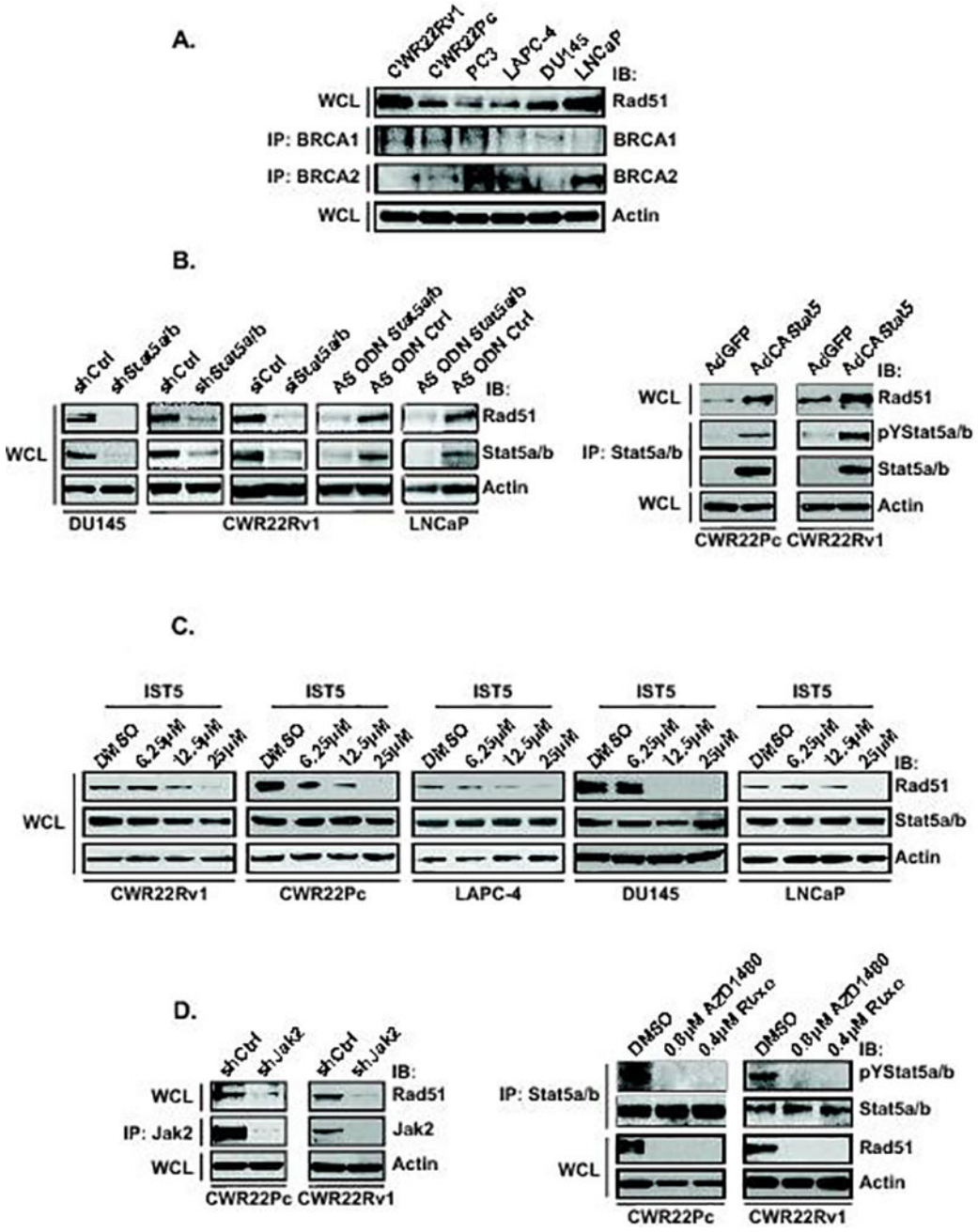
TRANSLATIONAL RELEVANCE

Radiation therapy is a key treatment option for both organ-confined and locally advanced prostate cancer (PC). However, irradiation is often associated with significant toxicities to the neighboring tissues, which can cause debilitating side-effects. In the present study, we demonstrated proof-of-concept that targeting Stat5a/b signaling sensitizes PC to radiation through regulation of DNA repair. Our results provide, for the first time, mechanistic evidence that Jak2-Stat5a/b signaling is critical for Rad51 expression and Homologous-Recombination DNA repair in PC. Using human PC cell lines, xenograft tumors and *ex vivo* culture of clinical PCs, we show that genetic or pharmacological inhibition of Stat5a/b sensitizes PC to irradiation while not affecting the radiation sensitivity of the surrounding tissues. These findings provide a strong rationale for development of Stat5a/b inhibitors as adjuvant therapy for radiation treatment of PC.



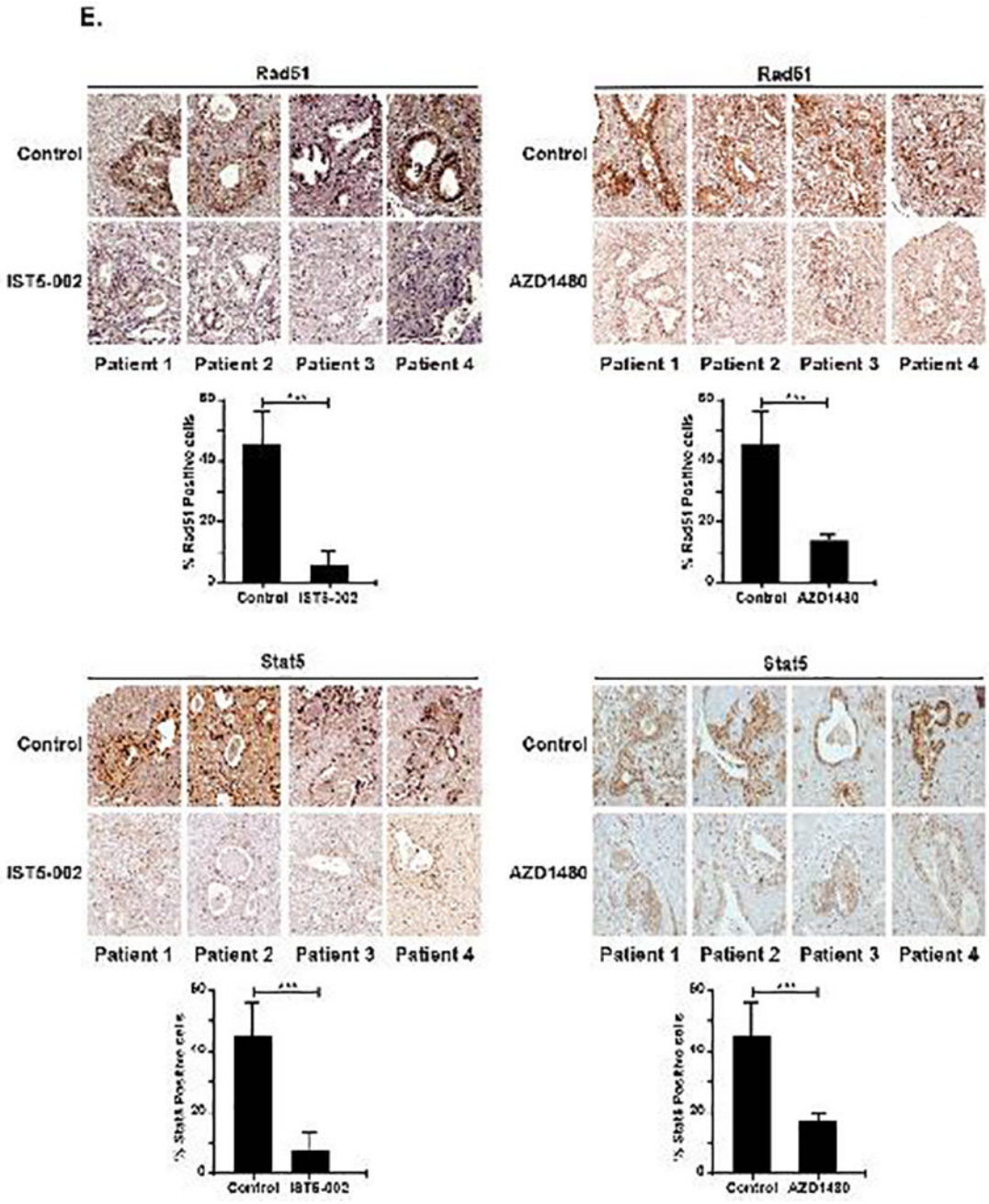


Figure 1. Stat5a/b drives Rad51 expression in human PC

A, Basal levels of Rad51, BRCA1 and BRCA2 in exponentially growing CWR22Pc, CWR22Rv1, LNCaP, DU145 and PC-3 cells shown by immunoblotting (IB) of whole cell lysates (WCL) or immunoprecipitates (IP), as indicated. **B,** Immunoblotting of Rad51 in DU145 cells and CWR22Rv1 cells with lentiviral expression of Stat5 shRNA (shStat5a/b) vs. control shRNA (shCtrl) or transfection with Stat5a/b siRNA vs. control (Ctrl) siRNA for 48 h in CWR22Rv1 cells. Alternatively, CWR22Rv1 and LNCaP cells were transfected with Stat5a/b or control (Ctrl) antisense oligodeoxynucleotides (AS ODN) for 48 h followed by immunoblotting for Rad51. Conversely, Stat5a/b signaling was increased by adenoviral (Ad)

expression of constitutively active (CA) Stat5a/b or GFP (control) followed by Western blot analysis of immunoprecipitated (IP) Stat5a/b. Effective genetic depletion of Stat5a/b, increased active Stat5a/b levels and equal loading are demonstrated by immunoblotting with anti-Stat5a/b, anti-PYStat5a/b and anti-actin antibodies of WCLs or IPs, as indicated. **C,** Western blot analysis of Rad51 expression in PC cells treated with 0, 6.25, 12.5, or 25 μM Stat5a/b inhibitor IST5-002 for 72 h. **D,** Jak2 was inhibited by lentiviral expression of Jak2 shRNA (shJak2) or control shRNA (shCtrl) in CWR22Pc and CWR22Rv1 cells. Alternatively, cells were treated with Jak2 inhibitors AZD1480 (0.8 μM) and ruxolitinib (Ruxo) (0.4 μM) (72 h), followed by Western blot analysis of Jak2 and active Stat5a/b. **E,** Stat5a/b inhibition decreases Rad51 expression in patient-derived PCs *ex vivo* in tumor explant cultures. Five clinical PCs were cultured for 7 days in the presence of IST5-002 (25 μM) or AZD1480 (25 μM) vs. vehicle, and Rad51 and active nuclear Stat5a/b levels were analyzed by immunohistochemistry followed by quantification (***P<0.001).

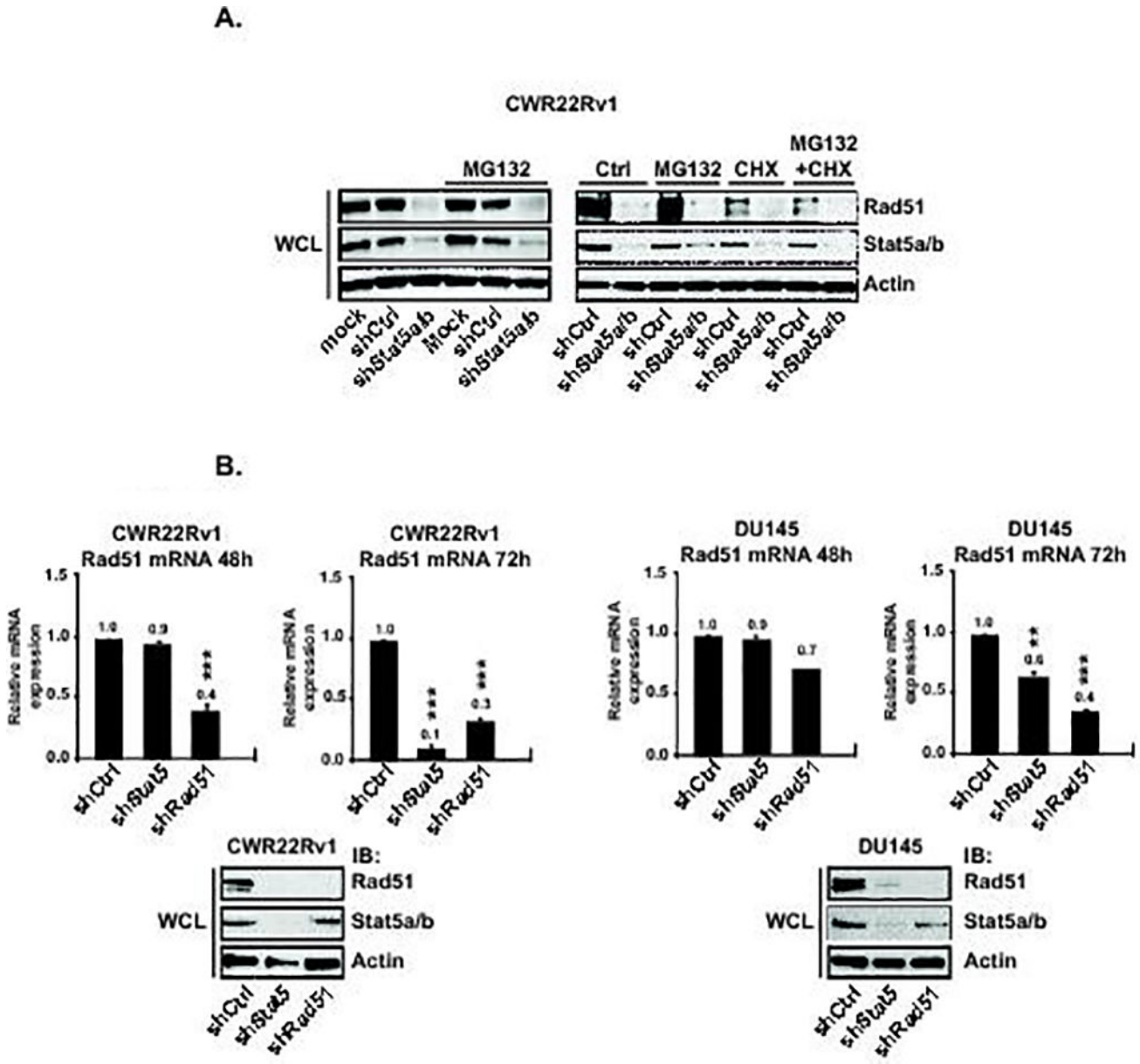
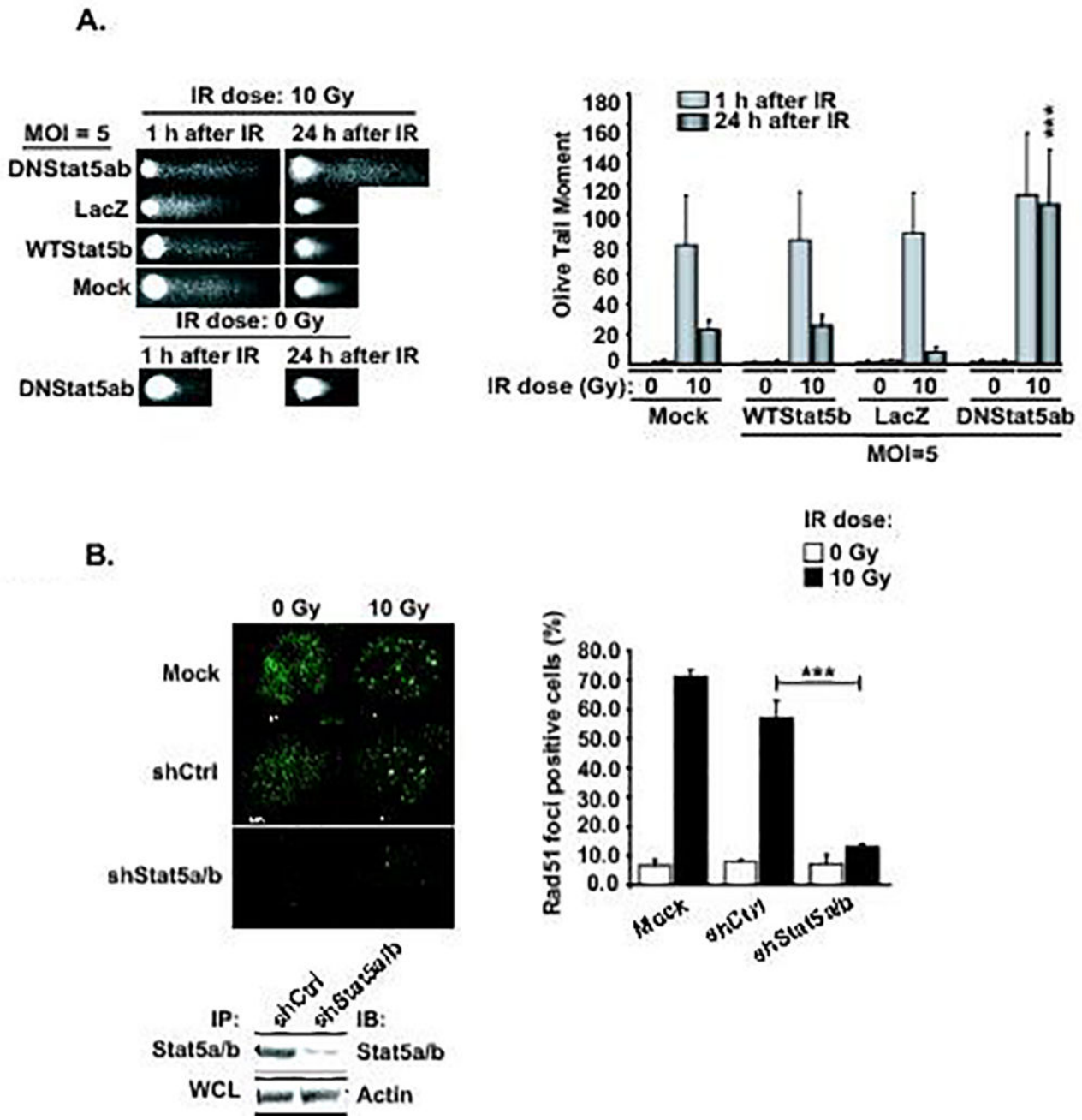


Figure 2. Stat5a/b inhibition suppresses Rad51 expression at the mRNA rather than protein level in PC

A, Stat5a/b was inhibited by lentiviral expression of Stat5a/b shRNA (shStat5a/b) or non-target shRNA (shCtrl) in CWR22Rv1 for 72 h, followed by treatment with MG132 (10 μM) proteasome inhibitor and/or cycloheximide (CHX) protein synthesis inhibitor (35 μM) for 6 h, as indicated. Rad51 and Stat5 levels were analyzed by immunoblotting of whole cell lysates (WCL). **B,** Stat5a/b was inhibited as described in (A) for 48 h and 72 h, followed by quantification of Rad51 mRNA levels by qRT-PCR (***P*<0.01, ****P*<0.001). Stat5a/b and Rad51 levels were analyzed from parallel wells by immunoblotting of WCLs with actin blotting as loading control.



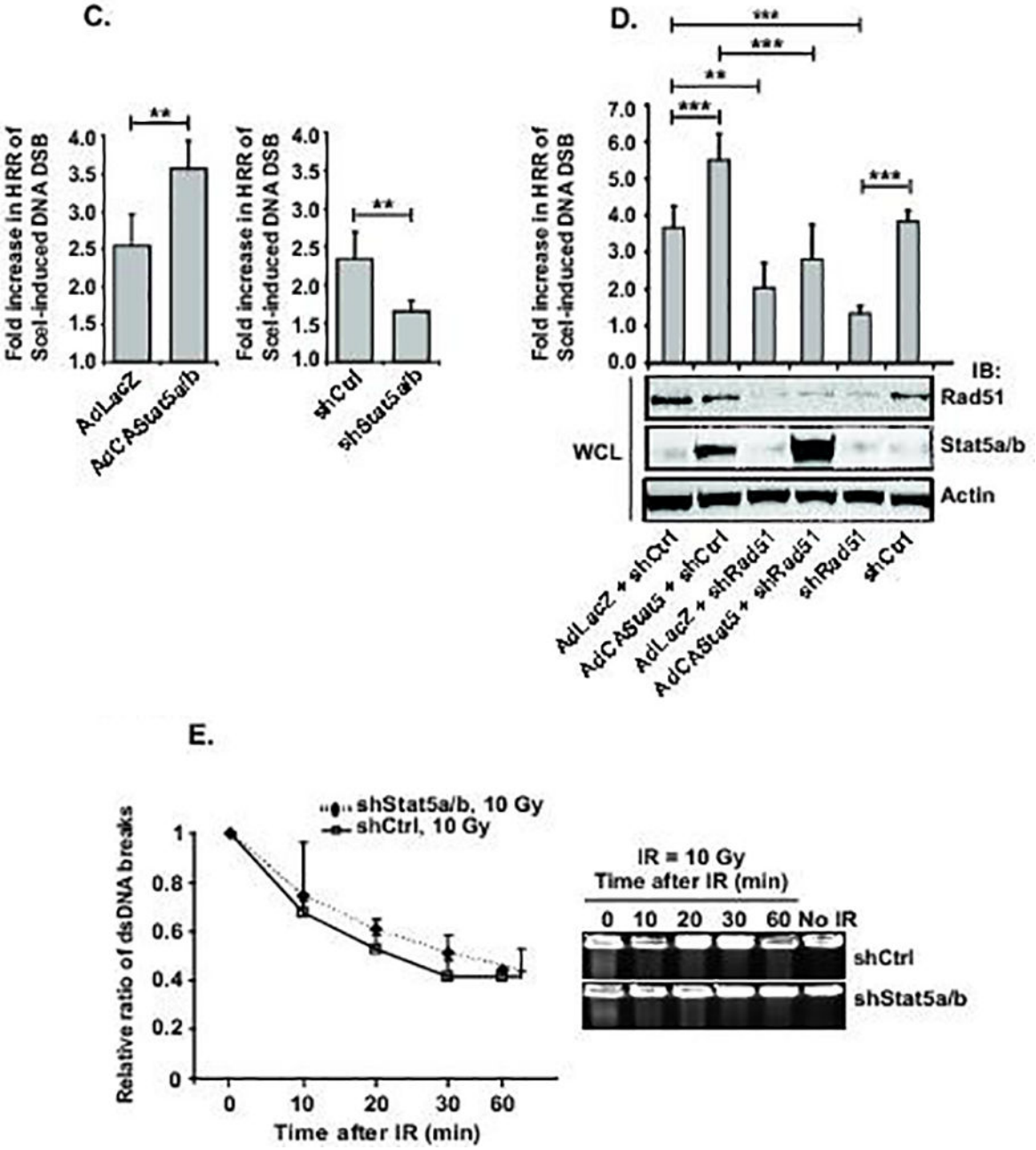


Figure 3. Stat5a/b inhibition suppresses homologous recombination (HR) repair of radiation-induced DSBs, while not affecting non-homologous end-joining (NHEJ) DNA repair in PC cells

Stat5a/b was inhibited by adenoviral (Ad) expression (MOI=5) of dominant-negative (DN) Stat5a/b in CWR22Rv1 cells with wild-type (WT) Stat5b, LacZ and mock-infection as controls for 48 h followed by neutral comet assay 1 h and 24 h after ionizing irradiation (IR) (10 Gy). **A,** Olive Tail Moment (% DNA × distance of center of gravity of DNA) (left panel) is plotted on the Y axis as the indicator of dsDNA breaks per each treatment group at different time points and radiation doses (right panel). **B,** Stat5a/b was inhibited for 48 h by lentiviral expression of shStat5a/b vs. non-target shRNA (shCtrl) in CWR22Rv1 followed by irradiation with 0 or 10 Gy and immunocytochemical analysis of Rad51 foci using Rad51

pAb and fluorescein-conjugated anti-rabbit secondary antibody. Approximately 200 nuclei for each treatment group were scored in each experiment, and a threshold of 5 foci/cell was considered positive. **C**, *Left panel:* Constitutively active (CA) Stat5a/b was expressed in CWR22Rv1/pDR-GFP cells by adenoviral (Ad) vector (MOI=5) with AdLacZ as control (left panel). *Right panel:* Stat5a/b was inhibited by lentiviral expression of shStat5a/b with non-target shRNA (shCtrl) as control. The amount of functional GFP (reflecting homologous recombination DNA repair) was analyzed by FACS. **D,** CAStat5a/b was expressed in CWR22Rv1/pDR-GFP cells with AdLacZ as control, as described in (C). At the same time, Stat5a/b was inhibited by lentiviral expression of shStat5a/b with non-target shRNA as control (shCtrl). In indicated groups, Rad51 was inhibited by lentiviral expression of Rad51 shRNA (shRad51), followed by transfection with pCbA-SceI and analysis of functional GFP by FACS (** $P<0.01$, *** $P<0.001$). **E,** Pulsed-field electrophoresis analysis of NHEJ DNA repair in CWR22Rv1 cells 0, 10, 20, 30 and 60 min after irradiation with 10 Gy. Stat5a/b was inhibited by lentiviral expression of shStat5a/b vs. shCtrl, as depicted. Relative ratios of intensity are plotted over time, and stained PFGE gel shows representative intensities of released, broken DNA following each treatment. Error bars indicate the standard deviations of the average values from three independent experiments.

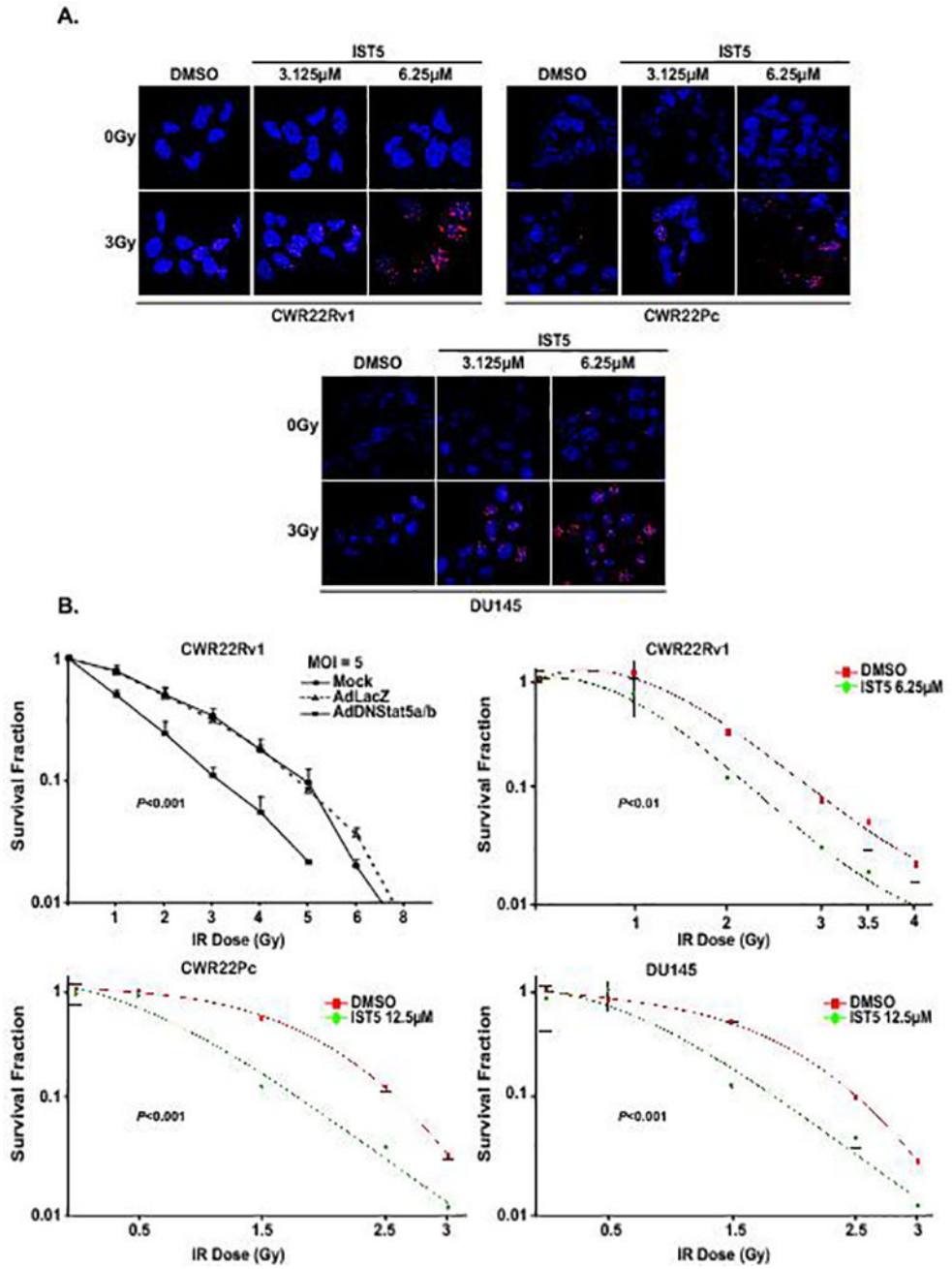
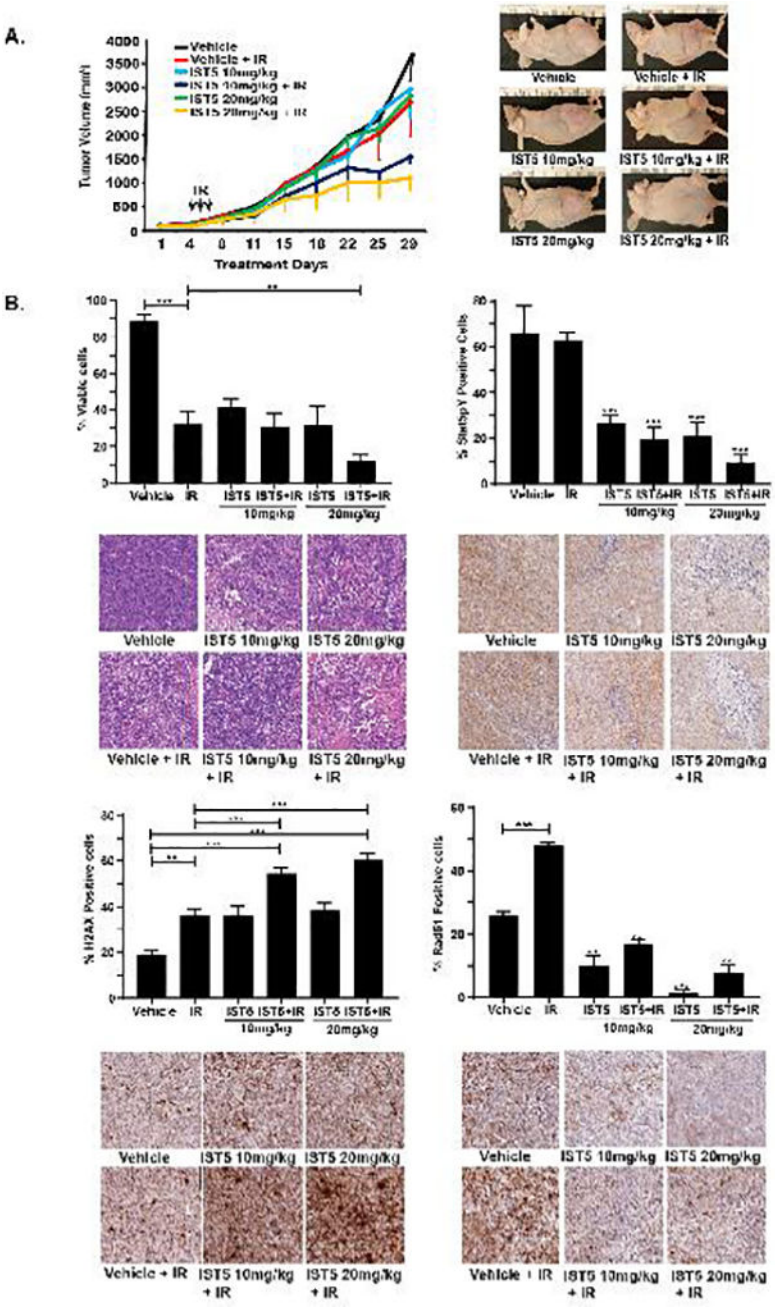


Figure 4. Stat5a/b inhibition increases accumulation of dsDNA breaks and suppresses clonogenic survival of human PC cells in response to radiation

A, Immunofluorescence staining of γH2AX indicating dsDNA breaks in CWR22Rv1, CWR22Pc, or DU145 cells treated with IST5-002 (3.1 or 6.3 μM or vehicle) followed by irradiation with 0 or 3 Gy (DAPI, blue; γH2AX, red). **B,** Clonogenic survival of CWR22Rv1 cells expressing DNStat5a/b or LacZ by adenoviral vector (Ad) (MOI=5) vs. mock-infected cells. Alternatively, CWR22Rv1, CWR22Pc and DU145 cells were treated with 6.25 μM or 12.5 μM IST5-002 or vehicle, as indicated. After 48 h cells, were irradiated with 0, 1, 2, 3, 4, 5 or 6 Gy. Colonies with >50 cells were counted and plating efficiency (colonies counted / cells seeded × 100) and survival fraction [colonies counted / cells seeded

× (plating efficiency / 100)] were calculated for each group. Survival fraction is plotted on a log-based scale versus IR dose.



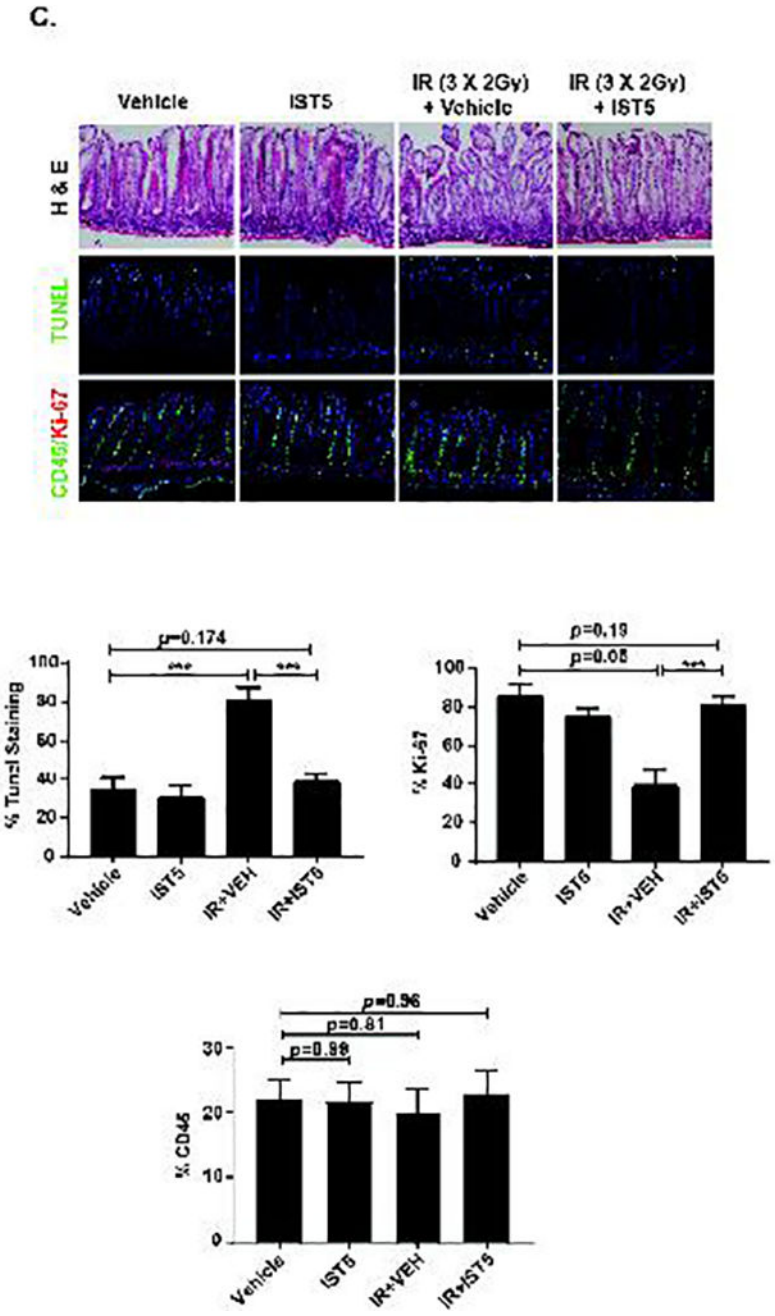


Figure 5. Pharmacological Stat5a/b inhibition sensitizes PC xenograft tumors to radiation, increases accumulation of dsDNA breaks and suppresses Rad51 levels, while not affecting viability of gastro-intestinal mucosal epithelium in irradiated mice

A, CWR22Rv1 cells grown as subcutaneous xenograft tumors in athymic nude mice were treated with vehicle (control), 10 mg/kg IST5-002 or 20 mg/kg IST5-002 i.p. daily until the largest tumor reached 2000 mm^3. On treatment days 3–5, tumors were irradiated (IR) with 0 or 2 Gy. Tumors were measured twice weekly and tumor volumes plotted over time. **B,** Hematoxylin and eosin staining of the CWR22Rv1 tumor sections showed a loss of viable tumor cells and accumulation of dead cells in irradiated tumors from mice treated with IST5-002 (20 mg/kg). Active nuclear Stat5a/b, γH2AX, and Rad51 nuclear were analyzed

by immunohistochemical analysis of CWR22Rv1 tumor sections treated with IST5-002 (10 mg/kg or 20 mg/kg) with or without irradiation (2 Gy) and quantified, as indicated. Statistical significance was calculated using a linear mixed effects model with empirical standard errors. **, $P < 0.01$; ***, $P < 0.001$. **C,** Inhibition of Stat5a/b alone, or in combination with radiation, does not affect viability or structure of the gastro-intestinal mucosa. Lower abdomens of male athymic mice, treated i.p. daily with vehicle or IST5-002 (20 mg/kg) for 6 days, were irradiated with 0 or 2 Gy for 3 consecutive days. The structure and cell viability of the small intestines were analyzed by hematoxylin and eosin staining, (top), apoptotic cells by TUNEL (middle, green stain) or leukocyte infiltration by CD45 immunostaining and proliferation by Ki-67 (bottom, CD45, green; Ki-67, red), and quantified. ***, $P < 0.001$.

Table 1

Characteristics of patient-derived prostate cancers cultured *ex vivo* in tumor explant cultures (Fig. 1E)

		IST5-002-treated PCs (n=4) Median (range)	AZD1480-treated PCs (n=4) Median (range)
Gleason score		**n (%)**	**n (%)**
	4	0 (0)	0 (0)
	5	0 (0)	0 (0)
	6	0 (0)	0 (0)
	7	2 (50)	3 (75)
	8	2 (50)	0 (00)
	9	0 (0)	1 (25)
	10	0 (0)	0 (0)
Metastases detected		**n (%)**	**n (%)**
	Yes	0 (0)	0 (0)
	No	4 (100%)	4 (100%)
	Unknown	0 (0)	0 (0)