Supplementary Information

Supplemental Materials and Methods

Radiosensitivity assay. Standard radiation clonogenic survival assays were performed as described previously (1) after exposure to various doses of x-rays. Different numbers of cells were seeded in 6-well dishes after treatments with NF-κB activation inhibitor JSH-23 (5 μM; Calbiochem, La Jolla, CA) or β1-integrin inhibitory antibody AIIB2 (0.1 μg/μl; Argon Biosciences, Morgan Hill, CA) with or without radiation. Each treatment was performed in three wells, and all experiments were repeated in triplicate. The treated and control cells were cultured for 14 days, and colonies with more than 50 cells were scored and normalized to the plating efficiency of each cell line.

Western blotting. Cells propagated in 3D lrECM were first treated with ice-cold PBS/EDTA [0.01 mol/L sodium phosphate (pH 7.2) containing 138 mmol/L sodium chloride and 5 mmol/L EDTA] to isolate the cells and then lysed in radioimmunoprecipitation assay (RIPA) buffer as previously described (2). For the preparation of whole tumor lysates, mouse tumors were homogenized in 500 μl of extraction buffer (10 mM Tris, pH 7.6, 5 mM EDTA, 50 mM NaCl and 1% Triton X-100) with protease inhibitor cocktail (set I, Calbiochem) and phosphatase inhibitor cocktail (set I and II, Sigma). The homogenates were centrifuged at 12,000 × g for 20 min at 4°C and the supernatants were stored at -80°C until needed. Equal amounts of protein were loaded onto reducing SDS gels (Invitrogen, Carlsbad, CA). After transfer onto PVDF membrane (Millipore, Temecula, CA), blots were blocked with 5% nonfat milk and probed. Primary antibodies used include β1-integrin, clone 18 (1:1,000; BD Transduction Laboratories, Lexington, KY), phospho-β1-integrin (1:1,000; Biosource International, Camarillo, CA); p65 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA); p50 (1:2,000); α5-integrin (1: 500;

Millipore, Temecula, CA); β-actin, clone AC-15 (1:5,000; Sigma, St. Louis, MO). Blots were washed, incubated with secondary antibody, and then visualized using the ECL Western blotting detection system (Thermo Scientific, Rockford, IL).

Immunofluorescence. This assay was performed as described (3) using the primary antibody against NF-κB p65 (Santa Cruz), α6-integrin (Millipore) or β-catenin (BD Biosciences). Fluorescent images were acquired by a Zeiss LSM 710 inverted laser scanning confocal microscope.

NF-κB DNA binding assays. Cells in 2D cultures were exposed to sham or 4-Gy x-ray and nuclear fractions were harvested 1, 4 and 24 h post-IR using the nuclear extraction kit (Thermo Scientific, Rockford, IL) following the manufacturer's suggestions. The protein concentration was determined using the DC Protein Quantitation Kit (Bio-Rad, Hercules, CA). NF-κB DNA binding activity was quantitatively assessed using the TransAM^{TM} NF-κB TF assay kit (Active Motif, Carlsbad, CA) per the manufacturer's protocol. To each well containing NF-κB consensus binding sites, 10 μg of nuclear extract in cell binding and cell lysis buffer were added in each well in triplicates. We used 5 μg of nuclear extract of Raji cells (a Burkitt's lymphoma cell line) as the positive control. To assess DNA binding specificity, excess wild-type NF-κB consensus oligonucleotide was added (20 pmol/well) to compete for the binding, as compared with other wells to which was added an inactive mutated consensus oligonucleotide. Plate was washed and the absorbance was measured at 450 nm by iMark^{TM} Microplate Absorbance reader (Bio-Rad).
To dissect the specific binding activity of NF-κB p65 to its cognate promoter sequence, we performed NF-κB binding assays using promoter-specific DNA oligonucleotides in TransAM^{TM} Flexi NF-κB TF assay kit (Active Motif). The 50 bp oligonucleotides contains a 10 bp core consensus sequence of NF-κB binding motif (wild type and mutated) of the β1-integrin promoter

were custom synthesized and biotinylated at the far end from the NF-κB consensus sites by the Midland Certified Reagent Company (Midland, TX). Oligos were duplexed by incubating 1 pmol/μl of each oligonucleotide together at 95°C for 5 min and ramping back to 4°C by decreasing 1°C/min. Twenty micrograms of whole cell extract prepared from T4-2 and S1 cells in 3D lrECM at day 7 and 9, respectively (Fig. 2*A*), 50 μl binding buffer, and 1 pmol of biotinylated oligonucleotide were incubated for 30 min at room temperature prior to placement in a well on streptavidin-coated 96-microtiter plates. After washing, wells were incubated sequentially with a primary antibody for p65 NF-κB, followed by anti-rabbit peroxidase-conjugated antibody. After substrate addition, peroxidase activity was measured by reading 450 nm in a SpectraMax® Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA) or iMark™ Microplate Absorbance reader (Bio-Rad).

Real-time PCR analysis. Total RNA was purified with TRIzol (Invitrogen) according to the manufacturer's instructions and then 5 μg of each sample was reverse transcribed using the SuperScript™ III First-Strand Synthesis System (Invitrogen). PCR was carried out with 2 μl of cDNA from each sample using QuantiTect® SYBR® Green PCR Kit (Qiagen) and CFX96™ Real-Time PCR Detection System (Bio-Rad). Primers used to amplify β1-integrin and GAPDH were bought from Qiagen. Fragments were amplified with the following protocol: 95ºC for 15 min (initial denaturation) and 40 amplification cycles (94ºC for 15 s, 55ºC for 30 s and 72ºC for 30 s). β1-integrin mRNA was normalized to the corresponding GAPDH and averaged from three independent experiments. Melting curve was analyzed to verify the presence of a single PCR product.

***In vivo* caspase-3/7 activity assay.** Caspase-3/7 activity in human T4-2 breast cancer cells was assessed with the Caspase-Glo® 3/7 Assay Kit from Promega according to the manufacturer's

instructions. Briefly, single cells dissociated from lrECM at day 7 (Fig. 2*A*) were incubated with Caspase-Glo® 3/7 reagent at room temperature for one hour and the luminescence was measured using a plate-reading luminometer (Modulus™ II Microplate Multimode Reader; Turner Biosystems, Madison, WI). Before the evaluation of caspase activity, cells were incubated with Z-VAD-FMK peptide (40 μM) with or without JSH-23 (40 μM) and AIIB2 (0.15 μg/μl) at day 4.

Tumor inhibition assay in nude mice. Animal experiments were performed under federal guidelines and approved by the Institutional Animal Care and Use Committee at LBNL. Exponentially growing T4-2 cells in 250-ml flasks were exposed to 4-Gy x-ray 4 h post-JSH-23 treatment and then suspended at a density of 5×10^6 cells 24 h post-IR in 100 μl medium containing 50% DMEM/F12 medium and 50% Matrigel. Cell suspension was subcutaneously injected into the rear flank of 5-week-old athymic female NCR nude mice (two sites/mouse; nu/nu, Simonsen Laboratories, Gilroy, CA). Mice were divided into three groups (n = 8 per group): vehicle, JSH-23 and JSH-23 + IR. Tumor dimensions were measured twice weekly with a caliper, and the volume of a tumor was calculated according to the standard formula: tumor volume in mm3 = length × width × height. Mice were sacrificed on day 28 and tumors were dissected for Immunohistochemical and Western blot analyses.

Immunohistochemistry stainings. Immunohistochemistry was performed on 5-μm-thick formalin-fiwas paraffin-embedded mouse tumor tissue sections following standard procedures. Deparaffinization and rehydration were followed by Antigen retrieval using a microwave oven with Citrate buffer (pH 6.0) for β1-integrin and with Glycin buffer (pH 9.0) for NF-κB p65. Primary antibodies were diluted 1:50: CP26, clone 4B7, Calbiochem for β1-integrin and sc-372, Santa Cruz for NF-κB p65 detection. Secondary antibodies were purchased from Vector

Laboratories (Burlingame, CA). Staining was developed with diamonobenzine tetrahydroxychloride solution (Sigma) as the peroxidase substrate. The sections were counterstained with hematoxylin. Photomicrographs were taken with Zeiss Axioskop Imaging platform and AxioVision 4.7 Software.

Immunoprecipitation. Cell extracts were prepared from T4-2 and S1 cells in 3D lrECM at day 7 and 9, respectively (Fig. 2*A*), using lysis buffer containing 1% Brij 98, 150 mM NaCl, 25 mM HEPES, 5 mM MgCl2 and 1% eukaryotic proteinase inhibitor cocktail (Calbiochem, La Jolla, CA). Extracts were precleared by 1 h treatment with normal Rat immunoglobulin G and 25 µl of Dynabeads® protein G (Invitrogen) at 4°C. Precleared protein lysates were incubated with 3 µg of anti-β1-integrin antibody (Argon Bioscience, Morgan Hill, CA) at 4°C for overnight with gentle rocking followed by the addition of 25 µl of Dynabeads® protein G and further 1 h incubation. Beads were collected, washed four times with buffer, boiled in SDS gel loading buffer, fractionated on 4-20% Novex® Tris-Glycine gel (Invitrogen), and blotted using a specific antibody.

Statistics. Data were analyzed by Student's t test; the *p* value was derived to assess the statistical significance. Results are expressed as mean ± SD of the number of replicates indicated in the figure legends. The experiments shown are representative three experiments with similar results.

Supplemental References

1. Ahmed KM, Dong S, Fan M, Li JJ. Nuclear Factor-{kappa}B p65 Inhibits Mitogen-Activated Protein Kinase Signaling Pathway in Radioresistant Breast Cancer Cells. Mol Cancer Res 2006;4(12):945-55.

2. Weaver VM, Petersen OW, Wang F*, et al.* Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol 1997;137(1):231-45.

3. Nam JM, Onodera Y, Bissell MJ, Park CC. Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin. Cancer Res 2010;70(13):5238-48.