Supplementary Methods

S1: Cell Culture

U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 degrees at 5% CO2 in 10 cm tissue culture dishes. To generate microscope slides for irradiation, cell cultures at a low passage number and 90% confluence were washed with phosphate buffered saline and trypsinized with 0.05% trypsin in DMEM. 400,000 cells were plated onto single chamber microscope slides (ThermoFisher Scientific, Waltham, MA) and cultured in DMEM, 37 degrees Celsius, 5% C02 overnight.

S2: Dosimetry and Irradiation Conditions

Both the IBA proton beam and the Varian True Beam Linear accelerator are calibrated for clinical treatment conditions. For photons, this is with an SAD set up in accordance with Protocol TG-51 [1]. Dosimetry of the proton beam was verified in the middle of a spread out Bragg peak with range of 16 cm, modulation 10 cm in accordance with the TRS 398 code of practice. NIST traceable chambers were used for all dosimetry measurements.

S3: Flow Cytometry

After incubation, cells were washed with PBS and trypsinized with 0.05% Trypsin in DMEM. Samples were then placed in triplicate into a 96 well plate and centrifuged. The supernatant was discarded, and samples were re-suspended in 1% paraformaldehyde on ice. After incubation, samples were again centrifuged, resuspended in ETOH with EDTA, and stored at -20 degrees Celsius. After overnight incubation, samples were centrifuged and washed in PBS supplemented with bovine serum albumin and Triton X-100. Samples were treated with primary antibody directed against a phosphorylated histone at the site of DNA double strand breaks (Anti-phospho-Histone H2A.X (Ser139) clone JBW301, EMD Millipore, Billerica, MA) and incubated. Samples were then washed and treated with secondary antibody (Alexa Fluor 488 F(ab')2 Fragment of Goat Anti-Mouse IgG, IgM, Life Technologies, Grand Island, NY).

After incubation, samples were stained with propidium iodide. The resulting samples were passed through a filter cap, and median fluorescent intensity (MFI) of the total γH2AX was assessed via flow cytometry.

S4: Clonogenic Survival Assay

U2Os cells at a low passage number and 90% confluence were trypsinized and counted. 400,000 cells were plated onto single chamber microscope slides and cultured in DMEM, 37 degrees Celsius, 5% C02 overnight. Cells were then irradiated with protons or photons as described above. After irradiation, cells were harvested and serially diluted in 4 mL DMEM, ranging from 500 – 8000 cells per sample. Seeding numbers were selected based on prior tests for plating efficiency after irradiation conditions, with the goal of 50-100 colonies per well. Samples were plated in triplicate onto 6 well tissue culture dishes and incubated 13-21 days at 37 degrees at 5% CO2. After the incubation period, media was removed and colonies were stained with methylene blue dye. Colonies containing at least 50 cells were counted. Samples with overgrown colonies or with contamination were excluded from data analysis. Plating efficiency in non-irradiated samples was determined and surviving fractions in irradiated samples were subsequently calculated.

References:

1. Almond PR, Biggs PJ, Coursey BM, Hanson WF, Huq MS, Nath R, Rogers DW. Aapm's tg-51 protocol for clinical reference dosimetry of high-energy photon and electron beams. *Medical physics* 1999;26:1847-1870.