Mass Spectrometry

A. Protein extraction, digestion, labeling and pooling

Tumors resected 48hr after treatment were snap-frozen in liquid nitrogen and homogenized using a bead mill (Omni Bead Ruptor 24) with 10 cycles of 30 s on 30 s off, power level 8, and chilled to 4ºC with liquid nitrogen. One milliliter ice-cold, fresh lysis buffer (50 mM triethylammonium bicarbonate, pH 8.5, 0.5% deoxycholate, 12 mM sodium lauroyl sarcosine, protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail containing 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and 50 mM beta-glycerophosphate) was used for tumor homogenization. Tumor homogenates were centrifuged at 450 x*g* for 5 min at 4ºC and supernatants were sonicated for 10 min, 30 s on 30 s off, at 4ºC using Bioruptor Pico (Diagenode) followed by heating at 95 ºC for 5 min. Protein concentrations of lysates were quantified by the BCA protein assay (Thermo Fisher Scientific), and 1 mg total protein from each sample was carried through the rest of the sample preparation. Protein disulfides were reduced with 5 mM dithiothreitol (final concentration) for 30 min at 37ºC, followed by alkylation with 10 mM chloroacetamide (final concentration) for 30 min at room temperature in the dark. Excess alkylating agent was quenched by adding the same amount of dithiothreitol as in the previous step and incubating for 5 min at room temperature. Each sample was then diluted 1:5 using 50 mM triethylammonium bicarbonate, pH 8.5, containing 10 µg trypsin (Promega) and digested at 37 ºC for 4 h. A second aliquot of 10 µg trypsin was spiked into the samples and digested overnight at 37 ºC. The reaction was quenched and detergents were extracted with 1:1 (v:v) ethyl acetate containing 1% trifluoroacetic acid. The samples were vortexed vigorously and centrifuged at 16,000xg for 5 min. The lower aqueous phase was transferred to new microcentrifuge tubes and dried using a SpeedVac vacuum concentrator. The samples were reconstituted in 2% acetonitrile with 0.1% trifluoroacetic acid, desalted on Oasis HLB 10 mg cartridges (Waters), and dried using a SpeedVac. Samples were resuspended in 200 mM EPPS,

pH 8.5, and peptide concentration was obtained using the Pierce Quantitative Colorimetric Peptide Assay. Four hundred microgram peptides were labeled with 10-plex TMT reagents at a 1:2 reagent : peptide concentration following the manufacturer's instruction, and dried by SpeedVac. Samples were reconstituted in 2% acetonitrile with 0.1% trifluoroacetic acid, and 1 µg total peptides from each sample were pooled and desalted using SDB StageTips. The pooled sample is used to assess the TMT ratios for fine adjustments to the TMT reagent and sample volume to achieve better uniform ratios among the samples. One microgram of total peptide from this pooled sample was acquired via nanoLC-MS/MS on a QExactive Plus (Thermo) using a 3h gradient. The raw data were processed using Proteome Discoverer v2.2 (Thermo) (see **E.**). Each sample was normalized to the protein median fold change compared to the 126 m/z TMT channel and ~300 µg of each sample was pooled accordingly. The pooled peptide sample was desalted on an Oasis HLB 200 mg cartridge (Waters) and dried by SpeedVac.

B. Phosphopeptide enrichment by immobilized metal affinity chromatography

For phosphopeptide enrichment using Fe-IMAC 3 mg of dried peptides were solubilized in 900 µL of phosphopeptide binding solution (80% acetonitrile and 0.1% TFA). One hundred fifty microliters peptide aliquots were mixed with 165 µL of Fe-IMAC beads and incubated at room temperature for 30 min with shaking. The supernatant and all washes were collected, dried by SpeedVac, desalted on an Oasis HLB 200 mg cartridge (Waters) and used for peptide fractionation and total protein quantification via nanoLC-MS/MS (see D.). The phosphopeptides were quickly eluted from beads with 100 µL of phosphopeptide elution solution (70% acetonitrile and 1% ammonium hydroxide), passed through C8 StageTip and acidified with 30 µL 10% formic acid. The phosphopeptide eluents were dried by SpeedVac and desalted using SDB StageTips.

C. Offline basic pH reverse phase liquid chromatography

The complete phosphopeptide-enriched sample and 70 µg of the non-phosphopeptide sample were each solubilized in 3 µL buffer A (10 mM ammonium bicarbonate, pH 10, and 2%

acetonitrile) and separated on a Zorbax 300Extend-C18 column (3.5 µm particle size, 0.3 mm × 150 mm, Agilent) using an Agilent 1260 capillary pump and µWPS autosampler equipped with an 8 µL sample loop. Ninety-six fractions were collected with a 60 min gradient from 5-60% buffer B (90% acetonitrile, 10 mM ammonium bicarbonate, pH 10, flow rate of 6 µL/min) into pre-deposited 20 µL of 5% formic acid. The samples were concatenated with an interval of 24 to form 24 final fractions (e.g., fractions 1, 25, 49, and 73 combined, fractions 2, 26, 50 and 74 combined, and so on). The concatenated fractions were desalted using SDB StageTips and dried by SpeedVac.

D. Acidic pH reverse phase liquid chromatography coupled with tandem MS

The dried peptide fractions for whole proteome analysis were reconstituted in 11 µL of 2% acetonitrile and 0.15% formic acid. For phosphopeptide analysis, the dried peptide fractions were reconstituted in 6 uL of 2% acetonitrile and 0.15% formic acid. Five microliters of sample were loaded on a laser-pulled reverse phase column (150 µm × 20 cm, 1.8 µm C18 resin with 0.5 cm of 5 µm C4 resin at the laser-pulled end, Acutech Scientific, San Diego, CA) interfaced with an Eksigent 2D nanoLC, Phoenix S&T dual column source, and QExactive Plus MS (Thermo). Peptides were eluted using 5-40% buffer B gradient in 3h (buffer A: 2% acetonitrile, 0.15% formic acid; buffer B: 98% acetonitrile, 0.15% formic acid, flow rate of 0.5 µl/min). The column was heated to 60°C by a butterfly portfolio heater (Phoenix S&T) to reduce backpressure. The mass spectrometer was operated in data-dependent mode with a survey scan from 350-1500 m/z (70,000 resolution, 3 × 10 6 AGC target and 100 ms maximal ion time) and 10 MS/MS scans with starting fixed m/z of 100 (35,000 resolution, 2 × 10 5 AGC target, 120 ms maximal ion time, 32 normalized collision energy, 1.2 m/z isolation window, and 30 s dynamic exclusion).

E. Identification and quantitation of peptides using Proteome Discoverer v 2.2

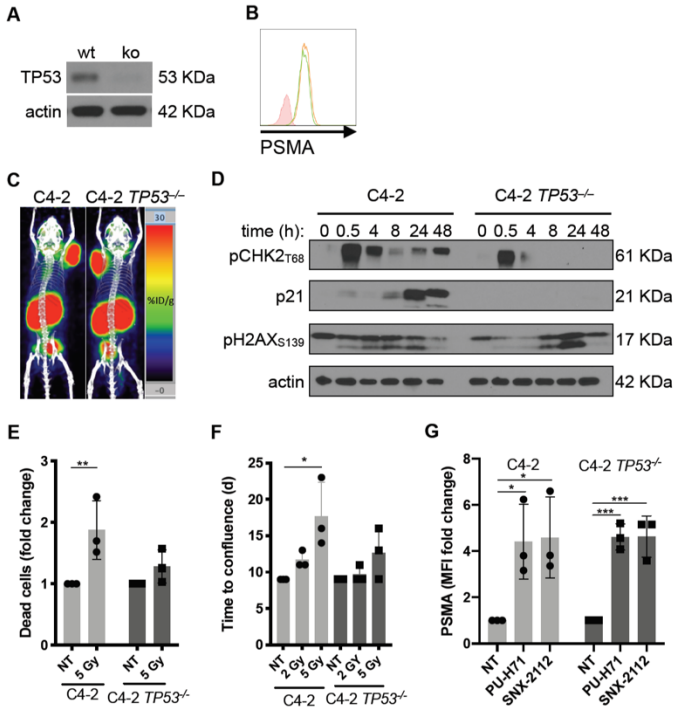
The acquired MS/MS raw files were searched by the Sequest algorithm against a forward and reverse target/decoy database to estimate FDR. The target protein database was downloaded from the Uniprot human database (reference and additional sequences, 93,320

protein entries; downloaded in March 2018) and the decoy protein database was generated by reversing all target protein sequences. A contaminate protein database was included in the searches (244 protein entries; downloaded from MaxQuant 1.6.0.16). Spectra were searched with ± 10 ppm for precursor ion and ± 0.02 Da product ion mass tolerance, fully tryptic restriction, static mass shift for TMT-tagged N-terminus and lysine (+229.16293), carbamidomethylation on the cysteine (+57.021), dynamic mass shift for oxidation of methionine (+15.995), deamidation of asparagine and glutamine (+0.984), acetylation of protein N-terminus (+42.011), phosphorylation of serine, threonine and tyrosine (+79.96633, only for phosphopeptide-enriched fractions), two maximal missed cleavages, three maximal modification sites, and the assignment of b and y ions. Putative peptide spectra matches were filtered by Percolator using 1% FDR. Post-translational modifications were site localized using ptmRS software. TMT reporter ions were quantified using the most confident centroid with reporter ion mass tolerance at 20 ppm.

F. Differential expression analysis of the proteome and phosphoproteome

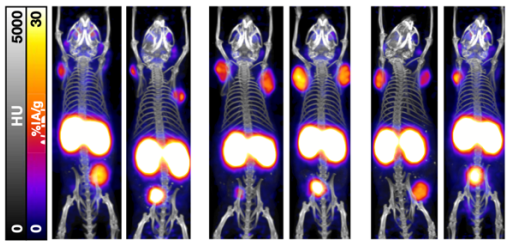
Differential expression events were defined by identifying proteins/phosphopeptides with between-treatment variance significantly larger than within-replicate variance using one-way ANOVA (analysis of variance). Significantly altered proteins/phosphopeptides were filtered using Benjamini-Hochberg (BH) procedure at 5% FDR. All statistical analysis, principle component analysis, and unsupervised hierarchical clustering was performed using Python. Kinase-substrate enrichment analysis (KSEA) was performed using the KSEA App (https://casecpb.shinyapps.io/ksea/). Briefly, the significantly-altered phosphopeptides were submitted and respective kinases were assigned using the PhosphositePlus database and NetworKin. Kinases were filtered with 5% FDR (n=512 significant phosphopeptides for ^{177}Lu and 405 for ^{225}Ac).

Supplemental Figures

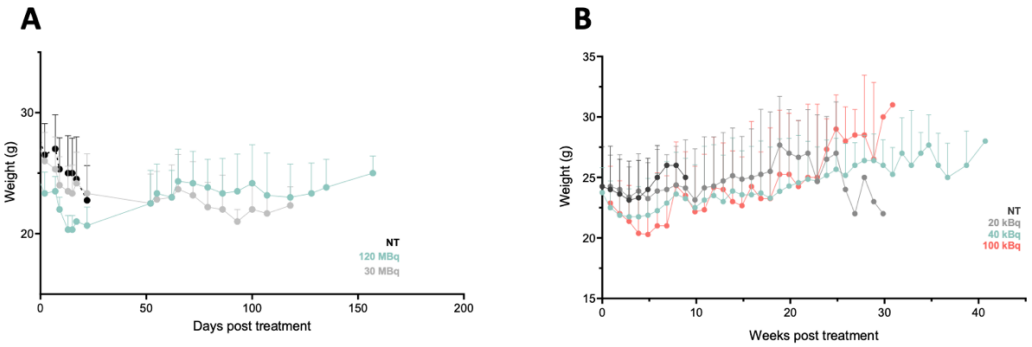


Supplemental Figure 1. Functional validation of C4-2 *TP53*$^{-/-}$ cells. (a) Confirmation of Crispr/CAS9 mediated *tp53* deletion in C4-2 cells by immunoblot (anti-p53, 7F5, dilution 1:1,000, #2527; anti-β-actin, 8H10D10, 1:10,000, #3700; Cell Signaling) (n=1). **(b, c)** Comparable PSMA expression levels in wt (red) and ko (green) cells as assessed by flow cytometry using an anti-hPSMA-APC antibody (1:10 dilution, REA408, Miltenyi; BD LSR II flow cytometer). Filled red curve: unstained control (n=1) (**b**), or by ^{68}Ga-PSMA PET/CT images (1 representative mouse out of 5 is shown per group) (**c**). (**d-f**) Loss of TP53 increases resistance of C4-2 cells to radiation *in vitro*. (**d**) Immunoblot analysis of DNA damage response activation *in vitro* following irradiation with 5 Gy using antibodies detecting phospho-Chk2 (Thr68; C13C1, 1:1,000, #2197), p21 (12D1; 1:1,000, #2947), phospho-Histone H2A.X (Ser139; 20E3, 1:2,500, #9718), and β-actin (8H10D10, 1:10,000, #3700; all antibodies Cell Signaling) (n=1). (**e**) Cell death following irradiation with 5 Gy was quantified by flow cytometry (propidium iodide). Data were normalized to untreated controls. (**f**) Cells were seeded on 6-well plates (100,000 cells/well) and irradiated with 2 Gy or 5 Gy. The days it took cells to reach confluence was recorded. (**g**) Flow cytometric quantification of PSMA

expression following treatment with HSP90 inhibitors for 72h. Data were normalized to untreated controls. In (**e-g**), mean±standard deviation are shown for 3 individual experiments. Significant differences are indicated by asterixes. ko - C4-2 TP53$^{-/-}$; wt - C4-2 (parental); NT - not treated.



Supplemental Figure 2. C4-2 murine model validation. [^{68}Ga]Ga-PSMA-11 PET/CT confirms PSMA expression of C4-2 tumors one day before treatment. HU = Hounsfield Unit; %IA/g = injected activity per gram



Supplemental Figure 3. Mouse weights. (A) [^{177}Lu-]LuPSMA-RLT study (n=6 mice/group). (**B**) [^{225}Ac]Ac-PSMA-RLT study (n=8 mice/group). Data represent mean and standard deviation.