Deciphering the Acute Cellular Phosphoproteome Response to Irradiation with X-rays, Protons and Carbon Ions*§

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Radiotherapy is a cornerstone of cancer therapy. The recently established particle therapy with raster-scanning protons and carbon ions landmarks a new era in the field of high-precision cancer medicine. However, molecular mechanisms governing radiation induced intracellular signaling remain elusive. Here, we present the first comprehensive proteomic and phosphoproteomic study applying stable isotope labeling by amino acids in cell culture (SILAC) in combination with high-resolution mass spectrometry to decipher cellular response to irradiation with X-rays, protons and carbon ions. At protein expression level limited alterations were observed 2 h post irradiation of human lung adenocarcinoma cells. In contrast, 181 phosphorylation sites were found to be differentially regulated out of which 151 sites were not hitherto attributed to radiation response as revealed by crosscheck with the PhosphoSitePlus database.

Radiation-induced phosphorylation of the p(S/T)Q motif was the prevailing regulation pattern affecting proteins involved in DNA damage response signaling. Because radiation doses were selected to produce same level of cell kill and DNA double-strand breakage for each radiation quality, DNA damage responsive phosphorylation sites were regulated to same extent. However, differential phosphorylation between radiation qualities was observed for 55 phosphorylation sites indicating the existence of distinct signaling circuitries induced by X-ray versus particle (proton/carbon) irradiation beyond the canonical DNA damage response. This unexpected finding was confirmed in targeted spike-in experiments using synthetic isotope labeled phosphopeptides. Herewith, we successfully validated uniform DNA damage response signaling coexisting with altered signaling involved in apoptosis and metabolic processes induced by X-ray and particle based treatments.

In summary, the comprehensive insight into the radiation-induced phosphoproteome landscape is instructive for the design of functional studies aiming to decipher cellular signaling processes in response to radiotherapy, space radiation or ionizing radiation per se. Further, our data will have a significant impact on the ongoing debate about patient treatment modalities. Molecular & Cellular Proteomics 16: 10.1074/mcp.M116.066597, 855–872, 2017.

More than 50% of all cancer patients receive radiotherapy during their course of treatment (1). Conformal X-ray irradiation techniques such as intensity-modulated radiation therapy (IMRT) combine the application of a curative dose to the tumor while sparing adjacent radiosensitive organs keeping side-effects to healthy surrounding tissue within tolerable limits (2). Contrary to X-rays, which show an exponential dose decrease with increasing tissue depth, charged particles deposit most of their energy to a small region within the tissue with a sharp dose fall-off after the so-called Bragg peak (3). This precise dose localization enables further dose escalation within the tumor while sparing healthy tissue (4). Worldwide, more than 135,000 patients have been treated with particles until the end of 2014 and the number is growing steadily (5).

An important term in the field of radiobiology is the relative biological effectiveness (RBE) being defined as the ratio of...
X-ray dose to an alternative radiation quality dose which produces the same biological effect such as cell survival or other surrogates such as the number of double-strand breaks (DSBs). Although the RBE for protons is assumed to be comparable to X-rays, carbon ions are more effective in inducing unrepairable DNA damage (6) and may therefore efficiently eradicate formerly radioresistant tumors (7–9). This is in part because of the fact, that carbon ions predominantly induce clustered and direct DNA damage, which is considered to be less dependent on cell cycle stage, oxygen level, genetic background and is less well repaired by DNA repair mechanisms (3).

So far, limited studies exist comparing X-rays to particle based radiations and are mainly focused on investigating the alterations of single or panel genes (10, 11). However, cells are equipped with versatile signaling cascades mainly transduced by post-translational modifications (PTMs) in order to minimize adverse effects of DNA damage (12). Post-translationally modified proteins are key targets especially to improve the understanding of acute signaling events. In DNA damage response (DDR), protein phosphorylation plays a crucial role to trigger a chain of events, starting with sensing DNA lesions, signaling cascade activation, cell cycle arrest and recruitment of DNA repair factors (13). Although phosphoproteome data exist elucidating the cellular response to X-rays per se (13) as well as to various X-ray dose levels (14, 15), radiation quality dependent phosphoproteome studies are urgently needed.

In the present study, we aimed to systematically decipher acute signaling events induced by different radiation qualities using high-resolution mass spectrometry based proteomics. To this end, we irradiated SILAC-labeled human lung adenocarcinoma cells (A549) with X-rays, protons and carbon ions in clinical-like setting. A549 cells have an intact DNA damage repair machinery (e.g. p53 wild-type) leading to a relatively low background of DNA damage foci compared with a panel of well-known cancer cell lines (6). Therefore, A549 cells are particularly suitable to study radiation induced DNA damage response. Proteomic and phosphoproteomic analyses performed 2 h post irradiation showed extensive alterations of the phosphorylation status whereas the protein expression itself remained largely unaffected. Phosphorylation events behaved similar upon proton and carbon irradiation, however a distinct number of sites responded differentially to X-rays versus particle based treatments. To validate selected candidates, we used synthetic isotope labeled phosphopeptides in a targeted spike-in experiment as recently proposed by Kennedy et al. as an alternative to extensive Western blotting, which requires phosphosite specific antibodies (16). By this approach, we validated the prevailing pattern of phosphorylation sites associated with DDR to be regulated in equal measure by all radiation qualities. Moreover, phosphorylation sites responding differentially to X-ray and particle based treatments were confirmed. Herewith, we demonstrate the existence of radiation quality dependent signaling events in the acute cell response.

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In summary, we established a robust and efficient workflow for the investigation and validation of phosphorylation changes in response to X-rays and particle based irradiations. We show for the first time, that differential acute signaling events are triggered by different radiation qualities. With this we provide valuable information to better understand molecular effects of radiation qualities and offer potential drug targets for modulation and optimization of cancer radiotherapy.

**EXPERIMENTAL PROCEDURES**

**Public Access to Mass Spectrometry Data—**All LC-MS/MS data of the discovery and targeted spike-in experiments have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) (17) via the PRIDE partner repository (18) with the data set identifier PXD004817. Entire MaxQuant folders are included as well as Skyline files for re-evaluation and detailed inspection of the results.

**Materials—**Triton X-100 (#A1388), Na-deoxycholate (#A1531) and dithiothreitol (DTT, #A2948) were purchased from AppliChem (Darmstadt, Germany). Water (H2O, #232141), acetonitrile (ACN, #12041G5), trifluoroacetic acid (TFA, #202341), methanol (MeOH, #136141GS) and formic acid (FA, #069141A8) were obtained from Biosolve ( Valkenswaard, Netherlands). Ammonium bicarbonate (ABC, #09830) and trizma base (#93349) were obtained from Fluka (Steinheim, Germany). Iron(III) chloride (FeCl3, #1.03943.0250), ammonia solution (NH3, #1.05428.0250) and acetic acid (AA, #1.00063.2511) were obtained from Merck (Darmstadt, Germany). L-proline (L5850), lodoacetamide (IAA, #11149), glycolic acid (#124737) and hexafluoro-2-propanol (HFIP, #10522) were obtained from Sigma (St. Louis, MO).

Further chemicals were purchased from different suppliers: SDS (#23262.2, Roth, Karlsruhe, Germany), sequencing grade trypsin (#9002-07-7, Promega, Madison, WI), chloroform (#22711.290, WWR, Radnor, PA), EDTA (#11278.01, SERVA, Heidelberg, Germany), tita- nium dioxide (TiO2, #5020–75000, GL Sciences, Tokyo, Japan).

**Cell Culture and Irradiation—**Human lung adenocarcinoma cells (A549, #CCL-185, ATCC, Manassas, VA) were cultured at 37 °C with 5% CO2 in SILAC Dulbecco’s Modified Eagle medium (DMEM, SILANTES GmbH, Munich, Germany) containing 10% dialyzed fetal bovine serum (FBS, SILANTES GmbH) and supplemented with either 0.798 mm15C0,15N0-lysine and 0.398 mm13C0,15N0-arginine or 0.798 mm15C0,15N0-lysine and 0.398 mm13C0,15N0-arginine (SILANTES GmbH). In order to prevent arginine-to-proline conversion additional 200 mg/l proline were added to the cell culture medium (19).

To ensure full incorporation of the SILAC labels, cells were cultured for at least six cell doublings and incorporation was checked using a recently published R script (20) to be ≥95%.

H2AX, histone H2AX; HFIP, hexafluoro-2-propanol; HIT, Heidelberg Ion Beam Therapy Center; KPN2, importin subunit alpha-1; LET, linear energy transfer; MAP1B, microtubule-associated protein 1B; MKI67, antigen Ki-67; MOPS, 3-(N-morpholino)propanesulfonic acid; NHEJ, nonhomologous end joining; NUMA1, nuclear mitotic apparatus protein 1; PRKDC, DNA-dependent protein kinase catalytic subunit; RAD50, DNA repair protein RAD50; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; RRPBP1, ribosome-binding protein 1; SF30, survival fraction 30%; SILAC, stable isotope labeling by amino acids in cell culture; SPE, solid phase extraction; SRC, proto-oncogene tyrosine-protein kinase Src; STMN1, stathmin; TP53BP1, tumor suppressor p53-binding protein 1; VIM, vimentin.
Cells were irradiated with different radiation qualities at 65-75% confluence in T75 cell culture flasks (app. 8-10^6 cells). Cells treated with X-rays were irradiated with 6 Gy at 320 keV and a dose rate of 110 cGy/min using the XRAD320 X-ray device (Precision X-Ray, North Branford, CT). Field size for the X-ray irradiation was 20 × 20 cm to fully cover the flasks and the source to surface distance was 50 cm. A filter unit comprised of 0.75 mm tin, 0.25 mm copper and 1.5 mm aluminum (half value layer ~3.7 mm Cu) was used. Cells treated with particles were positioned in the middle of a 1 cm wide spread-out Bragg peak centered at about 3.5 cm water-equivalent depth in a field size adjusted to cover two T75 cell culture flasks next to each other. The planned physical dose levels were 3.5 Gy for protons (5.5 keV/μm) and 2 Gy for carbon ions (95.2 keV/μm) at dose rate ~0.5 Gy/min. Irradiation with particles was performed at the Heidelberg Ion Beam Therapy Center (HIT). Detailed information on particle irradiation protocol and dosimetry are described in Dokic et al. (6).

After treatment, all cell culture flasks were returned to the incubator for 2 h at 37 °C and 5% CO2. Control cells were sham irradiated and handled in the same way as irradiated cells. Finally, cells were washed twice with chilled PBS and harvested with a cell scraper prior to cell lysis. Conegenic Survival Experiments—To determine radiobiological equivalent doses cells were irradiated with dose series of X-rays, protons and carbon ions and the survival fraction measured by clonogenic survival assays as described (6). Briefly, cells were seeded as single cells in triplicates in six-well plates. After attachment, cells were irradiated at different doses or left untreated (sham) and incubated. After colonies were formed, they were fixed with 75% methanol and 25% acetic acid for 10 min at room temperature and labeled with 0.1% crystal violet for 15 min. Colonies with more than 50 cells were counted as survivors to determine isoeffective doses.

Antibody Staining for Microscopy—Cells were plated onto 12-mm glass slides in 24-well plate format, irradiated 24 h later and incubated for desired time periods. Cells were fixed and stored in 70% ethanol at −20 °C for at least 24 h. For staining, cells on cover glasses were first permeabilized and blocked in PBS containing 0.1% (v/v) Triton-X100 and 3% (w/v) serum albumin for 20 min. Primary antibody (TP53BP1, Cell Signaling Technologies (CST), Danvers, MA #4937) was applied at 1:200 in PBS containing 0.02% (v/v) Triton-X100 and 0.6% (w/v) serum albumin at 4 °C overnight. Cells were washed twice with PBS containing 0.02% (v/v) Triton-X100 and 0.6% (w/v) serum albumin. Secondary antibody (Alexa-555, Invitrogen, A-21428) was applied at 1:400 in PBS containing 0.02% (v/v) Triton-X100 and 0.6% (w/v) serum albumin at 4 °C for 4 h. Washing was performed as before, followed by nuclear counter-staining with 1 μg/ml DAPI. Cells were washed once with PBS and then with H2O. Glass slides were fixed in Fluoromount G. Imaging was performed on an Olympus Cell Vivo microscope with a 20x objective. ImageJ (version 1.48) was used for image processing. TP53BP1-foci were quantified by performing background subtraction followed by global thresholding and watershed. Foci larger than 3 pixels and with a minimum circularity of 0.5 were measured.

Cell Lysis and Protein Quantification—Lysis was performed using RIPA buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.5% Na-deoxycholate; 0.1% SDS; 1% DTT) supplemented with miniComplete EDTA-free protease inhibitor mixture tablet and PhosSTOP phosphatase inhibitor mixture tablet (Roche Diagnostics, Mannheim, Germany) (21). Additionally 1% benzonase (Merck) was added to remove nucleic acids. After incubation on an orbital shaker for 1 h on ice the lysates were cleared by centrifugation at 15,000 rpm, 30 min at 4 °C. Protein concentration of supernatants was measured in triplicate using 2-D Quant Kit (GE Healthcare Bio-Sciences, Uppsala, Sweden).

Proteome Analysis - SDS-PAGE and Protein Digestion—For proteome analysis 15 μg of each light and heavy labeled protein lysates were combined and separated on a NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen, Carlsbad, CA) using a MOPS-buffer system. The gel was stained with colloidal Coomassie Blue and each lane was cut into 27 slices. Gel slices were washed once with 100 μl H2O and 100 μl H2O/ACN 50/50 (v/v) and incubated for 5 min at 37 °C on a thermal mixer at 600 rpm. For reduction of disulfide bonds, 100 μl of 10 mM DTT in 40 mM ABC were added for 1 h at 56 °C followed by a washing step with 100 μl H2O for 5 min at 37 °C. For alkylation of cysteine residues 100 μl of 55 mM IAA in 40 mM ABC were added and gel slices were incubated for 30 min at 25 °C in the dark. Subsequently, slices were washed alternately three times with 150 μl H2O and 150 μl H2O/ACN 50/50 (v/v) at 37 °C. Before adding trypsin, slices were incubated for 1 min in 100% ACN for dehydration. ACN was removed and slices were dried for 10 min. Gel pieces were rehydrated with 10 μl 40 mM ABC containing 150 ng trypsin and finally incubated overnight at 37 °C.

After overnight digestion the supernatants were collected in PCR tubes while gel pieces were subjected to four further extraction steps. Gel pieces were sonicated for 5 min in ACN/0.1% aqueous TFA 50:50 (v/v). Following centrifugation the supernatant was collected and gel pieces were sonicated for 5 min in ACN. After collecting the supernatant gel pieces were sonicated for 5 min in 0.1% TFA followed by another extraction step with ACN. The combined supernatants were evaporated to dryness in a speed-vac concentrator at 37 °C. Peptides were dissolved in 5 μl 0.1% TFA/2.5% HFIP by sonication for 5 min and subsequently analyzed by nanoLC-ESI-MS/MS.

StageTip and SPE Purification—10 μl TipOne Rtp tips (STARLAB GmbH, Hamburg, Germany) were blocked with C18 Empore plugs (Varian, Paolo Alto, CA) and filled with sufficient amount of Oligo R3 (Life Technologies, Carlsbad, CA) material. Reverse phase material was activated by adding 100% ACN and subsequently equilibrated with 2.5% FA. Sample was loaded by applying constant pressure using a 5 ml syringe. One wash step was performed using 2.5% FA. Finally, desalted peptides were eluted with 80% ACN/0.6% AA and evaporated to dryness in a speed-vac concentrator at 37 °C.

For desalting prior to phosphopeptide enrichment SPE cartridges were manually prepared using Bond Elut empty SPE cartridges (Agilent Technologies, Santa Clara, CA). For phosphopeptide enrichment we used a combination of IMAC and TiO2 enrichment. First, IMAC material was prepared as previously described, with minor modifications (23). All solutions were prepared with ultrapure water (Biosolve). Ni-NTA material was extracted from Qiagen spin columns (Qiagen, Venlo, Netherland) and incubated for...

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20 min under constant rotation in 100 mM EDTA (pH 8, adjusted by NH₃) followed by two washing steps with 100 mM EDTA. Subsequently, the material was washed two times with water followed by three times 0.6% AA. For loading with iron(III)-ions the material was incubated three times with 100 mM FeCl₃ in 0.6% AA for 20 min under constant rotation. To remove excessive iron(III)-ions, the material was washed four times with 0.6% AA and stored in 0.6% AA at 4 °C.

To enrich for phosphorylated peptides we incubated the samples with the IMAC material in a sample/IMAC ratio of 1:10 for 1.5 h under constant rotation at room temperature. The supernatants were transferred to new IMAC material and incubated again under the same conditions. The IMAC material of both steps was washed three times with 60% ACN/0.6% AA.

Peptides were eluted by adding 1 mM glycolic acid solution in 80% ACN/5% TFA. Both supernatants were incubated separately with TiO₂ (sample: TiO₂ ratio of 1:8) for 15 min under constant rotation. TiO₂ material was washed with 1 mM glycolic acid solution in 80% ACN/5% TFA, followed by 80% ACN/5% TFA and 20% ACN/0.2% TFA. Finally, the phosphopeptides were eluted from the TiO₂ material using 1% NH₄OH. Prior to LC-MS/MS analysis, samples were desalted using StageTips as described above and stored at −20 °C.

**Liquid Chromatography—Mass spectrometry—** Peptide mixtures were separated using a nanoAcquity UPLC system. For trapping we used a C18 precolumn (180 µm × 20 mm) with a particle size of 5 µm (Waters GmbH, Eschborn, Germany). Liquid chromatography separation was performed on a BEH130 C18 main-column (100 µm × 100 mm) with a particle size of 1.7 µm (Waters GmbH). Peptide mixtures were loaded on the trap column at a flow rate of 5 µl/min and were eluted with a gradient at a flow rate of 0.4 µl/min. The proteome samples were separated by a 1 h gradient. This was set as follows: from 0 to 4% B in 1 min, from 4 to 40% B in 39 min, from 40 to 60% B in 5 min, from 60 to 85% B in 0.1 min, 6 min at 85% B, from 85 to 0% B in 0.1 min, and 5 min at 0% B. The phosphoproteome samples were separated by a 3 h gradient, which was set as follows: from 0 to 4% B in 1 min, from 4 to 25% B in 25 min, from 25 to 40% B in 15 min, from 40 to 85% B in 10 min, 5 min at 85% B, from 85 to 4% B in 2 min, and 5 min at 4% B. Solvent A contained 98.9% water, 1% ACN and 0.1% FA, solvent B contained 99.9% ACN and 0.1% FA. The nanoUPLC system was coupled online to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The mass spectrometer was operated in data-dependent mode to automatically measure MS1 and MS2. Data were acquired by scan cycles of one mass spectrometer (Thermo Scientific, Bremen, Germany). Peptide mixtures were separated using a nanoAcquity UPLC system. For trapping we used a C18 precolumn (180 µm × 20 mm) with a particle size of 5 µm (Waters GmbH, Eschborn, Germany). Liquid chromatography separation was performed on a BEH130 C18 main-column (100 µm × 100 mm) with a particle size of 1.7 µm (Waters GmbH). Peptide mixtures were loaded on the trap column at a flow rate of 5 µl/min and were eluted with a gradient at a flow rate of 0.4 µl/min. The proteome samples were separated by a 1 h gradient. This was set as follows: from 0 to 4% B in 1 min, from 4 to 40% B in 39 min, from 40 to 60% B in 5 min, from 60 to 85% B in 0.1 min, 6 min at 85% B, from 85 to 0% B in 0.1 min, and 5 min at 0% B. The phosphoproteome samples were separated by a 3 h gradient, which was set as follows: from 0 to 4% B in 1 min, from 4 to 25% B in 25 min, from 25 to 40% B in 15 min, from 40 to 85% B in 10 min, 5 min at 85% B, from 85 to 4% B in 2 min, and 5 min at 4% B. Solvent A contained 98.9% water, 1% ACN and 0.1% FA, solvent B contained 99.9% ACN and 0.1% FA. The nanoUPLC system was coupled online to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The mass spectrometer was operated in data-dependent mode to automatically measure MS1 and MS2. Data were acquired by scan cycles of one FTMS scan with a resolution of 60,000 at m/z 400 and a range from 300 to 2000 m/z in parallel with six MS/MS scans in the linear ion trap of the most abundant precursor ions.

**Data validation and classification—** LC-MS/MS raw data were processed with MaxQuant (version 1.5.3.8) (24). Peptide identification was performed using the Andromeda search engine integrated into the MaxQuant environment (25) against the human UniProt database (downloaded: 06/07/2015; 146,661 sequences). SILAC quantification was enabled using ³¹C⁶/¹⁵N₂-arginine (+10.0083) and ³¹C⁶/¹⁵N₂-lysine (+8.0142) for the heavy state and their ³¹C⁴/¹⁴N-containing counterparts for the light state. Cysteine carbamidomethylation (+57.0215) was used as fixed modification; methionine oxidation (+15.9949), protein N-terminal acetylation (+42.0106), deamidation of asparagine as well as glutamine (+0.9840) and additionally for the phosphoproteome data set serine, threonine, and tyrosine phosphorylation (+79.9663) as variable modifications.

A false discovery rate (FDR) of 0.01 for proteins, peptides and sites, a minimum peptide length of 7 amino acids, a mass tolerance of 7 ppm for precursor and 0.5 Da for fragment ions were required. Enzyme specificity was set to trypsin/p with an allowed maximum of two missed cleavages for the proteome analysis and four missed cleavages for the phosphoproteome analysis. Additionally, the “quantify” and “match between runs” features were implemented with default settings to increase the number of peptides which could be used for quantification.

**Data transformation and evaluation** was performed with the freely available Perseus software (version 1.5.2.4), which includes all necessary functionalities. Contaminants as well as proteins identified by site modification and proteins derived from decoy database containing reversed protein sequences were strictly excluded from further analysis. Protein and phosphopeptide ratios were normalized, so that the median of their logarithms is zero, according to (24). For confident protein identification at least two unique peptides were required.

**Experimental Design and Statistical Rationale**—To define the molecular changes induced by ionizing radiation, we analyzed three biological replicates of SILAC labeled A549 cells. After treatment, SILAC states were mixed, processed and analyzed by LC-MS/MS. MaxQuant was used to identify proteins and phosphopeptides in the raw files with an FDR < 1%. Additional filtering for quantitative values was applied to have at least two valid values in the three biological replicates of each sample group (X-rays, protons, carbon ions). To define significantly regulated proteins/phosphorylation sites between irradiated samples and control samples one-sample t test was performed to be different from 0 (p value: 0.01). In addition, proteins/phosphorylation sites were only considered to be regulated if their abundance was changed more than 1.5-fold compared with the control. In order to find proteins/phosphorylation sites being differentially regulated between radiation qualities we applied a two-sample t test (p value: 0.01). In addition, they were only considered to be significantly regulated if their abundance was changed more than 1.5-fold between the two radiation qualities considered.

**Software Tools for Functional Analysis**—Significant hits were isolated and transformed by z-scoring prior to hierarchical clustering. Principle component analysis was performed using default settings in Perseus. Remaining data visualizations were performed in R (https://www.r-project.org/index.html, last accessed February 9, 2016). Majority of the plots were created using the ggplot2 package (https://cran.r-project.org/web/packages/ggplot2/index.html, last accessed February 9, 2016). Heat maps were created with heatmap.2 function from the gplots package (http://cran.r-project.org/web/packages/gplots/index.html, last accessed February 9, 2016).

For interpretation of the data GO term enrichment was performed with the Cytoscape 3.2.1 (26) plugin ClueGO (version 2.2.3) (27) and CluePedra (version 1.2.3) (28). Phosphoproteins significantly enriched in the data set were analyzed against the ontologies reference set. Significance level was set to p < 0.01 as determined by right-sided hypergeometric test and Bonferroni step down correction. Enrichment was performed using the GeneOntology databases: GOBP, GOCC, and GOMF (29).

To gather information about the phosphorylation sites identified in the data set we consulted the PhosphoSitePlus database (PSP, http://www.phosphosite.org/homeAction.action, last accessed February 15, 2016) (30). For identification of over-represented kinase motifs phosphopeptide sequences being differentially regulated by ionizing radiation were submitted to the Motif-X software (31). Sequence stretches were centered on the considered phosphosite and extended to 13 amino acids (±6 residues). Entire set of phosphopeptides identified in this study was used as background. Significance threshold was set to p < 0.01 and the occurrence threshold of motifs in the data set was set to 20. Enriched consensus sequences were assigned to kinases with matching substrate motifs according to the Human Protein Reference Database (http://www.hprd.org/serine_motifs, last accessed February 9, 2016). NetworKIN algorithm 3.0 in KinomeXplorer was used to predict kinases responsible for selected regulated phosphorylation sites (32, 33). Cutoff for the Net-
workIN score was set to 3. To illustrate connections between kinases and their targets we applied the Cytoscape plugin PhosphoPath (34).

**Synthetic Peptides**—HPLC purified stable isotope-labeled phosphopeptides were obtained from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). Peptide quality control included HPLC chromatograms and MALDI mass spectra. Stock solutions were produced at a concentration of 1 mg/ml in 3% ACN/1% FA and stored at −80 °C until use. Cysteine residues were carbamidomethylated using iAA. Stable isotopes were introduced in each peptide using one of the following amino acids during synthesis: 13C9,15N1-phenylalanine (+10.0272), 13C6,15N1-isoleucine (+7.0172), 13C6,15N1-leucine (+7.0172) or 13C9,15N1-valine (+6.0138).

**Targeted Phosphopeptide Validation**—Parallel reaction monitoring was performed using a list of 28 synthetic phosphopeptides selected from the discovery data set. Peak integration was accomplished using Skyline (35) and all integrations were manually checked for correct peak detection and absence of interferences. Spectral libraries were created in Skyline by injecting a 50 fmol standard mixture containing all synthetic phosphopeptides. Top4 transitions were selected on the presence of high abundant y- or b-ions. If necessary, additional transitions were selected that enable the differentiation between potential phosphorylation sites on the peptide. MS/MS spectra for all synthetic peptides with the selected transitions can be found in supplemental Fig. S2. Optimal spike-in amount for each peptide was obtained by setting up a standard curve between 0.1 fmol and 500 fmol and adjusting the spike-in level to the maximal intensity obtained in the discovery experiment. Information concerning the synthetic phosphopeptides can be found in supplemental Table S3. Corresponding spike-in amounts of each phosphopeptide were added to enriched phosphopeptide samples and analyzed with the LC-MS/MS method described above with minor modifications. Most important is the usage of a parent mass list for data dependent MS/MS acquisition. Six MS/MS scans of the most abundant precursors from this parent list were performed in parallel with one FTMS scan. The parent mass list contained three entries for each phosphopeptide: light (endogenous), medium (synthetic) and heavy (endogenous) peptide m/z.

Confirmation of the phosphopeptides was performed by verifying coelution of the phosphopeptide precursors as well as MS/MS transition profiles combined with the inspection of the relative abundance proportion of the fragment ions between endogenous and synthetic peptides. Information for each peptide can be found in supplemental Fig. S3.

**Western Blot Analysis**—Proteins from whole cell lysates were separated under reducing conditions on a NuPAGE 4–12% Bis-Tris gradient gel (Invitrogen) using a MOPS-buffer system and transferred to 0.45 μm nitrocellulose membranes (GE Healthcare). After blocking with 5% nonfat milk or 5% bovine serum albumin, the membranes were probed with primary antibodies against proteins or phosphorylation sites of interest. Secondary antibodies with horseradish peroxidase conjugate were used for visualization with enhanced chemiluminescence reagent (GE Healthcare). Commercial antibodies were purchased and used at the manufacturer’s recommended dilutions: ATM (CST, #2873), pATM (S1981) (CST, #4526), CHK2 (CST, #3440), pCHK2 (T68) (CST, #2681), RAD50 (CST, #3427), pRAD50 (S639) (CST, #14223), H2AX (CST, #2595), γH2AX (S139) (CST, #5438), NUMA1 (CST, #3888), pNUMA1 (S395) (CST, #3429), SRC (CST, #2109), pSRC (S75) (Abcam, Cambridge, UK, ab79308) and GAPDH (Merck Millipore, CB1001). For protein quantification, lysates were subjected to SDS-PAGE, gel slicing and subsequent in-gel digestion using trypsin. For phosphoproteome analysis lysates were tryptically digested in-solution and enriched using a combination of IMAC and
Subsequently, peptide mixtures were analyzed by high-resolution mass spectrometry and obtained raw data were processed using MaxQuant (24).

This discovery data set was used to select phosphorylation sites of interest to be confirmed in a targeted spike-in experiment. To this end, selected phosphopeptides were synthesized as medium isotope labeled counterparts. As indicated in Fig. 2A, they were added to the samples after the phosphopeptide enrichment in order to prevent overloading of the affinity material and associated loss of endogenous phosphopeptides. As we used the synthetic phosphopeptides for identity confirmation only and not for quantification, spike-in after the phosphopeptide enrichment was suitable. Usage of these synthetic phosphopeptides enabled to verify chromatographic coelution of the endogenous and the synthetic phosphopeptide and compare their fragmentation pattern, including fragments.

**Fig. 1. Determination and validation of radiobiological equivalent doses.**

A, Clonogenic survival assay for A549 cells exposed to dose series of X-rays, protons and carbon ions. Doses were set to achieve 30% survival fraction leading to the following doses used throughout the study: 6 Gy X-ray, 3.5 Gy protons and 2 Gy carbon ions. Data represents mean ± S.D. of three independent experiments.

B, Representative images of TP53BP1 foci (red) initial (1h) and residual (24h) after irradiation with the different radiation qualities. Cell nuclei were counterstained with DAPI (blue). Selected nuclei are magnified to emphasize the foci pattern following the individual treatments.

C, Percentage of cells containing none, one or more residual TP53BP1 foci per nucleus 24h after irradiation. Foci were counted in two independent experiments (n>2000 cell nuclei per condition and experiment) using ImageJ and mean ± S.D. is illustrated.

D, Western blot analysis of phosphorylation sites and their basal protein expression in response to different radiation qualities 2h after irradiation. Selected sites represent key player in the DNA damage signaling and are known to respond to ionizing radiation dependent DNA damage. GAPDH was used as loading control.

E, DNA damage response network, illustrating the quantitative Western blot results using Cytoscape plugin PhosphoPath (34). Additional proteins were added to complement the network. Functional analysis of the network using the ClueGO plugin within Cytoscape revealed three predominant biological processes demonstrating their affiliation to the DNA damage response as well as equal regulation by the different radiation qualities.
necessary for exact assignment of the phosphosite localization. These parameters were verified comprehensively using Skyline as described (35).

Descriptive Results of the Proteome and Phosphoproteome Analyses—A total of 3851 proteins and 5107 phosphorylation sites were identified with a false discovery rate (FDR) of less than 1% as depicted in Fig. 2B. Conservative thresholds were applied in order to use only high confident features during statistical analysis. Proteins and phosphorylation sites required identification in at least two out of three replicates of

Fig. 2. Experimental workflow for the analysis of radiation induced changes of proteome and phosphoproteome. A, SILAC labeled A549 cells were irradiated with radiobiological equivalent doses and harvested 2h after treatment. Heavy labeled samples irradiated with X-rays, protons and carbon ions respectively, were mixed with light labeled control samples for accurate quantification. SDS-PAGE with subsequent in-gel tryptic digestion was performed for proteome analysis and a combination of IMAC and TiO2 phosphopeptide enrichment after in-solution digestion for phosphoproteome analysis. Resulting samples were analyzed by LC-MS/MS and data processing was conducted with MaxQuant. Selected phosphorylation sites were subsequently validated using synthetic medium isotope labeled phosphopeptides in a targeted spike-in approach. B, Descriptive results of the proteome and phosphoproteome profiling of irradiated A549 cells (‘Regulation between irradiated and control samples; §According to PhosphoSitePlus). C, Distribution of phosphorylated serine, threonine, and tyrosine residues among quantified phosphosites. D, Distribution of singly and doubly phosphorylated peptides among quantified phosphosites.
each sample group (X-rays, protons, carbon ions). Overall 2634 proteins and 2818 phosphorylation sites were subject of further statistical evaluations. Among these, 2724 (97%) phosphorylation sites were mapped with high confidence (class I (39)). Predominant phosphorylated amino acid was phosphoserine with 2536 phosphorylation sites (90%), followed by phosphothreonine represented by 269 sites (9.5%) and phosphotyrosine for which 13 sites (0.5%) were detected (Fig. 2C). Majority of these phosphopeptides were singly phosphorylated (2764, 98.1%) and only minority of peptides showed phosphorylation at two residues (54, 1.9%), whereas no higher phosphorylated peptides were quantified (Fig. 2D).

Normalized log2-transformed ratios were plotted for proteome and phosphoproteome in order to estimate reproducibility and extent of regulation in the different sample groups (supplemental Fig. S1). These plots indicate minor changes at protein expression level. At phosphoproteome level each experiment showed similar spread of log2-ratios and good correlation between the replicates. Moreover, the standard deviation is almost equal between the replicates as well as between the radiation qualities ($\sigma_{\text{X-rays}}$: 0.48, $\sigma_{\text{Proton}}$: 0.50, $\sigma_{\text{Carbon}}$: 0.50) confirming the determined radiobiological equivalent doses used in this experimental setup.

On the protein level, only eight features were significantly regulated by ionizing radiation in our data set (supplemental Table S1C). Thus, only negligible changes occur at protein expression level following this short period of time after irradiation. Additionally, none of these eight regulated proteins contains phosphorylation sites in our data set. Because of these findings we focused on investigating phosphorylation changes in subsequent studies of acute radiation response.

Regulated Phosphorylation Sites in Response to Ionizing Radiation—In line with the fast protein phosphorylation kinetics of the cellular post translational modification machinery we identified many differentially regulated phosphorylation sites 2 h after irradiation. Overall, 181 sites were regulated on 141 proteins, whereas 2637 sites on 1269 proteins showed no regulation. The majority of identified phosphorylation sites (4702 out of 5107; >90%) were annotated in the PhosphoSitePlus database (30). This database contains 732 phosphorylation sites previously reported to be regulated by ionizing radiation which are predominantly derived from one comprehensive study by Bennetzen et al. (13). However, only 30 of the 181 regulated phosphorylation sites in our study were already described in radiation context. Hence our study considerably increases the content of the database by 151 novel phosphorylation sites that were found to be associated with cellular response to ionizing radiation per se.

Hierarchical cluster analysis of the significantly regulated phosphorylation sites is displayed as heatmap in Fig. 3A. Similar numbers of phosphorylation sites were up- and down-regulated indicating that next to phosphorylation events also dephosphorylation plays an important role in the response to ionizing radiation. The dendrogram of the treatment arms indicates that the radiation qualities are mainly separated into two clusters, namely X-rays (X) and particle based irradiations ($^1$H: proton and $^{12}$C: carbon). This is intriguing, considering that proton irradiation is believed to elicit similar biological effects compared with X-rays for planning patient treatment.

Functional Analysis of Regulated Phosphorylation Sites—To better understand ongoing molecular processes triggered by ionizing radiation we used the Cytoscape (26) plugin ClueGO (27) to search for enriched GO terms (29) in the 141 proteins, containing regulated phosphorylation sites. Enriched biological processes in this data set contain mainly terms involved in the DDR: DNA repair, DNA metabolic process, DNA conformation change, DSB repair via NHEJ, base-excision repair and execution phase of apoptosis (Fig. 3B). These enriched biological functions are consistent with current concepts considering DNA damage as hallmark of acute cellular response to ionizing radiation. Accordingly, most of these phosphoproteins are located in or translocated into the nucleus. This is accompanied by their biological functions, to repair damaged DNA and prevent chromosomal instability. Enriched molecular functions of the regulated phosphoproteins contain mainly nucleic acid binding motifs indicating that these phosphoproteins bind to DNA damage sites or at regulatory transcription sites for their respective function. A key representative of these findings is TP53BP1, which is located in the nucleus, involved in DNA damage response by regulating repair pathway choice and directly recruited to damaged chromatin by recognition of a specific histone code (40).

Kinase Motif Analysis of Regulated Phosphorylation Sites—One advantage of not using motif specific antibodies for phosphopeptide enrichment is independency of the consensus sequence surrounding the phosphorylation site. We applied a combination of IMAC and TiO2 both being affinity-based techniques independent of the amino acids adjacent to the phosphorylation site. This led to an unbiased population of phosphopeptides and enabled the identification of enriched consensus sequence stretches in the data set. In order to identify kinase motifs we analyzed a 13-amino acid sequence stretch surrounding the central phosphorylated residue for all regulated phosphosites compared with nonregulated sites using Motif-X (31).

Five consensus sequences were identified to be significantly enriched within the set of radiation regulated phosphorylation sites: p(S/T)P, pSPXXK, RXXpS, pSXSE, and pSQ as illustrated in Fig. 4. All five kinase motifs were previously characterized to be involved in different cellular signaling processes (41). To compare our findings with the 732 ionizing radiation dependent sites described in the PhosphoSitePlus database these sites were enriched in the same manner. All motifs identified by us were also present in the motifs enriched in the database, implying consistency between our study and the previously described ionizing radiation dependent phosphorylation sites. Next, we matched the enriched motifs to potential kinases described in the Human Protein
Reference database and assigned the radiation regulated phosphopeptides to each motif. Thereby we were able to identify three kinase motifs being mainly associated with phosphorylation events and three mainly with dephosphorylation. Highly upregulated are phosphorylation sites of the three phosphoinositide-3-like kinases (PIKK): ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase catalytic subunit (PRKDC), with the joint sequence motif p(S/T)Q. This finding correlates well with their proposed involvement in the DDR following treatment with ionizing radiation (42, 43).

In contrast, the motifs p(S/T)P and pSPXXK show mainly dephosphorylation in response to ionizing radiation. These represent consensus motifs of cyclin-dependent kinases (CDKs), glycogen synthase kinase 3 (GSK-3) and DNA-dependent protein kinase catalytic subunit (PRKDC), with the joint sequence motif p(S/T)Q. This finding correlates well with their proposed involvement in the DDR following treatment with ionizing radiation (42, 43).

Sites of the RXXS and SXXE motifs cannot be clearly assigned to one direction of regulation however upregulation is slightly pronounced. RXXS is a target of three potential kinases: protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and RAC-alpha serine/threonine-protein kinase (Akt) with joint functions including cell survival and proliferation (44–46). Casein kinase II represented by the SXXE motif also regulates important cellular functions as cell proliferation, differentiation and death (47).

**Differentially Regulated Protein Phosphorylation by X-ray Versus Particle Irradiation**—A central goal of our study was to decipher potential differences in acute radiation response to irradiation with X-rays, protons and carbon ions. Importantly, we aimed to exclude that these differences arise from distinct cellular fates resulting from variation in prescribed doses. In line with current standard approach to define biologically equivalent dose, the clonogenic survival was considered as the key readout to determine isoeffectiveness as described in Fig. 1. Based on current understanding of the radiobiology the two less densely ionizing radiations, X-ray and the low-LET protons were anticipated to show similar cellular responses versus the high-LET carbon irradiation. However, to our surprise, both unsupervised hierarchical clustering (Fig. 3A) and principle component analysis (Fig. 5A) predominantly discriminated between X-ray and particle irradiations (proton/carbon). Based on this data we searched for phosphorylation sites being differentially regulation between radiation qualities and identified 55 corresponding sites listed in supplemental Table S2D. Differences between X-rays and particle radiations
were more pronounced whereas protons and carbon ions induce very similar phosphosite regulation as indicated in the volcano plots in Fig. 5B. A second level separation is apparent between the low-LET protons versus high-LET carbon ions in the second PCA component although not as pronounced as the first level separation. These results indicate that differences in acute signaling events induced by different radiation qualities exist.

Validation of mass spectrometry based proteomic results can be achieved by Western blotting. For three distinct phosphorylation sites where phosphosite specific antibodies were commercially available we confirmed the SILAC based quantifications: DDR associated sites NUMA1 (S395) and RAD50 (S635) are shown in Fig. 1D and differentially phosphorylated site on SRC (S75) is shown in Fig. 5C. Comparison of the two orthogonal analyses revealed excellent correlation at phosphorylation as well as basal protein level indicating the high impact of the presented discovery data set and the power of SILAC based quantitative proteomics (Fig. 5D).

**Fig. 4.** Phosphorylation consensus sequences and putative kinases of radiation regulated phosphorylation sites. Six consensus sequences were identified using the Motif-X software to be overrepresented in the set of 181 regulated phosphorylation sites compared with nonregulated sites during the response to ionizing radiation. Putative kinases were assigned and the heatmaps show the phosphorylation sites belonging to the different motifs (GSK-3: glycogen synthase kinase 3, ERK1/2: mitogen-activated protein kinases, CDK5: cyclin-dependent kinase 5, PKA: cAMP-dependent protein kinase, CaMKII: Ca\(^{2+}\)/calmodulin-dependent protein kinase II, Akt: RAC-alpha serine/threonine-protein kinase, ATM: ataxia telangiectasia mutated).

were not yet described to be regulated by ionizing radiation in the PhosphoSitePlus database (30). Additionally, nine phosphopeptides were selected with known regulation by ionizing radiation in order to confirm the experimental workflow. Moreover, the selected candidates contain phosphorylation sites that are mainly responsible for the separation in the principle component analysis presented above between X-ray and the particle based irradiation. Because TP53BP1 was identified to play a central role in cellular response to radiation with significant enrichment of its phosphorylation sites across all radiation induced alterations (p value: 2.3e-6, Fisher’s exact test), we decided to particularly focus on validating radiation induced TP53BP1 regulation by tracking ten phosphorylation sites on this protein.

Initially, optimal spike-in amount was determined for each synthetic peptide. This was conducted by performing linear regression between 0.1 fmol and 500 fmol on column and comparing received intensities to the maximum intensity obtained in the discovery data set for the corresponding endogenous phosphopeptide. This led to a mean spike-in level of 35.3 \pm 54.3 fmol on column (in detail listed in supplemental Table S3). The optimized peptide amounts were spiked into the enriched phosphopeptides and analyzed simultaneously by LC-MS/MS as indicated in the experimental workflow (Fig. 2A).

This strategy enabled validation of 28 selected candidates as exemplarily shown in Fig. 6 for the phosphosite S56 on VIM. In Fig. 6A three parameters were checked to confirm the peptide characteristics: (i) quantity in the samples differentially treated, (ii) identification by comparing proportion of
selected transitions of the light (sham), medium (synthetic), and heavy (irradiated) peptides, and (iii) retention time comparison between the phosphopeptide identified in the discovery experiment and the corresponding synthetic peptide. Detailed results for each of the 28 selected phosphopeptides can be found in supplemental Fig. S3. For elucidation of the procedure additional information is given for VIM (S56) in Fig. 6B. Left, three mass spectra are shown, one for each of the three radiation treatments. It can be seen, that the light ($) and medium ($) peptide are comparable between the treatments but heavy (#) shows clear aberrations. For X-rays the heavy peptide has almost same abundance as the light peptide and for the two particle irradiations almost no heavy peptide can be detected which precisely confirms the quantification illustrated in Fig. 6A. On the right of Fig. 6B representative fragment spectra are additionally shown for the three peptide species. Exemplarily, the zoom shows the y8-ion for each isotope label. Combination of the selected transitions confirms identity and exact site of phosphorylation for each of the phosphopeptides.

A heatmap containing all 28 validated candidates is illustrated in Fig. 7A. The heatmap is divided into two clusters containing phosphorylation sites mainly comprising two distinct sequence motifs. Cluster 1 represents the sites of the p(S/T)Q-motif and few nonassigned sites which show intense upregulation and additionally uniform regulation across all radiation qualities. These sites are significantly enriched in the nuclear parts of the cell going along with their associated biological functions in the DDR (Fig. 7B). Thus, basic DDR functions maintaining genome integrity are similarly regulated by the different radiation qualities.

Phosphorylation sites associated to cluster 2 comprise sites of the p(S/T)P-motif which are dephosphorylated in response to irradiation. Several of these sites show differential
regulation between X-ray and the particle radiations. Potential reason for this may be deactivation of common upstream regulator leading to uniform dephosphorylation pattern of corresponding target sites. Putative kinases for these sites are illustrated in Fig. 7. Six sites have cyclin-dependent kinases (CDKs) as regulatory kinase in common emphasizing their potential role as upstream regulator of this differential signaling. Enriched GO terms in cluster 2 (Fig. 7B) highlight their role in apoptosis and DNA metabolic processes as well as their localization in the outer parts of the cell (cytoplasm, cytoskeleton, cytosol).

TP53BP1 with its crucial role in the DDR links the two clusters with several sites in both of them. Seven TP53BP1 residues show strong upregulation induced by the ATM/ATR kinases (S398, S580, S831, T855, S1068) and PRKDC kinase (S3205), respectively. In contrast, three TP53BP1 sites showed downregulation correlating with decreased CDK1 activity (S294, S1094, S1101) (Fig. 7D). Herewith, we underline the versatile mediator role of TP53BP1 in response to ionizing radiation. Allocation of these regulated sites at TP53BP1 is depicted schematically in Fig. 7E. This illustrates an enrichment of regulated phosphorylation sites

### Table I: List of phosphopeptides validated in the spike-in experiment using synthetic isotope labeled peptides.

| Gene symbol | Modified peptide sequence | Modified site | Protein name | Known IR response |
|-------------|---------------------------|---------------|--------------|-------------------|
| BRCA1       | NYPS(ph)QEEL$IK^a         | S1524         | Breast cancer type 1 susceptibility protein | + |
| CDK1        | I^aGEGTY(ph)GVY$K^a       | Y^15          | Cyclin-dependent kinase 1 | + |
| ELAVL1      | NVALLSQ^L^YH$S(ph)PAB^a   | S202          | ELAV-like protein 1 | |
| FLNB        | LDV^9TLS$S(ph)PSR^a       | S983          | Filamin-B | |
| KPNA2       | NVSSFPDDAT$S(ph)PLQ$ENR^a | S52           | Importin subunit alpha-1 | |
| LMNA        | S$S$ph)VGGSGGGSGFD$DL^9$VTR^a | S526 | Lamin-A/C | |
| MAPT1B      | SV^9NFSLT$S(ph)PNEIK^a    | T^1282        | Microtubule-associated protein 1B | |
| MCM6        | (ac)MDLAAAT$EPEGAGS$S(ph)QHL^8$EVRA^a | | DNA replication licensing factor | + |
| MCM6        | EIESEIDS$S(ph)EEEI$N^K^a  | S762          | DNA replication licensing factor | MCM6 |
| MKI67       | ELE^65QT(ph)PDHTEESTTDDK^a | T^2003        | Antigen Ki-67 | |
| NUMA1       | LSQLEHLS$S(ph)QLQ$DNPQPEK^a | S395 | Nuclear mitotic apparatus protein 1 | + |
| PLEC        | AOL$S$EVAS(ph)PAK^a        | S1435         | Plectin | |
| PRKDC       | LTL$S$PEDNS$S(ph)MNV^DQQGDPSDR^a | S3205 | DNA-dependent protein kinase catalytic subunit | + |
| RAD50       | LFDVCGS$S(ph)QDFESDL$DR^a | S335          | DNA repair protein RAD50 | |
| RRBP1       | NTDV$S$AQ$S(ph)PEAKQ$OEPAK^a | S515 | Ribosome-binding protein 1 | |
| SRC         | LFGGF$S$SSD$TVTS$S(ph)PQR^a | S75 | Proto-ontogene tyrosine-protein kinase Src | |
| STMN1       | ESSVPEF$S$Sp$S(ph)PPK^a   | S38           | Stathmin | |
| TOP2A       | TQMAEVPS$S(ph)PB^a         | S1213         | DNA topoisomerase 2-alpha | + |
| TP53BP1     | Sp$S$ph)PEPEVLSQEDLE$DQSNK^a | S294 | Tumor suppressor p53-binding protein | + |
| TP53BP1     | QDK$S$PM$S(ph)EDGEGEP$S$QK^a | S398 | Tumor suppressor p53-binding protein | + |
| TP53BP1     | FVPAENSDILMNPADQEVALS$S(ph)QNDD$K^a | S580 | Tumor suppressor p53-binding protein | + |
| TP53BP1     | SGTAETPEVQDS$S(ph)SQPSL$Q$VR^a | S830 | Tumor suppressor p53-binding protein | + |
| TP53BP1     | SGTAETPEVQDD$S(ph)QPSL$Q$VR^a | S831 | Tumor suppressor p53-binding protein | + |
| TP53BP1     | ADDPLRLQELQ$QOPQT(ph)QE$K^a | T^855 | Tumor suppressor p53-binding protein | + |
| TP53BP1     | GNLH$S$E$P$S$S(ph)QGEEEK$K^a | S1068 | Tumor suppressor p53-binding protein | + |
| TP53BP1     | QSQQPMK$S$Sp$S(ph)PVK$DPV$S$S(ph)P$ASQK$K^a | S1101; S1094 | Tumor suppressor p53-binding protein | + |
| TP53BP1     | LPGDPTGS$S(ph)$EEEEEFL$EIP$E$K^a | S1759 | Tumor suppressor p53-binding protein | + |
| VIM         | SLYASS$S(ph)PGGV$YATR^a    | S56           | Vimentin | |
in the N-terminal p(S/T)Q repeats responsible for interaction with telomere-associated protein RIF1 and PAX-interacting protein 1 (PTIP) as well as a single site in the BRCA1 C-Terminal domain (BRCT) associated with tumor antigen p53 and PWWP domain-containing protein MUM1 (EXPAND1) interaction mediating different cellular functions (40). Intriguingly, we identified a number of regulated phosphorylation sites on TP53BP1 for which no functional information is currently available at the PhosphoSitePlus database (30).

**DISCUSSION**

Precision radiotherapy such as particle radiation exhibits physical and biological advantages in comparison to conventional X-ray therapy (3). The application of particle radiation is especially of interest for difficult to reach tumors such as those in close proximity to organs at risk, where highly precise conformity of irradiation beams is needed and tumors being resistant to conventional X-ray therapy (i.e. hypoxic tumors) (48–50). Although particle radiation is already applied for patient treatment, there is still only limited knowledge about the
FIG. 7. Validated response to different radiation qualities. A, Phosphosite regulation following irradiation with X-rays, protons \((^1\text{H})\) and carbon ions \((^{12}\text{C})\) plotted as heatmap for 28 phosphopeptides validated using synthetic isotope labeled phosphopeptides in a spike-in experiment. Phosphopeptides are grouped into two clusters. Cluster 1 contains peptides of the p(S/T)Q and nonassigned motifs. Cluster 2 contains the peptides of the p(S/T)P motif. Values represent the normalized log\(_2\)-transformed ratios for the change in phosphorylation status between irradiated and control samples. B, Functional analysis using the ClueGO plugin within Cytoscape to identify enriched GO terms in the set of validated phosphopeptides represented by their corresponding protein. Analysis was performed in the two illustrated clusters separately to uncover their particular characteristics concerning cellular localization and biological function. Illustrated are the five most significant terms in each cluster by their corresponding \(p\) value. C, NetworKIN algorithm in KinomeXplorer \((32, 33)\) was used to predict associated kinases in order to estimate potential upstream regulators of the identified alterations. Kinase prediction for phosphorylation sites being differentially regulated between the radiation qualities. Regulation of the phosphosites as well as predicted kinases are illustrated using Cytoscape plugin PhosphoPath \((34)\). D, Sites on TP53BP1 show likewise up- and downregulation. Upregulations are conducted by the phosphoinositide-3-like kinases (PIKK); ATM, ATR and PRKDC. Concurrently, three phosphorylation sites are downregulated by decreased CDK1 activity. Regulation of the phosphosites as well as predicted kinases are illustrated using Cytoscape plugin PhosphoPath \((34)\). E, Schematic graph of TP53BP1 \((1,972\) amino acids) including two noteworthy structural features containing radiation regulated phosphorylation sites identified in this study: N-Terminal clusters of the p(S/T)-Q motif and two C-terminal BRCT domains. Domains not affected by our results are left out. The two depicted domains are responsible for TP53BP1 interaction with RIF1/PTIP and p53/EXPAND1 respectively \((40)\).
molecular and cellular effects induced by the different radiation qualities. In our study, we compared the acute proteome and phosphoproteome response of human lung adenocarcinoma (A549) cells 2h after irradiation with X-rays, protons and carbon ions, respectively. Our goal was to characterize the triggered signaling events and decipher aberrant signaling following treatment with the applied radiation qualities.

The depicted study represents a robust and efficient strategy to elucidate proteomic and phosphoproteomic changes in response to ionizing radiation. Alterations of protein abundance were observed at this acute time point for eight proteins only. In contrast, the prevailing pattern of cellular response was mediated by phosphorylation as a fast mechanism of signal transduction. In line with our observation, 6 Gy X-ray irradiation of GM00130 cells was also found to elicit limited alterations in proteome abundance within a relatively short time frame (5min - 8h) as recently published by Bennetzen et al. (13) confirming our decision to focus on changes at phosphorylation level. Single or fractionated high radiation doses as employed in the area of stereotactic ablative radiotherapy (SABR, radiosurgery) may result in higher number of differentially expressed proteins as suggested by Cho et al. (51). In contrast, in this study we focused on investigating dose ranges relevant to clinical routine applications.

Applying SILAC based phosphoproteomics, we identified a total of 5107 phosphorylation sites, including 2818 quantified sites that were subject of statistical analysis because of stringent exclusion thresholds. A phosphopeptide enrichment protocol was adopted which required only small sample amount (150 μg per SILAC state). Other enrichment strategies such as extensive sample fractionation would enhance the number of identifications and deepen the coverage of regulated signaling pathways (52–55), however this would require a larger amount of starting material. Moreover, our enrichment protocol was optimized for peptides containing one phosphorylation site only. Enhancement of multiple phosphorylated peptides would necessitate adjustments of the protocol such as reduction of IMAC material during enrichments (56). An additional extension by a single-step immunoaffinity purification would enlarge the proportion of phosphotyrosine residues (57, 58). However, as irradiated sample material was very limited because of restricted time slots for researchers caused by privileged beam time for patient treatment we could not randomly extend sample amount to apply additional enrichment strategies.

To examine cellular signaling events differentially regulated by the radiation qualities, application of radiobiological equivalent doses is a prerequisite. Therefore, we determined the RBE utilizing the gold standard clonogenic survival assay. At isoeffective doses of all three radiation qualities, resulting in 30% survival fraction, we were able to identify a large number of regulated phosphorylation sites and most importantly could show for the first time differential signaling induced by X-rays versus the raster-scanning particle irradiations (proton/carbon). Statistical analyses were conducted to: (i) identify phosphorylation sites with common regulation pattern across all radiation qualities and (ii) identify sites with differential phosphorylation pattern between the three radiation qualities. A total of 181 phosphorylation sites are regulated in response to ionizing radiation. One hundred twenty-six sites thereof are regulated regardless of the radiation quality, whereas 55 show differential regulation between the qualities. We found enrichment of five different kinase sequence motifs in our data and could confirm similar enrichment of motif families by analysis of previously published radiation dependent phosphoproteome alterations deposited in the PhosphoSitePlus database.

A consensus radiation quality independent phosphorylation pattern of p(S/T)Q sites was found mainly enriched for sites in the DDR, in line with current knowledge that DDR is a hallmark of ionizing radiation (59, 60), which is induced and anticipated by the usage of cell kill based isoeffective doses. Observed downregulation of p(S/T)P and pS/PXXK sites representing motifs of the CDK, GSK-3 and MAP kinases have crucial role in the cell response to ionizing radiation per se. CDKs play a key role in maintaining genome integrity (61). Our finding that radiation induced DNA damage affect protein substrates of CDKs hence provides a plausible explanation for the phenomenological observation of cell cycle arrest after irradiation. Indeed, radiation induced cell cycle arrest is important for proper DNA repair (62, 63). Recent findings indicate that CDKs additionally contribute to further upstream events such as checkpoint control and DNA repair (64). GSK-3 executes heterogeneous signaling functions including both cell death and survival signals dependent on the signaling context (65). The MAP kinases ERK1/ERK2 likewise conduct regulation of cell proliferation, migration and death (66, 67). The prevalent downregulation of phosphorylation sites belonging to these kinase motifs implies that cells enter a state of proliferative arrest to enable proper DNA repair and maintain genomic stability. These findings together emphasize, that radiation induced acute signaling pathways are dominated by DNA repair functions, cell cycle regulation and cell survival/death signals as previously reported (68). Our data enables the assignment of phosphorylation sites to distinct cellular functions and facilitate further investigations of exact phosphosite functionality.

Despite employing depicted isoeffective doses, 55 phosphorylation sites were identified to respond differentially between the three radiations. We identified distinct p(S/T)P sites with radiation quality dependent regulation being mostly reflected by minor downregulation of phosphorylation sites after X-ray treatment and pronounced downregulation following particle irradiation. This coherent pattern leads to the deduction that a common upstream regulator is responsible for these regulations. Our data points to the CDK family with its versatile regulation of cellular processes including most importantly cell cycle and transcription regulation (69). We could show that particle irradiation leads to dephosphorylation of proteins involved in cell mitosis such as MKI67, MAP1B,
RRBP1, and KPNA2 to a larger extent than X-ray irradiation. Sites on STMN1, FLNB, and VIM that are involved in cell migration and invasion show a similar pattern. These observations support the previously published data on functional analysis of different cell lines after proton and carbon irradiation in comparison to X-rays, suggesting altered regulation of angiogenesis (10, 11, 70), cell migration (71, 72), and mitosis (73) by different radiation qualities. We identified phosphorylation sites corresponding to these processes that were not yet described in the context of ionizing radiation. However, their exact role within the context of radiation biology remains to be elucidated.

For validation purposes, we selected phosphorylation sites of interest and confirmed their identity in an unbiased spike-in approach using synthetic isotope labeled phosphopeptides. Compared with Western blotting, the use of synthetic peptides has tremendous advantage: (i) no phosphosite specific antibodies are needed, (ii) uncomplicated and inexpensive production of synthetic peptides, (iii) misinterpretation of Western blot signals because of cross-reactivity of the antibodies is avoided (74), and (iv) distinct multiplexing possibilities in a targeted spike-in approach (16, 75). Moreover, in future experiments the same pool of synthetic peptides can be used as internal standards in tissue samples or in vivo experiments (76). Easy handling of synthetic peptides makes a distribution between laboratories possible. The availability of synthetic isotope labeled phosphopeptide pools, similar to antibody arrays, would be a valuable tool to study complete signaling pathways with high confidence and accurate quantification in a fast and reproducible manner. When the amount of each synthetic peptide is adjusted to its ionization efficiency as conducted in our study and a list of suitable transitions for the identification is included, it would enable a fast and easy usage of these synthetic phosphopeptide pools.

Our pool contained 28 synthetic phosphopeptides covering a broad range of characteristic, in order to confirm multiple hypotheses in a single experiment. We confirmed that the DNA damage responsive phosphorylation sites of the (S/T)Q motif show similar regulation pattern between the radiation qualities. Among these, sites on TP53BP1, PRKDC, and RAD50 with well-known DDR activity were similarly phosphorylated by the different radiation qualities. Moreover, our results indicate TP53BP1 to be a key regulator in response to ionizing radiation, supporting the recently published data which highlighted TP53BP1 in a central position to maintain genome integrity (40). In this work, we validated nine phosphopeptides containing ten phosphorylation sites on TP53BP1, which are regulated by crucial kinases simultaneously (ATM, PRKDC, and CDK1), underlining the versatile role of TP53BP1 as one of the main genome guardian molecules (40).

Moreover we confirmed ten phosphorylation sites with differential regulation pattern between the radiation qualities which is because of the radiations mode of action. Although X-ray induced damage is mostly a consequence of increased ROS production and indirect damage, high-LET carbon ion irradiation induces direct complex DNA damages (6). Hence differences between these two radiation qualities were anticipated. In contrast, proton irradiation was so far thought to elicit similar biological effects to X-rays reflected by the currently fixed proton RBE of 1.1 for patient treatment. Surprisingly, unsupervised clustering and principle component analysis clearly separated X-ray irradiation from proton and carbon irradiation. Hence, the low-LET proton irradiation shared more common phosphoproteome characteristics with high-LET carbon than with conventional X-ray irradiation. This is an interesting unexpected finding, however in line with more recent data indicating potential less appreciated radiobiological differences attributed to protons beyond the classical cell killing effect (11). Further, in the field of radiotherapy the paradigm of fixed proton RBE of 1.1 is currently controversially debated and increasing body of data imply that improved radiobiological models are urgently needed to better characterize the exact effect of proton irradiation (77). Our observations at phosphoproteome level indicate that differences in radiobiology between X-ray and proton irradiation exist even at doses that produce the same level of cell kill.

In summary, we present a versatile workflow for identification, quantification and validation of phosphorylation sites in response to ionizing radiation that could be employed for a broad spectrum of applications in life sciences. Our data stimulate radiation research at novel frontiers of intracellular signaling and cell-cell communication. These are instrumental for a better understanding of discrepancies found on tissue level responses to different radiation qualities. Further exploration in this direction will supplement and adjust our view on the so far predominantly tumor cell kill centric modeling of radiobiological effects. Especially for the ongoing debate about proton RBE in patient treatment we offer exclusive data. Moreover, the presented phosphorylation sites, that are differentially regulated, offer attractive targets for modulation and improvement of radiotherapy in the clinical setting.

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