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Low-dose radiation differentially regulates protein acetylation and histone deacetylase expression in human coronary artery endothelial cells

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Abbreviations: CVD, cardiovascular disease, Gy, Gray; EC, endothelial cell; human coronary artery endothelial cell, HCAEC ; histone deacetylase, HDAC; SIRT, sirtuin; ER, endoplasmic reticulum

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Abstract

Purpose: Ionizing radiation induces cardiovascular disease, the endothelium being the main target. The exact mechanism of the damage is unclear but the involvement of multiple signaling pathways is probable. Reversible lysine acetylation is a posttranslational protein modification that regulates activity across a broad range of signaling pathways. The goal of this study was to determine if a low radiation dose results in acetylome alteration in endothelial cells.

Materials and Methods: Human coronary artery endothelial cell line was irradiated with Cs-137 gamma-rays (0.5 Gy) and proteomics analysis was performed using enriched acetylated peptides and all peptides. Data were validated using immunoblotting, deacetylase activity assay, and RhoA activity assay.

Results: Nearly a hundred proteins were found to have an altered acetylation status 24 h after irradiation, primarily due to an overall decrease in acetylation. The expression of specific deacetylases was significantly increased, coinciding with an enhancement in global deacetylase activity. Proteins changed in their acetylation status belonged to several pathways including protein synthesis, cytoskeleton-related processes, protein folding and calcium signaling. The predicted changes in the RhoA/actin cytoskeleton pathway were validated by immunoassay.

Conclusions: This study shows that protein acetylation is an important mediator of radiation responses in human cardiac coronary endothelial cells. Increased knowledge of the endothelial response to radiation is crucial for the development of normal tissue sparing modalities during radiation therapy.

Key words: Acetylation; ionizing radiation; proteomics; endothelial cell; sirtuin, cardiovascular disease

Introduction

The vascular endothelium, a single layer of cells lining the inside of all blood vessels, is one of the most vulnerable and radiation-sensitive components in the body (Azimzadeh et al. 2015, Menendez et al. 1998, Soloviev et al. 2003, Sugihara et al. 1999). The earliest data indicating increased risk for radiation-induced cardiovascular disease (CVD) originated from studies of long-term outcomes after radiation therapy treatment of breast cancer (Darby et al. 2003) or Hodgkin´s disease (Hancock et al. 1993). A recent case-control study among women who underwent radiation therapy for breast cancer showed a significantly increased risk for ischemic heart disease even with doses smaller than 2 Gy (Darby et al. 2013). Although the risk for CVD at low and moderate doses has been extensively analyzed in numerous epidemiological studies (Little et al. 2012, Little et al. 2008, Little et al. 2010), the multiple contributory risk factors for CVD make it difficult to detect increased risk at low doses. Low-dose studies using endothelial cell models are necessary to estimate the risk and to allow the development of normal tissue sparing modalities.

Numerous forms of posttranslational modifications (PTM) regulate protein function (Karve and Cheema 2011). Acetylation of lysine residues occurs during cellular stress (Choudhary et al. 2009). Modification of lysine residues in core histone tails by histone acetyltransferases or histone deacetylases (HDACs) can modulate gene expression (Lee and Workman 2007, Shahbazian and Grunstein 2007) but lysine acetylation is also essential for other important pathways including cell cycle control, cytoskeletal organization, protein synthesis and folding, and RNA splicing (Choudhary, et al. 2009). Around 3,600 unique protein acetylation sites have been identified so far. They are suggested to contribute to the regulation of almost all nuclear functions and a large array of cytoplasmic functions (Choudhary, et al. 2009). However, the biological relevance of lysine acetylation is not known for most proteins as this

PTM means activation for some proteins but inactivation for others (Barjaktarovic et al. 2015).

The 18 mammalian HDACs are grouped into four classes. Classes I, II and IV require zinc as a co-factor for catalytic activity, while class III HDACs are dependent on nicotinamide adenine dinucleotide (NAD+) (Ferguson and McKinsey 2015). The zinc-dependent mammalian HDAC family comprises 11 enzymes, which have specific and crucial functions in tissue development and homeostasis (Delcuve et al. 2012). The sirtuin Class III deacetylase family contains seven members in humans (Frye 2000). The best characterized of these is sirtuin 1 (SIRT1) (Morris 2013), involved in cell cycle progression, cell survival, senescence and inflammation (Vijg et al. 2008). Sirtuin 3 (SIRT3) is located in mitochondria and has been implicated in regulating metabolic processes (He et al. 2012, Onyango et al. 2002).

We have already shown that cardiac endothelial cells respond to relatively high doses (2 Gy) of radiation by increasing the expression of sirtuins (Barjaktarovic, et al. 2015). Simultaneously, marked alteration was found in the acetylation status of several proteins that were members of biological pathways such as protein synthesis, general stress signaling, RhoA/cytoskeleton signaling and RNA metabolism (Barjaktarovic, et al. 2015). The aim of this study was to decipher the role of protein acetylation in a human coronary artery endothelial cell line (HCAEC) following a dose of 0.5 Gy that also represents the significance limit for the increased risk of radiation-induced CVD in irradiated populations (Shimizu et al. 2010). This study shows that protein acetylation is an important mediator of low-dose radiation response in endothelial cells.

Materials and methods

Cell culture, irradiation and harvesting of cells

HCAEC (Lowe and Raj 2014) were cultured in T175 culture flasks at 37°C with 5 % CO_2 in air in MesoEndo Endothelial Cell Growth Medium (Cell Applications, INC) as recommended by the supplier. For the acetylome analysis, exponentially growing cells were irradiated with 0.5 Gy using a Caesium-137 gamma source (HWM-D 2000, Waelischmüller, Germany) operated at a dose rate of 0.49 Gy/min. Control cells were sham-irradiated. Cells were harvested 4 and 24 h after irradiation by scraping and rinsed once with ice cold 10 mM Tris-250 mM sucrose (pH 7.0). Detached cells were centrifuged 3 min at 220 g (4°C), rinsed again with 1 ml of 10 mM Tris-250 mM sucrose (pH 7.0) and pelleted by centrifugation as before. The cell pellets were immediately stored at -80°C.

Acetylome analysis

Cells were lysed with a lysis buffer containing 1% Triton X100, 100 mM Tris HCl pH 7.6, and protease and phosphatase inhibitor cocktails (Roche), lysine deacetylase inhibitors MS275, and suberoylanilide hydroxamic acid (Alexis Biochemicals) according to manufacturer´s instructions. Protein lysates (300 µg) from control and irradiated HCAEC (4 h and 24 h post-irradiation) in 1% Triton, 100 mM Tris buffer, pH 7.6, were subjected to overnight in-solution tryptic digestion as described previously (Azimzadeh et al. 2010). Acetylated peptides were enriched from the lysates using agarose-conjugated anti-acetyl lysine antibody (#ICP0388, ImmunChem) and eluted using 0.1% TFA as described previously (Choudhary, et al. 2009). Peptides were separated by reversed phase chromatography (PepMap, 15 cm×75 µm ID, 3 µm/100 Å pore size, LC Packings) operated on a nano-HPLC (Ultimate 3000, Dionex) with a nonlinear 170 min acetonitrile (ACN) gradient in 0.1% formic acid (FA) in water at a flow rate of 300 nL/min. The gradient settings

were subsequently: 0−140 min: 5−31% ACN, 140−150 min: 31−93% ACN, followed by equilibration for 20 min at starting conditions (Hauck et al. 2010). The nano-LC was connected to a linear quadrupole ion trap- Orbitrap mass spectrometer (LTQ Orbitrap XL, ThermoFisher, Bremen, Germany) equipped with a nano-ESI source. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full scan mass spectra (from *m/z* 300 to 1500) were acquired in the Orbitrap with a resolution of R=60,000 at *m/z* 400. This method allowed up to 10 of the most intense ions to be isolated sequentially, depending on signal intensity, for fragmentation on the linear ion trap using collision-induced dissociation. High resolution MS scans in the Orbitrap and MS/MS scans in the linear ion trap were performed in parallel. Target peptides already selected for MS/MS were dynamically excluded for 60 s. The acquired spectra (Thermo raw files) were loaded into the Progenesis QI software (version 2.0, Nonlinear Dynamics) and label-free quantification was performed as described before (Azimzadeh et al. 2012, Hauck, et al. 2010). Briefly, the profile data of the MS scans as well as MS/MS spectra were transformed to peak lists with Progenesis QI and then stored in peak lists comprising *m/z* and abundance. One sample was set as a reference, and the retention times of all other samples within the experiment were aligned by automatic alignment to create maximal overlay of the two-dimensional feature maps. At this point, features with only one charge or more than 7 charges were excluded from further analyses and all remaining features were used to calculate a normalization factor for each sample that corrects for experimental variation. Samples were then allocated to their experimental group (control or irradiated).

MS/MS spectra were exported from the Progenesis QI software as Mascot Generic file (mgf) and used for peptide identification with Mascot (version 2.5) in the ENSEMBL Human database (release 75, 40047886 residues, 105288 sequences) using the MASCOT search

engine (version 2.5; Matrix Science).The following search parameters were used: 10 ppm peptide mass tolerance and 0.6 Da fragment mass tolerance, one missed cleavage was allowed, carbamidomethylation (C) was set as the fixed modification and oxidation (M), deamidation (N,Q) as well as acetylation (K and protein N-terminus) were allowed as variable modifications. A MASCOT- integrated decoy database search returned FDR of 0.01 when searching was performed on the concatenated mgf files with an ion score cut-off of 30 and a significance threshold of $p \leq 0.01$.

For quantification, all unique peptides (with Mascot score ≥30 and $p<0.01$, see above) of an identified protein were included and the total cumulative abundance was calculated by summing the abundances of all unique acetylated peptides derived from the respective proteins. No minimal thresholds were set for the method of peak picking or selection of data used for quantification.

Three biological replicates were used to quantify the acetylation and protein expression changes by mass spectrometry. Protein expression changes were measured using non-acetyl-enriched samples, and acetylome alterations after enrichment. The median ratios of acetylated peptides in controls and irradiated samples were calculated. Peptides and corresponding proteins showing a significant change ($p<0.05$, unpaired Student´s test) between the two groups were considered to be significantly changed in their acetylation status. The amount of each acetylated peptide was normalized to the total content of the corresponding protein.

Bioinformatics analysis

To analyze the significantly deregulated pathways associated with radiation-responsive proteins, all identified proteins with their corresponding accession numbers were imported into Ingenuity Pathway Analysis (IPA) (Ingenuity System, http://www.ingenuity.com). Only

pathways that had a p-value of ≤0.05 (Fischer´s exact test) were reported. Proteins changed in the acetylation status and their interacting HDAC partners were analyzed using BioGRID and GeneMania databases in Cytoscape software.

Histone deacetylase activity

The histone deacetylase activity was measured using the Sirt-Glo assay kit (Promega) at 4 h and 24 h after irradiation, in the presence or absence of inhibitors of Sirtuin class (Sirtinol: Sigma-Aldrich) or/and of HDAC classes I and II (Trichostatin A; Promega).

Immunoblotting

For expression alterations of HDACs protein family, 20 µg protein extract was separated on 8% or 12% SDS polyacrylamide gels according to Laemmli (Laemmli 1970). Proteins were transferred to nitrocellulose membranes (GE Healthcare) using a semidry blotting system at 100 mA for 90 min. Membranes were saturated for one hour with 5% advance blocking reagent (GE Healthcare) in TBS (50 mM Tris.HCl, pH 7.6 and 150 mM NaCl) containing 0.1% Tween 20 (TBS/T). Blots were incubated overnight at +4°C with antibodies against either SIRT1 (#9475), SIRT3 (#5490), SIRT7 (#5360), HDAC1 (#9928), HDAC2 (#9928), HDAC3 (#9928), HDAC4 (#9928), HDAC6 (#9928) (all from Cell Signaling), and HDAC5 (#1439, Abcam), and alpha-tubulin (#GTX72360, GeneTex) as the loading control. After washing three times in Tris-buffered saline/Tween 20 TBS/T, blots were incubated for one hour at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-goat secondary antibody (Santa Cruz Biotechnology) in blocking buffer (TBS/T with 5% w/v advance blocking reagent). Immunodetection was performed either with ECL Advance Western blotting detection kit (GE Healthcare) or 1-stepTM NBT/BCIP method (ThermoFisher) following standard procedures. The protein bands were quantified using

ImageQuant 5.2 software (GE Healthcare) by integration of all pixel values in the band area after soft-suggested background correction, and normalized to the alpha-tubulin expression. The level of alpha-tubulin was demonstrated not to be affected by irradiation in endothelial cells and was therefore used for normalization (Sriharshan et al. 2012). The controls were normalized to value one with standard deviation, without compromising the statistics, for a better overview of the results. Three biological replicates of controls and irradiated samples were used to analyze the expression changes by immunoblotting. Proteins showing significant change by un-paired t-test ($p<0.05$) between each treatment and respective control were considered to be affected by the response to the radiation.

RhoA activity assay

The G-LISA Rho activation assay (BK124-S, Cytoskeleton Inc) was performed as recommended by the manufacturer. Three biological replicates of control and irradiated cells at each time point were used for quantification.

Statistical analysis

All immunoblots and assays were performed using at least three biological replicates. The differences were considered significant if they reached a p-value of ≤ 0.05 (unpaired student´s t-test, $n \geq 3$).

Data deposition of proteomics experiments

The protein/peptide identifications and quantifications can be found under http://www.storedb.org/store_v3/study.jsp?studyId=1039.

Results

Irradiation induces changes to the HCAEC acetylome

Radiation-induced effects on the HCAEC acetylome were investigated using label-free proteome quantification after immune enrichment of acetylated peptides. Of 1,183 identified peptides 251 were found acetylated (21%) (Tables A1 and A2). The list of identified and quantified acetylated peptides is shown in Table A3. Four hours after irradiation, 48 acetylated peptides were significantly changed ($p<0.05$) (Table A4). Of the corresponding proteins, 45 possessed one unique acetylation site whilst three had multiple acetylation sites. Ten proteins showed an increased level of acetylation, whilst 38 had decreased acetylation status. Twenty-four hours after irradiation, 81 peptides had a changed acetylation status. Four proteins had two different acetylated peptides, two proteins had three different acetylated peptides, and one protein (vimentin) showed four different acetylation sites ($p<0.05$) (Table A5). Of these, 10 proteins showed significant increase in acetylation and 71 decreased acetylation. One protein (keratin 7) showed both increasingly acetylated and deacetylated peptides after irradiation. The ratios of acetylated proteins were further normalized to the total content of corresponding proteins. After normalization all acetylated proteins remained deregulated with corrected ratio value (Tables A4 and A5). All identified peptides, unique acetylated peptides and their quantification at both time points are shown in Tables A1-A6. A great majority (90%) of all deregulated proteins from the 4-hour time point were also found deregulated at the 24-hour time point and they showed the same direction of change in their acetylation status.

All proteins with a changed acetylation status were examined using Ingenuity Pathway Analysis (IPA). The proteins showing radiation-induced changes involved members of pathways including EIF2 signaling, mTOR signaling, protein folding, unfolding and

assembly (proteins of the ER, heat shock proteins, 14-3-3 proteins), glycolysis and integrin signaling (Table 1). Most of the proteins showing late (24 h) alterations in their acetylation status belonged to canonical pathways of actin cytoskeleton signaling, RhoA, RhoGDI, junction (adherence, tight and gap junctions) and calcium signaling (Table 1).

Irradiation induces immediate changes in the expression and activity of deacetylases

As the majority of modified proteins exhibited a decrease in acetylation after irradiation, the activity of cellular deacetylases was measured. Global deacetylase activity was significantly increased 4 h after irradiation but returned to pre-irradiation levels after 24 h (Figure 1). After adding Sirtinol, an inhibitor of sirtuin-type deacetylases, the radiation-induced increase in deacetylase activity was only partially reduced. This indicated that also other deacetylases were activated by irradiation. Indeed, Sirtinol together with Trichostatin A, an inhibitor of HDAC classes I and II, decreased the radiation-stimulated deacetylase activity down to the control level (Figure 1).

Proteins showing a radiation-induced change in acetylation were examined for known interactions with HDAC proteins using the BioGRID and GeneMania databases. SIRT1, SIRT7, HDAC5 and HDAC6 showed the greatest numbers of interactions with the deregulated proteins found in this study (Figure 2).

The results of the bioinformatics analysis prompted us to investigate radiation-induced changes in the protein levels of SIRT1, SIRT3, SIRT7, HDAC1, HDAC2, HDAC3, HDAC4, HDAC5 and HDAC 6 by immunoblotting. The expression of SIRT1 and SIRT7 was increased at 4 h in the irradiated cells whilst the level of SIRT3 decreased at this time point (Figure 3; Supplementary Figure). The level of HDAC3 showed an increase 24 h after the irradiation whilst HDAC6 showed a significantly increased expression at 4 h but only a tendency of increase at 24 h (Figure 3). Increased expression of SIRT1, SIRT7 and HDAC6

at 4 h was in agreement with the increased deacetylase activity seen at that time point. The HDACs showing protein expression changes either at 4-hour or 24-hour time point were further investigated 7 days after the treatment. Interestingly, HDAC3 was still significantly up-regulated at this late time point (Figure 3).

Radiation-induced activation of RhoA

Alterations in actin cytoskeleton are mediated by RhoA signaling (Maekawa et al. 1999). As RhoA signaling pathway proteins were found to be altered in the acetylome analysis, the activity of RhoA was measured at both time points. RhoA activity was increased significantly 24 h after irradiation ($p<0.05$; Figure 4) but not at the earlier time point (4 h). This was in agreement with the canonical pathway analysis (Table 1) showing significant induction of RhoA and RhoGDI signaling pathways only at 24 h.

Discussion

This study suggests that the radiation dose of 0.5 Gy is able to trigger marked changes in the endothelial acetylome. Most of the affected proteins had decreased acetylation status after irradiation and this was accompanied by a transient increase in the level of cellular deacetylase activity. Inhibitor studies revealed that both sirtuins and zink-dependent HDAC classes were responsible for the increase in deacetylation. In contrast to the transient nature of the changes in HDAC enzyme activity, the decrease in protein acetylation itself appeared to be relatively stable, remaining detectable in many proteins for at least 24 h after irradiation. Further, the number of proteins that were changed in their acetylation status increased between 4 and 24 h, again with the decrease in acetylation events being more pronounced compared to increase in acetylation events. This confirms previous observations showing that radiation-induced changes in protein acetylation occur relatively slowly but are retained in

comparison to short-lived radiation-induced phosphorylation events (Barjaktarovic, et al. 2015, Beli et al. 2012).

The decrease in acetylation events in this study is associated with multiple pathways such as protein synthesis, cytoskeleton-related processes, protein folding, calcium signaling, and glycolysis. Similar to our previous study (Barjaktarovic, et al. 2015), the pathway with the highest number of proteins altered by acetylation was protein synthesis (EIF2 signaling). Except for eukaryotic translation elongation factor 2, all ribosomal proteins and translation factors that showed changes were found with decreased acetylation status. The radiation-induced increase in the expression of SIRT7 is in agreement with this. SIRT7, the deacetylase with most predicted interactions with (de)acetylated proteins in this study, has been shown to play a role in ribosome biogenesis and its downregulation may contribute to the inhibition of protein synthesis (Tsai et al. 2014).

Several cytoskeletal proteins that demonstrated changes in their acetylation status are components of the RhoA/cytoskeleton signaling pathway necessary for the formation of adherence junctions, tight junctions and focal adhesions (Popoff and Geny 2009). These proteins include myosin, tropomyosin, vimentin, plectin, drebrin, alpha-integrin and tubulin. The precise regulation between acetylated and deacetylated forms of myosin and tropomyosin is important for the affinity to distinct actin structures within the cell. Each form has an effect upon the shape and integrity of the polymeric actin filaments (Coulton et al. 2010, Gupta et al. 2008, Samant et al. 2011). Additionally, the acetylation status of vimentin, plectin and drebrin (all found with decrease acetylation status in this study) plays an important role in modulating actin cytoskeletal dynamics (Ishizuka et al. 2014, Uehara and Uehara 2010, Valencia et al. 2013).

The radiation-induced activation of RhoA/cytoskeleton signaling, predicted from protein analysis, was confirmed using an activity ELISA assay. One possible cause for the increased

activity is Ca^{2+} -induced activation of the RhoA pathway (Masiero et al. 1999). We find several members of calcium-dependent pathways changed in their acetylation status: annexins A1, A2 and A5, S100 calcium binding proteins A6 and A10 and calmodulin 2. These proteins have the requisite properties to link the Ca^{2+}-signaling with RhoA-mediated actin dynamics (Hayes et al. 2004). Their acetylation status affects the cytoskeletal structure and contact to neighboring endothelial cells in a Ca^{2+} -dependent manner (Jackson et al. 1987, Psatha et al. 2007, van Nieuw Amerongen et al. 2000).

Activation of the RhoA pathway is associated with endothelial dysfunction through impaired control of the vasoconsriction and vasodilatation processes (Hennenberg et al. 2006). RhoA activation has also been linked to the decrease in available NO via reduced expression and PTM of the NO synthase (eNOS) (Priviero et al. 2010). We have shown previously that high doses (8 or 16 Gy) of ionizing radiation result in persistently reduced levels of NO in the serum of mice locally irradiated to the heart (Azimzadeh, et al. 2015).

Several proteins of the chaperone class are changed in the acetylation status after irradiation. These include heat shock proteins, protein disulphide isomerases, cyclophilins and 14-3-3 proteins. HSP90 is a highly abundant and ubiquitous molecular chaperone which is a key player in maintaining cellular homeostasis (Jackson 2013, Li and Buchner 2013). HDAC6, upregulated in our study, has been shown to deacetylate HSP90, thereby activating it (Kekatpure et al. 2009, Kovacs et al. 2005, Rao et al. 2008). The presumed acetylation-dependent activation of HSP90 in this study may be associated with unfolded protein response caused by ionizing radiation (Marcu et al. 2002). Similarly, HSPA5, a member of the heat shock protein 70 (HSP70) family, is found to have decreased acetylation levels in this study.

Cyclophilin A (CyPA) was also found with decrease in acetylation in this study. It is a pro-inflammatory mediator, the expression and secretion of which is coupled to its acetylation

and triggered in response to increased intracellular ROS levels (Fan et al. 2014, Soe et al. 2014). The increased deacetylation found here may be a response to increased oxidative stress due to irradiation.

14-3-3 proteins form a family of highly conserved proteins capable of binding to more than 200 different, mostly phosphorylated proteins, involved in cellular processes such as cell cycle, apoptosis, and cellular adhesion (Cui et al. 2014, Heverin et al. 2012, Niemantsverdriet et al. 2008, Zuo et al. 2010). Acetylation of conserved lysine residues in 14-3-3 proteins is necessary for the binding of phosphoproteins and thereby for proper functioning (Choudhary, et al. 2009). The 14-3-3 proteins YWHAZ and YWHAE had decreased acetylation levels 24 h after irradiation. Decrease in acetylation may suggest radiation-induced functional impairment of the 14-3-3 family and negatively affect the capacity of these proteins to interact with phosphorylated proteins (Choudhary, et al. 2009).

In addition to its role as a regulator of the HSP90 (Kekatpure, et al. 2009), HDAC6, the expression of which shows rapid (4 h) but transient upregulation, is involved in protein ubiquitination and subsequent protein degradation (Kawaguchi et al. 2003). With the involvement in both acetylation and ubiquitination it is at the crossroads between cytoskeleton and cell signaling (Boyault et al. 2007).

Conclusions

Taken together, many of the biological pathways known to be affected by ionizing radiation in different endothelial cell models coincide with those found in this study. These include RhoA signaling, cytoskeletal signaling, EIF2 signaling, mTOR signaling, and glycolysis. This suggests that acetylation/deacetylation process is an important mediator and coordinator of radiation-induced endothelial stress.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure Legends

Figure 1. Alterations in total deacetylase activity in HCAEC 4 and 24 h after irradiation. Deacetylase activity V (dL/dT) was investigated using SirtGlo assay which measures the total deacetylase activity in the sample. Sirtinol, an inhibitor of sirtuins, was added in a concentration of 50 µM and Trichostatin A, an inhibitor of class I and II HDACs, was added in a concentration of 1 µM. The error bars represent standard deviation. L, luminescence; T, time; OD, optical density; c, control; IR, irradiated (0.5 Gy); (t-test; *p< 0.05; **p<0.01, n= 3).

Figure 2. Cytoscape software representation of potential deacetylation targets of HDAC proteins. BioGRID and GeneMania databases were used for identification of interaction partners. HDACs are shown in yellow and the interacting proteins in blue.

Figure 3. Immunoblot analysis of HDAC proteins. The levels of SIRT1, SIRT3, SIRT7, HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, and HDAC6 are shown in control and irradiated samples at 4-hour and 24-hour time point. The HDACs showing deregulation were also measured 7 days after irradiation. The error bars represent standard deviation. (t-test; *p< 0.05; **p<0.01, n= 3).

Figure 4. RhoA activity measurement by ELISA assay. The columns represent the average ratios of RhoA activity in control and irradiated samples. The RhoA activation assay measures the level of GTP-loaded RhoA (active form). The level of activation is measured with absorbance set at 490 nm. The error bars represent standard deviation. (t-test; *p< 0.05; n=3).

Table 1. The most significant canonical pathways regulated by radiation-induced protein acetylation using Ingenuity Pathway Analysis. The p-values represent the significance of the pathway: high –log(p) corresponds to high significance. The ratios represent the number of deregulated proteins divided by the total number of proteins allocated to the corresponding pathway. The arrows indicate the time point when the pathway is most significant (4 h or 24 h).

Canonical Pathways	**-log(p-value) 4 h**	**Ratio 4 h**	**-log(p-value) 24 h**	**Ratio 24 h**
EIF2 Signaling	8.085	8/184	11.637 ↑	12/184
mTOR Signaling	5.385	6/145	6.485 ↑	8/187
Glycolysis I	4.603 ↑	3/25	2.444	2/25
Endoplasmic Reticulum Stress Pathway	2.980 ↑	2/21	2.594	2/21
Integrin Signaling	2.934	4/202	5.101 ↑	7/202
14-3-3-mediated Signaling	2.614	3/117	4.601 ↑	5/117
Unfolded Protein Response	2.167 ↑	2/54	1.794	2/54
eNOS Signaling	1.383	2/141	1.848 ↑	3/141
Epithelial Adherens Junction Signaling	1.356	2/146	7.286 ↑	8/146

Gap Junction Signaling	1.309	2/155	4.707 ↑	6/155
RhoA Signaling	0.612	1/122	5.390 ↑	6/122
RhoGDI Signaling	0.483	1/173	4.439 ↑	6/173
Actin Cytoskeleton Signaling	0.405	1/217	5.968 ↑	8/217
Calcium Signaling	0	0/178	4.639 ↑	6/178
Tight Junction Signaling	0	0/167	3.477 ↑	5/167