Quantitative Top-Down and Bottom-Up Profiling of the Proteomic Response to Ionizing Radiation

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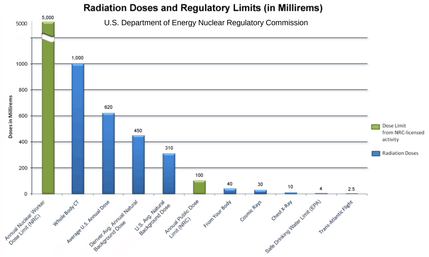
Overview

- **Goal: identify response mechanisms to ionizing radiation using top-down and bottom-up mass spectrometry.**
- Response mechanisms and epigenetic effects of exposure to radiation are not completely understood.
- Bottom-up protein quantification of subcellular fractionated skin cell models treated with varying doses of ionizing radiation.
- Top-down histone analysis with 2D RP-HILIC coupled to Orbitrap Velos CID/ETD high resolution scans.
- Combination of bottom-up whole cell analysis plus top-down histone PTM identification will provide insight to response mechanisms to varying doses of ionizing radiation at a cellular level.

Introduction

- Humans are exposed to various levels of ionizing radiation on a daily basis.[1]

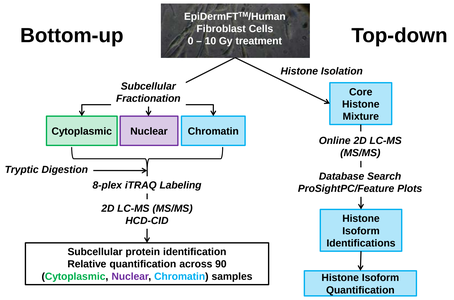


- Large doses of ionizing radiation cause severe tissue and cell damage leading to disease, cancer and death.
- The direct mechanisms of response to low doses of ionizing radiation and epigenetic effects remain poorly understood.
- Radiation activates cell signaling pathways including DNA damage repair, cell cycle regulation and apoptosis.
- Many low dose effects (altered transcription and epigenetic regulation, DNA damage repair, genomic instability) can be linked to altered chromatin states.
- Histone post-translational modifications regulate chromatin function, structure and accessibility.
- We seek to identify activated pathways and cellular response mechanisms to ionizing radiation by monitoring protein expression ratios and translocation after exposure to ionizing radiation. In addition, change in histone post-translational modifications will reflect response at the chromatin level and is investigated using a top-down approach.

Methods

EpidermFT™ (MatTek, Ashland, MA), were treated with ionizing radiation doses of 0, 0.1, 2.0 and 10 Gy, and fractionated into cytoskeletal, nuclear and chromatin fractions. Tryptic digests of the subcellular fractions labeled with 8-plex iTRAQ tags for quantification. Peptides were analyzed using online 2D (SCX-RP) LC coupled to an LTQ-Orbitrap Velos (Thermo Scientific).

Sequential HCD/CID fragmentation was performed, using SEQUEST for peptide identification, and in-house developed software for iTRAQ-based peptide quantification. Peptide identifications were filtered at 1 X 10^{-10} spectral probability using MSGF.[2] For significant quantitative results, peptide identifications were required in at least two biological replicates.



Subcellular Fraction 1

Tag	Bio Replicate #1	Tag	Bio Replicate #2	Tag	Bio Replicate #3
113	3 hr 0Gy	113	3 hr 0Gy	113	3 hr 0Gy
114	8 hr 0Gy	114	8 hr 0Gy	114	8 hr 0Gy
115	24 hr 0Gy	115	24 hr 0Gy	115	24 hr 0Gy
116	3 hr 2Gy	116	3 hr 2Gy	116	3 hr 2Gy
117	8 hr 2Gy	117	8 hr 2Gy	117	8 hr 2Gy
118	24 hr 2Gy	118	24 hr 2Gy	118	24 hr 2Gy
119	pool 1*	119	pool 1	119	pool 1
121	pool 2**	121	pool 2	121	pool 2

Tag	Bio Replicate #1	Tag	Bio Replicate #2	Tag	Bio Replicate #3
113	3 hr 0Gy	113	3 hr 0Gy	113	3 hr 0Gy
114	8 hr 0Gy	114	8 hr 0Gy	114	8 hr 0Gy
115	24 hr 0Gy	115	24 hr 0Gy	115	24 hr 0Gy
116	3 hr 10Gy	116	3 hr 10Gy	116	3 hr 10Gy
117	8 hr 10Gy	117	8 hr 10Gy	117	8 hr 10Gy
118	24 hr 10Gy	118	24 hr 10Gy	118	24 hr 10Gy
119	pool 1	119	pool 1	119	pool 1
121	pool 2	121	pool 2	121	pool 2

*Pool 1: equal combination of ALL 0 Gy samples
**Pool 2: equal combination of ALL non-0 Gy samples

Low Dose (0.1 Gy)

Subcellular Fraction 1
Tag
113
114
115
116
117
118
119
121

Tag	
113	8 hr 0Gy Rep 1
114	8 hr 0Gy Rep 2
115	8 hr 0Gy Rep 3
116	8 hr 0.1 Gy Rep 1
117	8 hr 0.1 Gy Rep 2
118	8 hr 0.1 Gy Rep 3
119	pool 1
121	pool 2

Human fibroblasts were treated with 0, 0.1, and 2 Gy of ionizing radiation, lysed and fractionated. Isolated core histones were separated using a custom 2D (RP-HILIC) nanoLC coupled to an LTQ-Orbitrap Velos.[3] The top-5 precursor masses were selected for fragmentation with sequential CID/ETD analysis, all spectra were acquired in the Orbitrap.

Data were searched using ProSightPC 2.0[4] for isoform identification, and quantification was done using in-house software. Histone isoforms were filtered by requiring the "Number of Best Hits" to be equal to one, and a p-score of less than or equal to 1 X 10^{-5}.

Results

Bottom-up

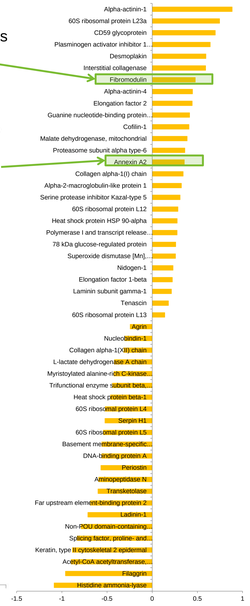
Preliminary 1D LC-MS/MS analyses of the cytoplasmic fraction demonstrated removal of keratins and cytoskeletal proteins that are highly abundant in unfractionated skin. Labeling efficiency was calculated to be >97%, and inter-label variation was minimal. 2D analysis increased the dynamic range of peptide sampling, while reducing MS/MS multiplexing.

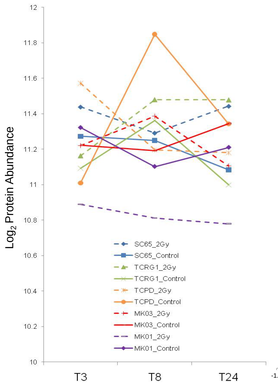
2D LC-MS/MS acquisitions of cytoplasmic and nuclear subcellular fractions identified and quantified a total of 12,402 and 2236 unique peptides and proteins, respectively, with the largest number of proteins found in the 2 Gy cytoplasmic fraction.

			Peptides			Proteins		
Subcellular Fraction	Total Peptides	Total Proteins	0.1 Gy	2 Gy	10 Gy	0.1 Gy	2 Gy	10 Gy
Cytoplasmic	9972	1759	2752	8075	4803	942	1660	1144
Nuclear	5969	1452	1301	4939	4405	486	1295	1052

A decrease of **fibromodulin** was observed in the cytoplasm, indicating translocation to the nucleus.

Annexin A2 was independently confirmed to translocate to the nucleus as a possible tumor suppressor after low dose radiation exposure.





Subset of nuclear protein abundance over time with respect to 2 Gy treatment.

Nuclear 8 h, 0.1 Gy differential protein abundance with respect to control.

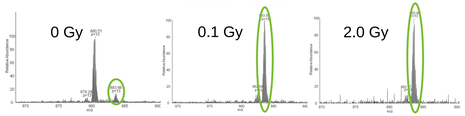
Top-down

Over 1100 histone isoforms were identified from two technical replicates of histones isolated after 0, 0.1 and 2Gy treatments of ionizing radiation; ( ) = number of phosphorylated isoforms.

	H4	H2B	H2A	H3
0 Gy	144(28)	105(15)	94(39)	418(25)
0.1 Gy	159(26)	136(24)	75(25)	249(12)
2 Gy	146(17)	85(9)	84(32)	309(16)
Total	254(41)	192(30)	118(48)	596(34)

Several modifications were identified that are known to be important for DNA damage repair regulation including acetylation on H4 at K5,K8, K12 and K16 and methylation at K20.

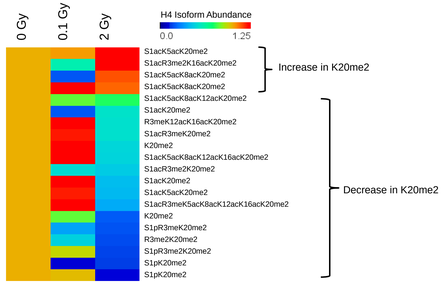
H4 S1acK5acK8acK12acK16acK20me2
883.59 *m/z* 13^+



	Maximum Intensity	TIC Base Peak Intensity	Intensity/TIC Intensity
0 Gy	7.08E+04	2.41E+07	2.94E-03
0.1 Gy	1.74E+05	1.19E+07	1.46E-02
2.0 Gy	9.60E+04	7.91E+06	1.21E-02

An increase of H4-S1acK5acK8acK12acK16acK20me2 was observed after treatment, potentially indicating activation of repair mechanisms in response to damage caused by ionizing radiation.

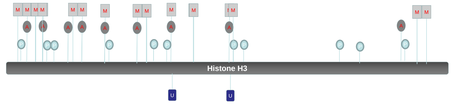
No change in total K20me2 levels. Analysis at the intact protein level demonstrates both increase and decrease of K20me2 combined with additional modifications.



Quantitative results for H4 modifications involved in DNA damage repair(K20me2) from extracted ion chromatograms of charge states 11^+ through 16^+.

Conclusions

- Online 2D LC analysis of 8-plex iTRAQ samples reduces multiplexing and increased protein identifications from minimal sample (<10 µg).
- A total of 2236 proteins from the cytoplasmic and nuclear fractions were identified and quantified using 8-plex iTRAQ.
- Proteins associated with ionizing radiation response mechanisms were identified as up-regulated after treatment.
- 1160 unique histone isoforms were identified from three radiation treatments. Histones are a complex class of proteins due to the presence of multiple modifications across the whole protein sequence.
- Histone isoform quantification across multiple charge states demonstrated an increase of modifications that are associated with DNA repair.
- Identification and quantification of specific modification sites is complicated by the number of co-occurring modifications.



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