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A New Paradigm for Radiation-Induced Persistent Cellular Stress and Genomic Instability in Lung Carcinogenesis

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14. ABSTRACT We study cellular stress responses to sublethal doses of 1 or 2 Gy X-Rays that persist for up to a week in cultures of an immortalized normal bronchial epithelial cell line, HBEC3KT. We have found that p38MAPK is a driver and genomic instability and cell transformation an outcome of these responses, thus potentially involved in the development of radiogenic cancer. Further studies into these responses revealed altered levels of ubiquitination associated to chromatin, which can be modulated by interfering with p38MAPK activity. The goal of this project is to evaluate whether dysregulated chromatin ubiquitination is involved in driving persistent genomic instability and is the target for p38MAPK regulation. The main findings of the concluding period are that the predominant usage of the homology dependent DNA repair pathway by irradiated cells is homeostatic as demonstrated by experiments of deubiquitinases overexpression. We also found that the cell cycle defects and HR usage are probably due to increased replication stress.					
15. SUBJECT TERMS genomic instability, cellular stress, cell transformation, DNA repair, ubiquitination					
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1. Introduction:

We study cellular stress responses to sublethal doses of 2 Gy X Rays that persist for up to a week in cultures of an immortalized normal bronchial epithelial cell line, HBEC3KT. We have found that p38MAPK is a driver and genomic instability and cell transformation an outcome of these responses, thus potentially involved in the development of radiogenic cancer. Further studies into these responses revealed altered levels of ubiquitination associated to chromatin, which can be modulated by interfering with p38MAPK activity. The goal of this project is to evaluate whether dysregulated chromatin ubiquitination is involved in driving persistent genomic instability and is the target for p38MAPK regulation. The main findings of the concluding period are radiation caused alterations in cell cycle progression, the potential identification of persistent DNA repair foci as 53BP1 bodies, predominant usage of the homology dependent repair pathway and that treatment of HBEC3KT cells with a p38MAPK inhibitor reduces deubiquitinase (DUB) activity.

2. Keywords:

X rays, genomic instability, protein ubiquitination, cell cycle, p38MAPK, deubiquitinase, human bronchial epithelial cell, cell transformation, DNA repair, cellular stress.

3. Accomplishments:

a. Major goals of the project:

Specific Aim 1: To determine whether exposure to IR disrupts chromatin associated ubiquitin signaling leading to persistent genomic instability and cellular stress.

Specific Aim 2: To evaluate whether p38MAPK mediated stress responses promote genomic instability and carcinogenesis by regulating chromatin associated ubiquitination.

b. Accomplishment of tasks listed in the SOW to be completed during the second year:

For Specific Aim 1:

Major Task 1: Establish methods to evaluate outcomes potentially affected by exposure to IR and by the chromatin associated ubiquitination pathway.

In the previous period, we reported our finding that irradiated cells exhibit a higher rate of repair via HR (homologous recombination) and lower usage of NHEJ (non-homologous end joining), with no difference in the usage of the alternative NHEJ pathway. We consider that this result constitutes the milestone outcome for major task 1. This finding also guided many of the experiments performed during this reporting period.

Major Task 2: Evaluate the functional consequences of Trip12 and BRCC36 dysregulation at day 7 post-exposure to IR.

Subtask 1: Optimize transfection to increase and reduce the expression of the chromatin ubiquitination regulators Trip12 and BRCC36 by tag-cDNA and siRNA transfection. Verify overexpression by Western Blot for the tag and downregulation by RT-PCR.

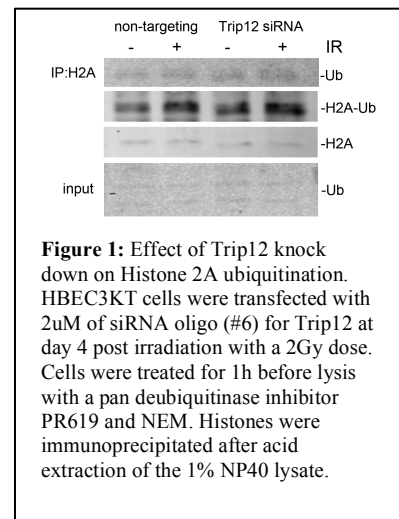


Figure 1: Effect of Trip12 knock down on Histone 2A ubiquitination. HBEC3KT cells were transfected with 2uM of siRNA oligo (#6) for Trip12 at day 4 post irradiation with a 2Gy dose. Cells were treated for 1h before lysis with a pan deubiquitinase inhibitor PR619 and NEM. Histones were immunoprecipitated after acid extraction of the 1% NP40 lysate.

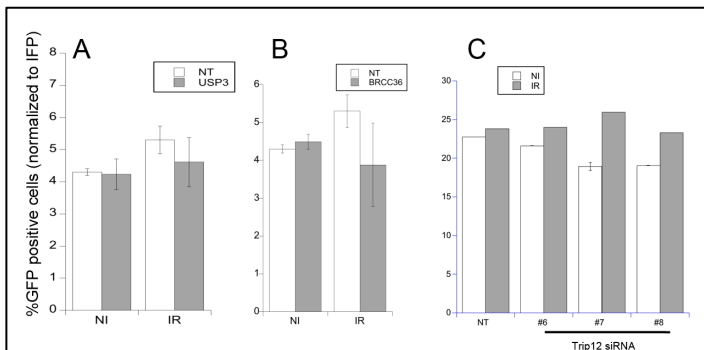


Figure 2: GFP conversion assay in U2OS reporter cell lines for homologous recombination repair. DRG-U2OS cells were transfected at day 3 post-irradiation with IFP-ISce1 and plasmid DNA with cDNAs for **A** USP3, **B** BRCC36, or with **C** siRNA oligos non targeting a specific sequence (NT) or to knock down Trip12 expression. At day 5 post irradiation the cells were collected for flow cytometry analysis of the GFP positive cells (successful HR repair) within the cell population expressing Sce-1 (IFP positive). The bars represent triplicate assays with standard deviation.

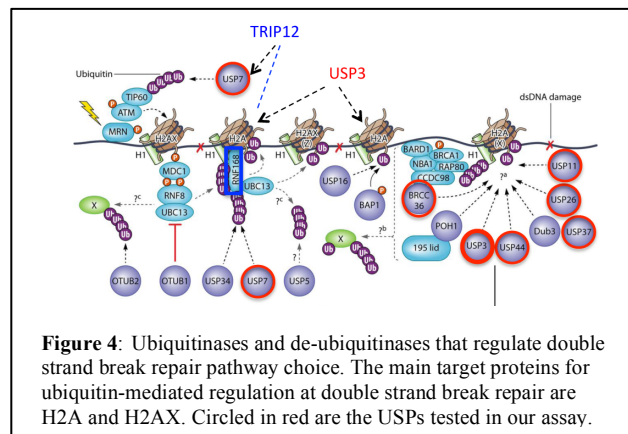
In the previous period we attempted BRCC3 and Trip12 cDNA overexpression, but we were not able to detect the expression of the cDNAs by western blot. In this period, we attempted knock-down for Trip12, which is elevated in irradiated cells. Because we are unable to detect the expression of these proteins, we attempted to report their activity by examining the ubiquitination status of their direct molecular targets, namely histones H2A and H2AX. Figure 1 shows a sample experiment to detect ubiquitination levels of Histone 2A following Trip12 knock down. While we were able to detect increased ubiquitination in irradiated cells, we did not detect an effect of Trip12 knockdown, even though we did detect a mild phenotype in our HR reporter assay (see Figure 2).

Subtask 2: With the conditions established in Subtask 2.1, test the effect of varying Trip12 or BRCC36 expression on assays developed in Major Task 1.

Figure 2 depicts sample results obtained with the HR reporter assay following USP3 (a), BRCC36 (b) overexpression and TRIP12 knock down (c). Overexpression of USP3, which is known to reduce H2A, H2B and γ H2AX ubiquitination induced by RNF168, ameliorated HR repair usage as expected (1). In the case of BRCC36, our results reproduced published data (2) and are consistent with our hypothesis as overexpression is reducing HR pathway usage and therefore ameliorating the effects of IR. However, the observed effects were generally small and currently don't have supporting western blot data confirming changes in expression of the proteins.

Subtask 3: With the conditions established in Subtask 2.1, test the effect of varying *Tripl2* or *BRCC36* expression on assays to measure genomic instability, cellular stress (ROS, cytokine release) and growth in soft agar.

As a sensitive and quantitative assay to report genomic instability, we measured micronucleus formation following overexpression of BRCC36 and USP3. As shown in Figure 3, overexpression of de-ubiquitinases that reduce HR usage increase genomic instability, indicating that HR is an homeostatic mechanism to reduce or resolve damage. Thus it might not be beneficial to manipulate ubiquitination and repair via homologous recombination.

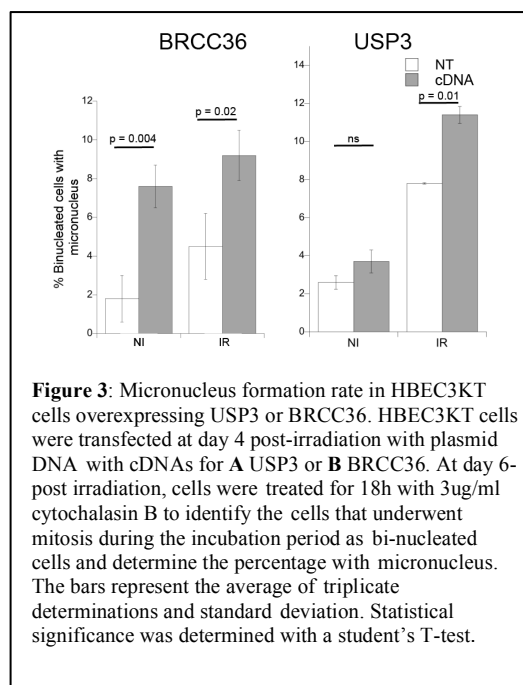


Subtask 2: Employing the conditions established in subtask 3.1, evaluate chromatin-associated ubiquitin following RNF168, USP3 or USP44 overexpression by western blot and immunofluorescence employing chain specific antibodies and FK2 antibody.

In the previous reporting period, we proposed to evaluate ubiquitination of histone H2A as a reporter for changes in RNF168 and USP3 activity at the chromatin level. Figure 5 shows a sample experiment of successful histone immunoprecipitation, however overexpression of USP3 and IR reduce H2A levels making it difficult to interpret the results due to low sensitivity of the assay.

Subtask 3: Evaluate the functional consequences of expressing the ubiquitin levels modulator identified in subtask 3.2 employing assays developed in Mayor Task 1.

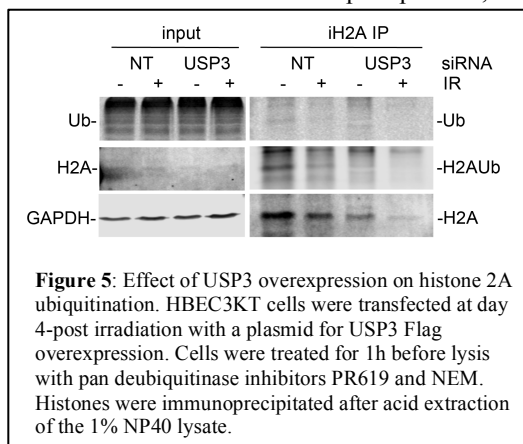
We have tested the effects of overexpressing other USPs regulating effector proteins regulating pathway choice (as shown in Figure 4) employing the homology dependent repair assay. As shown in Figure 6, USP7 appears to have an effect similar to USP3 or BRCC36 overexpression, reducing homology dependent repair. In contrast, USP37 increased homology dependent repair, which is consistent with published studies reporting that this USP counteracts RAP80 dependent recruitment of BRCA1 in the BRCA1-A complex, favoring the participation of



Major Task 3: For aim 1c to evaluate whether chromatin-associated ubiquitination can be altered to affect outcomes.

Subtask 1: Optimize RNF168, USP3 and USP44 overexpression by tagged cDNA transfection and anti tag western blot analysis.

In our previous reporting period we tested USP3 and USP44 over expression and measured increased micronuclei formation in irradiated cells. During this reporting period we tested the expression of other USPs that regulate the function of proteins recruited to DNA at the moment of pathway choice as summarized in the diagram of Figure 4 (diagram taken from (3)).



BRCA1 in complex with PALB2, BRCA2 and RAD51 facilitating HR (4).

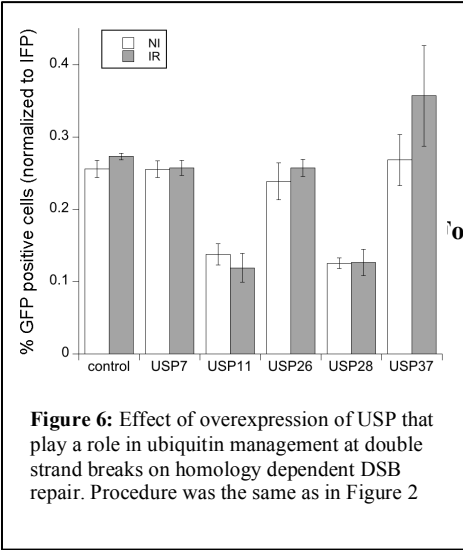


Figure 6: Effect of overexpression of USP that play a role in ubiquitin management at double strand breaks on homology dependent DSB repair. Procedure was the same as in Figure 2

Subtask 4: Evaluate the functional consequences of expressing the ubiquitin levels modulator identified in subtask 3.2 on assays to measure genomic instability, cellular stress (ROS, cytokine release) and growth in soft agar.

In the next period we will test whether USP37 overexpression ameliorates genomic instability phenotypes.

For Specific Aim 2:

We have not made much progress with Major Tasks 1 and 2 because of findings narrated in Major Task 3, which lead to a significant change in direction.

Mayor Task 3: Aim 2c To identify p38MAPK dependent nuclear proteins that promote genomic instability.

Subtask 2: Global proteome analysis of nuclear fractions isolated from irradiated cells at day 7-post exposure treated or not with p38MAPK inhibitor (triplicates of each condition=12 samples).

Instead of proceeding with an unbiased proteomic approach to detect potential molecules altered by the p38MAPK inhibitor, we decided to analyze our samples by reverse phase protein arrays, analysis provided by the Functional Proteomics Core at MD Anderson. Small amounts of samples are required and are interrogated for 413 different proteins with altered function or expression in cancer. Additionally appealing to us, the panel includes several proteins and phosphorylation of proteins that participate in homologous recombination. In the literature this array has been used to pinpoint proteins causing defects in the activity of this pathway.

We submitted for analysis triplicates samples of the following: A) naïve HBEC-3KT cells and day 5 or 7 post-irradiation to identify changes occurring overtime in this cell type. B) the same set as A) but the cells were treated for 24h with 5uM SB 203580 to investigate changes that can be attributed to p38MAPK activity. C) Naïve and 5-day post-irradiation U2OS cells to compare with HBEC-3KT, to test the hypothesis that proteins that change in both cell types might mediate common phenotypes.

Subtask 3: Analysis of the data obtained in subtask 2.

We have attached heatmaps of the data following unsupervised hierarchical clustering for both samples and antibody for the p38MAPK inhibitor treatment samples. The data employed are the relative protein level data points normalized for protein loading and transformed to linear values. The linear values were transformed to log2 values and then median-centered for hierarchical clustering analysis employing Cluster 3 (analysis provided by the facility). The main conclusions from the

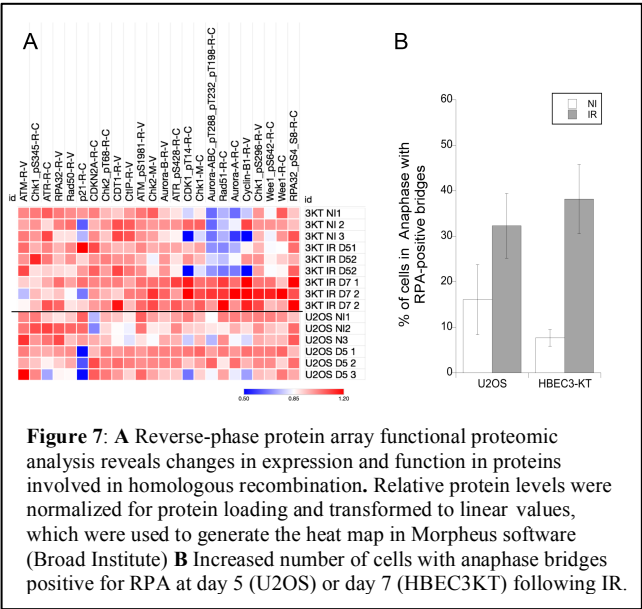


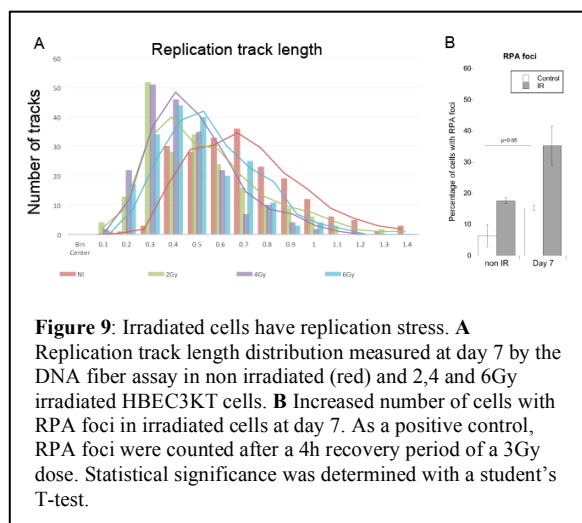
Figure 7: A Reverse-phase protein array functional proteomic analysis reveals changes in expression and function in proteins involved in homologous recombination. Relative protein levels were normalized for protein loading and transformed to linear values, which were used to generate the heat map in Morpheus software (Broad Institute) **B** Increased number of cells with anaphase bridges positive for RPA at day 5 (U2OS) or day 7 (HBEC3KT) following IR.

functional proteomics analysis are: a) In HBEC 3KT cells, the phenotypes emerge over-time, indicated by unsupervised clustering analysis showing that day 7 samples are significantly different from non irradiated or day 5 samples. To our surprise and disappointment, the p38MAPK inhibitor introduced widespread changes, in more proteins than the ones affected by radiations (shown in appendix). This result makes us question whether proteomics approaches will be useful to achieve further understanding of the mechanisms for p38MAPK inhibitor and whether the effects of the inhibitor are selective. Nonetheless, the comparison between both cell lines revealed several proteins activated during replication stress and alterations in the homologous recombination pathway as shown in Figure 7. We will explore these molecular changes in the coming period, because the accumulation of unresolved intermediaries of homologous recombination at the moment of mitosis generates genomic instability (5). We obtained further confirmation that this may be a potential mechanism by quantifying RPA-positive anaphase bridges formed during mitosis. As shown in figure 7B, both cell types have a higher frequency of this event during

mitosis following exposure to IR. The panel also points to replication stress, but without strong checkpoint activation. Mild replication stress, below the threshold that activates checkpoints, can lead to genomic instability and would also explain the preferred usage of homology dependent repair.

Subtask 4: Validation of top candidate proteins by western blot or RTPCR.

We confirmed replication defects in irradiated cells employing the DNA fiber assay, which reveals alterations in DNA replication. We found reduced replication speed with increasing dose of the initial exposure, reflected as shorter replication tracks (Figure 8A). We validated increased replication stress in irradiated cells by immunofluorescence staining for RPA, which accumulates where single stranded DNA becomes available either because of uncoupling of helicases and replication polymerases during triplication or due to increased end resection activity (Figure 8B). We measured acute RPA foci formation (at 3h recovery of a 4Gy dose) as a positive control for the assay. Replication stress could explain many of the phenotypes we observe in irradiated cells such as increased usage of homologous recombination for repair, changes in cell cycle progression (reported last period) and defects during mitosis (micronuclei, 53BP1 bodies in G1 cells and anaphase bridges). We also identified a candidate effector in mediating changes of expression of proteins involved in homologous recombination. For the next period, we plan to solidify these findings to generate a publication.



b. Training and professional development opportunities provided by the project during the reporting period: Dr. Zhentian Li authored an abstract submitted to the Radiation Research Society. He will attend this meeting and benefit from activities directed to new young investigators.

c. Result dissemination to communities: Results generated during this period were disseminated in joint lab meetings with the Yu lab and in our monthly seminars involving multiple laboratories from Emory and Georgia Tech with common research interest in nucleic acid metabolism, damage and repair. An abstract will be presented at the 2019 Meeting of the Radiation Research Society (included in appendix). A manuscript has been submitted for publication at Scientific Reports (included in appendix). We are working on the revisions. A revised version of the manuscript will be submitted in July.

d. Plans to accomplish the project goals in the next period: we will complete experiments to generate a publication with the findings of our experiments. To accomplish this, Dr. Werner and Dr. Li will maintain their efforts.

4. Impact:

a. Impact on the development of the principal discipline(s) of the project:

Impact on the discipline was effected by publication of a peer reviewed article (included in the appendix) and submission of a meeting abstract.

b. Impact on other disciplines: Nothing to Report.

c. Impact on technology transfer: Nothing to Report.

d. Impact on society beyond science and technology: Nothing to Report

5. Changes/ Problems:

a. Changes that had a significant impact on expenditures: the consumption rate of fungibles has changed as the direction of research is changing.

b. Changes that caused delays in the activities and progress: these are detailed in the narrative pages.

c. Changes in approach these are detailed in the narrative pages.

6. Products:

a. Publications: Ionizing Radiation induction of cholesterol biosynthesis in Lung tissue.

Erica Werner, Andrew Alter, Quidong Deng, Eric B. Dammer, Ya Wang, David S. Yu, Duc M. Duong, Nicholas T. Seyfried and Paul W. Doetsch. Under revision for Scientific Reports. Manuscript included in Appendices
Acknowledgement of DoD support

b. Abstracts: presentation for the Radiation Research Society 2019.

7. Participants and Other Collaborating Organizations:

a. Individuals working on the project during the reporting period:

Name: Erica Werner PhD

Project Role: Principal Investigator.

Nearest person month worked: 9CM .

Contribution to the Project: experimental design and planning, implementation and execution of experiments involving cell culture, transfections and outcome analysis, data analysis, interpretation of results, manuscript and report preparation as well as communication of findings at meetings.

Funding Support: NIH-NIGMS 1RF1AG060285 "Neuronal Mechanisms of Copper Transport and Toxicity"

PI: V. Faundez. The major goal of this project is to study the molecular mechanisms of childhood neurodegeneration in Menkes and Wilson disease using human, mouse and Drosophila models.

Co-Investigator: E. Werner 0.6CM

Role: study mitochondrial metabolism and dynamics in aim 2

No research overlap.

Name: Zhentian Li Ph.D

Project Role: Post-doctoral fellow

Nearest person month worked: 3CM

Contribution to the Project: flow cytometry analysis of the experiments with U2OS reporter cell lines and cell cycle, data analysis and interpretation of results.

Funding Support: no other external funds.

b. Changes in active other support during the reporting period: Nothing to Report.

c. Other organizations involved: Nothing to Report.

8 **Special Reporting Requirements**: Nothing to Report

9 **Appendices**:

a. Bibliography:

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2. Ng HM, Wei L, Lan L, Huen MS. The Lys63-deubiquitylating Enzyme BRCC36 Limits DNA Break Processing and Repair. *J Biol Chem* 2016; 291:16197-207.
3. Kee Y, Huang TT. Role of Deubiquitinating Enzymes in DNA Repair. *Mol Cell Biol* 2016; 36:524-44.
4. Typas D, Luijsterburg MS, Wiegant WW, Diakatou M, Helfricht A, Thijssen PE, et al. The de-ubiquitylating enzymes USP26 and USP37 regulate homologous recombination by counteracting RAP80. *Nucleic Acids Res* 2016; 44:2976.
5. Chan YW, Fugger K, West SC. Unresolved recombination intermediates lead to ultra-fine anaphase bridges, chromosome breaks and aberrations. *Nat Cell Biol* 2018; 20:92-103.

b. Meeting Abstract:

Exposure to a 2Gy ionizing radiation dose is sufficient to induce alterations in the homologous recombination pathway and replication stress in the progeny of irradiated cells.

Zhentian Li, Paul W. Doetsch and Erica Werner

Following a single exposure to a 2 Gy dose of ionizing radiation, which is the dose delivered during fractionated radiotherapy, proliferating human bronchial epithelial cells (HBEC-3KT) display persistent genomic instability, after the initial DNA damage has been repaired. We hypothesized that a driving mechanism might be the dysregulation of DNA repair pathways. By screening U2OS reporter cell lines for activity of major double strand break repair pathways, we found that at day 5-post exposure to a 2Gy dose, U2OS reporter cells employ homologous recombination to repair a double strand break introduced by the meganuclease I-SceI with a higher frequency than non-irradiated cells. To gain further understanding on how the activity of this pathway may be affected, we surveyed changes in the abundance and function of proteins in lysates from U2OS and HBEC-3KT by functional proteomics employing a Reverse Phase Protein Array probing for 431 proteins dysregulated in cancer and enriched for proteins involved in DNA repair. This analysis showed altered expression of multiple proteins participating in DNA repair and homologous recombination as well as activation of ATR and Chk1 kinases suggesting ongoing replication stress. The DNA fiber assay revealed a decrease in replication speed that was proportional to the initial dose of ionizing radiation. Furthermore, irradiated cells exhibited additional phenotypes associated with altered function of the homologous recombination pathway and persistent replication stress, such as increased number of micronucleus formation, anaphase bridges positive for RPA, 53BPB1 foci in G1 cells (Cyclin A

negative) and increased number of cells with RPA positive foci. These findings are informative for the rational design and timing of combination therapies for cancer treatment and for the management of adverse effects following radiation therapy. This work is funded by a DoD award W81XWH-17-1-0187 (LC160329)

- c. Publication: pages 10-29.
- d. Heatmap of unsupervised clustering of RPPA result for HBEC3KT cells non irradiated or day 5 and 5 post-irradiation treated or not for 24h with 5uM SB203580. Page 30

Ionizing Radiation induction of cholesterol biosynthesis in Lung tissue

Erica Werner^{1#}, Andrew Alter², Quidong Deng³, Eric B. Dammer², Ya Wang¹, David S. Yu¹, Duc M. Duong³, Nicholas T. Seyfried^{2,3} and Paul W. Doetsch^{1,2,4}

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Abstract

While evidence supporting the notion that exposures to heavy ion radiation increase the risk for cancer and other disease development is accumulating, the underlying biological mechanisms remain poorly understood. To identify novel phenotypes that persist over time that may be related to increased disease development risk, we performed a quantitative global proteome analysis of immortalized human bronchial epithelial cells (HBEC3-KT) at day 7 post exposure to 0.5Gy Fe ions (600 MeV/nucleon, LET= 175 keV/ μ m). The analysis revealed a significant increase in the expression of 4 enzymes of the cholesterol biosynthesis pathway. Elevated expression of enzymes of the cholesterol pathway was associated with increased cholesterol levels in irradiated cells and in lung tissue measured by a biochemical method and by filipin staining of cell-bound cholesterol. Radiation-increased cholesterol levels were reduced by treatment with inhibitors affecting the activity of enzymes in the biosynthesis pathway. While a 1Gy dose of Fe ion was sufficient to induce a robust response, a dose of 5Gy X-Rays was necessary to induce a similar cholesterol accumulation in HBEC3-KT cells. To examine the implications of this finding for radiotherapy exposures, we screened a panel of lung cancer cell lines for cholesterol levels following exposure to X-rays. We identified a subset of cell lines that increased cholesterol levels in response to 5Gy X-rays. Survival studies revealed that statin treatment is radioprotective, suggesting that cholesterol increases are associated with cytotoxicity. In summary, our findings uncovered a novel radiation-induced response, which may modify radiation treatment outcomes and contribute to risk for radiation-induced cardiovascular disease and carcinogenesis.

Introduction

Understanding the scope of biological responses to radiation is of increasing importance as exposures to radiation of human populations are rising due to medical diagnostics and treatment, potential radio-nuclear accidents, and future space travel ¹.

The most significant known effect of low to moderate doses of radiation, which cause DNA damage that is readily repairable in normal cells, is an increased risk of cancer ². While the mechanisms remain largely unknown, current models for radiation-induced carcinogenesis propose that radiation increases the probability of initiating mutations as well as the sequential accumulation of driver mutations in susceptible cell populations ³. However, cancer as a disease results from a process influenced by multiple factors such as environmental and genetic

constitution, and therefore could be affected by additional cellular responses elicited by radiation not related to mutated DNA ⁴. Furthermore, non-DNA damage-related radiation responses might also play a role in the emergence of non-cancer pathological effects, including cardiovascular disease, fibrosis, cataracts and central nervous system effects.

Initial DNA damage caused by radiation triggers mechanisms to restore genetic stability and cellular homeostasis. We and others have previously shown that a single exposure to a moderate dose of radiation in the range of 1Gy, which causes DNA damage that is readily repaired by normal cells, can induce cellular stress responses that persist for weeks ⁵. Such stress response associated with DNA damage repair processes could influence later outcomes by affecting the establishment and permanency of chromosomal aberrations and DNA mutations, as well as modulating cell fate and function in the exposed cell population. Persistent responses to radiation may provide molecular targets to mitigate the emergence of late deleterious effects as well as sources for potential biomarkers to monitor responses to radiation at the individual level and across an extended period of time.

We analyzed the effects of exposure to high mass and energy (HZE) particle radiation, because of its overall higher biological effectiveness and carcinogenic potential ⁶. Furthermore HZE particle radiation is a ground-based model for space radiation and relevant for particle beam therapy of human cancers. To gain biological knowledge about the scope of the cellular processes altered following exposure to radiation, we analyzed quantitative changes in the proteome, which captures the effects of changes in gene expression, translation and post-translational regulation and is a close reflection of cellular phenotype. We employed the human bronchial epithelial cell line HBEC3-KT, a non-tumorigenic immortalized cell line as an in vitro model for lung epithelia, which is a radiosensitive organ susceptible to radiation-induced cancer and late toxicity.

Results:

We exposed HBEC3-KT to moderate radiation doses ranging between 0.5 and 5Gy, doses within a therapeutic range and known to increase cancer risk in a normal human population ⁷. We have previously shown that at day 7, cells that have been exposed to 1Gy of low or high LET radiation are actively proliferating within the context of numerous altered cellular processes such as oxidative stress, genomic instability and pro-inflammatory cytokine production ^{5,8,9}. To discover novel relevant cellular phenotypes that are persistently affected, we conducted a label-free global proteome analysis of cells at day 7 post-exposure to 0.5Gy Fe ions. Analysis of triplicate samples

revealed that among 2706 proteins identified and quantified in all 6 samples, radiation exposure changed the expression of 51 proteins at a statistically significant level (Supplementary Table 1), as visualized in a volcano plot (Fig 1A). Among the top three proteins induced by Fe ion exposure is IL-1 α , which we have previously identified by ELISA as a radiation-induced cytokine driving the production of IL-8 and other inflammatory molecules⁸. Thus, the current approach detects some of the responses we have previously identified by biochemical methods. Other proteins induced are Fatty Acid Desaturase 1 and 2 (Supplementary Table 1), enzymes that regulate the synthesis of saturated fatty acids and therefore indirectly control the availability of polyunsaturated fatty acids, precursor molecules for the pro-inflammatory mediators eicosanoids and docosanoids¹⁰, pointing to a broad inflammatory phenotype that comprises cytokines and lipids metabolites.

The significantly altered proteins were functionally annotated and mapped to biological processes employing the bioinformatics DAVID annotation tool. The analysis revealed a significant increase of proteins involved in tissue repair and remodeling such as molecules promoting cell proliferation, angiogenesis, wound healing, chemotaxis, and the cellular interaction with the extracellular matrix (Fig 1B). Most interestingly, the analysis revealed 4 enzymes involved in the *de novo* cholesterol biosynthesis pathway (Fig 1C).

Cellular levels of cholesterol are tightly regulated to balance availability of essential metabolites for cellular functions with toxicity. Therefore, to test whether the observed changes in enzyme levels are sufficient to affect cellular cholesterol levels, we measured total cholesterol content in HBEC3-KT following exposure to radiation employing two methods: an assay based on the enzymatic oxidation of cholesterol coupled to Amplex Red fluorescence and another based on filipin III from *Streptomyces filipenses*, a polyene antifungal antibiotic with a chromophore that undergoes a shift in fluorescence when bound to non-esterified cholesterol¹¹. Both assays detect cell-associated cholesterol levels, which were proportional to cell number (not shown) and sensitive to treatment with inhibitors of cholesterol synthesis (Fig 2B,D) or chelation with methyl-beta-cyclodextrin (Fig 2A,C). We detected increased levels of cholesterol in HBEC3-KT cells at day 7-post exposure to 5Gy X-rays but not at an earlier time point or at a lower dose of 2Gy (Fig 2A), while we readily detected an increase at day 7 following 1 Gy Fe ion exposure (Fig 2B). Thus, based on these measurements, we estimate a relative biological effectiveness (RBE) of 5 for the response induced by particle radiation. Basal and radiation-induced cholesterol levels were reduced in cells treated with inhibitors targeting multiple enzymes in the cholesterol biosynthetic pathway when added 18h prior to the measurements (Fig 2B,D). While different inhibitors showed variable efficiency, all reduced the basal cholesterol content in non-irradiated cells and the increase induced

by radiation. An increase in cell-associated cholesterol induced by exposure to Fe ion and a reduction in cells treated with statins was also evident with filipin staining, which showed increased labeling of the plasma membrane and intracellular compartments (Fig 2C). Thus, these results indicate that *de novo* synthesis contributes to the total pool of cholesterol in HBEC3-KT cells and that exposure to low and high linear energy transfer (LET) radiation increases the biosynthesis of cholesterol.

To test whether our in vitro model reproduces phenotypes induced in lung tissue, we stained frozen lung tissue sections extracted from whole body irradiated C57BL/6 mice that were sacrificed at day 7 post-exposure to 1Gy Fe ions. Increased filipin fluorescence was observed in the epithelial cells lining small airways and in cells located in the adjacent respiratory compartments (Fig 3A). Quantification of the integrated fluorescence of both regions (Fig 3B) demonstrates that exposure to Fe ions increases cholesterol levels in both compartments, indicating that this response is not unique to HBEC3-KT cells and may be shared by multiple cell types in the lung. A significant increase in cholesterol levels in lung tissue homogenates was also detected employing an enzymatic assay (Fig 3C).

Because of the potential toxic effects of cholesterol on cells, we evaluated next whether this response is elicited by low LET radiation in cancer cell lines and modulates radiosensitivity in a dose range relevant to radiation therapy. We measured cholesterol levels in a panel of lung cancer cell lines employing filipin staining in a 96-well plate assay format 48h following exposure to 5Gy X-rays. In several of the 8 cell lines tested, X-rays increased cholesterol levels in variable magnitudes, while in two of them (A549 and H226) the levels were not affected (Fig 4A). Cholesterol induction did not correlate with p53, Ras or EGFR mutation status of the cell lines. Employing the Amplex Red assay we quantified cholesterol increases in Calu-1, H522 and H1650 which were the cell lines most responsive to radiation demonstrating increases of 10% in H522, 20% in H1650 and 30% in Calu-1 compared to no change in A549 (Fig 4B).

To test whether cholesterol synthesis modulates cellular radiosensitivity we measured the biological effects of pathway inhibition targeting 3-Hydroxy-3-Methyl glutaryl-CoA Reductase with water- and lipid-soluble statins and Squalene Synthase with YM5603. An 18h treatment with these inhibitors reduced the radiation-induced upregulation of cholesterol levels (Fig 5A), supporting our previous finding that exposure to radiation induces *de novo* synthesis of cholesterol. The inhibitors reduced basal levels of cholesterol in two cell lines, Calu-1 and H522, indicating that *de novo* synthesis contributes to the steady-state pool of cholesterol in these cell lines. When we assessed the effect of the inhibitors on cell growth by MTT reduction, only Calu-1 and H522 were sensitive to

Pitavastatin (IC₅₀ of 1.35uM+/-0.34 SE and 0.2uM+/-0.1 SE respectively), and only H522 was sensitive to the Squalene Synthase inhibitor YM5603 with an IC₅₀ of 4.25uM +/-0.72 SE (Fig 5B). These results highlight the heterogeneity of lung cancer cell lines reliance on different sources for cholesterol and variably dependency on the cholesterol biosynthetic pathway for survival.

To test whether cholesterol synthesis modulates cell survival following exposure to radiation, we first examined the effects of pathway inhibition on colony survival following X-ray exposure in two cell lines able to grow in sparse conditions required to measure clonogenic growth. Inhibition of Squalene Synthase increased the clonogenic survival of H522 cells but not of A549 (Fig 5C), suggesting a contribution of cholesterol accumulation to radiation toxicity. We tested the effects of modulating cholesterol synthesis with statins on cell growth at an earlier time point employing the MTT assay to monitor cell number following exposure to a radiotherapy relevant dose of 2Gy. Treatment of the cells with a dose close to the IC₅₀ of Pitavastatin increased the survival of Calu-1 and H522, but not of A549 or H1650 (Fig 5D).

Discussion:

An unbiased analysis of the proteome of cells that have been exposed to radiation revealed that induction of the cholesterol biosynthesis pathway is a major response persisting in irradiated lung epithelial cell lines and in lung tissue. Radiation upregulated the expression of four enzymes in the cholesterol biosynthesis pathway with functional consequences as demonstrated by increased cellular content of cholesterol following exposure to low and high LET radiation. A radiation dose as low as 0.5 Gy of high LET was sufficient to induce this response, which persisted at day 7-post exposure *in vitro* and *in vivo*.

The mechanism for cholesterol accumulation, which we demonstrate by two different methods, is by inducing cholesterol biosynthesis and is supported by increases in biosynthetic enzyme expression levels and by sensitivity of the phenotype to inhibitors targeting four different enzymes of the cholesterol biosynthetic pathway. The enzymes detected in the proteome are genes transcriptionally upregulated by SREBP and downregulated by cholesterol as part of a feedback loop that maintains cholesterol homeostasis^{12 13}. Therefore, it is intriguing that we can detect increased cholesterol levels at day seven after irradiation. While we did not perform an extensive time course analysis to determine when this phenotype emerges and how long it persists, our findings suggest the activity of an additional modification that alters the normal negative feedback mechanisms contributing to the expression of this phenotype long after the initial damage to DNA has been repaired. Known mechanisms that disrupt feedback regulation of cholesterol are

inflammation, metabolic syndrome and mTOR activation¹⁴ and as part of lipid reprogramming associated with cancer progression ^{15,16,17}. A similar signature involving the same enzymes of the cholesterol synthesis pathway and FADS1 was previously shown to strongly correlate with body mass index in a large population study, however very few studies have addressed gene and protein expression with cholesterol measurements. In support for a potential influence from other concurrent phenotypes induced by radiation, we have previously shown production of IL1 α and several other inflammatory cytokines ⁸ as well as oxidative stress and genomic instability. Thus in this work we provide evidence for a novel condition (exposure to radiation) that alters cholesterol homeostasis by inducing biosynthesis and, potentially, loss of feedback regulation.

While the radiation-induced increases in cholesterol levels are apparently modest (between 10 and 30%), the magnitude of the increases detected is well within the range of changes that affect protein and cellular function. The majority of the cell's cholesterol (60 to 90%) is concentrated in the plasma membrane ¹⁸, where it regulates protein function indirectly by affecting the biophysical properties of the lipid bilayer or directly by binding to specific motifs in target proteins. This cholesterol pool is regulated around a set point with a narrow range ^{18,19} and is sensitive to small changes (10-20%) in concentration ²⁰. The activity of proteins sensitive to cholesterol, which include ABC sterol transporters, the Na,K-ATPase, nicotinic acetylcholine receptors, three potassium ion channels, the ligand-gated ion channel GABA receptor, and the NMDA glutamate-gated ion channel, respond to modest changes of cholesterol in their local membrane environment. Furthermore, every G protein-coupled receptor has binding sites for cholesterol or their function is affected by sterol (reviewed in ²¹). Therefore, we can safely speculate that the changes we detected ranging between 10 and 30% in cholesterol levels are within a physiological range and are expected to modify the function of susceptible proteins.

We detected increased cholesterol levels induced by radiation in multiple systems, including non-tumorigenic cells, cancer cell lines and in lung tissue from a whole body irradiated mouse, where a generalized increase in cholesterol levels is observed in multiple compartments of lung tissue. This finding suggests that a similar response could occur in other post-mitotic tissues that are high risk for radiation exposures such as the cardiovascular and central nervous systems. The effects of low dose radiation on cardiovascular disease are still controversial, however increased incidence of cardiovascular disease is observed in the atomic bomb survivors cohort ²² and cardiovascular disease is a known side effect of radiation therapy ²³⁻²⁵. Another potentially susceptible tissue is the central nervous system, where cholesterol cannot be transported across

the blood-brain barrier, and therefore is synthesized *de novo* ^{26,27}. Oxysterols have also been used as biomarkers in neurodegenerative diseases ²⁸.

We also show that exposure to X-rays at doses equivalent to a fractionated radiation therapy dose is sufficient to increase cholesterol levels in a subgroup of lung cancer cell lines. This observation in combination with our cell growth and survival studies here is consistent with previous reports showing that oxysterols, downstream products of the cholesterol biosynthetic pathway, have selective cytotoxicity in tumor cell lines by inducing apoptosis in some of the tested cell lines ²⁹. Our studies support the conclusion that accumulated cholesterol contributes to radiation toxicity in cell lines that are dependent on this pathway for cell growth. While our findings support statin use as a therapeutic strategy in a selective fashion because they affected cell growth in some of the cell lines, they also suggest that statins may not be effective in combination with radiation as they reduced radiosensitivity in others.

Statins are safe, well tolerated and have broad pleiotropic effects, including immunomodulatory, anti-inflammatory and neuroprotective effects ³⁰⁻³². Simvastatin and Lovastatin have been shown to reduce radiation induced inflammatory and pro-fibrotic markers without affecting DNA repair in normal tissues ^{33 34}. Thus, statin use poses many advantages as a mitigation agent by, in addition to reducing cholesterol synthesis, targeting other radiation-induced alterations in addition to reducing cholesterol synthesis ³⁵.

Materials and Methods

Irradiation experiments

Experiments utilizing mice followed the American Association for Laboratory Animal Science policies and were approved by the Emory University and Brookhaven National Laboratory Institutional Animal Care Use Committee (IACUC). A six week old C57BL/6 female mice (Jackson Laboratory, Bar Harbor, ME) was whole-body irradiated with a dose of 600MeV/nucleon Iron (LET=175keV/ μ m), 50cGy dose rate at the NASA Space Radiation Laboratory (NSRL) Brookhaven National Laboratory. The NSRL dosimetry group calibrated the ion energy. Data was collected from triplicate irradiations in experiments over 3 different campaigns. Low-LET irradiation was performed at the Winship Cancer Institute of Emory University using an X-RAD 320 X-ray machine (320 kV, 10 mA Precision X-Ray, North Branford, CT).

Cell culture

The human bronchial epithelial cell line (HBEC3-KT) was a gift from Dr. Story (UT Southwestern)

and was authenticated by karyotyping. Cells were cultured in Keratinocyte serum free media (Invitrogen) supplemented with antibiotics, Epidermal growth Factor and Bovine Pituitary extract. For Fe ion irradiations, cells were plated in triplicate T25 flasks at 30% confluence. Each flask was sub-cultured 1:3 on day 4 and plated for the assays on day 6 post-exposure. A549, H1650, H1975, H522, H226, H1792 and HCC827 cell lines were maintained in RPMI-1640 media supplemented with 10% FBS, and were obtained from the Winship Cancer Institute (Atlanta, GA, USA).

Label-free quantitative global proteomic analysis:

Three T25 flasks for each experimental condition were independently mock or 0.5Gy Fe irradiated, passaged at day 4 and the cells were harvested at day 7 and stored frozen until analysis. Samples were homogenized in 8M urea buffer and 100 µg of protein homogenates were diluted with 50 mM NH_4HCO_3 to a final concentration of less than 2M urea and proteins were reduced, alkylated and digested with LysC/trypsin as previously described ³⁶. Desalted peptides (2µg) were analyzed by nanoliquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) on a Fusion Orbitrap (Thermo Fisher Scientific, San Jose, CA). Elution was performed over a 120 minute gradient at a rate of 300nl/min with buffer B ranging from 1% to 65% (buffer A: 0.1% formic acid in water, buffer B: 0.1 % formic in acetonitrile). The machine was set to collect at top speed for cycles of 3 seconds where ms1 scans were collected in the Orbitrap at 120,000 resolution with an automatic gain control (AGC) of 200,000, a m/z range of 400-1600, and a max injection time of 50ms. Precursor ions were fragmented at 32% normalized collision energy (NCE) in the Higher-energy C-trap dissociation (HCD) cell and collected in the ion trap with an AGC of 10000 and a max injection time of 35ms. Other parameters include S-lens RF of 60%, dynamic exclusion of 20s, exclusion of +1 and greater than +7 charged species, and quadrupole isolation window of 0.7 Da. The spectra were searched using the Andromeda search engine against a human Uniprot database (downloaded April 2015 – 90270 target sequence). The search parameters include full tryptic enzymatic cleavage, 0.6 Da fragment mass tolerance, dynamic methionine oxidation (+15.994915), N-terminal acetylation (+42.010565), and static cysteine carbamidomethyl (+57.021464). Peptide-specific ion current intensities were extracted and compared using MaxQuant (version 1.5.2.8) label-free quantification (LFQ) according to standard procedures as described ³⁷. Accurate peptide mass and retention time were defined by MaxQuant derived signal intensity for every peptide across LC-MS/MS runs for each sample. For each pair-wise cohort comparison, GenePattern (The Broad Institute) was employed to produce a statistically analyzed list of proteins, each ranked by the degree of change and p-value significance of the abundance change it exhibits between specified

cohorts as we have previously shown ³⁸. From a total of 2706 proteins quantified and identified, 51 proteins varied significantly after filtering for the following criteria established previously: a) a cut-off of $> 2SD$ for upregulated and $0.5 <$ for downregulated proteins (outside 95% confidence interval), b) with a p value < 0.05 and c) passed a threshold set at 5% for FDR estimated by utilizing the power of technical replicates and a null experimental comparison to quantify false positives under current filtering criteria ^{38,39}. The list of proteins shown in supplementary material was analyzed employing the functional annotation tools with default settings in the Database for Annotation, Visualization and Integrated Discovery (DAVID). Data is available at Synapse.org, DOI: 10.7303/syn18081952 <https://www.synapse.org/#!Synapse:syn18081952/files/>

Cholesterol content measurements:

Amplex red cholesterol assay:

100,000 cells were plated 24h before lysis in 100 μ l Buffer A 0.5% Triton X100 supplemented with antiproteases (Complete, Roche). Lysates from frozen lung tissue were prepared in Buffer A 0.5% Triton X100 with 10 strokes of a Dounce homogenizer, sonicated 3 times for 10 seconds at 6watts and centrifuged at maximum speed in a refrigerated tabletop centrifuge. Protein concentration was equalized in all samples at 2.5 μ g/ μ l for tissue lysates and at 1 μ g/ μ l for cell lysates. Seventy five microliters of lysate were extracted with 300 μ l of a 3:1 mixture of chloroform: isopropanol. A hundred and fifty microliters of the organic phase were recovered and the solvents evaporated at room temperature in a speed-vac. The lipids were resuspended in 50 μ l of reaction buffer and cholesterol was measured as recommended by the Amplex® Red Cholesterol Assay kit (Invitrogen). This is an enzyme coupled fluorimetric assay that detects both free cholesterol and cholesteryl esters.

Filipin staining:

Cells plated on glass coverslips, in 96 well black wall plates or frozen tissue sections were fixed with 4% Paraformaldehyde in PBS. After 4 washes with PBS, aldehyde groups were quenched with 10mM Glycine in PBS for 10 minutes. Filipin from *streptomyces filipensis* (Sigma) was added at a dilution of 0.05mg/ml in PBS containing 10% fetal bovine serum and incubated for 2h at room temperature in the dark. After four washes with PBS, nuclear DNA was counterstained with 5 μ M Draq5 (eBioscience) for fluorescence microscopy assays or SybrGreen (Invitrogen) for plate assays. Cell associated filipin (350nm em/450nm ex) and SybrGreen (480nm ex/580nm em) fluorescence was measured in a Molecular Dynamics Plate reader. Cells and tissue sections were imaged in a

Olympus epifluorescence inverted microscope and a Zeiss LSM510META confocal microscope (Thornwood, NY, USA) using a 20×Plan-Apo objective (NA=0.75). Images were processed using contrast/brightness enhancement only. Filipin fluorescence in the images was quantified employing Fiji (Image J) recording the integrated fluorescence intensity for filipin and for Draq5 fluorescence, a far-red cell-permeable DNA intercalating probe to correct for cell density. In tissue images, two ROI of defined area (4x4 arbitrary units) were placed on the bronchial epithelium lining small airways or on the adjacent respiratory portion in four different images. In cultured cells, the cell-associated pixel intensity of fillipin fluorescence was recorded for a total of 50-70 cells per sample in replicate irradiations.

Tissue preparation and staining

Lung tissue was collected from mice euthanized at day 7 post-irradiation, and snap frozen in liquid nitrogen to preserve molecular integrity of the cells and for laser scanning microscopy imaging. The tissues were embedded in OCT Compound (Tissue-Tek) and cut frozen into five micrometer thick sections. Frozen sections were thawed in 3% PFA and stained with filipin as described above.

Cell growth and survival assays

To measure cell growth, cells were plated in 96 well plates and irradiated and/or treated with inhibitors 18h after plating. Cell growth was measured by quantification of reduced MTT and IC50 were calculated with GraphPad Prism employing non-linear regression of an equation for the logarithm of the inhibitor concentration vs. % survival with a variable slope and a top constrain of less or equal to 100. Cell survival to radiation was measured by the colony survival assay 12 days following exposure. Inhibitors were added within one hour of exposure to radiation and washed after 3 days.

Statistical analysis

The statistical test applied in each case is stated in the figure legend. Excel was used for paired two-tailed student's t-test analysis assuming equal variance of the samples. Graph Pad Software (San Diego, CA, USA) was used for repeated measures analysis of variance employing a Bonferroni (repeated measures) or Dunett's (one way) post-hoc test.

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Competing Interest:

The authors declare no competing interests.

Contributions:

E.W., N.S. and P.D. conceived and designed the project. E.W and A.A. carried out experiments and data analysis. Q.D. and D.D. performed the proteomics experiments. Q.D., E.D. and N.S. performed proteomics data analysis. Y.W. provided essential animal tissue samples. E.W., D.Y. and P.D. drafted the manuscript. All authors reviewed and approved the manuscript.

Data Availability:

Mass spectrometry raw data is available at Synapse.org,

DOI: 10.7303/syn18081952 <https://www.synapse.org/#!/Synapse:syn18081952/files/>

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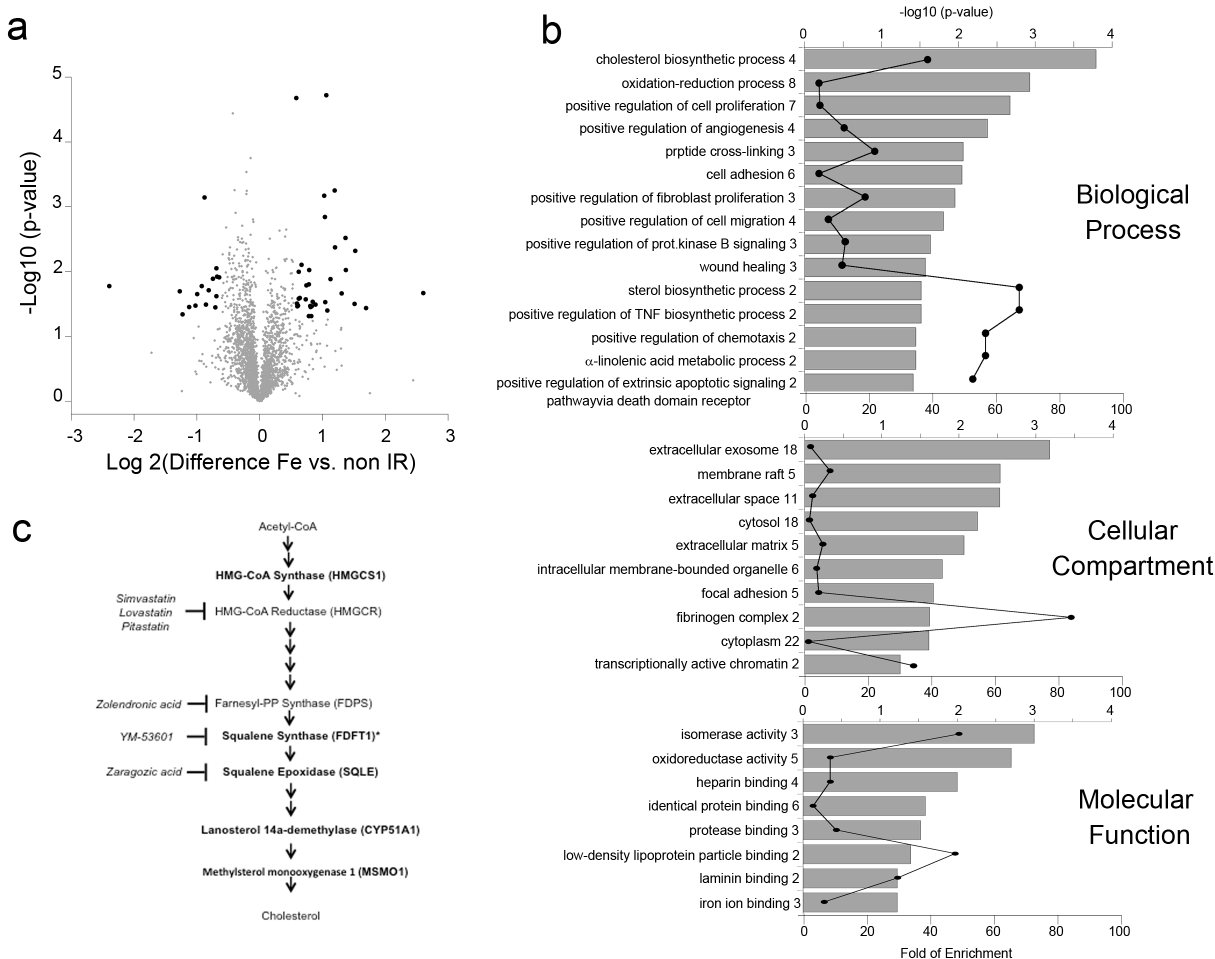


Figure 1

Figure 1: Quantitative global proteomic analysis of the cellular response at day 7 following a 0.5 Gy Fe ion exposure.

A) Volcano plot displaying the distribution of the proteins identified in all samples with proteins differentially regulated significantly by particle radiation exposure highlighted in bold.

B) Top GO terms identified for the list of differentially expressed proteins following annotation analysis in DAVID. The graphs display the significance (grey bar) and the relative enrichment (line graph) of proteins in the list compared to a random sample. Next to the GO term, the number indicates the number of proteins in the list included in the category.

C) Five of the significantly induced proteins (gene symbol in parenthesis) belong to the cholesterol biosynthetic pathway and are highlighted in bold. *= FDFT1 was induced two-fold, but did not pass the FDR filter setting of our analysis. The diagram includes the inhibitors employed in the experiments.

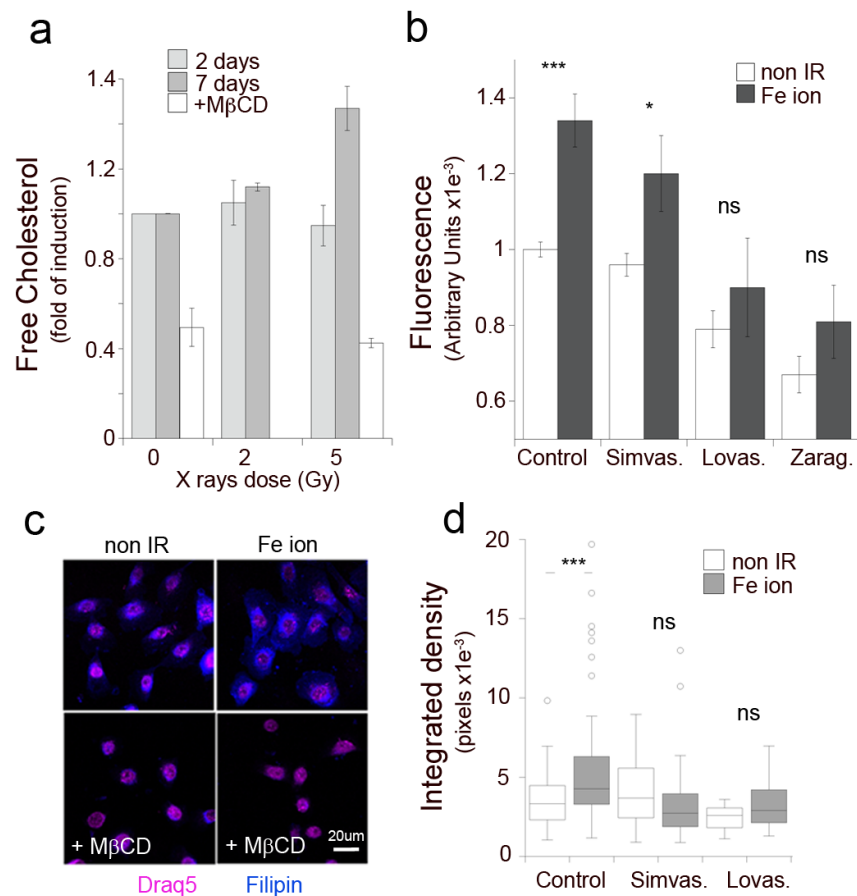


Figure 2

Figure 2: Exposure to Fe ion radiation increases cell-associated cholesterol

A) Free cholesterol measured with the Amplex Red assay in HBEC-3KT cells at day 2 or 7 following exposure to 2 (light gray) or 5Gy (dark gray) dose X-rays. White bars represent samples that were treated with 10 mg/ml Methyl beta cyclodextrin for 1h prior to analysis. Error bars represent standard error of 2 replicate experiments.

B) Free cholesterol measured with the Amplex Red assay in HBEC-3KT cells at day 7 following mock irradiation (white bars) or exposure to 1Gy Fe ions (gray bars). Some samples were incubated with vehicle (Control), 10 μ M Simvastatin, 10 μ M Lovastatin or 40 μ M Zaragozic acid for 18h prior to analysis. Error bars represent standard error of 3 replicates. T-test *= p <0.05, ***= p <0.005.

C) Filipin fluorescence microscopy at day 7 post-irradiation of HBEC-3KT cells pre-treated or not for 1h with 10mg/ml methyl beta cyclodextrin before fixation. Draq 5 is a cell permeable DNA binding fluorescent probe with far red emission employed as a counterstain.

D) Quantification of cell-associated filipin fluorescence of HBEC-3KT cells at day 7 post-exposure to 1Gy Fe ions. Some cultures were treated for 18h prior staining with vehicle and 10 μ M Simvastatin or Lovastatin. Box plots indicate the mean value and standard deviation of measurements in 35 or more cells per condition. Paired T-test, ****= p <0.001.

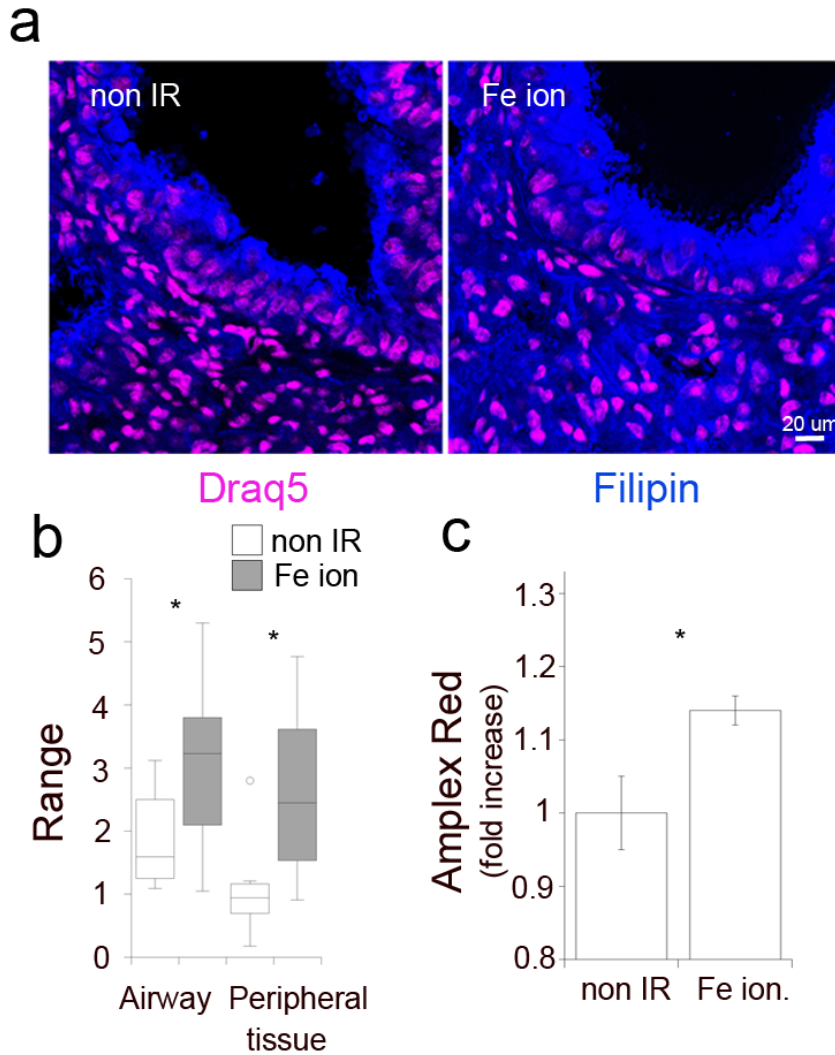


Figure 3

Figure 3: Exposure to Fe ion radiation increases lung tissue associated cholesterol.

A) Confocal fluorescence microscopy of lung tissue sections from a non-irradiated or a 1Gy Fe ion whole body irradiated mice, stained for cell-associated cholesterol with filipin (blue) and nuclear DNA with Draq 5 (purple). Arrows point to the airway bronchial epithelium and respiratory tissue.

B) Average fluorescence intensity of filipin staining in the bronchial epithelium (Airway) and the nearby respiratory tissue (Peripheral tissue) was quantified employing Fiji/Image J. The box plots represent measurements in 8 different areas. Paired T-Test, $*=p<0.05$.

C) Total cholesterol content measured with the Amplex Red assay in lysates prepared from frozen lung tissue isolated from mock and 1Gy Fe ion whole body irradiated mouse. Average of duplicate measurements in two different tissue samples. Paired T-Test, $*=p<0.05$.

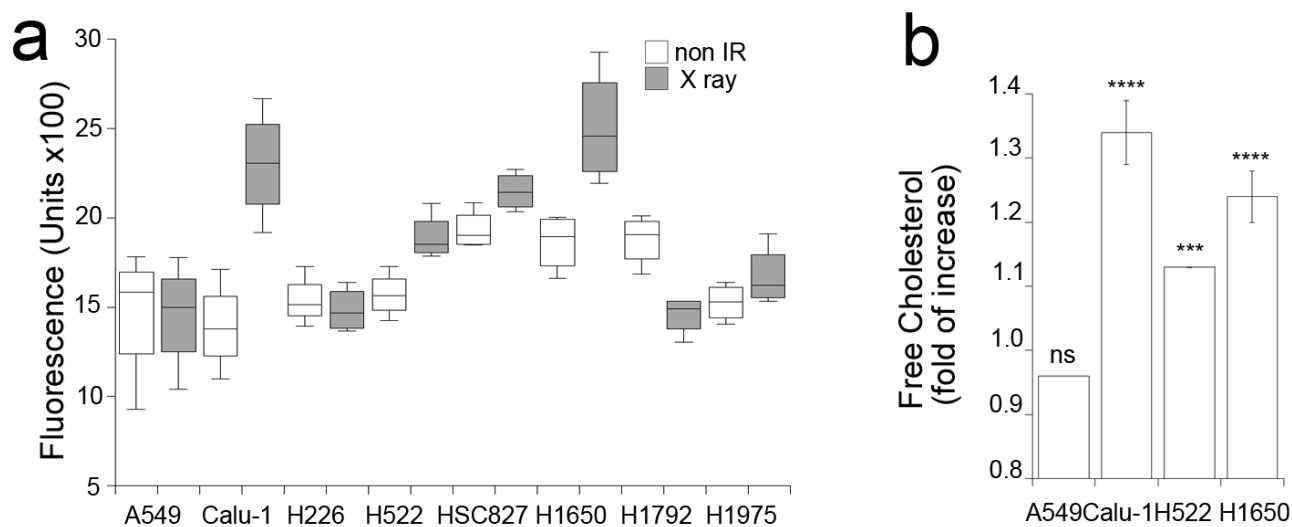


Figure 4

Figure 4: X-rays increases cell-associated cholesterol in a subgroup of non-small cell lung cancer cell lines.

A) Average fluorescence intensity of filipin staining in a panel of lung cancer cell lines 48h following mock or 5Gy X-rays exposure. (1 of 2 replicate experiments shown). Error bars represent standard deviations of triplicate measurements.

B) Total cholesterol content measured with the Amplex Red assay in cell lines that increase cholesterol content following exposure to 5Gy X-Rays Error bars represent standard error of 3 replicate experiments. One-way ANOVA with Dunnett post test $F=16$, $p<0.005$. ***= $p<0.005$.

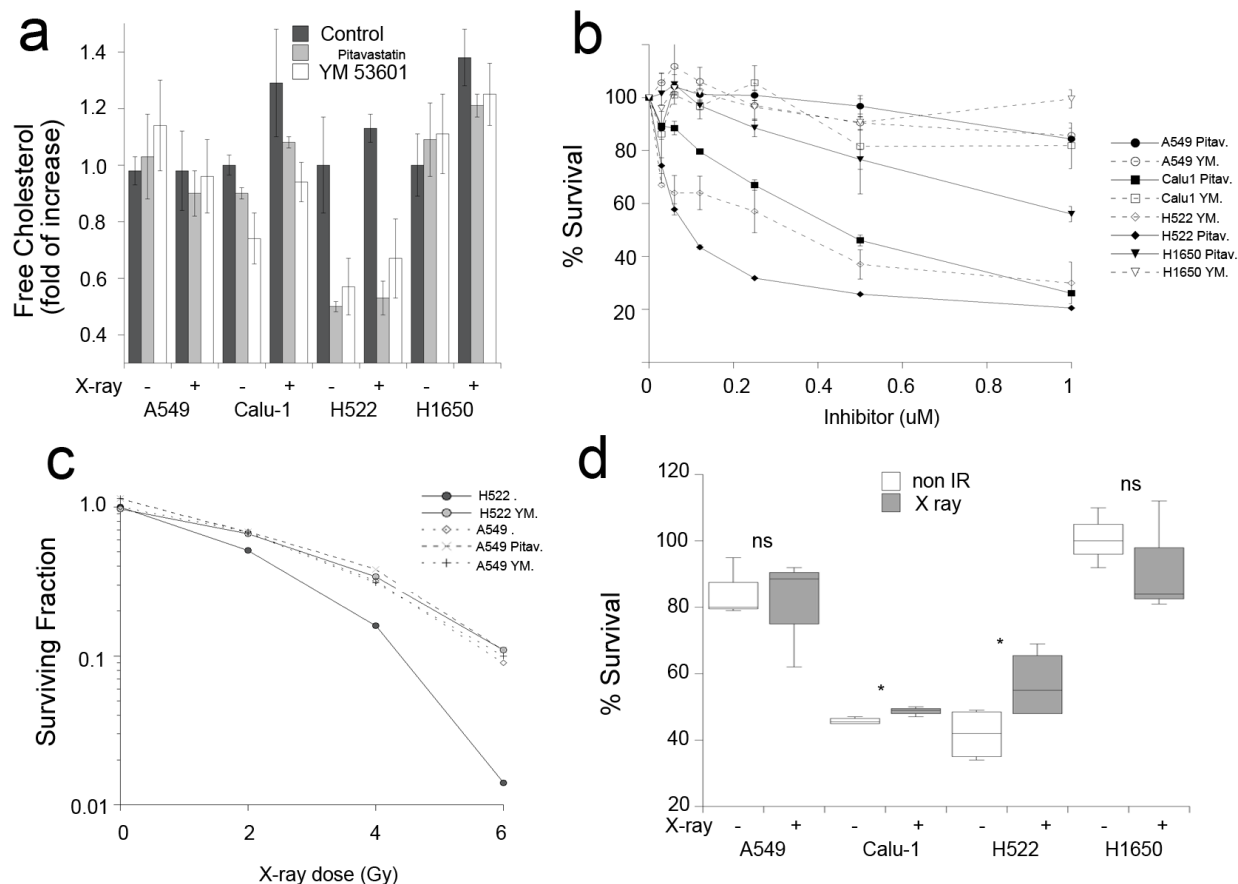


Figure 5

Figure 5: Effect of cholesterol biosynthesis inhibition on cell growth and radiosensitivity.

A). Cellular cholesterol levels measured with the Amplex Red assay are reduced by an 18h treatment prior the assay with vehicle (black bars), 1 μ M Pitavastatin (grey bars) or 2 μ M YM-53601 (empty bars).

B) MTT survival assay. In the 3 cell lines were radiation induced cholesterol synthesis, statins are cytotoxic, while only in H522 both inhibitors reduce cell growth. One of two experiments is shown. Error bars represent standard deviation of triplicate samples.

C) Effect of the cholesterol synthesis inhibitor YM-53601 on A549 (dotted line) or H522 (full line) cell survival measured by the clonogenic assay following exposure to X-rays. 1 of 2 experiments is shown. Error bars represent standard deviation of triplicate samples

D) Cells were irradiated with 2Gy X rays and within the hour treated with 1.25 μ M (A549, Calu1 and H1650 or 0.3 μ M (H522) Pitavastatin. Cell growth was measured by MTT reduction after 72h and compared to non-irradiated cells. The average of 3-4 experiments is shown. Paired t-test. NS= not significant, $p < 0.05$.

Gene sym	Name of the protein	f01	f02	f03	n01	n02	n03	log.p	Pvalue	thresho	Difference	fold chang	Fold change (F/N)
ALDH3A1	Aldehyde dehydrogenase 3 fam	7.49601	7.84454	7.977853	8.417332	8.714462	8.347096	-1.7726924	0.0168775	TRUE	-0.72016	5.250043	0.190474627
HSPB1	Heat shock protein beta 1	9.16436	9.19343	9.147317	9.370143	9.720893	9.559373	-1.6939785	0.0202312	TRUE	-0.38177	2.408609	0.415177377
BCAM	Basal cell adhesion molecule	7.47551	7.49633	7.546669	7.687858	8.117105	7.819794	-1.340311	0.0456761	TRUE	-0.36875	2.337496	0.427808205
CA2	Carbonic anhydrase 2	7.66075	7.63757	7.461586	7.871243	8.095293	7.805113	-1.450559	0.0354357	TRUE	-0.33725	2.173935	0.45999533
PTK7	Protein Tyrosine Kinase 7	7.74636	7.99712	7.859868	8.095634	8.303409	8.127904	-1.4709523	0.0338102	TRUE	-0.30786	2.031725	0.492192528
SPRR1B	Cornifin B	7.97578	7.86863	7.941932	8.146939	8.381223	8.156849	-1.6501529	0.0223793	TRUE	-0.29956	1.993225	0.501699589
AKR1C2	Aldo-keto reductase family 1 m	7.82009	7.86302	7.891428	8.011392	8.241179	8.155465	-1.7775264	0.0166907	TRUE	-0.27783	1.895976	0.527432848
PCK2	Phosphoenolpyruvate carboxyk	8.33044	8.30079	8.361753	8.566416	8.63726	8.578897	-3.1437527	0.0007182	TRUE	-0.2632	1.833137	0.545512821
CTSD	Cathepsin D	9.12262	9.18861	9.148591	9.297621	9.560039	9.375912	-1.4908061	0.0322994	TRUE	-0.25791	1.810984	0.552186083
HBB	Hemoglobin beta	7.8013	7.63995	7.777107	7.951092	7.936134	8.065057	-1.7126691	0.019379	TRUE	-0.24464	1.756484	0.569319206
HIST1H1C	Histone H1C	9.6761	9.85201	9.729881	9.986335	9.975047	9.966918	-1.8872365	0.0129647	TRUE	-0.22344	1.672775	0.597808895
DNAJC2	DnaJ homolog subfamily C merr	7.53481	7.30962	7.433984	7.601212	7.643989	7.668785	-1.4466018	0.0357601	TRUE	-0.21186	1.628764	0.613962332
FAHD1	Acylpyruvase	7.77815	7.93035	7.84824	8.028826	8.137071	8.013326	-1.6167834	0.0241667	TRUE	-0.20749	1.612476	0.620164204
ECH1	Delta(3,5)-Delta(2,4)-dienoyl-Cc	8.04727	8.15382	8.087913	8.264217	8.364087	8.283021	-2.048961	0.0089339	TRUE	-0.20744	1.612273	0.620242514
H1FX	Histone 1x	8.34009	8.39209	8.368031	8.628084	8.483706	8.599569	-1.9214322	0.0119831	TRUE	-0.20372	1.59852	0.625578501
MUT	Methylmalonyl-CoA mutase	7.286	7.19645	7.151587	7.409667	7.437645	7.366316	-1.9080242	0.0123588	TRUE	-0.1932	1.560255	0.640920995
SDCBP	Syntenin-1	8.16473	8.15053	8.153642	7.981589	7.969698	7.991442	-4.6751526	2.11E-05	TRUE	0.17539	0.667743	1.497581278
TTI1	TELO2-interacting protein 1 hor	7.75163	7.68076	7.636516	7.42459	7.568774	7.53772	-1.5032512	0.0313869	TRUE	0.17927	0.661801	1.51102738
SCARB1	Scavenger receptor class B men	7.77851	7.88609	7.794887	7.569036	7.620359	7.72761	-1.4606334	0.0346232	TRUE	0.18083	0.659439	1.516441115
RBMS2	RNA-binding motif, single-stran	7.89264	7.70224	7.790762	7.585558	7.602346	7.646168	-1.4726086	0.0336815	TRUE	0.18386	0.654854	1.527058564
TTC37	Tetrapeptide repeat protei	7.91382	7.77405	7.868972	7.62935	7.735947	7.628441	-1.581594	0.0262063	TRUE	0.1877	0.649082	1.540637381
LAMC2	Laminin subunit gamma-2	9.23567	9.22277	9.175372	9.096184	8.992242	8.980719	-1.9941609	0.0101354	TRUE	0.18822	0.648302	1.542490745
WAPL	Wings apart-like protein homol	7.0271	6.93759	7.043208	6.889082	6.733056	6.805645	-1.5907514	0.0256595	TRUE	0.19337	0.640663	1.56088354
LPXN	Leupaxin	8.45487	8.56063	8.527952	8.263428	8.32974	8.349566	-2.1009949	0.0079251	TRUE	0.20024	0.630612	1.585761648
CSK	Tyrosine-protein kinase CSK	7.96223	7.92024	7.990674	7.710042	7.645349	7.853402	-1.5714088	0.0268282	TRUE	0.22145	0.600552	1.665135934
SPATS2	Spermatogenesis-associated sei	8.74995	8.73723	8.699421	8.412823	8.600951	8.49878	-1.7851231	0.0164012	TRUE	0.22468	0.596101	1.67756728
CDKN2AIP	CDKN2AIP N-terminal-like prote	8.22037	8.19229	8.018579	7.880547	7.828195	8.015329	-1.3113005	0.0488314	TRUE	0.23572	0.581137	1.720763835
CYP51A1	Lanosterol 14-alpha demethyla	8.6308	8.51433	8.585984	8.387842	8.389822	8.245002	-1.7998786	0.0158534	TRUE	0.23615	0.580568	1.722452463
FSTL1	Follistatin-related protein 1	6.7959	6.819	6.775575	6.657062	6.524059	6.498845	-2.0231383	0.0094812	TRUE	0.23684	0.579648	1.725185495
ATP6V1H	V-type proton ATPase subunit t	7.97132	8.13196	8.028333	7.837368	7.883834	7.685023	-1.4705797	0.0338392	TRUE	0.2418	0.573063	1.74500746
HIGD1A	HIG1 domain family member 1/	8.03587	8.22262	8.062221	7.77548	7.85936	7.954576	-1.4518478	0.0353307	TRUE	0.24377	0.570472	1.752933791
AAAS	Aladin	7.88056	7.76012	7.678916	7.642569	7.521206	7.412703	-1.3104854	0.0489232	TRUE	0.24771	0.565317	1.768919229
THOC3	THO complex subunit 3	8.1398	8.04642	8.121279	7.853941	7.979895	7.723377	-1.4624956	0.034475	TRUE	0.2501	0.562218	1.778669295
FADD	FAS-associated death domain p	8.41752	8.23417	8.382799	8.019776	8.061236	8.19179	-1.5320099	0.0293758	TRUE	0.25389	0.557322	1.794295021
UCK2	Uridine-cytidine kinase 2	7.94037	7.93367	7.854081	7.797959	7.570473	7.555239	-1.4916354	0.0322377	TRUE	0.26815	0.539327	1.854163148
CDK4	Cyclin-dependent kinase 4	9.8375	9.80622	9.830274	9.557507	9.454906	9.533965	-3.1698016	0.0006764	TRUE	0.3092	0.490678	2.037998143
MSMO1	Methylsterol monooxygenase 1	7.90859	8.02447	7.968439	7.698072	7.627812	7.638474	-2.8397557	0.0014463	TRUE	0.31238	0.487105	2.052945655
SQLE	Squalene monooxygenase	8.02901	7.89673	7.88959	7.68943	7.723385	7.456663	-1.5272845	0.0296972	TRUE	0.31528	0.483857	2.066727879
FN1	Fibronectin	7.99319	7.96399	7.953356	7.645268	7.64161	7.662577	-4.7217677	1.90E-05	TRUE	0.32036	0.478235	2.091021195
DUT	Deoxyuridine 5'-triphosphate n	8.434	8.46489	8.370584	8.234882	7.892043	8.167315	-1.3973612	0.0400533	TRUE	0.32508	0.473068	2.113860201
RFTN1	Raft-linking protein, isoform CR	7.79599	7.82111	7.771662	7.456599	7.320892	7.5927	-1.8833179	0.0130822	TRUE	0.33952	0.457588	2.185369638
IPO11	Importin-11	8.2006	8.22814	8.142639	7.881839	7.801511	7.807637	-3.2474239	0.0005657	TRUE	0.36013	0.436384	2.291558486
FADS2	Fatty acid desaturase 2	8.61977	8.50359	8.552562	8.266077	8.096497	8.230707	-2.3714233	0.0042518	TRUE	0.36088	0.435635	2.295501987
TRIP12	E3 ubiquitin-protein ligase TRIP	7.76426	7.9081	7.633933	7.44938	7.230966	7.448999	-1.6616565	0.0217943	TRUE	0.39231	0.405216	2.467818195
THBS1	Thrombospondin-1	9.15713	9.06907	9.104838	8.804893	8.602079	8.689427	-2.5202789	0.003018	TRUE	0.41155	0.387663	2.579560707
HMGCS1	Hydroxymethylglutaryl-CoA syn	9.4041	9.37662	9.383731	9.098643	8.804267	9.021466	-2.0215204	0.0095166	TRUE	0.41336	0.386047	2.590360912
FADS1	Fatty acid desaturase 1	7.83135	7.52822	7.768061	7.107992	7.192799	7.45991	-1.4996493	0.0316483	TRUE	0.45564	0.350233	2.855239486
IL1A	Interleukin-1 alpha	8.08982	7.96655	7.976467	7.689152	7.516855	7.452335	-2.3176364	0.0048124	TRUE	0.45817	0.348204	2.8718831
F3	Tissue factor	8.33194	8.39688	8.203358	8.059397	7.521193	7.819461	-1.433267	0.0368751	TRUE	0.51071	0.308524	3.24123765
ENAH	Protein enabled homolog	7.30695	7.54952	7.766435	6.833144	6.435067	7.000438	-1.669676	0.0213956	TRUE	0.78475	0.164152	6.091901127

