Proteomic Analysis of Trypanosoma cruzi Response to Ionizing Radiation Stress

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Abstract

Trypanosoma cruzi, the causative agent of Chagas disease, is extremely resistant to ionizing radiation, enduring up to 1.5 kGy of gamma rays. Ionizing radiation can damage the DNA molecule both directly, resulting in double-strand breaks, and indirectly, as a consequence of reactive oxygen species production. After a dose of 500 Gy of gamma rays, the parasite genome is fragmented, but the chromosomal bands are restored within 48 hours. Under such conditions, cell growth arrests for up to 120 hours and the parasites resume normal growth after this period. To better understand the parasite response to ionizing radiation, we analyzed the proteome of irradiated (4, 24, and 96 hours after irradiation) and non-irradiated T. cruzi using two-dimensional differential gel electrophoresis followed by mass spectrometry for protein identification. A total of 543 spots were found to be differentially expressed, from which 215 were identified. These identified protein spots represent different isoforms of only 53 proteins. We observed a tendency for overexpression of proteins with molecular weights below predicted, indicating that these may be processed, yielding shorter polypeptides. The presence of shorter protein isoforms after irradiation suggests the occurrence of post-translational modifications and/or processing in response to gamma radiation stress. Our results also indicate that active translation is essential for the recovery of parasites from ionizing radiation damage. This study therefore reveals the peculiar response of T. cruzi to ionizing radiation, raising questions about how this organism can change its protein expression to survive such a harmful stress.

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Introduction

Chagas disease, a neglected tropical disease caused by the protozoan parasite Trypanosoma cruzi, is considered to be a public health problem [1,2]. Over 10 million people are infected in Latin America and more than 100 million individuals live at risk of infection by blood transfusion, congenital, or oral transmission [3]. Forty years after its introduction, benznidazole and nifurtimox continue to be the first choice of treatment for Chagas disease. However, chemotherapy based on nitroheterocyclic compounds has a limited efficacy for patients in the chronic phase of infection by blood transfusion, congenital, or oral transmission [3]. Forty years after its introduction, benznidazole and nifurtimox continue to be the first choice of treatment for Chagas disease. However, chemotherapy based on nitroheterocyclic compounds has a limited efficacy for patients in the chronic phase of infection by blood transfusion, congenital, or oral transmission [3].

Another organism that is extremely resistant to ionizing radiation is the bacterium Deinococcus radiodurans, which can withstand radiation doses of up to 15 kGy [14]. D. radiodurans...
presents a very robust DNA repair apparatus; nevertheless, the biological responses to genomic lesions depend on its proteome integrity. Considering that ionizing radiation also induces protein damage through oxidative stress, a protected functional proteome ensures an efficient cell recovery from this type of stress [15]. Using the classical proteomic approach of two-dimensional differential gel electrophoresis (2D-DIGE) coupled with mass spectrometry (MS), Basu & Apte observed in a time-course analysis that some classes of proteins have a strong influence on stress responses. These proteins are mainly involved in processes such as DNA damage repair, protein synthesis and folding, and responses to oxidative stress [16].

Proteome versus transcriptome analyses have been highly recommended for studies with tripanosomatids, as they have very peculiar molecular features concerning their gene expression control. As a kinetoplastid, *T. cruzi* transcription is polycistronic and gene regulation occurs mainly post-transcriptionally, with mature mRNAs being generated by trans-splicing and polyadenylation [17,18]. The processing and stabilization of mRNAs are extremely important in trypanosomatid gene regulation [19,20]. Furthermore, other dynamic control mechanisms, such as posttranslational modifications, are fundamental in the regulation of gene expression and need to be better characterized in these organisms [21–23].

A time-course microarray study previously carried out by our group analyzed the *T. cruzi* gene expression in response to gamma radiation [7]. Among the 273 differentially expressed genes, 160 were upregulated and 113 were downregulated. The majority of the genes with assigned functions was downregulated. Translation, protein metabolic processes, and the generation of precursor metabolites and energy pathways were affected. Four mitochondrial genes and Retrotransposon Hot Spot genes were upregulated; likewise, the tyrosyl-DNA phosphodiesterase 1, a gene involved in DNA DSB repair, was also induced [7]. Taking into account the *T. cruzi* gene expression peculiarities, analyses of proteome changes after irradiation in different time points may contribute to the understanding of the parasite response to such stress.

In this work, we performed quantitative proteomic analyses using 2D-DIGE to ascertain the parasite response to ionizing irradiation. A total of 543 protein spots were found to be differentially expressed considering all analyzed time points and 53 protein spots were identified by tandem mass spectrometry (MS/MS). The great majority of the identified proteins was represented by several isoforms, suggesting that post-transcriptional and/or post-translational modifications are occurring as a consequence of gamma radiation exposure. Overexpression of tryparedoxin after irradiation was also observed, indicating that the parasite may be responding to the oxidative stress caused by irradiation. We also compared the time-course microarray and proteomic analyses. Although some of the protein expression patterns confirmed the microarray results, the correlation between mRNA and protein levels of the genes identified in both studies was extremely poor. In addition, treatment of the parasites with translation inhibitors showed that the synthesis of proteins putatively involved in the parasite response to stress is essential for its recovery from such a harmful stress.

**Materials and Methods**

**Cell Culture and Gamma Irradiation**

In this work, we used *T. cruzi* epimastigote forms of the CL Brener strain, which were isolated and characterized by Brener & Chiari [24]. Clones have been maintained as frozen stocks at Universidade Federal de Minas Gerais. Parasites were grown at 28°C in liver infusion tryptose (LIT) medium pH 7.3, supplemented with 10% fetal bovine serum, streptomycin sulfate (0.2 g/L), and penicillin (200,000 units/L). Cultures in the exponential growth phase (2 × 10⁷ cells/mL) were exposed for 20 minutes to 500 Gy of gamma radiation (1,576 Gy/h) in a cobalt (60 Co) irradiator (Centro de Desenvolvimento da Tecnologia Nuclear – CDTN, Belo Horizonte, Brazil). Cells were counted daily after irradiation to generate the growth curve.

**Cycloheximide and Puromycin Treatments**

Parasites exposed or not exposed to 500 Gy of gamma radiation were treated with cycloheximide (Calbiochem) 50 µg/mL for 15 minutes or with puromycin (Sigma) 25 µg/mL for 1 hour. Both drugs were added to the parasite cultures 4 hours after irradiation. Parasites were washed twice in phosphate buffered saline (137 mM NaCl, 4 mM Na₂HPO₄, 1.7 mM KH₂PO₄, and 2.7 mM KCl), the LIT medium was replaced, and the cells were counted.

**Protein Extract Preparation and DIGE Labeling**

Protein extracts were obtained, simultaneously, in triplicate for each condition: non-irradiated control (NI), 4 hours, and 96 hours after irradiation. Parasites (2 × 10⁹ cells) were washed twice with LIT medium followed by centrifugation at 1,500 g for 5 minutes at 4°C. Each pellet was resuspended in 200 µL of lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, Tris base, and a protease inhibitor mix (GE Healthcare, USA). Samples were mixed on vortex every 30 minutes during 2 hours of incubation at room temperature and subsequently centrifuged at 14,000 g for 30 minutes. The supernatants were aliquoted and stored at −70°C for further use. For all samples, protein concentration was determined using the 2D Quant kit (GE Healthcare, USA), according to manufacturer’s instructions.

Before labeling, samples had their pH adjusted to 8.5 with NaOH 0.05 M (as recommended by the manufacturer’s protocol). To reduce biological variation, a pool of protein extracts obtained from triplicates was used. A total of 50 µg of protein from each pool (NI, 4, 24, and 96 hours after irradiation) was labeled with CyDye DIGE Fluor Minimal Labeling Kit (GE Healthcare, USA). The dye swap strategy was used to avoid label bias, where each sample was labeled with 400 pmol of either Cy3 or Cy5. A mixture of all protein extracts (12.5 µg of each pool sample) was labeled with Cy2 as the internal control. Reactions were carried out on ice for 30 minutes in the dark and then stopped by the addition of 10 mM lysine.

**Two-Dimensional Gel Electrophoresis**

**First dimension.** The isoelectric focusing (IEF) was performed using Immobiline Dry Strips (GE Healthcare, USA) 18 cm in size, with a pH ranging from 4–7. Strips were loaded with 30 µg of protein per CyDye (total of 150 µg) and sample buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol (DTT), 0.002% bromophenol blue, and 1% IPG buffer (pH 4–7; GE Healthcare, USA). Passive rehydration followed overnight, at room temperature, in a strip holder (GE Healthcare, USA). The IEF protocol used in the Ettan IPGphor3 (GE Healthcare, USA) instrument was as follows: 50 mA, 4–7 pH, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 125 mM DTT) and then alkylated.
for an additional 15 minutes in an equilibration solution containing 13.5 mM iodoacetamide instead of DTT.

Second dimension. Equilibrated strips were briefly washed in 1x running buffer (25 mM Tris, 192 mM glycine, and 0.2% SDS) and placed on top of 12% acrylamide/bis-acrylamide gels, overlaid with a 0.3% agarose solution. Protein separation was carried out at 10°C, in an Ettan Dalt Six Electrophoresis System (GE Healthcare, USA), 45 mA per gel, until the dye front reached the bottom of the gel. Labeled proteins in each gel were visualized using the Typhoon FLA 9500 scanner (GE Healthcare, USA) at 100 μm image resolution with excitation/emission wavelengths for Cy5 (532/580 nm), Cy5 (635/670 nm), and Cy2 (488/520 nm). Gel images were uploaded and cropped using Image Loader Software (GE Healthcare, USA), then imported to DeCyder 2D software, version 7.0 (GE Healthcare, USA).

DIGE Data Analysis
For spot detection, the Differential In-gel Analysis (DIA) module of DeCyder 2D software, version 7.0 (GE Healthcare, USA), was used. The DIA co-detection algorithm exploits the identical spot patterns from multiple samples in the same gel. After the removal of some artifacts from the gels, spot quantification was performed automatically by normalizing the spot volumes against the internal control. The following steps were performed in the Biological Variation Analysis module, which uses images processed in DIA and matches spots across gels. One-way ANOVA and Student’s t-test were applied to evaluate differential protein expression levels between the groups of study. Spots classified as significantly differentially expressed were manually inspected. Abnormal spots were excluded from the analysis when necessary and gels were re-matched.

Trypsin in-Gel Digestion, Mass Spectrometry, and Protein Identification
Differentially expressed protein spots were excised and trypsin in-gel digestion was carried out overnight at 37°C with 20 ng/μL of trypsin (Promega, Sequencing Grade Modified Trypsin, USA), diluted in 25 mM ammonium bicarbonate. After trypsin digestion, peptides were extracted from the gel by washing twice with 30 μL of 50% acetonitrile and 5% formic acid solution and shaking for 15 minutes. Peptides were then concentrated (Eppendorf Concentrator 5301) to 10 μL and desalted using Zip-Tip (C18 resin, Millipore Corporation, USA). Once the peptides were eluted with a 0.5% agarose solution, Protein separation was carried out at 10°C, in an Ettan Dalt Six Electrophoresis System (GE Healthcare, USA), 45 mA per gel, until the dye front reached the bottom of the gel. Labeled proteins in each gel were visualized using the Typhoon FLA 9500 scanner (GE Healthcare, USA) at 100 μm image resolution with excitation/emission wavelengths for Cy5 (532/580 nm), Cy5 (635/670 nm), and Cy2 (488/520 nm). Gel images were uploaded and cropped using Image Loader Software (GE Healthcare, USA), then imported to DeCyder 2D software, version 7.0 (GE Healthcare, USA).

Manual Curation and Statistical Analysis
Peptide sequences obtained from MASCOT were aligned to the T. cruzi annotated genome using the BLAST tool from TriTrypDB (http://www.tritrypdb.org). Protein annotation was reassigned particularly when partial sequences were chosen by MASCOT and full-length sequences were available at the TriTrypDB. Once a final annotated and curated set of upregulated and downregulated spots was available, it was possible to assess the protein species by their expected and observed weights (retrieved from the TriTrypDB and calculated from the position in the 2D-DIGE, respectively).

For spot detection, the Differential In-gel Analysis (DIA) module of DeCyder 2D software, version 7.0 (GE Healthcare, USA), was used. The DIA co-detection algorithm exploits the identical spot patterns from multiple samples in the same gel. After the removal of some artifacts from the gels, spot quantification was performed automatically by normalizing the spot volumes against the internal control. The following steps were performed in the Biological Variation Analysis module, which uses images processed in DIA and matches spots across gels. One-way ANOVA and Student’s t-test were applied to evaluate differential protein expression levels between the groups of study. Spots classified as significantly differentially expressed were manually inspected. Abnormal spots were excluded from the analysis when necessary and gels were re-matched.

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Results and Discussion
The Effects of Protein Synthesis Inhibition on the Growth of T. cruzi Epimastigote Cells Exposed to Gamma Radiation
Normal growth of epimastigote cells was affected by protein synthesis inhibition (using 50 μg/mL cycloheximide or 25 μg/mL puromycin) and by ionizing radiation treatment (500 Gy), as shown in Figure 1. However, irradiation promoted a more drastic growth arrest that persisted for approximately 96 hours; after this period, the parasites resumed normal growth, reaching the stationary phase 216 to 240 hours after irradiation (Figure 1). The treatment of NI cells with cycloheximide (Figure 1A) or puromycin (Figure 1B) retarded the cell growth by at least 24 hours when compared with non-treated cells, but did not lead to parasite death. Conversely, the combination of cycloheximide treatment and gamma radiation was lethal to 40% of the parasites. The remaining parasites resumed growth only 270 hours after irradiation, reaching the stationary phase 408 hours after irradiation (Figure 1A). For puromycin, a similar effect was observed, but treated cells resumed normal growth earlier when compared with cycloheximide-treated parasites (Figure 1B) and, in this case, no parasite death was detected.

These observations indicate that an active translation is important for the recovery of parasites from damage caused by ionizing radiation. Protein synthesis blockage is potentially impairing the translation of newly synthesized or pre-existing mRNAs that code for proteins involved in triggering cell proliferation. These proteins may accumulate within 24 hours after irradiation and act later after irradiation, when parasites have
their genome recomposed and are ready to resume growth. Interestingly, our previous work using microarray did not demonstrate upregulation of a variety of mRNAs coding for proteins involved in cell proliferation or cell cycle control, even 96 hours after irradiation, and only a cyclin transcript was found to be affected (1.67-fold change increase) 24 hours after irradiation [7].

Analysis of the Proteome Profile of T. cruzi Epimastigote Cells Exposed to Gamma Radiation

Since we have verified that newly synthesized proteins have an impact on parasite recovery from irradiation stress, we decided to analyze time-course T. cruzi changes in the proteome induced by irradiation. Protein extracts were obtained from control NI cells and 4, 24, and 96 hours after irradiation. No significant losses in the total protein content and integrity were observed by 1D-gel electrophoresis (Figure S1). Using the 2D-DIGE approach, six gels were produced following the experimental design specified in Table 1. This technique was chosen due to its greater sensitivity, reduced gel-to-gel variation, and its capacity for quantitative measurements of the relative abundance of each protein in a complex sample [25]. Figure 2 illustrates 2D-DIGE gels at all time points. An average of 2,186±140 spots was found when compared with the master gel. From those, 543 presented altered expressions after irradiation, considering all time points (one-way ANOVA, p<0.01) and 215 were identified by peptide mass fingerprint, corresponding to 53 different proteins (Table 2). Almost half of these proteins (26) were represented by more than one spot in the 2D gel (ranging from 2–12 spots per protein), indicating the presence of several isoforms for the same protein. These results suggest that post-translational modifications or protein processing are occurring during the response to gamma radiation stress. We have manually annotated the function of all 53 identified proteins via a literature search. Proteins were then manually assigned to 15 different classes according to their biological function (Figure S3).

Additionally, the Student’s t-test (p<0.01) was applied to verify which proteins were differentially expressed in each time point when compared with the NI sample. The overall and time-specific number of downregulated protein spots was higher than the
number of upregulated ones (Figure 3A). These findings are different from those described previously in our microarray study. Twenty-four hours post-irradiation, the number of downregulated genes decreases drastically, reaching only 6 down-expressed transcripts 96 hours after irradiation, while the number of upregulated genes increases [7]. A linear regression analysis between mRNA and protein levels from genes concomitantly identified in both studies was carried out for each time point. The correlation was extremely poor at all time points, starting with multiple R2 = 0.064 at 4 hours and reaching R2 = 0.27 and 0.24 at 24 and 96 hours post-irradiation, respectively (data not shown). Although a very low correlation was obtained, the result is in agreement with other studies performed in both prokaryotes and eukaryotes using classical methodologies such as microarray, Serial Analysis of Gene Expression (SAGE) and RNA-Seq for transcriptomic expression data, and 2-DE, Multi-dimensional protein identification technology, and MS for proteomics data [26–31]. This analysis reinforces the idea that transcriptomic and proteomic approaches are complementary, not confirmatory [29].

Moreover, changes in the T. cruzi proteome are more evident 24 hours after exposure to gamma radiation (Figure 3). This scenario suggests that epimastigote cells present an immediate but subtle response to gamma radiation characterized by 12 induced and 21 repressed protein spots 4 hours after irradiation (Figure 3A). Between 4 and 24 hours after irradiation, a more intense response to stress was observed and most of the induced

| Gel | NI | 4 h | 24 h | 96 h | Pool |
|-----|----|-----|------|------|------|
| 1   | Cy3| Cy5 | Cy5  | Cy2  | Cy2  |
| 2   | Cy3| Cy5 | Cy5  | Cy2  | Cy2  |
| 3   | Cy5| Cy3 | Cy5  | Cy2  | Cy2  |
| 4   | Cy3| Cy5 | Cy3  | Cy2  | Cy2  |
| 5   | Cy3| Cy5 | Cy5  | Cy2  | Cy2  |
| 6   | Cy3| Cy5 | Cy5  | Cy2  | Cy2  |

Each two-dimensional gel was loaded with 50 μg of total protein extract per sample, labeled either with Cy3 or Cy5. The internal control (a pool containing 50 μg of all time point proteins: NI, 4, 24, and 96 hours after irradiation) was labeled with Cy2.

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Figure 2. 2D-DIGE analysis of total protein extracts of irradiated and NI epimastigote cells. Gel images 1–6 (see the experimental design in Table 1) showing – in triplicate – parasite proteins from each time point, labeled either with Cy3 (green) or Cy5 (red). Proteins were separated in the first dimension along a pH gradient (pH 4–7, 18 cm Immobiline DryStrip (GE Healthcare, USA), and in the second dimension in a 12% polyacrylamide gel. The molecular weight marker (MW) is indicated in kDa.

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### Table 2. Protein data for the 53 proteins identified in this study.

| Description | TriTrypDB ID (TcCLB.) | Ref. Spot pl | MW | 4 hs | 24 hs | 96 hs | Anova | Peptides matched | Sequence coverage | Mascot score |
|-------------|-----------------------|--------------|----|------|-------|-------|-------|-----------------|------------------|-------------|
| 14-3-3 protein, putative | 511167.90 | 153 | 5.00/4.78 | 24.3/29.1 | −1.35 | 1.08 | −1.16 | 5.61E−5 | 2 | 12% | 73 |
| 40S ribosomal protein S12, putative | 508551.20 | 195 | 4.82/4.78 | 10.9/15.9 | 1.45 | 3.52 | 2.92 | 1.32E−5 | 2 | 24% | 110 |
| Actin, putative | 510571.39 or 510127.79 or 510571.30 | 66 | 5.75/5.46 | 45.9/41.2 | −1.64 | −14.92 | −12.65 | 8.20E−9 | 3 | 10% | 136 |
| Alpha tubulin, putative | 411235.9 | 69 | 6.07/4.7 | 44.7/49.8 | −1.01 | −12.65 | −10.53 | 2.00E−7 | 5 | 16% | 248 |
| ATPase beta subunit | 509233.180 | 26 | 5.15/5.07 | 58.6/55.7 | −2.42 | −21.04 | −18.44 | 7.26E−7 | 5 | 18% | 253 |
| Beta tubulin, putative | 506563.40 | 107 | 5.58/4.43 | 45.0/49.8 | −1.36 | −4.9 | −5.17 | 1.02E−7 | 3 | 7% | 156 |
| Calreticulin, putative | 510685.10 | 81 | 6.37/4.49 | 42.2/46.2 | −1.07 | 1 | 1.52 | 1.81E−5 | 2 | 6% | 89 |
| Chaperonin containing t-complex protein, putative | 511725.250 | 12 | 5.02/4.80 | 69.7/59.2 | −1.95 | −12.81 | −16.4 | 4.12E−7 | 3 | 9% | 113 |
| Chaperonin HSP60; mitochondrial precursur GroEL protein; heat shock protein 60 (HSP60) | 507641.290 or 507641.300 or 510187.551 | 17 | 5.44/5.14 | 66.2/59.2 | −4.9 | −19.19 | −19.86 | 1.27E−8 | 9 | 24% | 470 |
| 2.4 | 66.0/59.2 | −4.07 | −9.24 | −10.18 | 1.75E−8 | 3 | 20% | 111 |
| 2.0 | 65.8/59.2 | −3.99 | −8.83 | −9.9 | 1.25E−7 | 7 | 18% | 354 |
| 2.3 | 62.3/59.2 | −2.03 | −8.02 | −9.51 | 6.42E−8 | 7 | 18% | 191 |
| 2.4 | 62.2/59.2 | −1.72 | −11.52 | −13.96 | 9.42E−8 | 11 | 31% | 533 |
| 2.5 | 61.5/59.2 | −1.78 | −16.99 | −15.1 | 1.61E−7 | 6 | 16% | 205 |
| Description                                      | TriTrypDB ID (TcCLB.) | Ref. Spot pl | MW     | 4 hs  | 24 hs | 96 hs | Anova | Peptides matched | Sequence coverage | Mascot score |
|--------------------------------------------------|-----------------------|--------------|--------|-------|-------|-------|-------|------------------|-------------------|--------------|
| Observed/Expected                                |                       |              |        |       |       |       |       |                  |                   |              |
| 28                                               | 5.28/5.14             | 62.8/59.2    | -2.16  | -9.12 | -9.25 | 3.25E-8 | 5     | 14%             | 195              |              |
| 88                                               | 4.68/5.14             | 51.2/59.2    | -1.06  | -3.44 | -2.79 | 9.76E-7 | 3     | 18%             | 112              |              |
| 89                                               | 4.83/5.14             | 51.8/59.2    | -1.28  | -7.85 | -7.58 | 3.21E-7 | 3     | 18%             | 150              |              |
| 106                                              | 5.5/5.14              | 43.1/59.2    | 1.23   | -5.57 | -4.86 | 2.11E-7 | 6     | 15%             | 177              |              |
| 131                                              | 5.0/5.14              | 34.0/59.2    | -1.76  | -9.8  | -7.3  | 3.45E-8 | 1     | 5%              | 76               |              |
| 162                                              | 5.6/5.14              | 20.3/59.2    | 1.97   | 1.81  | 1.97  | 4.31E-7 | 2     | 10%             | 62               |              |
| 28                                               | Chaperonin; Tcomplex-protein 1; theta subunit; putative | 906247.50 | 16     | 5.42/5.12 | 68.9/58.3 | -2.42 | -4.81 | 5.44 | 6.21E-8 | 3 | 7% | 138 |
| 78                                               | Cystathionine beta-synthase, cysteine synthase, serine sulfhydrylase (CBS) | 508177.120 or 506905.50 | 2     | 1.06  | 3.44  | 2.79  | 9.76E-7 | 3 | 18% | 150 |
| 124                                              | Cytochrome c oxidase subunit IV; putative | 506529.360 or 501889.50 | 2     | 1.28  | 7.85  | 7.58  | 3.21E-7 | 3 | 18% | 150 |
| 200                                              | Cytochrome c oxidase subunit V, putative | 510565.30 or 508503.20 | 2     | 1.28  | 7.85  | 7.58  | 3.21E-7 | 3 | 18% | 150 |
| 200                                              | D-isomer specific2-hydroxycid dehydrogenase-protein | 510099.120 | 119    | 6.72/6.41 | 35.2/38.5 | -1.46 | -6.4 | -5.31 | 6.69E-7 | 12 | 36% | 103 |
| 197                                              | Dihydrolipoamide acetyltransferase precursor | 509717.20 and 510105.170 | 0     | 5.7/5.8 | 6.2/4.96 | -1.32 | -7.65 | -5.03 | 1.35E-7 | 1 | 3% | 61 |
| 197                                              | Dihydrolipoyl dehydrogenase; putative (GCVL-2) | 507089.270 or 511025.110 | 73    | 6.6/7.4 | 52.3/54.9 | 1.01  | -2.41 | -2.34 | 2.36E-7 | 3 | 7% | 98 |
| 29                                               | Dipetidyl-peptidase | 508601.141 or 509205.120 | 29    | 5.40/5.60 | 62.4/7.44 | -2.19 | -5.73 | -4.96 | 5.14E-7 | 2 | 3% | 58 |
| 30                                               | Drug resistance protein | 44477.10 | 123    | 5.54/5.45 | 37.2/50.3 | -1.18 | 1.68 | 2.34 | 5.72E-6 | 1 | 5% | 26 |
| 36                                               | Elongation factor 2, putative | 510963.90 | 36    | 6.3/5.56 | 55.4/94.2 | -1.82 | -6.16 | -5.58 | 5.78E-7 | 4 | 6% | 197 |
| 49                                               | Enolase | 504105.140 | 72    | 6.5/6.2 | 50.646.4 | -2.63 | -5.28 | -6.37 | 6.94E-8 | 2 | 7% | 73 |

T. cruzi Proteome after Gamma Radiation
| Description | TriTrypDB ID (TcCLB.) | Ref. Spot pl | MW | 4 hs | 24 hs | 96 hs | Anova | Peptides matched | Sequence coverage | Mascot score |
|-------------|-----------------------|--------------|-----|------|-------|-------|-------|-----------------|------------------|-------------|
| Eukaryotic translation initiation factor 6 (eIF-6); putative | 506679.70 | 168 | 5.04/6.09 | 20.7/33.2 | 1.55 | 1.31 | 1.27 | 3.90E–5 | 2 | 9% | 127 |
| Glucose-regulated protein 78, putative | 506585.40 | 2 | 5.19/4.82 | 76.9/71.3 | −2.45 | −23.64 | −16.54 | 1.02E–7 | 4 | 12% | 198 |
| Glutamamyl carboxypeptidase; putative | 507699.40 or 507657.20 or 507657.10 | 70 | 6.18/6.51 | 47.6/43.4 | −1.22 | −2.15 | −2.41 | 1.66E–6 | 2 | 6% | 92 |
| Glutamate dehydrogenase | 508111.30 | 212 | 6.72/8.05 | 15.9/45.0 | 1.62 | 1.64 | 2 | 663E–6 | 2 | 7% | 77 |
| Glycerate kinase, putative | 50741.170 | 199 | 5.50/5.85 | 44.9/70.9 | −0.16 | −0.76 | −0.86 | 1.75E–7 | 2 | 7% | 79 |
| Heat-shock protein 70kDa, putative | 509543.50 and 511257.10 | 1 | 5.14/4.55 | 76.1/70.0 | −2.15 | −9.69 | −10.51 | 3.28E–8 | 2 | 11% | 70 |
| Heat-shock protein 70kDa, putative | 506135.9 | 91 | 4.98/4.60 | 52.6/70.0 | −1.12 | −4.52 | −3.7 | 2.44E–7 | 4 | 18% | 205 |
| Heat-shock protein 70kDa, putative | 511211.160 | 7 | 5.55/5.85 | 72.7/70.9 | −1.34 | −4.53 | −3.34 | 1.15E–7 | 4 | 15% | 191 |
| Heat shock 70 kDa protein, mitochondrial precursor, putative | 507029.30 | 8 | 5.65/5.71 | 72.4/71.0 | −1.39 | −4.87 | −3.73 | 9.36E–9 | 2 | 4% | 81 |
| Heat shock 70 kDa protein, mitochondrial precursor, putative | 507029.30 | 9 | 5.77/5.71 | 72.7/71.0 | −1.42 | −5.3 | −4 | 9.88E–9 | 6 | 14% | 293 |
| Heat shock 70 kDa protein, mitochondrial precursor, putative | 507029.30 | 10 | 5.80/5.71 | 73.1/70.0 | −1.32 | −3.85 | −3.08 | 7.83E–8 | 3 | 7% | 85 |

*Note: The table continues with similar entries for various proteins and conditions.*
| Description | TrTrypDB ID (TcCLB.) | Ref. | Spot pI | MW | 4 hs | 24 hs | 96 hs | Anova | Peptides matched | Sequence coverage | Mascot score |
|-------------|----------------------|------|---------|----|------|------|------|-------|-----------------|-----------------|--------------|
| Heat-shock protein 85kDa, putative | 509643.130 or 507713.30 or 509105.140 | 93 | 4.89/4.79 | 47.6/80.7 | 1.43 | −1.5 | −1.15 | 3.25E−7 | 3 | 5% | 114 |
| Hypothetical protein, conserved | 505989.110 | 182 | 4.46/4.50 | 18.4/22.2 | −1.25 | 3.47 | 3.51 | 1.92E−5 | 2 | 11% | 114 |
| Hypothetical protein, conserved | 506605.120 or 511239.110 | 202 | 5.72/4.99 | 15.1/28.6 | 2.75 | 7.08 | 7.83 | 1.34E−9 | 4 | 22% | 174 |
| Hypothetical protein | 508817.20 or 503801.70 | 154 | 5.38/5.88 | 23.5/56.7 | 1.78 | −3.9.6 | 3.33 | 3.66E−6 | 9 | 41% | 92 |
| Nucleoside phosphorylase, putative | 508999.9 and 509569.100 | 121 | 6.90/6.42 | 34.2/37.0 | −1.18 | −5.59 | −4.38 | 2.96E−8 | 3 | 16% | 190 |
| Oligopeptidase B, putative | 503995.50 | 47 | 5.86/6.1 | 55.2/80.8 | −0.71 | −3.9.4 | −7.55 | 9.76E−9 | 2 | 3% | 68 |
| Oligopeptidase B, putative | 503995.50 | 63 | 5.86/6.1 | 52.0/80.9 | −1.12 | −2.26 | −2.47 | 8.01E−7 | 2 | 3% | 69 |
| Oligopeptidase B, putative | 503995.50 | 60 | 6.09/5.96 | 56.1/68.6 | 1.21 | −3.51 | −4.33 | 6.89E−8 | 1 | 2% | 24 |
| Oligopeptidase B, putative | 503995.50 | 39 | 5.57/5.19 | 55.4/51.2 | −1.74 | −5.55 | −7.07 | 8.47E−7 | 2 | 6% | 66 |
| Oligopeptidase B, putative | 503995.50 | 40 | 5.50/5.19 | 54.4/51.2 | −1.64 | −5.35 | −9.41 | 2.26E−8 | 1 | 3% | 31 |
| Peroxiredoxin; trypareredoxin peroxidase | 509499.14 | 189 | 5.16/6.92 | 18.1/25.5 | 1.11 | 1.42 | 1.73 | 7.81E−7 | 3 | 15% | 174 |
| Phosphoglycerate kinase, putative or 3-phosphoglycerate kinase, glycosomal (PGKA) | 511419.40 or 509999.90 or 511419.50 or 509999.100 | 74 | 6.76/7.4 | 51.9/54.90 | −2.99 | −5.56 | −5.35 | 2.46E−7 | 1 | 3% | 78 |
| Prostaglandin F2 alpha synthase (TcPGFS) | 508461.80 | 14 | 5.11/6.43 | 68.1/42.2 | −1.19 | −6.45 | −7.01 | 1.59E−6 | 4 | 14% | 169 |
| Prostaglandin F2 alpha synthase (TcPGFS) | 508461.80 | 111 | 5.91/6.43 | 40.0/42.2 | −1.31 | −11.76 | −7.75 | 1.74E−8 | 5 | 17% | 284 |
| Prostaglandin F2 alpha synthase (TcPGFS) | 508461.80 | 113 | 6.10/6.43 | 40.0/42.2 | −1.11 | −10.19 | −6.05 | 1.75E−8 | 8 | 38% | 116 |
| Prostaglandin F2 alpha synthase (TcPGFS) | 508461.80 | 114 | 6.09/6.43 | 38.7/42.2 | −1.12 | −6.24 | −5.05 | 3.12E−7 | 4 | 13% | 188 |
| Prostaglandin F2 alpha synthase (TcPGFS) | 508461.80 | 144 | 6.15/6.43 | 26.7/42.2 | 1.49 | −1.65 | −1.39 | 5.49E−8 | 4 | 13% | 217 |
| Description                                                                 | TriTrypDB ID (TcCLB.) | Ref. Spot pl | MW          | 4 hs | 24 hs | 96 hs | Anova          | Peptides matched | Sequence coverage | Mascot score |
|-----------------------------------------------------------------------------|------------------------|--------------|-------------|------|-------|-------|----------------|------------------|------------------|--------------|
| **Observed/Expected**                                                       |                        |              |             |      |       |       |                |                  |                  |              |
| Protein disulfide isomerase                                                 | 506247.10 or 507611.370 | 161          | 5.52/6.43   | 22.1/42.2 | 1.74 | 1.88 | 1.99 | 6.34E–8        | 9                | 31%             | 92            |
| Pyruvate dehydrogenase E1 beta subunit; putative                           | 510091.80              | 132          | 5.03/5.02   | 30.9/37.8 | 1.08 | -2.21 | -1.88 | 1.27E–6        | 5                | 20%             | 191           |
| Pyruvate kinase 2, putative                                                 | 507993.390 or 511281.60 | 133          | 4.62/5.02   | 40.7/37.8 | 1.02 | -4.58 | -3.83 | 4.13E–7        | 2                | 6%              | 108           |
| Pyruvate phosphate dikinase                                                 | 510101.140             | 194          | 5.00/8.27   | 13.5/100.8 | 1.63 | 10.51 | 7.42 | 7.88E–9        | 2                | 3%              | 94            |
| Receptor for activated C kinase 1, putative                                | 511211.120 or 511211.130 | 122          | 5.93/6.04   | 35.4/35.0 | -1.22 | -6.66 | -6.06 | 2.31E–9        | 3                | 11%             | 122           |
| S-adenosylhomocysteine hydrolase                                            | 511229.50 or 511589.200 | 193          | 5.25/6.64   | 12.9/48.4 | 1.41 | -1.23 | -1.1  | 3.74E–6        | 2                | 7%              | 80            |
| Seryl-tRNA synthetase                                                       | 511163.1 or 506777.80   | 140          | 8.53/5.41   | 28.9/25.7 | 1.4  | -1.07 | 1.28  | 3.36E–6        | 3                | 19%             | 97            |
| succinyl-CoA ligase [GDP-forming] beta-chain, putative                      | 507767.10              | 150          | 4.92/5.58   | 26.2/34.5 | 1.63 | 2.03  | 2.25  | 1.08E–6        | 2                | 7%              | 87            |
| Thiol–dependent reductase 1; putative; thiol transferase; putative; glutathione s-transferase putative | 509105.70 or 503419.30 | 62           | 6.00/5.83   | 51.9/50.7 | -1.18 | -2.07 | -1.56 | 4.56E–6        | 2                | 6%              | 86            |
| Trans-sialidase                                                             | 509927.10              | 158          | 6.21/5.83   | 21.5/30.7 | -1.32 | -2.17 | -2.64 | 4.48E–6        | 1                | 3%              | 32            |
| Trypanoxidin peroxidase                                                     | 504750.710 or 503645.10 | 210          | 6.24/6.75   | 11.5/22/4 | 2.17 | 3.18  | 3.42  | 8.05E–5        | 1                | 5%              | 33            |
| Tyrosine aminotransferase                                                   | 510187.20 and 510187.50 | 211          | 6.66/6.75   | 17.0/22.4 | -1.52 | -3.96 | -1.82 | 3.25E–8        | 3                | 22%             | 105           |
| Vacuolar ATP synthase subunit B                                             | 506025.50 or 511209.10  | 37           | 5.71/5.29   | 59.1/55.5 | -1.69 | -3.54 | -4.01 | 2.44E–7        | 6                | 21%             | 207           |
Exposure to Gamma Rays Increases the Levels of Shorter and/or Processed Proteins in Epimastigote Cells

When analyzing the set of upregulated proteins (especially 24 and 96 hours after irradiation), we observed a tendency for the overexpression of shorter molecules to the detriment of longer ones. The upregulated protein spots (red-colored dots) are mainly at the lower part of the gel (lower molecular weight), while the downregulated protein spots (green-colored dots) are more sparsely distributed across the gel (Figure 4A). In addition, low molecular weight protein spots tended to have larger fold changes when compared with those with molecular weights close to the expected value (Figure 4B). The Wilcoxon test was applied and confirmed that the median values of the molecular weight of downregulated and upregulated protein spots were different for each time point (p < 1e-9; median values of 55.45/19.39, 45.64/19.38, and 46.51/19.42 for 4, 24, and 96 hours, respectively; Figure 5A).

When we considered the expected molecular weight of the full-length isoform (predicted size obtained from the TriTryDB website) of both upregulated and downregulated proteins, we noticed a decrease in protein size in the former case and an increase in the latter case, thus showing the emergence of lower molecular weight protein isoforms after irradiation (Figure 5B). We then decided to confirm if the observed molecular weight of these proteins in the 2D gels was in agreement with their expected molecular weight. In the case of upregulated proteins, the observed molecular weight was significantly lower than expected (Figure 5B), indicating that these proteins might be processed, yielding shorter polypeptides. It is important to note that this result does not seem to be a consequence of protein degradation, since clear spots can be observed in the 2D gel, indicating the presence of a large amount of identical polypeptides in this region of the gel.

This would not be the case if proteins were degraded, considering that in this situation peptides of variable size would be generated and no clear spot would be observed in the gel. These results may indicate the emergence of new protein isoforms, as the result of protein processing, alternative splicing of mRNAs, and/or alternative translational start/stop sites after irradiation. Alternative splicing of transcripts has the potential to expand the repertoire of proteins. Recent studies have estimated that all multi-exonic human genes are able to produce at least two alternatively spliced mRNA transcripts by alternative splicing, generating different proteins isoforms with altered structures and biological functions [32]. In trypanosomatids, mature mRNAs are generated after two processing events: trans-splicing to add the spliced leader (SL) sequence to the 5′ end of transcripts and subsequent polyadenylation [17]. A genome-wide analysis comparing the SL addition site along the developmental cycle of the parasite suggests that alternative trans-splicing plays an important role in differential gene expression [33]. The occurrence of alternative trans-splicing could be an explanation for the presence of so many different isoforms in T. cruzi after radiation response.

A similar event has already been described in D. radiodurans, since different isoforms of the single-strand binding (SSB) protein were produced after ionizing radiation stress induction. SSB proteins are vital for cell survival due to their involvement in processes such as DNA replication, recombination, and repair. The SSB protein spots in the gel followed a dynamic pattern of appearance, indicating a progressive processing of the C-terminal acidic tail, perhaps upon its interaction with ssDNA. The observed isoelectric point (pI) and molecular weight of deinococcal SSB isoforms were in agreement with the in silico-predicted pI and molecular weight of the SSB proteins shortened from the C-terminal end [16].

Intriguingly, in most of the observed processed proteins, the identified peptide sequences were the same or nearly the same in all sequenced protein spots and, therefore, it was impossible to define the actual outcome of the protein processing. As a particular case of study, the protein annotated as prostaglandin F2 alpha synthase, which is similar to NADH-flavinoxidoreductase, is processed to a total of six different forms (Figure S2). While the expected molecular weight for the annotated sequence is of 40 kDa, only two isoforms are nearly this size (~40 kDa) and

Figure 3. Protein spots differentially expressed at all time points. A) Number of downregulated and upregulated protein spots per time point. B) Venn diagram showing the overlaps of 32 protein spots differentially regulated among the three time points and of the 428 protein spots between 24 and 96 hours.

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and repressed protein spots were still significantly altered until 96 hours (428 spots; Figure 3B). This finding indicates a sustained alteration in the abundance of specific T. cruzi proteins 24 hours after gamma radiation exposure. When analyzing the T. cruzi proteome 24 hours after irradiation, we found that, from the 59 exclusive spots, approximately 66% were repressed and 34% were induced. However, the majority of the 23 exclusive spots found 96 hours after irradiation were induced (approximately 61%).
were, in fact, the most downregulated isoforms. A third isoform has a predicted molecular mass of 68 kDa, greatly exceeding the expected protein size. As all of the MS/MS-identified peptides were mapped to the C-terminal portion of the isoforms, there is no information to characterize the N-terminal of this enlarged protein naturally present in the NI parasites and downregulated after exposure to gamma radiation. A smaller (29 kDa) protein is expressed in approximately equal levels before and after radiation exposure, while an even smaller (22 kDa) protein species is exclusively present in irradiated cells. This is an interesting example, representative of multiple cases, in which we have observed the emergence of shorter isoforms of a same protein after epimastigote irradiation. The list of processed proteins expressing shorter isoforms after irradiation includes alpha and beta-tubulin, D-isomer specific 2-hydroxyacid dehydrogenase-protein, elongation factor 2, glycerate kinase, pyruvate dehydrogenase E1 beta subunit, tyrosine aminotransferase, and several heat shock proteins (HSPs), such as HSP60, DnaK, HSP70s, and glucose-regulated protein 78. Apart from the previously discussed SSB proteins in *D. radiodurans*, very few references in the scientific literature mention the presence of shorter protein fragments after radiation exposure in any organism.

Interestingly, Parodi-Talice and collaborators [34] observed a similar pattern in *T. cruzi* for the proteins glutamate dehydrogenase (GluDH), HSP70, and alpha and beta-tubulins, where lower molecular weight isoforms were differentially expressed during metacyclogenesis when compared with isoforms with the predicted molecular weight. The transformation of epimastigotes into metacyclic trypomastigotes is a complex process of differentiation, requiring a controlled production of various proteins [34]. Similarly, a quantitative time-course proteome analysis for the schizont-stage of *Plasmodium falciparum* (34 to 46 hours after invasion) demonstrated that actin-I, enolase, HSPs, and eukaryotic initiation factor 4A and 5A presented more than one isoform. The isoforms also showed different expression patterns at the different time points analyzed. *P. falciparum* is characterized by a complex life cycle, undergoing extensive morphological and metabolic changes, which reflects its capacity to survive in different host environments [35]. According to the authors, post-translational modifications may be a very important strategy for the parasites to control gene expression during differentiation [34,35].

### Differentially Expressed Proteins after Gamma Radiation Exposure

Regarding the differentially expressed proteins, many of the listed proteins in Table 2 and Figure S3 are related to the protein synthesis process that seems to be upregulated, except for some protein spots of the elongation factor 2 that show a reduction in their levels. This may be a response to compensate for the processing of proteins that occurs after irradiation. This response may also enhance the synthesis of specific proteins that will possibly play a role in the stress response. The results obtained from the analyses of translation inhibition and proteomic profile after irradiation place de novo protein synthesis as an important
cellular response to gamma radiation. The same pattern is observed in *D. radiodurans*, where proteins related to translation/folding displayed either enhanced or de novo expression in the first hour of post-irradiation recovery. Proteins involved with DNA repair and oxidative stress alleviation were also induced in *D. radiodurans* under ionizing radiation stress [16].

Proteins involved in protein folding processes, such as chaperones, are mostly downregulated post-irradiation (Figure S3). This represents an unexpected result, since these proteins are classically involved with stress response by stabilizing newly synthesized protein molecules. Nevertheless, this result is in agreement with transcriptomic data observed in microarray experiments [7]. It is worth noting that, although HSPs are mostly downregulated, processed forms of these molecules are upregulated and may even be functional. Interestingly, the two chaperones localized in the endoplasmic reticulum (calreticulin and protein disulfide isomerase) are upregulated after gamma radiation exposure, which may indicate an important role of this compartment in the ionizing radiation stress response, suggesting the existence of an unfolded protein response-like in this condition [36].

Another unexpected result is the downregulation of proteins involved in the ATP metabolism (namely the beta subunit of ATP synthase and the subunit IV of cytochrome c oxidase), although another member of this class is upregulated (cytochrome c oxidase subunit V). The outcome of this result is not clear and a more in-depth study of the cell energy metabolism would be important.

Perhaps the most remarkable observation in the post-irradiation proteome investigated here is the putative decline in the activity of the glycolytic and amino acid metabolism pathways. Several important enzymes of glycolysis were downregulated after gamma radiation exposure. Accordingly, the only enzyme (pyruvate phosphate dikinase) from gluconeogenesis listed here was upregulated. Most enzymes involved in the amino acid metabolism were also downregulated, but shorter isoforms of the GluDH were upregulated after irradiation. They consist of three isoforms with experimental molecular weights (15 kDa) lower than the predicted values (45 kDa), suggesting once again the occurrence of post-transcriptional modifications/processing of important metabolic enzymes during the stress response. GluDH catalyzes the NAD- and/or NADP-dependent reversible deamination of L-glutamate to form alpha-ketoglutarate and is essential for the metabolism of amino nitrogen in organisms ranging from bacteria to mammals [37]. *T. cruzi* has a metabolism that is largely based on the consumption of amino acids, mainly, proline, aspartate, and glutamate, which constitute the main carbon and energy sources of the epimastigote forms. In *T. cruzi*, GluDH has NADP-specific

**Figure 5. Boxplots of peptide molecular weights.** A) Distribution of the observed molecular weight in downregulated (green) or upregulated (red) protein spots at each time point analyzed. B) comparison between the distribution of the expected (E) and observed (O) molecular weights among downregulated or upregulated protein spots 24 and 96 hours after irradiation. A single asterisk corresponds to p<0.05 and a double asterisk corresponds to p<0.001. doi:10.1371/journal.pone.0097526.g005
stress and continue reproduction [46].

ing those required for DSB repair, allowing them to recover from the cell and indicating the presence of oxidative stress. The nucleotide 8-oxo-dGMP, or another secondary metabolite produced by polymerases. Parasites overexpressing heterologous MutT also increase the levels of cytosolic and mitochondrial DNA by polymerases. Parasites overexpressing MutT hydrolyses 8-oxo-dGTP in the nucleotide pool, returning it to the monophosphate form so that it cannot be incorporated into DNA. MutT enzyme product, 8-oxod-GMP, can generate an oxidative stress signal, enabling the cells to overcome this stress. The MutT enzyme product, 8-oxod-GMP, can generate an oxidative stress signal, enabling the cells to overcome this stress. The hypothesis was that proteins with important roles after irradiation in T. cruzi would have an amino acid composition different than what is observed in the set of all T. cruzi proteins and in the respective orthologues in T. brucei (which is not radio-resistant).

When we compared T. cruzi proteins that were upregulated after radiation exposure with the entire set of annotated proteins from this parasite, we observed that the former have in general fewer polar, hydrophobic, and small amino acids (although some amino acids in these classes are more frequent). In addition, upregulated proteins have fewer aromatic amino acids (except for tyrosine, which is more frequent) and less sulfur-containing cysteine residues.

Conclusions

Using 2D-DIGE and MS, we have identified 543 protein spots differentially expressed after gamma radiation exposure. The presence of multiple isoforms was observed for more than half of the identified proteins, most of which are shorter than the annotated protein size in the T. cruzi genome. Additionally, there was a strong correlation for lower molecular weight peptide spots to be overexpressed. This result could be explained by de novo protein synthesis of different isoforms, protein processing, and/or modification events subsequent to radiation exposure. This observation indicates that post-translational control of gene expression have an important role in the parasite response to gamma radiation stress. The inhibition of protein synthesis in face of gamma radiation was shown to have a significant effect decreasing parasite growth and survival rates, highlighting the importance of active translation for parasite recovery after exposure to ionizing radiation.

We have annotated all 53 proteins identified by MS according to their biological roles. Several proteins were represented by multiple spots, and most of them had molecular weights lower than predicted. As a consequence of this observation, we cannot precisely state which biological processes are upregulated versus downregulated, since the different protein isoforms may not function in the same way as the full-length protein. Nevertheless, some tendencies could be observed in this study, including changes in the following biological processes: upregulation of the protein synthesis process, downregulation of protein folding (except for the upregulation of two endoplasmic reticulum chaperones), downregulation of the ATP generation pathway, glycolysis, and amino acid metabolism, and the upregulation of two tryparedoxins (which reduce hydrogen peroxide in response to oxidative stress).
Finally, taking into account the translation inhibition proteins obtained herein, together with the observed proteomic profile after irradiation, we can conclude that de novo protein synthesis is an essential cellular response to gamma radiation.

Supporting Information

Figure S1 Electrophoretic analysis of total protein extracts of irradiated and non-irradiated cells. Total protein extracts were obtained for each time point N1, 4, 24, and 96 hours after irradiation. Samples were subjected to 12% SDS-PAGE and stained with coomassie blue.

Figure S2 Differentially expressed isoforms of prostaglandin E2 alpha synthase. The upregulated protein spot (161) shows a lower molecular weight when compared with the downregulated proteins spots (14, 113, 113, 144, and 144).

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Figure S3 Time point expression of protein spots.

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Author Contributions

Conceived and designed the experiments: HGSV PG SFP AMM CRM HGSV. Performed the experiments: HGSV PG MB SFP HOH. Analyzed the data: HGSV PG MB SFP AMM CRM HMA GRF. Contributed reagents/materials/analysis tools: HGSV AMM CRM HMA GRF. Wrote the paper: HGSV PG MB SFP GRF.

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Author Contributions

Conceived and designed the experiments: HGSV PG SFP AMM CRM HMA GRF. Performed the experiments: HGSV PG MB SFP HOH. Analyzed the data: HGSV PG MB SFP AMM CRM HMA GRF. Contributed reagents/materials/analysis tools: HGSV AMM CRM HMA GRF. Wrote the paper: HGSV PG MB SFP GRF.
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