

Multi-Omics Mapping of DNA Damage Response Inhibition Reveals Distinct Systems-Level Adaptations to Photon and Carbon Ion Irradiation in Glioblastoma

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Abstract

Glioblastoma (GBM) is a highly aggressive and radioresistant brain tumor with limited therapeutic options. While conventional photon radiotherapy remains standard of care, high linear energy transfer (LET) carbon ion (CI) irradiation offers enhanced biological effectiveness through the induction of complex DNA damage. The DNA damage response (DDR), particularly via ATM and ATR kinases, plays a central role in mediating cellular responses to irradiation and represents a promising target for radiosensitization. However, the systems-level molecular adaptations underlying different radiation qualities and DDR inhibition remain poorly understood.

To address this, we performed an integrated multi-omics analysis combining metabolomics and phosphoproteomics in GBM cells exposed to X-rays or CI irradiation, with or without ATM inhibitor (ATMi) / ATR inhibitor (ATRi). Metabolic and phosphoproteomic data were analyzed using multivariate statistics, pathway enrichment, kinase inference, and integrative network approaches to identify coordinated molecular responses.

Our results reveal marked radiation quality-dependent differences. X-ray irradiation induced irradiation-driven metabolic suppression, particularly affecting central carbon metabolism, nucleotide biosynthesis, and mitochondrial function. In contrast, CI irradiation exhibited stronger interaction effects with DDR inhibition, leading to distinct metabolic adaptations involving redox balance, lipid remodeling, and enhanced protein turnover. Phosphoproteomic analyses showed extensive signaling reprogramming in both conditions, with ATR inhibition emerging as a dominant driver of phosphorylation changes under X-rays, while CI irradiation itself was the primary determinant under high-LET conditions. Distinct kinase networks and biological processes were identified, including RNA metabolism (ATRi + X-ray), RNA transport and stress granule dynamics (ATRi + CI), cytoskeletal regulation (ATMi + X-ray), and translational control (ATMi + CI).

This integrative multi-omics analysis demonstrated coordinated phosphometabolic networks, highlighting tight coupling between signaling pathways and metabolic rewiring. Network analysis identified key hubs linking amino acid metabolism, lipid dynamics, and phosphorylation-dependent signaling, with clear divergence between radiation modalities.

Introduction

Glioblastoma (GBM) remains one of the most aggressive and treatment-refractory primary brain tumors, characterized by profound radioresistance, extensive intratumoral heterogeneity, and rapid recurrence despite multimodal therapy [1, 2]. Radiotherapy (RT) constitutes a cornerstone of GBM management; however, therapeutic efficacy is limited by intrinsic resistance mechanisms and the need to spare surrounding normal brain tissue [3]. Particle therapy, particularly high linear energy transfer (LET) CI irradiation, has emerged as a promising alternative or complement to conventional photon-based approaches. High-LET particles generate densely ionizing tracks that induce clustered and complex DNA lesions, leading to increased biological effectiveness compared with low-LET X-rays [4, 5]. Beyond

enhanced tumor cell killing, radiation quality influences the architecture of DNA damage, checkpoint responses, and downstream cellular signaling networks, underscoring the importance of mechanistic studies that compare photon and particle irradiation within the same experimental framework.

Cellular responses to irradiation are orchestrated by the DNA damage response (DDR), a complex signaling network centered on sensor and effector kinases such as ATM and ATR [6–8]. These kinases regulate cell cycle checkpoints, DNA repair pathway choice, and survival signaling following genotoxic stress. Emerging evidence indicates that radiation quality profoundly modulates DDR activation dynamics. High-LET irradiation induces more robust and persistent DNA damage signaling than low-LET photons, consistent with increased lesion complexity and reduced repair efficiency [9, 10]. In GBM cells, comparative irradiation studies demonstrated enhanced phosphorylation of DDR effectors and stronger checkpoint activation after high-LET particle exposure relative to photon irradiation at comparable doses highlighting qualitative differences in signaling network engagement. Such qualitative signaling differences are likely to influence not only canonical DNA repair pathways but also broader stress response programs, including metabolic adaptation and inflammatory signaling.

Pharmacological inhibition of DDR kinases has emerged as a compelling strategy to overcome tumor radioresistance. ATR inhibition disrupts replication stress responses and abrogates radiation-induced G2 checkpoint arrest, whereas ATM inhibition modulates double-strand break signaling and checkpoint maintenance [11–14]. Recent studies demonstrated that both ATR and ATM inhibitors enhance radiosensitivity following photon as well as particle irradiation, challenging earlier assumptions that high-LET damage is largely irreparable and therefore insensitive to DDR targeting [15, 16]. In GBM models, ATR inhibition has been shown to abrogate G2 checkpoint arrest and modify DDR signaling after both X-ray and CI exposure, while ATM inhibition prolongs checkpoint activation and exerts more moderate radiosensitizing effects. These findings suggest that DDR inhibition interacts with radiation track structure to generate distinct cellular states with altered checkpoint dynamics and stress signaling.

Beyond direct cytotoxicity, radiotherapy can also trigger innate immune signaling pathways that influence tumor–immune interactions and therapeutic outcome [17, 18]. Type I interferon responses, frequently activated following cytosolic DNA sensing, represent a key axis connecting DNA damage, micronuclei formation, and antitumor immunity. Previous work demonstrated that ATR inhibition enhances interferon signaling in irradiated GBM cells across radiation modalities, with stronger responses observed when DDR inhibitors were combined with high-LET irradiation compared with photons [19]. ATM inhibition produced similar but less pronounced effects. These results indicate that radiation quality and DDR targeting collectively shape cellular signaling landscapes extending beyond canonical DNA repair pathways.

While previous studies have established that high-LET irradiation and DDR inhibition modulate checkpoint activation, radiosensitivity, and inflammatory signaling in GBM, the systems-level consequences of these perturbations remain largely unexplored. Cellular adaptation to radiation involves coordinated changes in metabolic fluxes, energy homeostasis, redox balance, and kinase-driven

signaling cascades [20, 21]. Because phosphorylation-dependent signaling events frequently regulate metabolic enzymes and stress response pathways, integrated analyses of metabolomic and phosphoproteomic alterations may provide a comprehensive view of how radiation quality and DDR inhibition reprogram tumor cell physiology. Building on prior evidence demonstrating differential DDR activation, checkpoint modulation, radiosensitization, and interferon signaling in GBM cells exposed to X-ray and high-LET particle irradiation with ATR or ATM inhibition [19], the present study aims, for the first time in GBM, to characterize the systems-level molecular consequences of these perturbations through integrated metabolomic and phosphoproteomic profiling. We hypothesized that (i) radiation quality generates distinct metabolic and phosphorylation landscapes reflecting differences in damage complexity and checkpoint control, (ii) ATR and ATM inhibition reshape signaling–metabolism crosstalk in a radiation-dependent manner, and (iii) combined DDR targeting and high-LET irradiation reveal coordinated molecular vulnerabilities that are not apparent from single-omics or phenotypic analyses alone. By integrating complementary omics layers, this work seeks to provide a comprehensive mechanistic framework linking radiation track structure, DDR modulation, and global cellular reprogramming in GBM.

Materials and Methods

Cell culture and treatment

U-251 MG human GBM cells were (300385, CLS Cell Lines Service GmbH) grown in Dulbecco's modified Eagle medium (DMEM) High Glucose (Biowest Industries, Nuaille, France), supplemented with 10% fetal bovine serum (Biowest, Nuaille, France) and 1% penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37°C in a humidified 5% CO₂ atmosphere. Cells were checked for mycoplasma contamination. The cells were seeded in T25 flasks at a density of 400 000 cells/flask for metabolomics, and 500 000 cells / flask for phosphoproteomics, about 48 h before irradiation/treatment.

The samples were exposed either to X-ray or CI. The X-ray experiments were conducted using a Primus Mevatron medical linear accelerator (Siemens Medical Systems Inc-Oncology Care Group, Germany, Department of Radiation Oncology of the “Colțea” Clinical Hospital, Bucharest, Romania). The irradiation setup consists of a vertical 6 MV high energy photon beam line and a 30 cm × 30 cm irradiation field. CI experiments were performed using the IRABAT 95 MeV GANIL beam line, CEA, Caen using a LET of 73 KeV/μm [22]. All irradiations were performed at 2 Gy/min, using a dose of 6 Gy. For all experiments control samples of 0 Gy (sham irradiation) were prepared and manipulated similarly.

DDR inhibition was induced using AZD1390 (Selleck Chemicals, Huston, US) – a selective ATMi and VE822 (berzosertib, Selleck Chemicals, Huston, US) – a selective ATRi. 1 hour before irradiation the inhibitors were added to the samples to a final concentration of 10 nM for ATMi and 100 nM for ATRi. Stock solutions of the inhibitors were diluted in DMSO to a final concentration of 0.01%. A corresponding volume of DMSO was added to the non-treated samples.

Experimental design

The experimental design employs a comprehensive 2*3 factorial approach, evaluating two radiation modalities - X-ray and CI RT - across three primary treatment conditions per modality: untreated control (Ctrl), single-agent drug (ATMi or ATRi), and irradiation alone. Additionally, two combination arms (irradiation + ATMi and irradiation + ATRi) are included for each radiation type (Table 1). Each group consist of four (metabolomic) or three (phosphoproteomic) biological replicates, enabling robust univariate and multivariate analysis to detect radiation and drug-specific perturbation. Such high-throughput approaches allowed to understand the complex rewiring underprint GBM's aggressive phenotype and resistance mechanisms.

Metabolomics

Samples were collected at 24h post-irradiation. For each condition 5 flasks were collected together to ensure an adequate cell number ($> 10^6$). Cells were trypsinized, counted and washed 2 times with cold PBS. Pellets were stored immediately at -80°C for further metabolomic analyses that were outsourced to the expert lab Metabolon (Metabolon, Inc; www.metabolon.com). Such as, global metabolic profiles for each sample were obtained via the Metabolon Platform that utilizes liquid chromatography-mass spectrometry (LC-MS) technology and advanced bioinformatics.

Phosphoproteomics extraction

Samples were collected at 3h post-irradiation. Culture media was removed and the flasks were washed with cold PBS and stored immediately at -80°C until further processing. GBM cells were lysed on ice using a homemade lysis buffer containing 25 mM Tris base, 120 mM NaCl, 10 mM Triton X-100, and 1 mM EDTA (pH 7.6). Immediately before use, the buffer was supplemented with 1 mM PMSF and a protease/phosphatase inhibitor cocktail (Merck, Darmstadt). Cells were mechanically disrupted using an insulin syringe (0.33 mm diameter), followed by ultrasonication for 30 s. The manual lysis and sonication steps were repeated twice. The lysates were centrifuged at $12,000 \times g$ for 30 min, and the supernatants were transferred to fresh tubes. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Illkirch) according to the manufacturer's instructions. For each phosphoprotein condition, approximately 75 μg of protein was adjusted to a final volume of 100 μL with Solution A (H_2O , 0.1% formic acid) and supplemented with 2.5 μL sodium bicarbonate buffer. Digestion, peptide purification, phosphopeptide enrichment and LC-MS analysis were performed as previously described [23, 24].

Phosphopeptide database search and statistical analysis

Quantification of proteoform abundance levels was performed using the summed area of the top three unique peptides. Proteins with a ≥ 1.2 -fold increase in relative abundance and a p-value < 0.05 (Student's t-test, calculated in PERSEUS) were considered significantly enriched. Gene Ontology enrichment analyses for biological process, cellular component, and molecular function categories were performed

using ShinyGO (<http://bioinformatics.sdstate.edu/go/>).. Kinase–substrate enrichment analysis was conducted using Kinase Enrichment Analysis 3 (KEA3) (<https://maayanlab.cloud/kea3/>), with differentially expressed phosphopeptides used as input to predict enriched kinases in the dataset. Upstream phosphatases for the downregulated kinases were identified using PhosphoSitePlus (www.phosphosite.org/).

All data were deposited in iProX public repository database, available with the link: <https://www.iprox.cn/page/project.html?id=IPX0019360000>

Results

To compare metabolomic and proteomic changes induced by X-ray versus CI radiation, we applied doses of 6 Gy for X-rays and 2 Gy for CI. These doses were selected based on the higher relative biological effectiveness (RBE) of CI, which is typically approximately threefold greater than that of X-rays, allowing comparison at doses expected to produce broadly similar biological effects. Additionally, we included the ATMi AZD1390 and the ATRi VE822, both as single treatments and in combination with each type of irradiation. The inhibitors were used at concentrations of 10 nM and 100 nM, respectively, as we have previously shown that these concentrations radiosensitize U251 cells to both X-ray and CI radiation [19]. While metabolomics analysis was performed 24 hours after irradiation to capture downstream metabolic changes, radiation-induced phosphorylation occurs rapidly, and phosphoproteomics analysis was therefore performed 3 hours post-irradiation.

Radiation Quality-Dependent Metabolic Reprogramming in GBM Cells

To formally assess the contribution of irradiation, DDR inhibition (ATMi or ATRi), and their interaction, we performed two-way ANOVA for each metabolite within each radiation cohort. This analysis revealed marked radiation quality dependent differences in response magnitude and interaction structure.

In the X-ray cohort, a substantial proportion of metabolites exhibited significant irradiation main effects, indicating broad irradiation driven metabolic alteration, largely independent of ATMi or ATRi. These changes were distributed across central carbon metabolism, amino acid metabolism, and lipid pathways, reflecting widespread metabolic reprogramming induced by low-LET radiation.

By contrast, the CI cohort displayed comparatively fewer dominant irradiation main effects but demonstrated significantly stronger irradiation × treatment interaction effects. This indicates that metabolic responses under high-LET CI exposure were more strongly modulated by DDR inhibition. In particular, several metabolites involved in the redox balance, nucleotide metabolism, and mitochondrial function showed significant interaction terms, suggesting enhanced dependency of high-LET induced metabolic remodeling on ATM/ATR signaling pathways.

Two-way ANOVA revealed markedly different response magnitudes between radiation types. X-ray irradiation induced broader irradiation-driven metabolic changes, whereas CI irradiation exhibited stronger irradiation × treatment interaction effects, indicating greater modulation by DDR inhibition.

To further characterize the biochemical basis of radiation-induced metabolic alteration, we examine different pathways levels. All results were presented in Fig. 1 and table 1.

De Novo Purine Synthesis (Fig. A, B)

The pentose phosphatase pathway (PPP) plays a central role in nucleotide biosynthesis and redox metabolism. Proliferating cells, including cancer cells, frequently redirect glucose-derived carbon flux through the PPP to sustain increased DNA and RNA synthesis demands [25]. The oxidative branch of the PPP converts the glucose-6-phosphate derived from glycolysis, into 6-phosphogluconate while generating NADPH required for reductive biosynthesis and maintenance of redox homeostasis. The pathway subsequently produces ribose-5-phosphate, a key precursor for nucleotides synthesis.

Ribose-5-phosphate can be interconverted with ribose-1-phosphate and is further metabolized to phosphoribosyl pyrophosphate (PRPP), an essential activated ribose donor in nucleotide biosynthesis [26]. PRPP is required for the formation of 5-aminoimidazol-4-carboxamide ribonucleotide (AICAR), an intermediate in the synthesis of inosine 5'-monophosphate (IMP), which serves as the central precursor for adenosine 5'-monophosphate and guanosine 5'-monophosphate (Fig. 1, A, B).

In the X-ray cohort, several metabolites involved in the *de novo* purine synthesis pathway were reduced relative to the non-irradiated control across multiple treatment conditions, including 6-phosphogluconate and ribose-1-phosphate, although not all changes reached statistical significance. These decreases were generally more pronounced in the combined treatment groups (X-ray + ATMi and X-ray + ATRi), suggesting that inhibition of the DDR may exacerbate X-ray-induced suppression of purine biosynthesis. Consistent with this observation, lower levels of purine nucleotides, including adenosine 5'-monophosphate (AMP) and guanosine 5'-monophosphate (GMP), were observed in several X-ray-treated groups relative to the non-irradiated control, with some comparisons trending toward statistical significance.

By contrast, the metabolic response in the CI cohort differed markedly from that observed following X-ray irradiation. In the ATRi, CI + ATMi, and CI + ATRi groups, levels of AICAR, inosine 5'-monophosphate (IMP), AMP, and GMP were elevated relative to the non-irradiated control. Not all increases were statistically significant. This pattern may reflect enhanced *de novo* purine synthesis. Alternatively, it could indicate reduced nucleotide utilization following CI irradiation in the presence of DDR inhibition. In the CI-only group, upstream metabolites of the pentose phosphate and purine synthesis pathways, including 6-phosphogluconate, ribose-1-phosphate, and AICAR, were increased relative to the non-irradiated control. By contrast, downstream purine nucleotides (IMP, AMP, and GMP) were reduced. This dissociation

between precursor and nucleotide levels may indicate impaired nucleotide synthesis or utilization, resulting in the accumulation of upstream intermediates.

Tryptophan–Kynurenine Metabolism (Fig. C)

Tryptophan acts as a precursor for several metabolic pathways, including serotonin synthesis, NAD⁺ production, and the kynurenine pathway. Indoleamine 2,3-dioxygenase (IDO) catalyzes the conversion of tryptophan to kynurenine and is induced by pro-inflammatory cytokines, such as IFN- γ and TNF- α . Although kynurenine is generated under inflammatory condition, it also exerts immunomodulatory effects and contributes to immune tolerance.

In the X-ray cohort, tryptophan levels were generally reduced in treated groups, with the largest decreases observed in the combined irradiation and ATM or ATR inhibition groups. Kynurenine levels increased in the ATMi and ATRi only conditions but were markedly reduced following X-ray irradiation, particularly in the X-ray + ATMi and X-ray + ATRi groups.

In contrast, kynurenate, formed by transamination of kynurenine, was strongly elevated following X-ray irradiation, with or without kinase inhibition. Notably, kynurenate levels increased substantially relative to non-irradiated controls (e.g., 7.49 in X-ray and 8.19 in ATRi vs 2.01 in control). The opposing changes in kynurenine and kynurenate suggest a redistribution of metabolic flux downstream of kynurenine rather than simple activation or suppression of the pathway. This complicates interpretation of inflammatory signaling based solely on single metabolite abundance and indicates selective pathway branch modeling following low-LET irradiation.

In the CI cohort, alterations in tryptophan metabolism were more modest. Tryptophan levels were slightly increased relative to controls. Kynurenine levels were elevated in the CI and CI + ATMi groups, although they remained relatively stable across other treatments. This contrasts sharply with the pronounced kynurenate accumulation observed after X-ray exposure, suggesting radiation quality-dependent regulation of kynurenate.

Glutathione Metabolism and Redox Homeostasis (Fig. D)

Oxidative stress arises from an imbalance between reactive oxygen species (ROS) production and antioxidant defense capacity. Reduced Glutathione (GSH) is the principal intracellular antioxidant responsible for maintaining redox homeostasis and protecting against oxidative damage. We therefore examined metabolites associated with glutathione metabolism altered following treatment

In the X-ray group, GSH exhibited differential regulation depending on DDR inhibition. GSH increased slightly in the X-ray + ATMi group (1.11 vs 0.64 in control) but decreased in the X-ray + ATRi group (0.50), suggesting inhibitor-specific modulation of redox balance.

Oxidized glutathione (GSSG) levels were lower in all X-ray-treated groups compared to control (0.51), indicating either reduced glutathione oxidation or altered redox cycling dynamics following irradiation. Similarly, cysteine-glutathione disulfide levels were lower in X-ray + ATMi (0.54) and X-ray + ATRi (0.46), relative to the control (0.78), suggesting decreased formation of oxidized glutathione conjugates.

Ophthalmate, a glutathione analog synthesized by the same enzymatic machinery and commonly used as a marker of glutathione biosynthetic activity, was reduced in all X-ray treated groups compared to controls (0.41), with the largest decrease observed in the X-ray + ATRi group (0.28). These coordinated changes suggest altered glutathione synthesis and redox adaptation following low-LET irradiation.

By contrast, CI irradiation produced a distinct redox profile. GSH levels in the CI and inhibitor-treated CI groups were similar to (or slightly higher than) the controls. GSSG levels were stable, or modestly increased, compared to the controls, indicating preserved or enhanced redox cycling capacity.

However, cysteine-glutathione disulfide levels were consistently lower in all CI-treated groups (0.54–0.76) compared to the controls (0.78) suggesting modifications in glutathione conjugation or thiol redox exchange rather than global depletion of antioxidant capacity. Additionally, cysteine levels were reduced in the CI group, potentially influencing GSH biosynthesis dynamics.

Ophthalmate levels remained stable or increased in CI-treated groups relative to controls, further supporting a radiation quality dependent difference in GSH production and redox adaptation.

Polyunsaturated Fatty Acids and Lipid-Derived Inflammatory Mediators (Table 1)

In condition of elevated oxidative stress, polyunsaturated fatty acids (PUFAs), including arachidonate (20:4n6) eicosapentaenoate (EPA), and docosahexaenoate (DHA), are susceptible to peroxidation. Oxidation of these lipids generates bioactive mediators such as eicosanoids and docosanoids, which participate in inflammatory signalling and redox regulation.

The oxidized linoleate derivatives 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) were detected as combined isobaric species (13-HODE + 9-HODE) on the analytical platform, and their individual contributions could not be distinguished. Functionally, these metabolites have been reported to exert partially opposing biological effects: 13-HODE is generally considered anti-inflammatory, whereas 9HODE is associated with pro-inflammatory signaling and has been implicated in macrophage maturation.

Across treatment conditions, trends in PUFA levels and combined 13-HODE + 9-HODE were variable and did not follow a uniform directional pattern following irradiation or DDR inhibition. Although some increases in arachidonate were observed in selected treatment groups, oxidized lipid derivatives did not consistently mirror these changes. These findings suggest that irradiation-induced oxidative stress may

alter lipid metabolism in context-dependent manner, potentially influenced by radiation quality and DDR signaling status.

Endocannabinoid Signaling (Table 1)

Endocannabinoids are fatty acid derived signaling molecules characterized by an amide linkage. They regulate diverse biological processes, including appetite, nociception, memory, stress responses, and immune function, particularly inflammatory signaling [27, 28].

In both radiation cohorts, levels of selected endocannabinoids, including palmitoylethanolamide and stearoylethanolamide, were reduced in multiple treatment groups relative to their respective non-irradiated controls, although not all comparisons reached statistical significance. Given their recognized anti-inflammatory and cytoprotective roles, these decreases may reflect altered lipid signaling dynamics following irradiation and/or DDR inhibition.

Protein Degradation (Table 1)

Proteins frequently undergo post-translational modifications (PTMs), including N-terminal acetylation and methylation, which regulate protein stability, localization, and function. During protein turnover, modified amino acids can be released and detected as free N-acetylated or N-methylated derivatives. Therefore, changes in the abundance of N-acetylated and N-methylated amino acids may reflect alterations in protein degradation dynamics. Additional indicators of protein remodeling include small peptides (e.g., dipeptides), which arise from proteolytic processing, and dimethylarginine species (ADMA + SDMA), which are generated during protein arginine methylation and subsequent proteolysis.

In the X-ray cohort, metabolites associated with lysine metabolism, including N6-acetyllysine and N6,N6,N6-trimethyllysine, were consistently reduced across treatment groups relative to the non-irradiated control. The strongest decreases were observed in the X-ray + ATRi group. Similarly, dimethylarginine (SDMA + ADMA) levels were lower in all X-ray treated groups, with the greatest reduction with ATRi.

Dipeptides, such as alanyl-leucine, glycylisoleucine, phenylalanylglycine, and threonylphenylalanine, were also decreased in X-ray treated conditions, particularly in the combined irradiation plus kinase inhibition groups. Together, these coordinated reductions in modified amino acids, methylarginine derivatives, and dipeptides suggest decreased protein turnover and catabolic activity following low-LET irradiation, with potential enhancement of this effect under DDR inhibition.

By contrast, the CI cohort exhibited a distinct pattern. While lysine-derived metabolites showed only modest changes, dipeptides were markedly increased in all CI treated groups relative to the control. The most pronounced increases were observed in the CI + ATRi groups, with phenylalanylglycine displaying the highest elevations.

This accumulation of dipeptides is indicative of enhanced proteolysis and protein turnover following high-LET irradiation, particularly in the presence of ATRi. The divergence between radiation types suggests that CI exposure induces greater proteostatic stress or damage-associated protein remodeling compared with X-ray irradiation.

Overall, these results suggest that X-ray irradiation, especially when combined with ATMi or ATRi, is associated with reduced protein degradation, whereas CI irradiation promotes increased protein turnover, with the strongest effects observed in ATRi-treated conditions.

Glycolysis and Branched-Chain Acid Catabolism (Fig. 1E)

Branched-chain amino acids (BCAAs), leucine, isoleucine, and valine serve as essential protein constituent and alternative energy substrates. Following transamination by branched-chain aminotransferase (BCAT), BCAAs are converted to their corresponding α -keto acids, which are subsequently metabolized in the mitochondria by the branched-chain α -keto acid dehydrogenase (BCKD) complex. These intermediates generate metabolites that enter the tricarboxylic acid (TCA) cycle or support anabolic pathways such as gluconeogenesis and lipid synthesis. Accumulation or depletion of α -keto and α -hydroxy derivatives can reflect altered BCAA flux, BCKD activity, or mitochondrial function.

In the X-ray cohort, intracellular levels of leucine, isoleucine, and valine were consistently reduced in irradiated groups compared to non-irradiated control. While decreases in the parent BCAAs were modest, their downstream α -keto acid derivatives, including 4-methyl-2-oxopentanoate and 3-methyl-2-oxobutyrate, were markedly diminished approaching minimal detectable levels in several treatment groups. The strongest were observed in the combined X-ray + ATMi and X-ray + ATRi conditions.

Similarly, the α -hydroxy acid derivative α -hydroxyisovalerate was substantially decreased, particularly in the co-treatment conditions. Collectively, these coordinated reduction across BCAA and their downstream intermediates suggest suppressed BCAA availability and/or reduced catabolic flux following X-ray irradiation with further attenuation under ATMi or ATRi. This pattern is consistent with reduced protein turnover, describe above and may reflect altered energetic or biosynthetic demands in the setting of DNA damage and may reflect altered energetic or biosynthetic demands in the setting of DNA damage combined with DDR inhibition.

In the CI cohort, BCAAs and their α -keto and α -hydroxy acid derivatives were also generally lower than controls. However, changes were smaller in magnitude and more heterogeneous compared with X-ray irradiation. Notably, α -keto acid derivatives remained at relative higher levels than those observed following X-ray exposure, indicating less pronounced disruption of mitochondrial BCAA metabolism under CI exposure.

Parallel analysis of glycolytic intermediates demonstrated reduced lactate levels in treated groups, particularly following X-ray irradiation, most prominently under combined DDR inhibition. This pattern suggests decreased glycolytic throughput downstream of glucose. Although enhanced aerobic glycolysis (Warburg metabolism) is frequently associated with tumor aggressiveness [29], interpretation

of intracellular glucose and lactate levels in vitro is limited by the substantially higher concentrations present in culture media. Direct assessment of glucose consumption and lactate secretion would therefore be required to confirm changes in glycolytic flux.

Fatty Acid Metabolism (Fig. 1E)

Fatty acids participate in energy production, storage, and membrane biosynthesis. Through β -oxidation, fatty acids are converted to acetyl-CoA, which enters the TCA cycle. Transport of long-chain fatty acids into mitochondria requires conjugation to carnitine; thus, changes in free carnitine and acylcarnitine species can reflect alterations in mitochondrial fatty acid oxidation. Ketone bodies such as 3-hydroxybutyrate (BHBA) further provide insight into β -oxidation and ketogenesis.

In the X-ray cohort, BHBA levels were markedly reduced (0.20–0.29 relative to control), consistent with impaired ketogenesis and diminished mitochondrial β -oxidation. Saturated fatty acids—including myristate, palmitate, and stearate—were also decreased, suggesting increased lipid utilization or oxidative remodeling.

Free carnitine levels were reduced (0.48), accompanied by decreases in long-chain acylcarnitines such as palmitoylcarnitine (C16). In contrast, medium-chain acylcarnitines particularly octanoylcarnitine (C8) accumulated under X-ray + ATR inhibition (1.40), a pattern consistent with incomplete β -oxidation or mitochondrial inefficiency.

In contrast, the CI cohort demonstrated partial preservation of BHBA (0.41–0.69), near-normal levels of saturated fatty acids, and increased free carnitine (up to 1.21), suggesting maintenance of mitochondrial fatty acid transport and oxidative capacity. Acylcarnitine profiles were comparatively stable, indicating less disruption of β -oxidation relative to X-ray treatment.

Collectively, these findings indicate that X-ray irradiation induces broad suppression of mitochondrial energy metabolism, encompassing BCAA catabolism, glycolytic output, and fatty acid β -oxidation, with further exacerbation under ATM or ATR inhibition. In contrast, CI irradiation produces a more moderate and heterogeneous metabolic response, with relative preservation of mitochondrial function despite DNA damage response inhibition.

These radiation quality-dependent differences suggest fundamentally distinct bioenergetic adaptations to low- versus high-LET irradiation, X-rays being associated with more pronounced suppression of mitochondrial metabolic flux under the conditions tested.

The metabolic alterations described above are unlikely to occur in isolation. Cellular energy metabolism, mitochondrial function, and amino acid catabolism are tightly regulated by intracellular signalling networks, many of which operate through phosphorylation-dependent mechanisms. In particular, the DDR kinases ATM and ATR exert broad regulatory control that extends beyond genome maintenance, influencing metabolic enzymes, nutrient sensing pathways, and mitochondrial activity.

The coordinated changes observed in glycolysis, β -oxidation, and branched-chain amino acid metabolism therefore suggest underlying remodelling of signalling pathways in response to irradiation and DDR inhibition. To define the signalling event associated with these metabolic adaptations, we performed a global phosphoproteomic analysis to characterize treatment-induced changes in protein phosphorylation following X-ray or CI irradiation, with or without ATM or ATR inhibition.

Phosphoproteomic Landscape Reveals Differential DDR and Signaling Pathway Activation

To characterize the signaling networks underlying treatment-induced metabolic adaptations, global phosphoproteomic profiling was performed across all experimental conditions, namely control (CTRI), kinase inhibition (ATRi or ATMi), irradiation alone, and combined irradiation with kinase inhibition. Both low-LET X-ray irradiation and high-LET CI irradiation were analyzed to determine whether radiation quality influences phosphorylation-dependent signaling responses.

Across all datasets, irradiation and DDR kinase inhibition induced substantial remodeling of the cellular phosphoproteome, indicating extensive modulation of signaling pathways associated with DDR and replication stress.

In the X-ray cohort, 211 protein accessions were significantly regulated ($p < 0.05$, Benjamini–Hochberg adjusted $q < 0.05$), indicating extensive remodeling of phosphorylation-dependent signaling pathways. Both ATR inhibition and X-ray irradiation contributed to these changes, consistent with activation of replication stress and canonical DDR pathways.

ATR inhibition strongly restructures phosphorylation networks (Fig. 2)

In the ATR inhibition datasets, PCA demonstrated pronounced treatment-dependent clustering.

For the X-ray cohort, the first principal component accounted for the majority of variance and clearly separated ATRi-treated samples from untreated controls (Fig. 2A). This indicates that ATR inhibition represents a dominant driver of global phosphorylation remodeling under low-LET radiation conditions. X-ray irradiation further structured the data along the second component, distinguishing irradiated from non-irradiated samples. Notably, the combined X-ray - ATRi condition occupied a distinct region of PCA space, suggesting that ATR pathway suppression generates a specific phospho-signaling state in the context of radiation-induced DNA damage.

In the CI cohort, PCA similarly revealed treatment-dependent clustering (Fig. 2C). However, in contrast to the X-ray dataset, irradiation itself represented the principal driver of variance, separating CI-irradiated samples from non-irradiated conditions. ATR inhibition further modulated the distribution of samples within irradiated and non-irradiated groups. The combined CI-ATRi condition formed a distinct cluster, indicating that ATR inhibition alters signaling responses triggered by complex DNA lesions induced by high-LET radiation.

ATM inhibition modulates DDR signaling responses

PCA of the ATM inhibition datasets also revealed clear treatment-dependent clustering, although the structure of variance differed from that observed for ATR inhibition.

In the X-ray cohort, ATM inhibition induced measurable changes in phosphorylation patterns relative to controls (Fig. 2B). Irradiation remained a major determinant of sample distribution, while the combined X-ATMi condition formed a distinct cluster relative to either treatment alone. These results suggest that ATM signaling contributes to the regulation of radiation-induced DDR signaling pathways.

In the CI cohort, PCA again demonstrated strong separation between irradiated and non-irradiated samples (Fig. 2D), confirming that high-LET CI irradiation drives substantial phosphoproteomic remodeling. ATM inhibition further modulated this response, generating a distinct clustering pattern in the CI-ATMi condition.

Hierarchical Clustering Reveals Treatment-Specific Phosphorylation Signatures

Unsupervised hierarchical clustering of regulated phosphosites further supported the PCA observations (Fig. 3). Heatmap analyses revealed clear grouping of samples according to treatment condition across all datasets.

In the ATR inhibition datasets, extensive phosphorylation remodeling was observed across multiple phosphosite clusters. ATR inhibition induced widespread changes consistent with its central role in replication stress signaling. In the X-ray cohort, irradiation alone produced moderate phosphorylation changes, whereas the combined X-ray-ATRi condition displayed amplified phosphoproteomic remodeling. In the CI cohort, irradiation induced stronger and more extensive phosphorylation changes, reflecting activation of signaling networks associated with complex DNA damage.

In the ATM inhibition datasets, hierarchical clustering also separated samples according to irradiation and inhibitor status. X-ray irradiation produced widespread phosphorylation changes relative to controls, while ATM inhibition modulated a subset of phosphosite clusters associated with DDR signaling pathways. CI irradiation induced broader phosphoproteomic remodeling, consistent with the increased biological impact of high-LET radiation.

Importantly, in both radiation types, the combined irradiation and kinase inhibition conditions displayed distinct phosphorylation signatures, suggesting non-additive signaling interactions between radiation-induced DNA damage and checkpoint pathway suppression.

Kinase activity inference and pathway enrichment analysis

To further investigate the signaling pathways underlying the phosphoproteomic changes induced by irradiation and kinase inhibition, kinase activity inference and pathway enrichment analyses were

performed based on the regulated phosphoproteins. This approach enabled the identification of kinase signaling signatures associated with the different treatment conditions (Fig. 4).

Kinase enrichment analysis revealed several candidate kinases potentially responsible for the observed phosphorylation changes. Among the most significantly enriched kinases were PINK1, MAPKAPK5, NEK4, GRK2, LATS2, PBK, GRK5, EPHA7, CAMK2A, and STK32C, as identified by the TopRank scoring method. These kinases are involved in diverse cellular signaling pathways, including stress response signaling, cell cycle regulation, and cytoskeletal organization.

Network-based ranking analysis further highlighted kinases such as GSK3A, CDK16, PAK1, ABL1, PDPK1, and RPS6KB2, which showed strong connectivity across multiple protein–protein interaction databases including BioGRID, STRING, MINT, and PTMSigDB. The presence of these kinases suggests coordinated regulation of signaling pathways associated with cell cycle progression, cytoskeleton dynamics, and growth factor signaling.

Several of the identified kinases are also known to participate in pathways related to MAPK signaling, mTOR signaling, and cellular stress responses, consistent with the activation of adaptive signaling mechanisms following irradiation. In particular, kinases such as MAPKAPK5 and PAK1 are well-established mediators of stress-activated signaling pathways, whereas RPS6KB2 and PDPK1 are associated with mTOR-dependent regulation of protein synthesis and cellular metabolism.

Gene Ontology enrichment analysis reveals distinct biological responses to ATR and ATM inhibition combined with X-ray or CI irradiation

To explore the biological processes affected by kinase inhibition during irradiation, Gene Ontology (GO) enrichment analysis was performed on the differentially expressed proteins identified after treatment with inhibitors targeting ATR or ATM combined with either X-ray or CI irradiation (Fig. 5).

In cells treated with ATR inhibition and X-ray irradiation (Fig. 5A), enriched GO terms in the Biological Process category were predominantly associated with RNA metabolism, including regulation of mRNA splicing via the spliceosome, RNA processing, and mRNA metabolic processes. Consistently, Cellular Component enrichment highlighted spliceosomal complex and nuclear-associated structures, whereas Molecular Function terms included RNA binding and translation initiation factor binding. Together, these findings suggest that ATR inhibition during X-ray irradiation strongly affects post-transcriptional regulation and spliceosome-mediated RNA processing.

In contrast, ATR inhibition combined with CI irradiation (Fig. 5B) resulted in the enrichment of biological processes related to RNA trafficking and nucleic acid transport, such as RNA export from the nucleus, mRNA transport, and RNA localization. Cellular component terms indicated enrichment in cytoplasmic ribonucleoprotein granules and stress-granule-related structures, while molecular functions included helicase activity and phosphatase regulator activity. These results suggest that the combination of ATR

inhibition with high-LET CI radiation preferentially disrupts RNA transport mechanisms and induces cellular stress responses linked to ribonucleoprotein granule formation.

A distinct pattern was observed following ATM inhibition combined with X-ray irradiation (Fig. 5C). Enriched Biological Process terms were mainly related to cytoskeletal organization and cell division, including assembly of the actomyosin apparatus involved in cytokinesis, actomyosin contractile ring assembly, and tight junction organization. Molecular Function enrichment revealed SH2 domain binding, cadherin binding, and actin binding activities. These observations indicate that ATM inhibition under X-ray irradiation predominantly affects cytoskeletal dynamics and cell division processes.

Finally, cells treated with ATMi and CI irradiation (Fig. 5D) showed enrichment in biological processes linked to protein synthesis and RNA metabolism, including cytoplasmic translation, RNA processing, and peptide biosynthetic processes. Cellular component terms were enriched for vesicular and secretory compartments such as cytoplasmic vesicle lumen and secretory granule lumen, while molecular functions included nucleotide binding and RNA binding activities. These findings suggest that ATM inhibition in the context of CI irradiation influences translational regulation and vesicle-associated cellular pathways.

Overall, GO enrichment analysis revealed distinct molecular signatures depending on both the inhibited kinase and the radiation modality. While ATR inhibition primarily impacted RNA processing and transport pathways, ATM inhibition was associated with cytoskeletal organization and translational regulation. Moreover, CI irradiation appeared to induce broader effects on RNA trafficking and protein synthesis compared with X-ray irradiation.

Integrated Multi-Omics Analysis Identifies Coordinated Metabolic and Phospho-Signaling Networks

To decipher the molecular mechanisms underlying treatment-specific responses, an integrative multi-omics analysis combining metabolomic and phosphoproteomic datasets was performed using DIABLO-based framework (<https://mixomics.org/mixdiablo/>).

This approach enabled the identification of correlated features across omics layers, revealing coordinated variation between metabolite abundance at 24 hours and earlier phosphorylation dependent signaling events at 3 hours (Fig. 6).

We performed a multi-omics integration using DIABLO to identify correlated features between the phosphoproteome and metabolome in samples treated with X-ray combined with ATRi or ATMi. For each treatment, the top contributing variables to components 1 and 2 were selected. Component 1 included ten phosphoproteins, such as E9PPJ0, TBC1D10B, and Q7Z5K2, along with ten metabolites showing strong negative correlations. Component 2 comprised ten phosphoproteins, including SF3B2, TBC1D10B, and WAPL, together with ten metabolites exhibiting strong positive correlations.

In the X-ray + ATRi samples (Fig. 6A), a heatmap of pairwise correlations between selected metabolites and phosphoproteins highlighted distinct co-regulated modules. Strong positive correlations, exceeding 0.8, were observed for several metabolite-phosphoprotein pairs, including 100000039 with SF3B2 ($r = 0.91$) and 313 with NUMA1 ($r = 0.92$), suggesting coordinated regulation between specific phosphoproteins and metabolic pathways. Conversely, negative correlations were detected for a subset of metabolites with certain phosphoproteins, such as metabolite 264 with SF3B2 ($r = -0.74$), indicating potential inhibitory or inverse relationships. These results reveal treatment-associated co-regulation and mechanistic links between signaling and metabolic responses to X-ray + ATRi.

Separately, in X-ray /ATMi samples (Fig. 6B), the interactions between metabolites and phosphoproteins also revealed key contributing variables for the first two principal components. Notably, phosphoproteins such as PAK2, CBX1, and SMC4 exhibited strong negative correlations with multiple metabolites, suggesting potential inverse regulatory relationships (e.g., PAK2 with metabolite 491, $r = -0.87$; CBX1 with metabolite 100000036, $r = -0.94$). In contrast, some phosphoproteins, including CCDC88A and AP3B1, displayed positive correlations with specific metabolites, indicating co-activation modules (e.g., CCDC88A with metabolite 264, $r = 0.75$; AP3B1 with metabolite 249, $r = 0.63$).

These findings demonstrate distinct clusters of phosphoproteins and metabolites with coordinated expression in X-ray + ATMi-treated samples, highlighting potential functional interactions between kinase signaling and metabolic pathways. Both treatment conditions reveal candidate modules for further functional investigation and pathway enrichment analysis, but each shows unique regulatory patterns that may reflect the specific mechanisms of ATRi versus ATMi in combination with X-ray treatment.

A correlation analysis between metabolites and phosphoproteins was performed to explore the molecular interactions in the CI /ATRi as well as CI/ATMi conditions.

In the CI/ATRi treatments (Fig. 6C), strong correlations were observed between specific phosphoproteins, such as PARN, CANX, and PEG10, and metabolites. These interactions suggest that these proteins play a key role in metabolic changes under these conditions. The heatmap analysis further revealed clustering of proteins and metabolites, indicating the activation of common biological pathways and potential interaction networks. Notably, PARN showed strong relationships with several metabolites, highlighting its involvement in cellular stress responses and potential post-transcriptional regulatory functions under CI and ATRi.

In contrast, the analysis of the CI/ATMi (Fig. 6D), condition revealed a more complex, synergistic network of correlations. The protein PAK2 showed strong positive correlations with PSMD2 ($r = 0.93$) and AHNK2 ($r = 0.91$), indicating its central role in regulating cellular responses under the combined treatment. Additionally, PAK2 exhibited a notable negative correlation with MARCKSL1 ($r = -0.88$), suggesting an antagonistic regulatory mechanism. Proteins involved in protein degradation regulation, such as VCIPI1 and HUWE1, also showed significant positive correlations with PAK2 ($r = 0.84$ and $r = 0.83$, respectively), suggesting their role in DNA repair and stress response pathways. Furthermore, CAPS and ANK2 ($r = 0.83$) exhibited positive correlations, suggesting coordination between cellular

signaling pathways and metabolic regulation. The negative correlation between PRPF38B and PARN ($r = -0.76$) further supports the post-transcriptional modulation driven by the combination of CI and ATMi, which influences key biological processes such as protein degradation and stress response.

Network analysis reveals structured phospho-metabolic interaction landscape

Integrative network analysis at a correlation cutoff of 0.5 uncovered distinct and structured patterns of phospho-metabolic interactions depending on radiation quality and DDR inhibition, highlighting a coordinated reprogramming of metabolism and signaling (Fig. 7).

Under X-ray irradiation, MCC-based centrality analysis identified highly connected nodes within metabolite–protein networks under both ATR and ATM inhibition, revealing key regulatory interfaces between metabolic pathways and phosphorylation-dependent signaling. Under ATR inhibition (Fig. 7A), central metabolites included 100000039, 2054 (ethylmalonate), 297 (3-methyl-2-oxovalerate), 313, 469, 806, 1113, 1134, 1547, and 275, indicating a predominant involvement of amino acid metabolism, intermediary metabolism, and energy-related pathways. In parallel, phosphoproteins such as SF3B2, SVIL, WAPL, SEC31A, NUMA1, LIMA1, TBC1D2B, CDCA3, TBC1D10B, and NMD3 emerged as central hubs, underscoring their roles in RNA processing, cytoskeletal organization, and genome stability.

Under ATMi (Fig. 7B), the metabolic landscape shifted toward central carbon metabolism and redox-associated pathways, with key metabolites including 2054 (ethylmalonate), 100000036 (3-methyl-2-oxovalerate), 100000551 (4-methyl-2-oxopentanoate), 338 (gluconate), 482 (lactate), 100000341 (6-phosphogluconate), 491 (pyridoxal), and 823 (pyruvate). These nodes point to coordinated regulation of branched-chain amino acid metabolism, glycolysis, gluconeogenesis, and the PPP. Concurrently, phosphoproteins such as PRPF40A, KRT82, SKIC2, CIC, PAK2, ANLN, CBX1, SMC4, AFAP1, and A0A096LP25 were identified as key signaling hubs, reinforcing the tight coupling between metabolic flux and phosphorylation-mediated regulatory mechanisms under X-ray stress.

In contrast, CI irradiation revealed a distinct phospho-metabolic architecture dominated by lipid remodeling and membrane-associated processes. Under ATRi (Fig. 7C), central metabolites included 428 (N,N,N-trimethyl-5-aminovalerate), 563 (1-oléyl-2-linoléoyl-GPC, O-18:1/18:2), 1023 (1-palmitoyl-2-pentadecanoyl-GPC, 16:0/15:0), 1114 (1-(1-en-oléoyl)-2-docosaheptaenoyl-GPC, P-18:1/22:6), and 1225 (sphingomyelin, d17:1/14:0; d16:1/15:0), highlighting extensive phospholipid and sphingolipid remodeling.

Under ATMi (Fig. 7D), central metabolites included 111 (3-hydroxyisobutyrate), 344 (guanidinoacetate), 100000054 (N,N,N-trimethyl-5-aminovalerate), 100000341 (1-oléyl-2-linoléoyl-GPC, O-18:1/18:2), and 100000454 (1-palmitoyl-2-pentadecanoyl-GPC, 16:0/15:0), indicating a convergence between amino acid metabolism and lipid remodeling pathways. In parallel, phosphoproteins such as PEG10, RNASEK-C17orf49, DLAT, ARID5B, and PANK2 (ATRi), and LMO7, MARCKSL1, RPL29, KDM2B, and HUWE1 (ATMi)

occupied central network positions, reflecting their involvement in post-translational regulation and stress adaptation.

Collectively, these results reveal a radiation quality–dependent reorganization of phospho-metabolic networks, where X-ray irradiation preferentially engages central carbon metabolism and redox pathways, while CI irradiation drives lipid remodeling and membrane dynamics. Importantly, across all conditions, the consistent co-centrality of metabolites and phosphoproteins highlight a robust and previously underexplored crosstalk between metabolic reprogramming and phosphorylation-mediated signaling. This integrative network framework uncovers key mechanistic nodes at the interface of metabolism and signaling, providing new insights into adaptive responses to genotoxic stress and identifying potential combinatorial therapeutic targets involving DDR inhibition and metabolic vulnerabilities.

Discussion

This study provides a comprehensive systems-level characterization of GBM cellular responses to irradiation by integrating metabolomic and phosphoproteomic analyses under conditions of photon and high-LET CI exposure, combined with pharmacological inhibition of key DDR kinases ATM and ATR. The results demonstrate that radiation quality is a primary determinant of cellular adaptation, not only at the level of DNA damage signaling but also across metabolic and phosphorylation-dependent regulatory networks. Importantly, the data reveal that DDR inhibition does not produce uniform radiosensitization effects but instead interacts with radiation track structure to generate distinct molecular states.

A central finding of this work is the divergence between low-LET X-ray and high-LET CI irradiation in shaping metabolic responses. X-ray exposure induced broad suppression of metabolic pathways, including central carbon metabolism, nucleotide biosynthesis, and mitochondrial energy production. This is consistent with previous reports indicating that photon irradiation promotes a generalized metabolic slowdown associated with DNA damage-induced cell cycle arrest and reduced biosynthetic demand [25, 29].

Pathways such as central carbon metabolism plays a critical role in supporting tumor cell adaptation to radiation-induced stress by coordinating ATP production, redox homeostasis and biosynthetic process. As highlighted by the studies targeting mitochondrial metabolism in radioresistant tumors [30], metabolic rewiring toward oxidative phosphorylation (OXPHOS) and PPP activity enables maintenance of DNA repair capacity and cellular survival following irradiation. Therefore, disruption of central carbon metabolism may comprise the metabolic flexibility required for an efficient response to genotoxic stress as suggested by the reduced level of central carbon metabolism associated metabolites observed in our study. In contrast to our findings, previous reports describe radiation induced metabolic adaptation characterized by increased ATP/GTP levels and maintenance of bioenergetic activity in GBM. This discrepancy likely reflects differences in metabolic plasticity and DDR capacity, where impairment of checkpoint signaling may prevent the adaptive metabolic rewiring required for tumor cell survival.

The observed decrease in purine synthesis intermediates and nucleotide pools further supports the notion that X-ray irradiation limits anabolic processes required for proliferation, potentially as a consequence of checkpoint activation and replication stress.

Focusing on the nucleotide biosynthesis pathways, cells obtained nucleotides by two routes: *de novo* pathway and salvage pathway. Tumor cell exploits both routes which allows tumors to exhibit route plasticity and evade single-agent therapy, when both gates are closed, tumor cells become nucleotide-starved, accumulate replication stress, leading to DNA damage and cell death. Savu et al., 2022, stated that IDH-mutant gliomas are particularly dependent on *de novo* pyrimidine synthesis due to a limited compensatory capacity of the salvage pathway. Accordingly, inhibition of this pathway induces nucleotide depletion, replication stress, and DNA damage, thereby suppressing tumor cell proliferation and viability.

Our findings are consistent with and extend these observations. We show that X-ray irradiation, particularly when combined with ATM/ATR inhibition, is associated with a reduction in nucleotide availability affecting purine metabolism, including decreased level of ribose-1-phosphate, 6-phosphogluconate, and purine nucleotides (AMP and GMP). These metabolic alterations are further exacerbated checkpoint inhibition, suggesting that impairment of DDR signaling amplifies radiation-induced metabolic stress.

Importantly, these results align with the work of Zhou et al. [31], who demonstrated that purine metabolism is not only essential for nucleotide supply but also activity regulates DNA repair capacity and contributes to therapy resistance in GBM. In this framework, the observed depletion of purine metabolites may compromise DNA repair efficiency while simultaneously limiting nucleotides availability for repair synthesis. Together, these effects likely converge to enhance replication stress, promote accumulation of unrepaired DNA damage, and ultimately increase tumor cell vulnerability to genotoxic stress.

By contrast, CI irradiation produced a more selective and dynamic metabolic response characterized by stronger interaction effects with DDR inhibition. Rather than a global suppression, high-LET exposure was associated with preserved or even enhanced mitochondrial function, sustained redox capacity, and increased protein turnover. These findings align with the known ability of high-LET radiation to induce complex, clustered DNA lesions that are more difficult to repair and that trigger prolonged and heterogeneous signaling responses [4, 9]. The increased accumulation of dipeptides and lipid remodeling observed in CI-treated cells suggests elevated proteostatic and membrane-associated stress, which may reflect ongoing damage processing and cellular adaptation rather than metabolic shutdown. Importantly, experimental studies in GBM models after IR exposition have been shown to promote lipid accumulation supporting membrane remodeling under stress condition [32]. In parallel, enhanced lipid metabolic plasticity have been identified as key adaptive mechanisms that allow GBM cells to maintain energetic homeostasis and resist therapy induced stress [33].

The differential regulation of redox homeostasis further illustrates the impact of radiation quality. X-ray irradiation was associated with decreased GSH-related metabolites and impaired redox cycling, whereas CI exposure maintained or enhanced antioxidant capacity. This distinction may be linked to differences in reactive oxygen species (ROS) generation and spatial distribution. While both radiation types induce oxidative stress, high-LET particles produce dense ionization tracks that may localize damage and allow preservation of global redox balance, whereas low-LET radiation induces more diffuse oxidative damage affecting broader cellular compartments. Such differences could influence sensitivity to DDR inhibition, as redox status is known to modulate both DNA repair efficiency and signaling pathway activation.

At the signaling level, phosphoproteomic analysis revealed extensive remodeling of kinase-driven networks in response to both irradiation modalities. ATR inhibition emerged as a dominant regulator of phosphorylation dynamics under X-ray conditions, consistent with its central role in replication stress signaling and S-phase checkpoint control [7, 8]. The strong separation of ATRi-treated samples in principal component analysis underscores the importance of ATR in coordinating cellular responses to low-LET-induced DNA damage. In contrast, under CI irradiation, the primary driver of phosphoproteomic variation was the radiation itself, with ATR inhibition acting as a secondary modulator. This suggests that high-LET-induced DNA lesions activate signaling pathways that are less dependent on canonical ATR-mediated replication stress responses and may instead involve alternative or more robust checkpoint mechanisms.

ATM inhibition, while also modulating DDR signaling, exhibited more context-dependent effects. Under X-ray irradiation, ATM inhibition influenced pathways related to cytoskeletal organization and cell division, supporting previous observations that ATM contributes to mitotic progression and structural integrity following DNA damage [7]. This is further reinforced by experimental evidence demonstrating that ATM directly regulates spindle assembly checkpoint fidelity through segregation during mitosis and maintaining genomic stability [34]. Under CI irradiation, ATM inhibition was associated with alterations in translational regulation and vesicle-associated processes, indicating a shift toward post-transcriptional and proteostasis-related adaptations. These findings highlight that ATM and ATR play non-redundant roles that extend beyond DNA repair and are differentially engaged depending on radiation quality.

One of the most significant contributions of this study lies in the integration of metabolomic and phosphoproteomic datasets, which revealed coordinated phospho-metabolic networks underlying treatment-specific responses. The identification of strongly correlated modules linking metabolites and phosphoproteins demonstrates that phosphorylation-dependent signaling is tightly coupled to downstream metabolic rewiring. For example, under X-ray irradiation, central carbon metabolism and redox pathways were closely associated with phosphoproteins involved in RNA processing and cytoskeletal regulation, suggesting a coordinated adaptation linking energy metabolism to gene expression and structural dynamics. This is consistent with phosphoproteomic analyses showing that X-ray irradiation induces large scale remodeling of phosphorylation networks, prominently affecting RNA processing, transcriptional regulation, and cytoskeletal organization, thereby linking DDR to gene expression and structural adaptation [35]. By contrast, CI irradiation preferentially engaged lipid

metabolism and membrane-associated signaling networks, reflecting a distinct mode of cellular reorganization.

These integrated network analyses provide mechanistic insight into how DDR signaling intersects with metabolic control. ATM and ATR kinases are increasingly recognized as regulators of metabolic enzymes and mitochondrial function, influencing pathways such as glycolysis, nucleotide synthesis, and oxidative phosphorylation [25]. In particular, it has been demonstrated that nuclear DNA damage can trigger ATM-dependent AMPK activation, which directly regulates the mitochondrial radiation response and cellular energy homeostasis [36]. The present data extends this concept by showing that inhibition of these kinases reshapes global metabolic architecture in a radiation-dependent manner. The strong interaction effects observed under CI irradiation suggest that DDR signaling becomes a critical determinant of metabolic adaptation when cells are challenged with complex DNA damage.

Few studies in cancer models combine metabolomics and phosphoproteomics with ATM/ATR inhibition plus irradiation. An integrated study addressing this combination is in a leukemia models where ATR inhibition radiosensitised the cancer cells altering DDR signalling, cell cycle pathways, replication stress signalling and metabolic kinase networks. The study also evidence suppression of mTOR signalling, nucleotide metabolism and oxidative stress pathways demonstrating that ATR inhibition plus irradiation rewires DNA damage signalling and central carbon/ nucleotide metabolism simultaneously. The authors proposed that impaired nucleotide recovery after irradiation is a potential contributor to radiosensitisation [37].

From a therapeutic perspective, these findings have important implications for the optimization of GBM radiotherapy. The enhanced interaction between DDR inhibition and CI irradiation supports the rationale for combining ATRi or ATMi with particle therapy to exploit context-specific vulnerabilities. Notably, the data suggest that ATR inhibition may be particularly effective in modulating signaling and metabolic responses under both radiation modalities, consistent with its role in abrogating the G2 checkpoint and enhancing radiosensitivity [8]. Similarly, ATM inhibition has been demonstrated to potentiate radiation induced cytotoxicity signaling by impairing DNA double break repair and disruption downstream signaling networks controlling cell cycle arrest and survival [38]. However, the distinct molecular states induced by high-LET radiation indicate that treatment strategies should be tailored to radiation quality, rather than assuming equivalent biological effects across modalities.

Furthermore, the identification of metabolic and phosphoproteomic signatures associated with specific treatment conditions raises the possibility of developing predictive biomarkers for patient stratification. For instance, alterations in nucleotide metabolism, redox balance, or lipid remodeling could serve as indicators of response to combined DDR inhibition and radiotherapy. Such biomarkers would be particularly valuable in the context of GBM, where therapeutic resistance and heterogeneity remain major challenges.

In conclusion, this work demonstrates that radiation quality and DDR inhibition jointly define the molecular landscape of GBM cells, driving distinct and coordinated changes in metabolism and

signaling. By integrating metabolomic and phosphoproteomic data, the study uncovers a complex network of adaptive responses that extend beyond canonical DNA repair pathways. These findings provide a foundation for the rational design of combination therapies and highlight the importance of systems-level approaches in understanding and overcoming radioresistance in GBM.

Declarations

Conflicts of Interest

The authors declare no conflicts of interest.

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Author Contribution

Conceptualization, R.G.S., D.I.S. and F.C.; methodology, O.B.D., A.G., M.T. (Mihaela Tudor), M.T. (Mihaela Temelie), B.B. and N.M.; formal analysis, F.C., A.G., M.T. (Mihaela Tudor), M.T. (Mihaela Temelie), B.B. and N.M.; writing—original draft preparation, O.B.D., M. T. (Mihaela Temelie), D.I.S. and F.C.; writing—review and editing, all authors; supervision, F.C., D.I.S., M.S., C.S. and R.G.S.; project administration, R.G.S., D.I.S. and F.C.; funding acquisition, R.G.S. and D.I.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability

All data were deposited in iProX public repository database, available with the link: <https://www.iprox.cn/page/project.html?id=IPX0019360000>

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Tables

Table 1 is available in the Supplementary Files section.

Figures

Figure 1

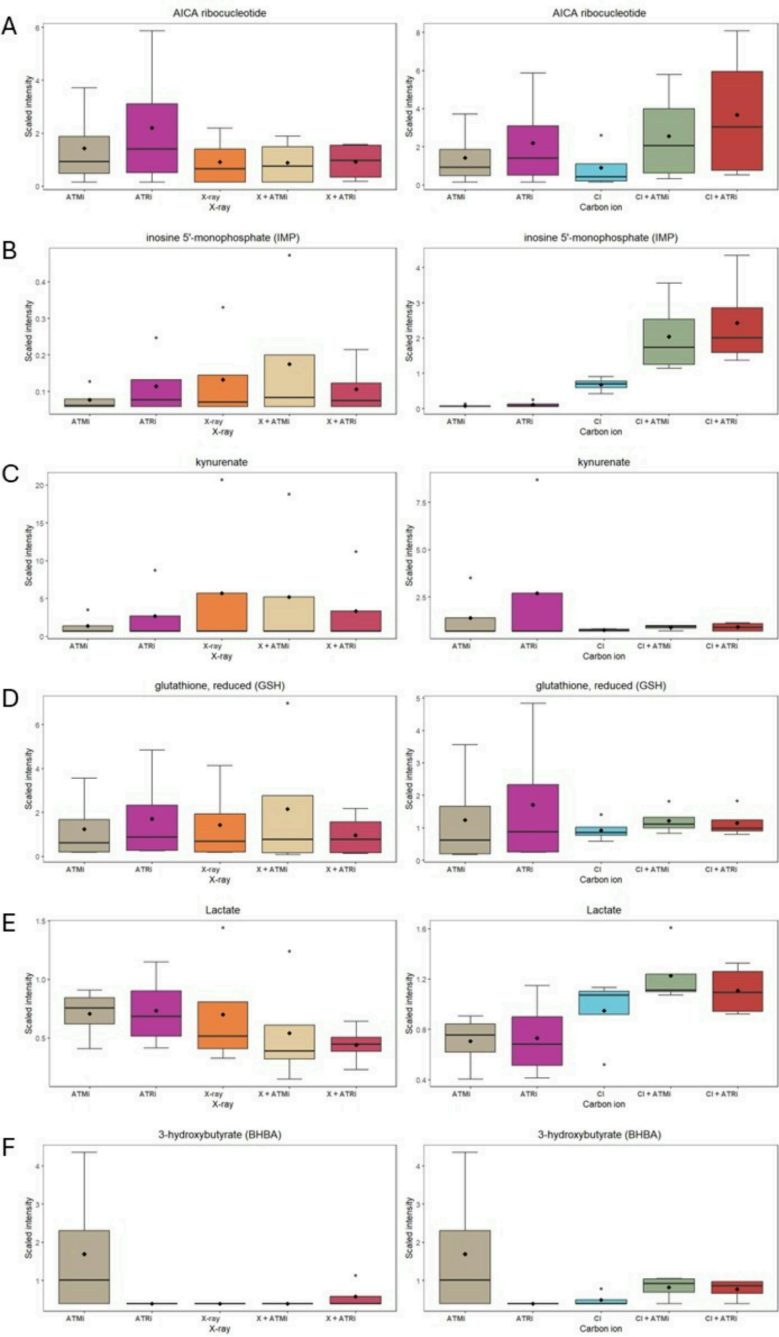


Figure 1

Radiation quality dependent alterations in different biological pathway with representative boxplots illustrating metabolites associated with AICA ribonucleotides (A), inosine'-monophosphate (B), kynurenate (C), reduced glutathione GSH (D), lactate (E) and 3-hydroxybutyrate (F). Boxplots display scaled intensity values for selected intermediates, across control, irradiation and combined DDR inhibition conditions for both X-ray and carbon ion cohorts.

Figure2

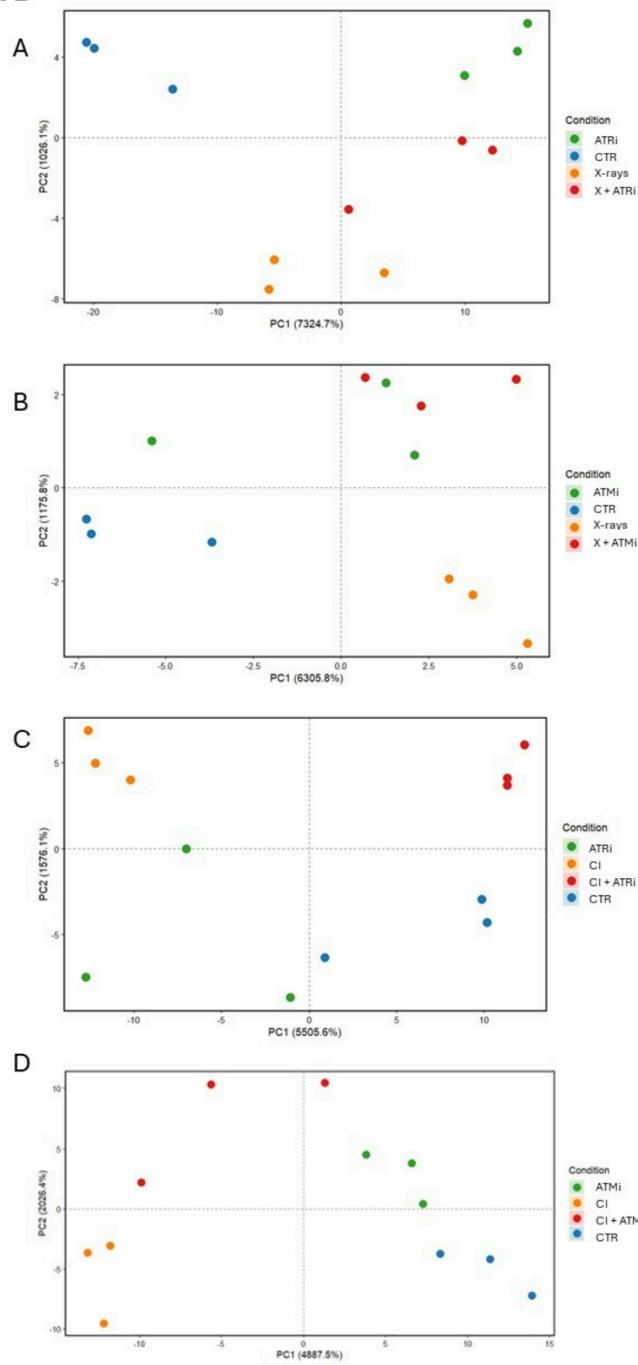


Figure 2

PCA plots of the X-ray and carbon ion irradiations, with the samples colored by treatment group (ATMi or ATRi) and experimental replicate.

Figure 3

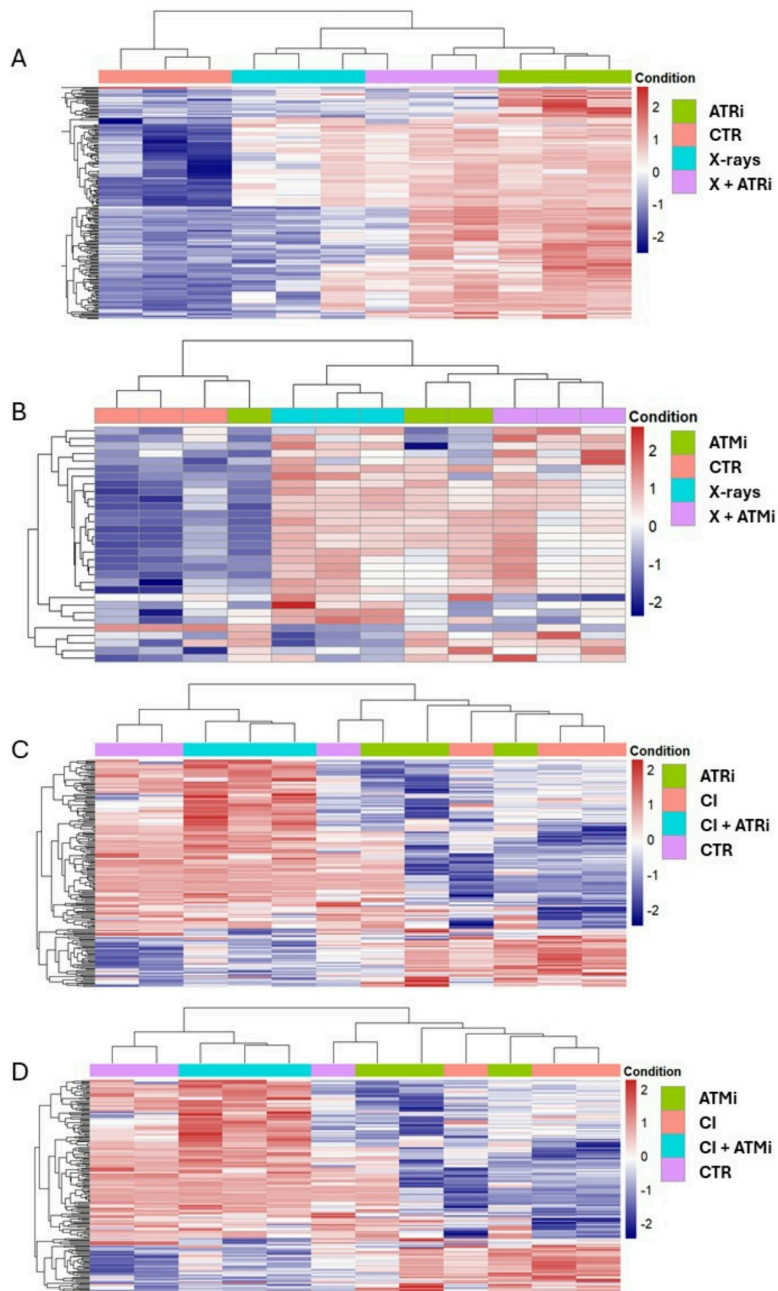


Figure 3

Heatmap of differentially regulated phosphosites across treatment conditions following X-ray irradiation and ATRi (A), X-ray irradiation and ATMi (B), carbon ion irradiation and ATRi (C), and carbon ion irradiation and ATMi. (D). Rows represent phosphosites and columns represent biological samples.

Figure 4

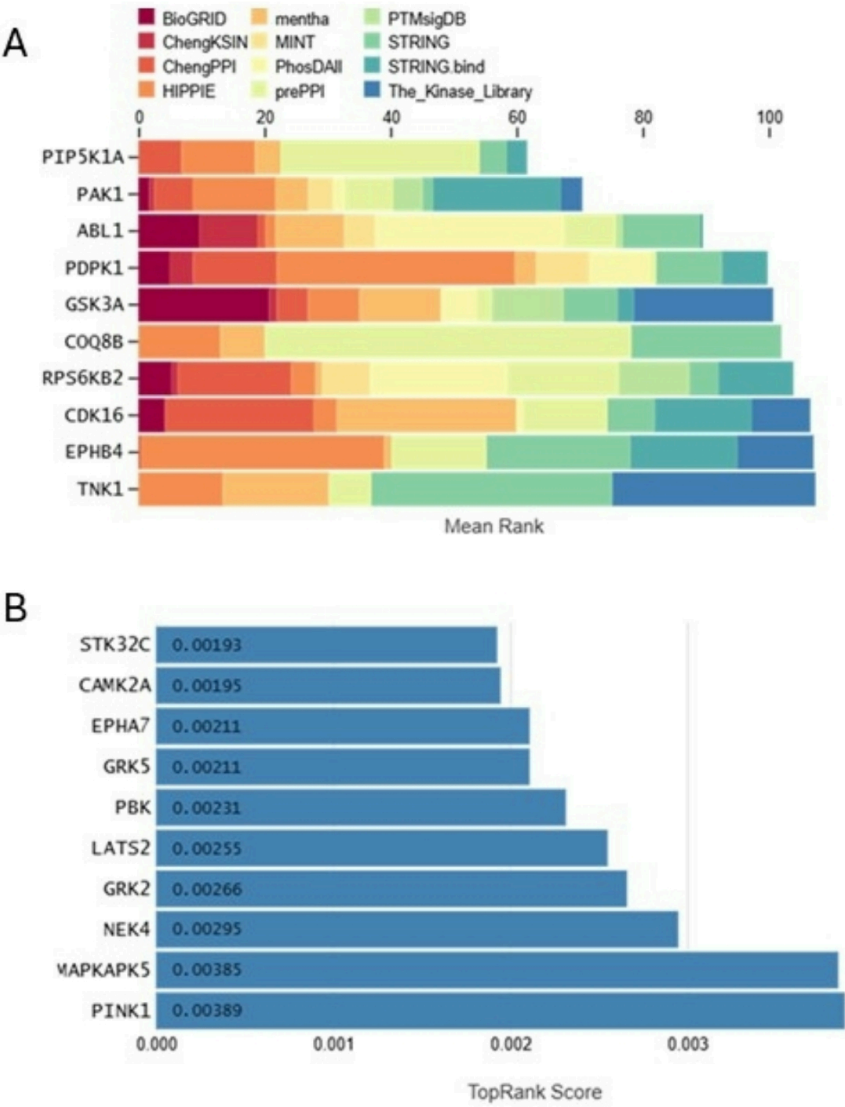


Figure 4

Kinase enrichment analysis.

A



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represents the fold enrichment of each GO term, while the color scale indicates statistical significance expressed as $-\log_{10}(\text{FDR})$.

Figure 6

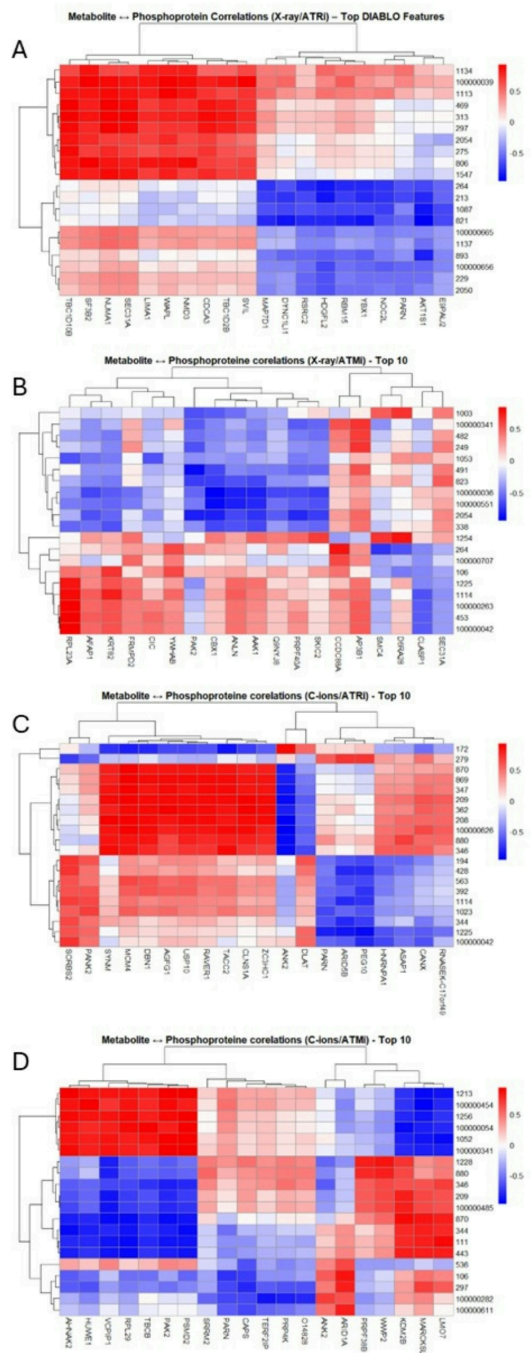


Figure 6

Heatmap of correlation between metabolites and phosphoproteins under different treatment conditions.

Figure 7

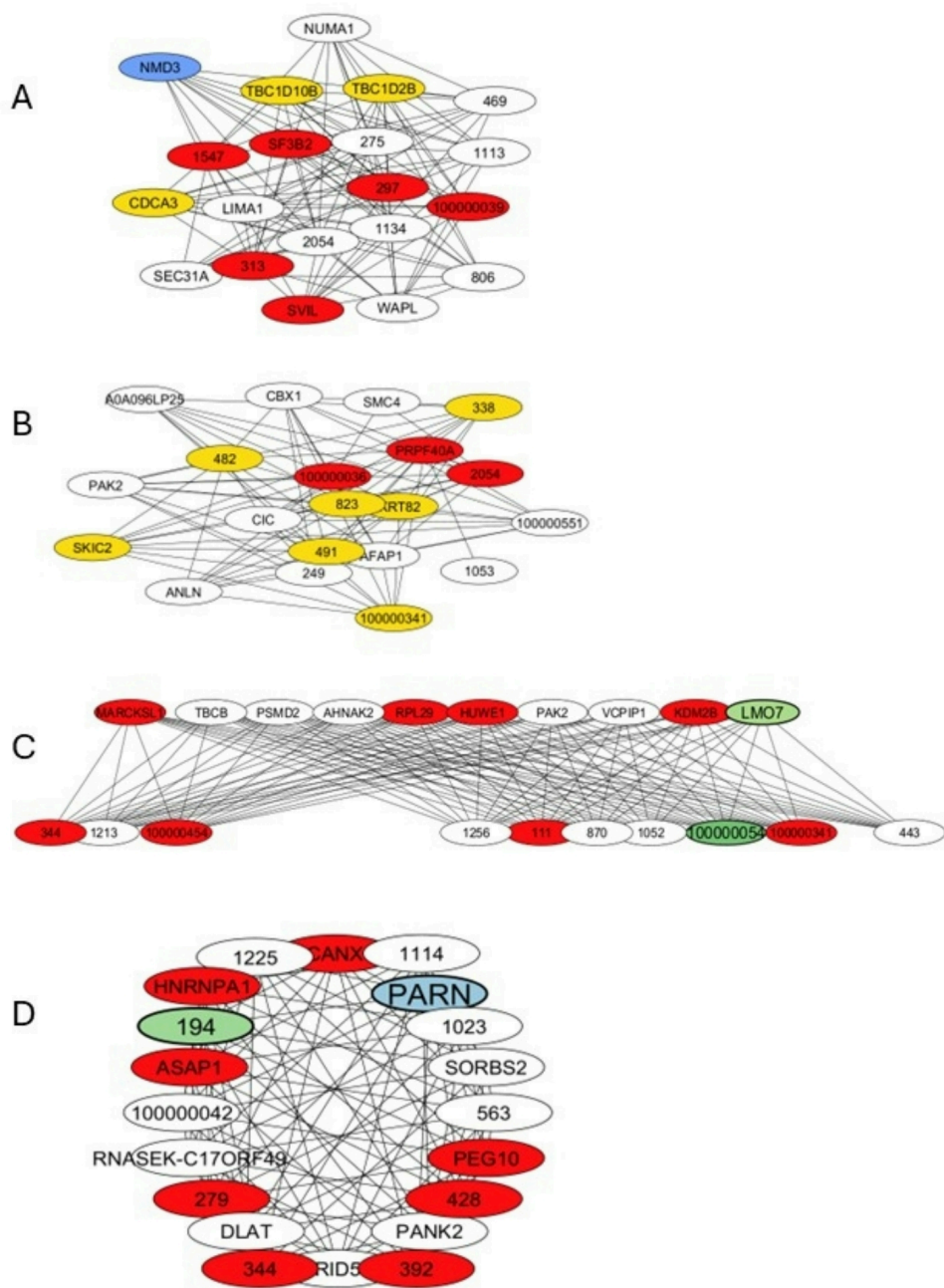


Figure 7

Interaction network of metabolites and phosphoproteins in response to treatment conditions.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.docx](#)