USP46 inhibits cell proliferation in lung cancer through PHLPP1/AKT pathway

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Abstract

Background: USP46 has been shown to function as tumor suppressor in colon cancer and renal cell carcinoma. However, its specific role in other cancers remains unknown. This study was aimed to investigate the role of USP46 in lung cancer tumorigenesis, and to identify the underlying mechanism.

Methods: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western Blotting (WB) were used to measure the expression levels of USP46 and PHLPP1 in lung cancer tissue and adjacent normal tissue from lung cancer patients. The functional role of USP46 in regulating proliferation in lung cancer cells were examined by cell proliferation assay, radiation assay, genetic overexpression and knock down and chemical inhibition of relevant genes. The underlying mechanisms were investigated in multiple lung cancer cell line models by co-immunoprecipitation and ubiquitination assays.

Results: This study identified strong downregulation of USP46 and PHLPP1 expression in lung cancer tissues relative to normal adjacent tissues. USP46 was further shown to inhibit lung cancer cell proliferation under normal growth conditions and during radiation induced DNA damage by antagonizing the ubiquitination of PHLPP1 resulting in the inhibition of AKT signaling. The effect of USP46 knock down on lung cancer cell proliferation was significantly reversed by exposure to radiation and AKT inhibition.

Conclusions: USP46 is down-regulated in lung cancer, and it suppresses proliferation of lung cancer cells by inhibiting PHLPP1/AKT pathway. AKT inhibition slows proliferation of USP46 down-regulated lung cancer cells exposed to radiation suggesting a potential therapeutic avenue for USP46 down-regulated lung cancer through a combination of radiation and AKT inhibitor treatment.

Background

Post-translational modifications in proteins play important role in regulating numerous biological processes, such as cell cycle, DNA damage repair, apoptosis and so on [1]. Ubiquitination is one of the most ubiquitous post-translational modifications that is dynamically regulated by ubiquitin ligases and deubiquiniases [2]. Ubiquitin-specific-protease 46 (USP46) is a member of cysteine proteases which functions as a deubiquitinase that has been associated with neurological disorders [3] and behavioral
abnormalities [4]. These observed phenotypes are likely due to an important role of USP46 in regulating neuronal signaling. For instance, USP46 has been shown to regulate glutamate receptor function by regulating the abundance of GLR-1 [5]. Likewise, USP46 has been shown to deubiquitinate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs) that leads to stabilization of the receptor [6]. Recent studies have identified other substrates of USP46 that have important function in cancer progression. In colorectal cancer, USP46 had been shown to deubiquitinate PH domain leucine-rich-repeats protein phosphatase 1 (PHLPP1) [7]. The PHLPP1 ubiquitination primes its degradation, and the deubiquitination thus protects PHLPP1 from degradation and enhances its activity in the cell [7]. One of the key substrates of PHLPP1 is Protein Kinase B (PKB, also called AKT), and the phosphatase removes activating phosphorylations in AKT proteins thus antagonizing the activity of AKT [8].

AKT family proteins are serine/threonine kinases that play critical role in cell proliferation, DNA damage repair and cell survival during stresses [9]. AKT family member genes are frequently mutated in different types of cancers, and inhibitors against the kinases are used in the treatment of several cancers [10]. Previously, USP46 has been shown to antagonize the activity of AKT by deubiquitinating PHLPP1 in colorectal cancer [7]. Consistent with the anti-proliferation activity of USP46, the protein is found to be down-regulated in colorectal cancer [7], suggesting that USP46 is an important tumor suppressor gene. The tumor suppressor activity of USP46 has been shown in renal cell carcinoma as well with a similar mechanism as described in colorectal cancer [11]. The role of USP46 in other cancers remains unclear.

Lung cancer is the most deadly form of cancer for both men and women with one of the worst survival rate [12]. About 1.6 million of people are estimated to die from lung cancer every year [13]. Recent advancements in cancer therapies have improved the survival rate of the lung cancer patients, however there are still unmet needs in terms of therapies. Elucidating the molecular mechanisms underlying the tumorigenesis is a key step towards developing effective therapies against lung cancer.

Here, we investigated the potential role of USP46 in lung cancer tumorigenesis, and found that USP46
expression is down-regulated in lung cancer along with PHLPP1. Mechanistically, we found that USP46 inhibits cell proliferation in lung cancer cells by inhibiting AKT pathway, in part by preventing DNA damage repair.

Methods

Human Tissues samples

A total of 30 pairs of lung cancer tissues and adjacent-matched normal tissues were used to examine mRNA levels and protein levels of different genes.

Cell culture

Cell lines (A549, H358, H446, H1299 and 16HBE) were obtained from cell bank of Shanghai Biology Institute (Shanghai (P.R. China)). Cells were cultured in DMEM medium (Trueline, Kaukauna (USA)) supplemented with 10% FBS (Thermo Fisher Scientific), 2 mM L-glutamine and 1% penicillin/streptomycin (Solarbio, Beijing (P.R. China)) and maintained under 5% CO2 atmosphere at 37 °C. The AKT inhibitor LY294002 (25 µmol/L; S1105, Selleck, USA) was dissolved in DMSO (D2650, Sigma USA) and added to cell culture.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNAs were isolated using TRIzol Reagent (Invitrogen, Waltham (USA)). cDNA was synthesized by using cDNA synthesis kit (Thermo Fisher Scientific, Waltham (USA)) following the manufacturer’s specifications. The thermal cycle was set as follows: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 45 seconds. All data represent an average of three replicates. Primers used were as follows: USP46: F 5’-AGAAGCCCAGAAAAGGATGAGG-3’, R 5’-CAAAAGCCAGAAGCCGTGAC-3’; PHLPP1: F 5’-AATGTGCCTGAGTGGGTATGTG-3’, R 5’-TCATCAGAAGGTTAGGTGGGAG-3’; Beta-actin: F 5’-CGTGGACATCCGCAAAGAC-3’, R 5’-TGCTGGGAGCCAGAGCAGC-3’.

Western Blotting (WB)

Whole protein lysates were extracted using Radioimmunoprecipitation assay (RIPA) lysis buffer (JRDUN, Shanghai (P.R. China)) containing EDTA-free Protease inhibitor Cocktail (Roche, Heidelberg (Germany)), followed by protein concentration measurement by an enhanced BCA protein assay kit (Thermo Fisher Scientific). 25 µg of proteins from each sample were ran in 10% SDS-PAGE gel and
transferred to a nitrocellulose membrane (Millipore, Billerica (USA)) overnight. After blocking with 5% nonfat dry milk for 1 hour at room temperature, the membranes were probed at 4 °C overnight with primary antibodies followed by secondary anti-mouse IgG antibody (1:1,000; Beyotime, Shanghai (P.R. China)) for 1 hour at 37 °C. Protein expressions were visualized by the enhanced chemiluminescence system (Tanon, Shanghai (P.R. China)). Detailed information of primary antibodies are as follows: anti-USP46 (Ab88795, Abcam (UK)), anti-PHLPP1(Ab135957 Abcam (UK)), anti-p-AKT(#9271, CST (USA)), anti-AKT (#9272, CST (USA)), anti-β-actin (#4970, CST (USA)).

Knockdown and overexpression of USP46

Lentiviral plasmids (pLKO.1) containing three siRNAs directed to different regions of human USP46 (NM_001134223.2) and a negative control siRNA (siNC) were synthesized (Major, Shanghai (People’s Republic of China)). USP overexpression plasmid was constructed by inserting the full-length human USP46 cDNA sequence into Lentiviral plasmid (pLVX-puro) while the vector plasmid was used as a negative control (oeNC). Experiments were performed 48 hours after the transit transfection of the plasmids into lung cancer cells using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer’s instruction. Detailed nucleotide sequences are as follows: siUSP46-1: (8–26; TCCGAAACATCGCCTCCAT), siUSP46-2: (17–35; TCGCCTCCATCTGTAATAT), siUSP46-3 (26–44, TCTGTAATATGGGCACCAA).

Cell Proliferation assay

Cell proliferation was analyzed by Cell Counting Kit-8 (CCK-8) (SAB, College Park, (USA)) following manufacturer’s protocol. Briefly, cells were seeded in 96-well plates for 0, 24, 48 and 72 hours. CCK-8 solution (1:10) was added to each well and incubated for 1 hour. The absorbance was measured by the microplate reader (Pulangxin, Beijing (P.R. China)) at 450 nm.

Immunofluorescence (IF)

Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. After that, samples were washed with PBS three times, 3 minutes each time, at 25 °C and blocked with 1% BSA (Solarbio, Beijing (People’s Republic of China)) for 1 hour at room temperature. Subsequently, cells were incubated with rabbit anti-γ-H2AX antibody (ab2893, abcam (UK)) in PBS overnight at 4 °C followed
by goat anti-rabbit IgG (H + L) (A0423, Beyotime, Haimen (People’s Republic of China)) for 1 hour at room temperature. Images were obtained by an ECLIPSE Ni microscope and a digital image analyzer (NIKON, Tokyo, Japan).

Co-Immunoprecipitation (Co-IP)

For IP, whole-cell extracts were prepared after transfection or stimulation with appropriate ligands, the lysates were incubated overnight with the appropriate antibodies conjugated to Protein A/G beads (Santa Cruz Biotechnology, (USA)). Beads were washed five times and separated by western blotting as described above.

Ubiquitination Assay

After 48 hours of transfection with oeNC or oeUSP46, the cells were lysed with 1% SDS-containing RIPA buffer, and sonicated. Subsequently, samples were incubated with IgG (sc-2027, Santa Cruz Biotechnology (USA)) or PHLPP1 antibody (PA5-34434, Invitrogen (USA)) overnight at 4°C, followed by incubation with Protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology (USA)) for 1 hour. The beads were centrifuged at 3000 rpm for 5 minutes at 4 °C and subsequently washed with wash buffer for four times. The purified proteins were separated by 4–20% gradient SDS-PAGE protein gel, and immunoblotted with anti-ubiquitin antibody (ab7780, Abcam, UK).

Data analysis

Data were shown as mean ± standard deviation (SD) based on three independent experiments. Statistical significance was determined by one-way ANOVA for multiple comparisons using GraphPad Prism software version 7.0 (USA). P-value < 0.05 was defined as statistically significant.

Results

USP46 and PHLPP1 were down-regulated in human lung cancer tissues

The expression of USP46 was measured in lung cancer tissues and adjacent normal tissues in 30 patients by qRT-PCR. We found that the cancer tissues had significantly lower level of USP46 messenger RNA (mRNA) (Fig. 1A). The protein level of USP46 was further evaluated in 8 patients with lung cancer tissues, and as expected the lower level of USP46 mRNA in the cancer tissue compared to the adjacent normal tissue correlated with the protein level (Fig. 1B). We also compared the
expression of USP46 in several human lung cancer cell lines to normal human bronchial epithelial (HBE) cells, and found that USP46 expression was greatly decreased both in mRNA level (Figure S1A) as well as in protein level (Figure S1B) in the lung cancer cell lines compared to HBE cells.

USP46 has been shown to increase the stability of PHPLL1 in colorectal cancer [7], hence we checked if USP46 expression is positively correlated with the protein level of PHPLL1. Indeed, the lower protein level of USP46 correlated with the lower level of PHLPP1 in lung cancer tissues compared to the adjacent cancer tissues (Fig. 1B).

**USP46 inhibited proliferation of human lung cancer cells**

Next, the role of USP46 in cell proliferation in several lung cancer cell lines was determined. We overexpressed USP46 in H1299 and measured their proliferation. The cells overexpressing USP46 (oeUSP46) showed significantly lower proliferation 48 hours after USP46 overexpression compared to the control cells (oeNC) (Fig. 2A). The inhibition of proliferation was even more prominent after 72 hours post transfection (Fig. 2A). To verify that the effect of USP46 in proliferation is general phenomena in lung cancer cells, we repeated the experiment in another lung cancer cell line, A549. As expected, oeUSP46 inhibited proliferation in A549 cell line as well (Fig. 2B).

Next, we sought to identify potential mechanism through which USP46 regulates cell proliferation in lung cancer cells. In colorectal cancer, USP46 has been shown to promote stabilization of PHLPP1 and subsequent inhibition of AKT pathway [7]. Hence, we checked the protein level of PHLPP1 and the activation status of AKT in H1299 USP46oe cells (Figures S1C and S1D, Fig. 2C). Indeed, oeUSP46 led to a significant increase in PHLPP1 protein level and significant decrease in the activating AKT phosphorylation without altering the protein level of AKT (Fig. 2C), demonstrating the role of USP46 in increasing protein level of USP46 and inhibiting the AKT signaling pathway. Similar results were observed in A549 cell as well (Figure S1C and S1D, Fig. 2D). Conversely, knock down of USP46 led to significant increase in the proliferation of H446 cell lines (Figures S1E and S1F, Fig. 2E), as well as decrease in PHLPP1 protein level and an increase in the activating AKT phosphorylation (Fig. 2F). Collectively, these results established the role of USP46 in inhibiting cell proliferation in lung cancer cells by promoting the stability of USP46 and subsequently inhibiting the AKT pathway.
USP46 promoted PHLPP1 protein stability via deubiquitination in human lung cancer cells

We investigated the mechanism through which USP46 causes an increase in PHLPP1 protein level. First, we checked if USP46 regulates the expression of PHLPP1 in the transcriptional level. We found neither overexpression nor knock down of USP46 has any effect in the mRNA level of PHLPP1 (Figs. 3A and 3B). These results suggested a potential regulation in the protein level. Hence, we checked physical interaction between USP46 and PHLPP1 by co-immunoprecipitation (co-IP). We found that PHLPP1 precipitated with USP46 and vice versa (Figs. 3C) demonstrating that USP46 and PHLPP1 physically interact with each other.

USP46 is a deubiquitinase so we hypothesized that USP46 deubiquinates PHLPP1 in lung cancer cells. To test this hypothesis, we overexpressed USP46, and measured the level of ubiquitination in PHLPP1 in H1299 and A549 cells. Compared to the control cells, overexpression of USP46 led to a decrease in ubiquitination in PHLPP1 (Fig. 3D), indicating that USP46 deubiquitinates PHLPP1 in lung cancer cell lines.

USP46 inhibited cell proliferation during DNA damage in human lung cancer cells

AKT family kinases play an important role in DNA damage repair [14]. More importantly, recent studies have suggested that AKT activity is a good predictive marker of radiation response in cancer treatment, and that AKT promotes cell survival post radiation by accelerating DNA damage repair (IR) [15–17].

Hence, we tested if USP46 is involved in inhibition of cell proliferation following DNA damage. We treated cells with varying degree of ionizing radiation (IR) to induce DNA damage and measured cell proliferation at different time points post IR. USP46oe cells showed significantly less cell proliferation compared to the control cells in both H1299 and A549 cells (Figs. 4A, B). The degree of reduction in cell proliferation in USP46oe cells correlated with the degree of IR (Figs. 4A, B).

DNA damage in the cells induces phosphorylation at Ser139 residue of histone H2AX forming gamma H2AX (Y H2AX) foci, hence Y-H2AX can be used as a marker of DNA damage in the cells [18]. Since PHLPP1 inhibits the activation of AKT, we checked the effect of USP46 expression in the formation of DNA damage dependent Y H2AX foci upon exposure of cells to IR. Overexpression of USP46 led to a
decrease in Y H2AX foci formation in both H1299 and A549 cells one hour post IR (Fig. 4C, D), and the foci formation was not restored even four hours post IR (Fig. 4C, D). Conversely, knock down of USP46 showed an increase in Y H2AX foci formation post IR (Fig. 4E). Collectively, these results indicate the role of USP46 in inhibiting lung cancer cell proliferation during DNA damage.

**AKT inhibition improved proliferation rate of USP46 knock down cells exposed to radiation**

Next, we tested the effect of an AKT inhibitor, LY294002, in lung cancer cell proliferation during DNA damage, and checked the role of USP46 in this context. As expected, we found that knock down of USP46 increased the proliferation of H446 cells following exposure to IR (Fig. 5A). However, proliferation of the cancer cells were significantly reduced when AKT kinases were inhibited by LY294002 (Fig. 5A). The inhibition in proliferation was partially rescued in the cells treated with LY294002 and exposed to IR in the USP46 knocked down cells (Fig. 5A). Collectively, these results suggest an important role of USP46 in antagonizing the activity of AKT in cell proliferation following DNA damage.

**Discussion**

In this study, we showed that USP46 expression is downregulated in lung cancer patients, suggesting a tumor suppressor role of USP46 in lung cancer, which is consistent with the reports from other studies in colorectal cancer [7] and renal cell carcinoma [11]. Functionally, we demonstrated USP46 inhibits cell proliferation in lung cancer cells under normal growth conditions as well as in cells exposed to ionizing radiation. Conversely, we showed down-regulation of USP46 increases proliferation of lung cancer cells, and exposure to radiation in combination with an AKT inhibitor suppresses proliferation of the lung cancer cells. These results have huge clinical significance as they indicate a potential therapeutic avenue in USP46 down-regulated lung cancers through a combination therapy involving radiation and AKT inhibitors.

Mechanistically, we showed the anti-proliferation activity of USP46 in lung cancer is mediated through inhibition of AKT activity via deubiquitination of PHLPP1. These findings form a solid foundation for in vivo experiments where the precise role of USP46 in lung cancer progression can be evaluated in more physiological conditions in mouse models. It will be interesting to test if USP46 is primarily
involved in the primary tumor growth or it is important for metastasis or both. These questions will be matter of future investigations.

Lung cancer is the most deadly cancer for both men and women in the United States with one of the lowest survival rates [19]. Although several treatments options are available for lung cancer patients, there is still a huge unmet clinical need. Our study has identified USP46 as an important negative regulator of cell proliferation in lung cancer cells. We also established an adverse clinical correlation of USP46 expression in lung cancer patients. These findings suggest that targeting pathways or proteins that increases expression of USP46 could be beneficial therapeutic avenue for lung cancer treatment. Our study along with previous studies strongly suggests the tumor suppressor role of USP46 in multiple cancers. It will be interesting to test if knock out of USP46 is sufficient to drive tumorigenesis by itself or in combination with other known mutations. Furthermore, it remains to be investigated if there are recurrent mutations in USP46 gene in cancer patients, and if so, how they correlate with other known driver mutations. Based on our study, it is very likely that there will be some correlation with mutations in PI3K/ AKT genes across multiple cancers. A thorough analysis of the genomic databases could easily provide more concrete answer to these important questions.

We also showed that USP46 antagonizes the activity of AKT pathway that affects the proliferation of lung cancer cells. Multiple classes of potent AKT inhibitors have been developed, and some of them are already used in clinics for different diseases and some are currently under clinical trials for the treatment of different cancers [20]. Drugs targeting AKT might benefit lung cancer patients with lower expression of USP46. It will be interesting to know if the patients who respond to the AKT inhibitors correlate with the expression level of USP46. Future work will shed more light into the functional ties between USP46 and AKT during cancer therapies.

Conclusion
This study establishes an important role of USP46 in antagonizing the activity of AKT in lung cancer cells that ultimately inhibit cell proliferation. Mechanistically, we showed that USP46 stabilizes PHLPP1 by deubiquitinating the protein, and thus negatively affect AKT activity. Clinically, we showed that USP46 and PHLPP1 are down-regulated in lung cancer patients.
Abbreviations
Quantitative Real Time-Polymerase Chain Reaction
qRT-PCR;
Western Blotting
WB
Ubiquitin-specific-protease 46
USP46;
Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPARs
PH domain leucine-rich-repeats protein phosphatase 1
PHLPP1

Declarations
Ethics approval and consent to participate
All patients had informed and written consent. This study was approved by the independent ethics committee of Affiliated Taizhou hospital of Wenzhou Medical University and was in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable

Availability of data and material
The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
The concept of the paper was devised and the first draft was written by HH. The experiment was done by WW and MC, DQ and SN analyzed the data. All authors read and approved the final manuscript.

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Supplementary Figure Caption

Figure S1: Expression of USP46 in human lung cancer cells was measured. A & B. Relative mRNA and protein levels of USP46 in normal human bronchial epithelium (HBE) cells and indicated human lung cancer cells were measured by qRT-PCR (A) and WB (B) respectively, and the protein levels were quantified. Mean ± SD, (n=3), *** means p < 0.001. C & D. Relative mRNA and protein levels of USP46 in indicated human lung cancer cells overexpressing USP46 were measured by RT-PCR (A) and WB (B) respectively, and the protein levels were quantified. USP46 mRNA level was normalized to beta-actin mRNA. Mean ± SD, (n=3), *** means p < 0.001. E & F. Relative mRNA and protein levels of USP46 in H446 cells upon USP46 knock down were measured by qRT-PCR (C) and WB (D) respectively, and the protein levels were quantified. Mean ± SD, (n=3), *** means p < 0.001.

Figures
USP46 and PHLPP1 were downregulated in human lung cancer tissues. A. mRNA level of USP46 in human lung cancer and adjacent-matched tissues was measured by qRT-PCR. USP46 mRNA level was normalized to beta-actin mRNA level. (n=30). ** means p < 0.01. B. Expression of USP46 and PHLPP1 in 8 pair’s human lung cancer and adjacent-matched tissues was measured by western blotting (left), and protein level were quantified according to the gray value after normalized to β-actin (n=8). *** means p < 0.001.
USP46 inhibited proliferation of human lung cancer cells. A & B. Cell proliferation was measured by CCK-8 kit in H1299 cells (A) and A549 cells (B), and the rate of proliferation was compared between cells overexpressing USP46 (oeUSP46) and control cells (oeNC). (n=3). *** means p < 0.001. C & D. Protein level or phosphorylation level of indicated proteins were measured by WB in H1299 cells (C) and in A549 cells (D) overexpressing USP46 (oeUSP46) cells and control (oeNC) cells, and their quantifications are shown. (n=3). *** means p < 0.001. E. Cell proliferation in H446 cells transfected with control siRNA (siNC) or USP46 siRNA (siUSP46-1 and siUSP46-2) were measured by CCK-8 kit. (n=3). *** means p < 0.001. F. Level of indicated proteins and phosphorylation were measured by WB in cells shown in E, and their quantifications are shown. (n=3). *** means p < 0.001.
USP46 promoted PHLPP1 protein stability via deubiquitination in human lung cancer cells. A & B. mRNA level of PHLPP1 in A549 and H1299 cells upon USP46 overexpression (A), and USP46 knockdown (D) was measured by RT-PCR. The data were normalized to beta-actin. Mean ± SD, (n=3). C. Interaction between USP46 and PHLPP1 was tested by co-IP followed by WB using indicated antibodies. D. USP46 dependent ubiquitination in PHLPP1 was measured by comparing ubiquitination in PHLPP1 in USP46 overexpressing cell (oeUSP46) and control cells (oeNC).
USP46 inhibited cell proliferation during DNA damage in lung cancer cells. A & B.

Proliferation of H1299 (A) and A549 (B) cells overexpressing USP46 (oeUSP46) and control vector (oeNC) exposed to radiation was measured by CCK-8 kit. Mean ± SD, (n=3). *** means p < 0.001 (vs. 4Gy + oeNC); ### means p < 0.001 (vs. 4Gy + oeUSP46). C & D. γ-H2AX foci formation in H1299 (C) and A549 (D) oeNC and oeUSP46 cells after exposure to radiation was assessed by IF at the indicated time points after 4 Gy radiation exposure. E. γ-H2AX foci formation in USP46 knock down H446 cells after exposure to 4 Gy radiation was assessed by IF.
AKT inhibition improved proliferation rate of USP46 knock down cells exposed to radiation.

Control cells (siNC) or USP46 knock down cells (siUSP46) exposed to 4 Gy radiation were treated with LY294002, and cell proliferation was measured by CCK-8 kit. Mean ± SD, (n=3), *** means p < 0.001 (vs. 4Gy + siNC); ### means p < 0.001 (vs. 4Gy + siUSP46).

Supplementary Files
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Figure S1.jpg