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**Irradiation of epithelial carcinoma cells upregulates calcium-binding proteins that
promote survival under hypoxic conditions**

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Running title: γ -ray induced pro-survival calcium-binding protein in hypoxia.

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Abstract

Hypoxia is thought to promote tumor radio-resistance via effects on gene expression in cancer cells which modulate their metabolism, proliferation, and DNA repair pathways to enhance survival. Here, we demonstrate for the first time that under hypoxic conditions, A431 epithelial carcinoma cells exhibit increased viability when exposed to low-dose γ -irradiation, indicating that radiotherapy can promote tumor cell survival when oxygen supply is limited. When assessed using iTRAQ quantitative proteomics and Western blotting, irradiated tumor cells were observed to significantly up-regulate the expression of calcium-binding proteins CALM1, CALU and RCN1, suggesting important roles for these mediators in promoting tumor cell survival during hypoxia. Accordingly, shRNA-knockdown of CALM1, CALU and RCN1 expression reduced hypoxic tumor cell resistance to low-dose radiation and increased apoptosis. These data indicate that γ -irradiation of hypoxic tumor cells induces up-regulation of calcium-binding proteins that promote cancer cell survival and may limit the efficacy of radiotherapy in the clinic.

Keywords: Hypoxia, cancer, radiotherapy resistance, calcium signaling, proteomics, LC-MS/MS, iTRAQ

Introduction

Developing tumors are subjected to a hostile microenvironment with a limited oxygen supply that promotes increased cancer cell resistance to radiotherapy¹. Low-oxygen conditions have previously been shown to significantly increase the radioresistance of tumor cells by up to 3-fold relative to cancer cells growing under aerobic conditions². These pro-survival effects of oxygen restriction or ‘hypoxia’ are thought to be achieved via modulation of DNA damage repair pathways in the developing tumor cells. Consequently, efforts to improve the efficacy of radiotherapy have explored many different approaches to modifying or sensitizing hypoxic tumor cells to radiation damage before a patient commences treatment³. Understanding the tumor cell response to radiation is therefore key to improving outcomes for human cancer patients undergoing radiation therapy. However, the tumor cell response to therapeutic radiation is complex and can range from only limited effects through to variable growth arrest and/or apoptotic death, and the factors that determine the balance between these diverse outcomes remain poorly understood.

The transcription factor hypoxia-inducible factor-1 regulates cellular responses to low-oxygen conditions via wide-ranging effects on gene expression, and has previously been implicated in the modulation of tumor radioresistance⁴⁻⁶. While a previous report has identified that radiation can stimulate HIF-1 expression levels in tumors to increase⁷, the authors observed complex effects on radiosensitivity and variable impact on cancer cell survival⁸. Indeed, ionizing radiation (IR) itself has been reported to activate cell signaling cascades that can modulate the viability of carcinoma cells, including the proliferation-enhancing Ras/Raf/ERK kinase pathway⁹⁻¹². Similarly, exposure to radiation has been shown to stimulate NF- κ B gene expression¹³⁻¹⁵, and induces a potent stress response accompanied by kinase activation in a variety of lineages exposed to IR doses ranging from 2–100 Gy^{12, 16-22}.

Calcium flux regulates diverse cellular processes including exocytosis, enzyme function, gene expression, cell proliferation, and major apoptosis pathways²³. Many proteins either interact with or require calcium ions to mediate signal transduction, but the possible configurations of calcium-binding sites in proteins are actually rather limited. The most common of these calcium-binding structures is the EF-hand motif²⁴, which mediates diverse functions including ion buffering in the cytosol, signal transduction between cellular compartments, and a wide range of critical metabolic processes^{25, 26}. A major sensor of intracellular calcium is the multifunctional protein Calmodulin (CaM) which can potently modulate the activity of

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many key signaling pathways^{27, 28}. CaM has previously been implicated in proliferative responses to low-dose radiation under aerobic culture conditions^{11, 29, 30}, but the ability of calcium-binding proteins to influence the survival of hypoxic tumor cells has not been described.

In the current study, we observed that low-dose γ -irradiation induced tumor cell expression of several calcium-binding proteins with EF-hand motifs, including CALM1, CALU and RCN1, and that targeted knockdown of these proteins prevented radiation-induced increases in the viability of hypoxic tumor cells. These data suggest that in low-oxygen environments such as in tumors, exposure to low-dose radiation can increase tumor cell viability via induction of calcium-binding proteins and signaling pathways that promote survival. These data may assist efforts to increase the efficacy of radiotherapy and improve outcomes for cancer patients in the clinic.

Experimental Procedures

Chemicals and reagents

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. GasPakTM EZ Gas Generating Pouch Systems with indicators were obtained from Becton Dickinson (Franklin Lakes, NJ). Antibodies against CALM1, CALU and RCN1 were purchased from Abcam (Cambridge, UK). Anti-actin (clone C4 | MAB1501) was purchased from Millipore (Billerica, MA). The antibiotic reagent G418 was acquired from PAA Laboratories (Piscataway, NJ). Protease inhibitor cocktail tablets were obtained from Roche (Basel, Switzerland).

Cell culture and induction of hypoxia

A431 epithelial carcinoma cells were purchased from ATCC and maintained in DMEM supplemented with 10% FBS for culture at 37°C with 5% CO₂. The cells were later seeded into 24-well plates (4×10⁴ cells per well) or 10cm dishes (3×10⁶ cells each) and incubated overnight, washed twice with 1X PBS, and washed twice more in serum-free medium. The cell pellets were then re-suspended in serum-free medium and transferred to a humidified CO₂ incubator for culture either under normoxic conditions (Nx: 21% O₂; 5% CO₂) or were subjected to hypoxia by sealing the culture plate/dish inside a single GasPakTM anaerobe pouch (EZ Gas Generating Pouch Systems) to generate a low-oxygen atmosphere (Hx: <0.7% O₂; >5% CO₂).

Gamma irradiation

After 24h incubation under normoxia, the A431 cells were exposed to 2-4Gy of γ -irradiation at a dose rate of 2.6Gy/min with a Cs-137 source (Biobeam 8000 γ -irradiator, Gamma-Service Medical GmbH, Germany). Hypoxic cells remained sealed in anaerobic pouches for the duration of this process. Thereafter, the cells were returned to their respective culture conditions for further 24h incubation.

MTT assay and colony formation

Cell survival under normoxia or hypoxia with/without gamma irradiation was determined by MTT assay or assessment of colony formation at the end of the 48h culture period. For MTT assay in different dose of gamma irradiation under hypoxia, the same amount of A431 cells (2 × 10⁴) were seeded in several 24-well plates. Next day the cells in some plates were used for MTT assay to determine the amount of cells as original control. And the cells in the other

plates were washed with PBS and sealed into hypoxic bags. After cultured for 24h, the cells were treated with different dose of gamma irradiation. With total 48h culturing under hypoxia, the amount of survival cells were also determined by MTT assay. For MTT quantification, the medium was replaced with 0.5mg/mL thiazolyl blue tetrazolium bromide (MTT) in DMEM and the suspension was incubated for 2h under aerobic conditions (5% CO₂). The MTT content of the cells was then solubilized in DMSO and quantified at A570nm, reference A630nm. The survival ratio of H48 was calculated by OD value of cells with only 48h hypoxia treatment divided by that of cells used for original control. The survival ratio of H48 cells was defined as 100% and the survival ratios of those cells with gamma irradiation were listed as compared with it. For colony formation assays, the A431 cells were re-suspended in DMEM containing 10% FBS and then cultured at 2,500 cells per well in 6-well plates for 2 weeks prior to fixation with 95% ethanol for 10min and final staining with 0.5% crystal violet for 30min. Excess dye was removed by washing with water. Once dry, each well was supplemented with 1ml 0.5% Triton X-100 in order to solubilize the cells during overnight incubation at room temperature. Colony quantification was performed by measuring absorbance at A_{595 nm} using a microplate reader (Tecan MagellanTM, Männedorf, Switzerland).

Sample preparation, iTRAQ labeling, and LC-MS/MS

Proteomic experiments were performed in triplicate. Four samples were used in the iTRAQ experiment. N48 sample was from cells incubated in normoxia for 48hr; N24+3G+N24 sample was from cells incubated under normoxia for 24h, followed by exposure to 3Gy of γ -irradiation and then incubated for another 24h in normoxia condition; H48 sample was from cells cultured under hypoxia condition for 48hr. H24+3G+H24 was similar to N24+3G+N24 except the cells were incubated in hypoxia conditions prior and after γ -irradiation. The cellular proteins obtained from each experiment were dissolved in 8M urea in 20mM TEAB (pH 8.5) solution containing protease inhibitor cocktail (1:50). Protein concentrations were measured using BCA assays. For each condition, a total of 100 μ g protein was reduced with 5mM TCEP for 3h at 30°C then alkylated with 10mM MMTS for 45min at room temperature in the dark. The samples were then diluted with 20mM TEAB to achieve a final concentration of 1M urea prior to digestion with trypsin overnight at 37°C (mass ratio trypsin 1:50 protein). The resultant solution containing tryptic peptides was adjusted to pH 2-3 with TFA and desalted using Sep-Pak C18 cartridges (Waters). The peptides were then dried using a Speedvac concentrator, dissolved in 1M TEAB (pH 8.5), and finally labeled with isobaric tags according to manufacturer's protocol (Applied Biosystems, Foster City, CA). The sample

from cells culture in normoxia for 48hr (N48) was labeled with 114; the N24+3G+N24 sample was labeled with 115; the H48 sample was labeled with 116; and the H24+3G+H24 sample was labeled with 117. The labeled samples were then pooled and desalted using Sep-Pak C18 cartridges.

The iTRAQ-labeled samples were fractionated on an ERLIC column (PolyLC, Columbia, MD; 4.6×200 mm, 5µm particle size, 300-Å pore size) using a Shimadzu Prominence UFLC system (Kyoto, Japan). Fractionation was performed using a gradient of buffer A (10mM CH₃COONH₄ in 85% ACN/1% FA) and buffer B (30% ACN/0.1% FA) conducted over a 60min period at a constant flow rate of 0.9ml/min (0% buffer B for 5 min, 0-28% B for 40min, 28-100% B for 5min, 100% B for 10min). A total of 30 separate fractions were collected and desalted using Sep-Pak C18 cartridges. Each fraction was then dried and reconstituted in a 40µl volume of 3% ACN, 0.1% FA for Q-Exactive LC-MS/MS analysis. The detailed parameters used were as previously described³¹.

Database searching

Protein identification and quantification were performed using Proteome Discovery 1.4 software with Sequest searches conducted against the UniProt human database (released on 2012_05, comprising 87881 protein sequences) combined with 115 common contaminants (cRAP protein sequences). The false discovery rate (FDR) was determined using an in-house decoy database search and was set to less than 1% for both peptide and protein identification. Trypsin was selected as the specific enzyme with a maximum of two missed cleavages permitted per peptide. Parameters included static modification: iTRAQ 4 plex (N-term), iTRAQ 4 plex (K), Methylthiol (C); dynamic modification: Oxidation (M), Deamidation (N, Q), iTRAQ 4 plex (Y). Data were searched with a peptide mass tolerance of 10ppm and a fragment mass tolerance of 0.02Da. The iTRAQ-quantified proteins were then exported by Proteome Discoverer software to a tab-delimited text file for further data analysis (Supplemental Table S1). The LC-MS/MS raw data were deposited in the ProteomeXchange Consortium via the PRIDE data repository under the dataset identifier PXD003937. The submission details are:

Project Name: gamma ray irradiation of epithelial carcinoma cells under hypoxic conditions

Project accession: PXD003937

Project DOI: Not applicable

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Western blotting

For Western blot (WB) analysis, the protein content of the cell lysates was first solubilized using 1% SDS in 40mM Tris-HCl (pH 8.0) then quantified by BCA assay prior to loading equal quantities of each sample onto a 12% SDS-PAGE gel. For native gels, protein complexes were extracted using a solution of 1% Triton X-100 in 20mM Tris-HCl (pH 7.4) and 150mM NaCl containing complete protein inhibitor cocktail (1:50). Proteins were then transferred onto nitrocellulose membranes and immunoblotted using antibodies against STAT1, p-STAT1, Ku70, Ku80, ITGA5 and ITGB1. Labeled proteins were then detected using the Invitrogen ECL system according to the manufacturer’s instructions.

Knockdown of calcium-binding protein expression in A431 cells

The primer sequences used to perform shRNA knockdown of CALM1, CALU and RCN1 (or luciferase-only control) were generated using the Invitrogen BLOCK-iT™ RNAi Designer (Supplemental Table S2). After annealing according to the manufacturer’s instructions, the fragments were inserted into a shRNA vector (pSuper-GFP-Neo), and plasmids with positive insertions were transfected into A431 cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s protocol. Cells were then selected by culture in standard medium containing 1mg/ml G418 antibiotic. After 1-2 weeks, monoclones were picked from the plates by trypsin digestion and positive clones were confirmed by WB. In the knockdown experiments, two different shRNA fragments were chosen to reduce the expression level of each gene and two positive monoclones with lowest expression level in all positive clones were picked up for function studies.

Statistical analysis

Data are expressed as mean ± SD. Paired t-tests were used to evaluate differences between groups. P-values <0.05 were considered significant.

Results and Discussion

Low-dose γ -radiation increases epithelial carcinoma cell survival under hypoxic conditions

Hypoxia is a well-established contributor to cancer radioresistance and has been reported to increase mammalian tumor cell survival ~3-fold relative to normoxic culture conditions. In the current report, we observed that under low-oxygen conditions, γ -radiation significantly increased the viability of A431 epithelial carcinoma cells compared with their non-irradiated counterparts. These data suggested that γ -radiation can elicit a cellular response that promotes cell survival in the hypoxic microenvironment of a developing tumor. As shown in Figure 1A, our MTT cell survival assays indicated that 1-2 min of low-dose γ -irradiation (2-4G) administered half-way through a 48h culture in low-oxygen conditions (H24+Gamma+H24) increased tumor cell viability compared with cells subjected to hypoxia alone (H48). Exposure to γ -irradiation was associated with a 13-17.5% increase in survival compared with non-irradiated cells (n=6 per group; p<0.05), with optimum viability being induced by treatment with just 3G of radiation, although this survival advantage was conferred only under hypoxic conditions (Fig.1B). Similar data were obtained when assessing tumor cell survival by colony formation assay, which indicated that 3G radiation treatment reduced the number of colonies formed under normoxia, but increased the number of colonies formed under hypoxic conditions (Fig.1C). Under conventional culture conditions, these longer-term assays are typically associated with increased rates of apoptotic cell death, but when we quantified colony staining we observed that a 3G dose of γ -irradiation increased the viability of hypoxic tumor cells by as much as ~25% (Fig.1D). Previous reports have indicated that A431 epithelial carcinoma cells exhibit increased radioresistance when over-expressing epidermal growth factor receptor (EGFR)³². Similarly, when transfected into OCA-I murine ovarian carcinoma cells, EGFR expression level correlated with tumor cell resistance to ionizing radiation, which might explain the radiosensitizing effect of treatment with the EGFR inhibitor erlotinib^{11, 14}. In addition, low-dose IR has been reported to activate VEGFR, and radiotherapy-induced enhancement of tumor growth and metastasis are reportedly blocked by administration of a VEGF receptor tyrosine kinase inhibitor immediately prior to IR exposure³³. According to a previous study conducted in A431 cells, just 2 Gy radiation induced phosphorylation of EGFR tyrosine and activated the mitogen-activated protein kinase (MAPK) and c-Jun NH(2)-terminal kinase (JNK) pathways¹². In our experiments, EGFR expression/activity was not altered by γ -irradiation, perhaps due to the longer culture period

used in our survival assays. We therefore cannot exclude a role for EGFR activation in the observed post-radiation increases in tumor cell survival under hypoxic conditions.

Calcium-binding protein induction by γ -radiation in both normoxic and hypoxic tumor cells

In order to elucidate the mechanism/s by which γ -irradiation enhances tumor cell survival in low-oxygen environments, we next used quantitative proteomic analysis to identify potential mediators of this increased viability. To do this, tumor cells were cultured under various conditions (N48; N24+3G+N24; H48; H24+3G+H24) and subjected to protein extraction, digestion, peptide labelling with iTRAQ reagents, and quantitative profiling by LC-MS/MS analysis using a Q-Exactive apparatus. Raw data were analyzed using Proteome Discovery 1.4 software and compared against the UniProt human database (downloaded on 14 June 2012, including 87187 sequences). Changes in protein expression level were determined by calculating the ratios of isobaric tags detected to assess the extent of peptide relative enrichment or depletion between culture conditions (N48: tag 114; N24+3G+N24:115; H48:116; H24+3G+H24:117). The protein identification and quantification result was summarized in Supplemental Table S1, which was a search result from 3 combined replicates of MS runs. A total of 3,383 proteins were identified with FDR<1% and with at least 2 PSMs (Peptide-Spectrum Match). In the proteins, only 21.4% were those with one unique peptide identified but with at least 2 PSM, which was similar with our other iTRAQ data from Q-Exactive LC-MS/MS quantification results (Supplemental Figure S1A). To evaluate the quantification quality, the distribution of ratios from peptides and proteins in 115/114 and 117/116 was plotted in Supplemental Figure S1B and 1C. The distribution pattern showed that most of the ratios for peptides and proteins were within 1.3 fold change ($\log_2$ ratio <0.38) respectively. Only around 2% and 5% of the detected proteins and peptides respectively were changed >1.3 fold cut off. Their normal distributions showed that our iTRAQ quantification were as expected and accurate, and thus the significantly changed proteins were used for further analysis. The rationale for using 1.3 fold cut-off is discussed in Supplementary Data S-1 file.

The modulated proteins were defined as those displaying a consistent iTRAQ reporter ratio indicative of either up- or down-regulation (ratio >1.3 or <0.77, with less than 20% variability) across 2 or more LC-MS/MS runs. Proteins modulated by γ -irradiation in normoxic and/or hypoxic tumor cells are summarized in Table 1 and Supplemental Table S1. Consistent with

previous studies, we detected substantial modulation of protein expression in tumor cells subjected to hypoxia, but only a select subset of 37 proteins was modified by exposure to γ -radiation in either aerobic or low-oxygen conditions. Among these was a distinct group of calcium-binding proteins that displayed consistent upregulation following γ -irradiation, irrespective of oxygen availability, whether assessed by quantitative proteomics (Figure 2A) or confirmed by Western blot (Figure 2B). As a major sensor of cellular Ca^{2+} across numerous species, calmodulin is reportedly up-regulated in a dose-dependent fashion in response to radiation exposure. Consistent with these data, overexpression of calmodulin has previously been shown to protect mouse macrophages against radiation-induced cell death. Other calcium-binding proteins have also been reported to influence cellular resistance to radiation, including a reported correlation between S100A4 mRNA expression levels and radioresistance of pancreatic cancer cells³⁴, and a ~6-fold up-regulation of calreticulin protein in the nuclei of irradiated SQ-20B cells³⁵. These previous data suggested that modulation of key protein regulators of calcium flux can enhance the survival of irradiated tumour cells in normoxia condition. In the current study, we observed that 3G γ -irradiation consistently induced the expression of multiple calcium-binding proteins, including calmodulin, calmodulin-like proteins 3, calumenin, reticulocalbin, and the catalytic subunit calpain-2 in both normoxia and hypoxia conditions. Our data demonstrate for the first time that under hypoxic conditions, A431 epithelial carcinoma cells exhibit increased viability when exposed to low-dose γ -irradiation by up-regulating the expression of calcium-binding proteins CALM1, CALU and RCN1. The results suggested important roles for these calcium signaling mediators in promoting tumor cell survival during hypoxia.

Knockdown of calcium-binding proteins in hypoxic tumor cells prevents radiation-induced increases in survival

To uncover the role of IR-induced calcium-binding proteins in promoting tumor cell survival under hypoxic conditions, we next used a shRNA-knockdown approach to disrupt A431 cell expression of the representative calcium-binding proteins CALM1, CALU and RCN1. Two knockdown fragments per gene were inserted into a shRNA-plasmid, followed by screening for stably-transfected cell lines, and confirmation of reduced target protein expression using WB. Using this approach, we obtained four clones displaying stable knockdown of all 3 target genes, including two clones from the same knockdown fragment which were therefore mixed and carried forward for functional assessment. Under aerobic conditions, A431 cells featuring stable knockdown of CALM1, CALU and RCN1 displayed no change in radioresistance

relative to luciferase-only knockdown control cells assessed in the same MTT assays (Fig. 3B). However, under hypoxic conditions, A431 cells deficient in these calcium-binding proteins were unable to increase their viability following exposure to 3Gy γ -irradiation (Fig. 3C). Indeed, while γ -irradiation of luciferase-only knockdown control cells induced a 18.6% increase in cell survival under hypoxic conditions, A431 cells depleted of the calcium-binding proteins CALM1, CALU and RCN1 displayed increased rates of cell death when subjected to the same dose of radiation and duration of hypoxia. In a previous study, Smallwood *et al.* reported that calmodulin mediates DNA repair responses to histone H2AX formation in macrophages exposed to low-dose radiation. However, irrespective of culture oxygenation, in the current study we were unable to detect any change in H2AX levels in A431 cells deficient in the calcium-binding proteins CALM1, CALU and RCN1 (data not shown). Further study of the IR-induced calcium-binding proteins identified in our study, together with assessment of their various downstream targets, should shed important new light on mechanisms of tumor radioresistance in human cancer patients.

Conclusion

For the first time, we report that exposure to γ -irradiation under hypoxic conditions elicits a cellular response that increases the viability of A431 epithelial carcinoma cells *in vitro*, and may also confer increased tumor cell survival in human patients *in vivo*. Our quantitative proteomic analysis indicated that a distinct group of calcium-binding proteins were consistently induced by cancer cell exposure to low-dose γ -irradiation, and that targeted knockdown of these mediators prevented increases in the viability of hypoxic tumor cells. These data support the concept that cancer cells can improve their survival in low-oxygen environments via IR-induced up-regulation of key regulators of calcium flux, and suggest that therapeutic targeting of specific calcium-binding proteins has the potential to improve outcomes for cancer patients receiving radiotherapy in the clinic.

Supporting Information:

Figure S-1: The quality evaluation of iTRAQ identification and quantification.

Table S-1: Proteins identified by Sequest in Proteome Discoverer version 1.4 with <1% FDR and > 2 PSM.

Table S-2: List of primers for CALM1, CALU and RCN1 shRNA knockdown.

Data S-1: The rationale for using 1.3 fold cut-off of iTRAQ reporter ratio.

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Table 1. Proteins modulated by γ -irradiation under normoxic and/or hypoxic conditions

	N24+3G+N24/N48 115:114	H48/N48 116:114	H24+3G+H24/N48 117:114	H24+3G+H24/H48 117:116
Ratio>1.3	67	376	372	37
Ratio<0.77	54	310	304	67

Figures:

Figure 1: Low-dose γ -irradiation enhances tumor cell survival under low-oxygen conditions. When assessed by MTT survival assay, hypoxic tumor cells displayed maximum viability when subjected to 3Gy radiation treatment (A), which was sufficient to kill 14% of cancer cells cultured under aerobic conditions (B). Similar data were obtained when assessing oxygenation/radiation effects on tumor cell viability by colony formation assay (C and D).

Figure 2: Calcium-binding protein induction by γ -irradiation of normoxic and hypoxic tumor cells. A) iTRAQ quantification revealed that a distinct group of calcium-binding proteins exhibited comparable up-regulation after tumor cell exposure to 3Gy γ -irradiation irrespective of oxygen availability. B) Tumor cell upregulation of CALM1, CALU and RCN1 following γ -irradiation under normoxia or hypoxia was confirmed by WB. CALM1: 16% SDS-PAGE gel; CALU and RCN1: 10% SDS-PAGE gel.

Figure 3: Knockdown of CALM1, CALU and RCN1 prevents radiation-induced increases in the survival of hypoxic tumor cells. Targeted shRNA knockdown of CALM1, CALU and RCN1 protein expression was confirmed by WB (A) and the impact on tumor cell survival under normoxia or hypoxia was determined by MTT survival assay (B and C). For each gene, two shRNA fragments were used for knockdown and named as Gene name-1/2. In each shRNA fragment transfection, two positive monoclonal cell lines were selected for further function studies and named as Gene name-1-1/2.

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