

## Down-regulation of *Betaig-h3* Gene is Involved in the Tumorigenesis in Human Bronchial Epithelial Cells Induced by Heavy-Ion Radiation

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High-energy (HZE) heavy ions, when compared to low-LET radiation, are highly effective in inducing gene mutation, chromosomal aberrations and neoplastic transformation. However, the underlying molecular mechanisms are not clearly understood. We have recently shown that the down-regulation of *Betaig-h3* expression is causally linked to the tumorigenic phenotype of papillomavirus-immortalized human bronchial epithelial (BEP2D) cells treated with high-LET  $\alpha$ -particle radiation. Using the BEP2D cell culture system, a radiation-induced transformation model has been established by a single 60-cGy dose of <sup>56</sup>Fe heavy-ion radiation. To determine whether the *Betaig-h3* gene is involved in <sup>56</sup>Fe ion-induced tumorigenesis, the expression levels of the *Betaig-h3* gene in tumorigenic cell lines and the ability of *in vivo* tumor suppression through the reintroduction of the *Betaig-h3* gene in tumorigenic cells were determined. We found that the expression level of this gene is markedly decreased in three tumorigenic cell lines (<sup>56</sup>FeT1–T3) compared with parental BEP2D cells. Ectopic expression of its cDNA in the <sup>56</sup>FeT2 tumorigenic cells significantly suppressed their tumorigenicity. Although biologically active TGF $\beta$ 1 is elevated in two of three tumorigenic cell lines, all these cell lines are resistant to the induction of *Betaig-h3* expression by incubating the transformed cells with exogenous TGF $\beta$ 1 relative to control cells. Our data strongly suggest that down-regulation of *Betaig-h3* expression results from the defect in the TGF $\beta$ 1 signaling pathway and plays a pivotal role in the tumorigenic process induced by <sup>56</sup>Fe heavy-ion radiation. © 2004 by Radiation Research Society

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### INTRODUCTION

Cancer induction by space radiation is a major concern for manned space exploration. Among the various particular components of ionizing radiation in space, high-energy (HZE) heavy ions have been of special concern to radiobiologists and NASA administrators mainly because they

contribute only about 1% of the particle radiation field in frequency yet produce a considerable amount of the whole dose equivalent because of their large energy deposition and high LET (1). In addition, these particles are densely ionizing radiation, which is effective in producing clustered damage in DNA along a single particle track (2). However, there is no epidemiological evidence for their carcinogenic effects. *In vitro* transformation systems based on human cells have been used to examine the biological effectiveness of heavy-ion radiation and are useful tools in delineating the molecular mechanisms of heavy-ion carcinogenesis (3, 4).

*Betaig-h3* is a secreted protein induced by transforming growth factor beta in human adenocarcinoma cells as well as other human cell types (5). Our recent data have shown that down-regulation of *Betaig-h3* expression is causally linked to the tumorigenic phenotype of papillomavirus-immortalized human bronchial epithelial (BEP2D) cells treated with either high-LET  $\alpha$ -particle radiation or asbestos fibers (6–8). In addition, the *Betaig-h3* gene is ubiquitously expressed in various normal human tissues with the exception of the brain, whereas decreased or loss of expression of the *Betaig-h3* gene has been found in 14 human tumor cell lines of diverse histological types (7). Our data provide strong evidence that the down-regulation of *Betaig-h3* expression is a frequent event and contributes to the acquisition of tumorigenic phenotype in BEP2D cells treated with  $\alpha$ -particle radiation or asbestos fibers.

*In vitro* assay systems such as oncogenic transformation have been used to examine the biological effectiveness of heavy-ion radiation (3, 9). Using the BEP2D cell model, malignantly transformed cells have been established by a single 60-cGy dose of <sup>56</sup>Fe heavy ions 6 months after irradiation (9). Transformed cells progress through sequential stages including altered growth kinetics and anchorage-independent growth before becoming tumorigenic and producing progressively growing subcutaneous tumors after inoculation into athymic nude mice. In addition, results from the fusion experiments demonstrated that the tumorigenic phenotype in BEP2D cells induced by <sup>56</sup>Fe ions could be completely suppressed by fusion with nontumorigenic BEP2D cells. The data suggest that loss of a tumor suppressor gene(s) is a likely mechanism for the carcinogenic

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effects of  $^{56}\text{Fe}$  ions (9). Therefore, the tumorigenic BEP2D cell model is useful for studying the genetic events involved in  $^{56}\text{Fe}$ -ion-induced tumor progression. We show here that *Betaig-h3* expression is significantly suppressed in three  $^{56}\text{Fe}$ -ion-induced and independently generated tumorigenic cell line ( $^{56}\text{FeT1-T3}$ ). Ectopic expression of this gene in  $^{56}\text{FeT2}$  cells, a highly tumorigenic cell line, significantly suppresses the *in vivo* tumorigenicity of the cells. Our findings strongly suggest that the loss of *Betaig-h3* expression plays a pivotal role in heavy-ion carcinogenesis.

## MATERIALS AND METHODS

### Cell Culture

Exponentially growing BEP2D cells were irradiated with a single dose of 60 cGy of 1 GeV/nucleon  $^{56}\text{Fe}$  ions accelerated with the Alternating Gradient Synchrotron at the Brookhaven National Laboratory (9). The irradiated cells were cultured for 12 weeks and then inoculated into nude mice. Each mouse was injected subcutaneously at one site with  $5 \times 10^6$  cells and examined for tumor growth once a week. Tumors larger than 1 cm in diameter were resected from nude mice and used to establish tumor cell lines. Five out of eight animals developed progressively growing tumors from which three tumor cell lines ( $^{56}\text{FeT1-T3}$ ) were derived. Human BEP2D cells and their radiation-induced tumorigenic variants were maintained in serum-free LHC-8 medium supplemented with various growth factors as described previously (4). The experiments involving mice were reviewed and approved by the Institutional Animal Care and Use Committee.

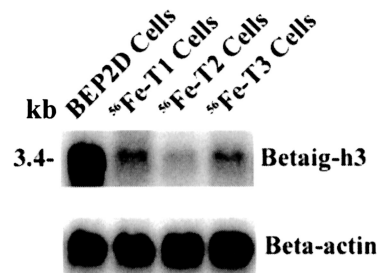
### Northern and Western Blotting

For Northern blotting, 2.5  $\mu\text{g}$  of mRNA was denatured and separated on a 1% denaturing agarose formaldehyde gel. The mRNAs were then transferred onto a nylon membrane (Millipore Corp., Bedford, MA) by downward capillary blotting in  $20\times$  SSC (3 M NaCl, 0.3 M  $\text{Na}_2\text{citrate}\cdot 2\text{H}_2\text{O}$ , pH 7.0) followed by UV crosslinking. Specific probes were generated by labeling of PCR-amplified cDNA fragments with [ $^{32}\text{P}$ ]dCTP using a random primed DNA labeling kit (Boehringer, Mannheim). The membranes were prehybridized for 30 min and then hybridized with the cDNA probes in ExpressHyb TM hybridization solution (Clontech) for 8–12 h at 68°C. The blots were washed twice in  $2\times$  SSC, 0.1% SDS at room temperature for 15 min and then washed twice in  $0.2\times$  SSC, 0.1% SDS at 55°C for 15 min. The membranes were exposed to Kodak BioMax film at  $-70^\circ\text{C}$  for 12–72 h. The band intensities were evaluated by phosphorimaging and normalized to the expression level of  $\beta$ -actin.

For analysis of TGFB1 and *Betaig-h3* protein expression, conditioned medium was collected from confluent cultures and the protein was then concentrated using SP Sepharose (Amersham) and eluted using lysis buffer by boiling for 5 min (10). Protein concentrations were measured using a Bio-Rad DC protein assay kit. Samples containing equal amounts of proteins were then fractionated by SDS-PAGE, transferred onto a Hybond membrane, and immunoblotted with a 1:1000 dilution of anti-*Betaig-h3* human polyclonal antibody (produced by Invitrogen) or anti-TGFB1 polyclonal antibody (Promega). Peroxidase-conjugated anti-rabbit IgG was used to detect *Betaig-h3* and TGFB1 levels by ECL procedures.

### Transfection with *Betaig-h3* Gene

$^{56}\text{FeT2}$  tumorigenic cells were plated at  $1.5 \times 10^6$  per 60-mm dish in serum-free LHC-8 medium. When they were 70–80% confluent, the cells were transfected with either pRc/CMV2-*Betaig-h3* or pRc/CMV2 (2  $\mu\text{g}$ /dish) for 24 h using lipofectamin (Gibco) according to the manufacturer's instructions. The cells were split at 1:10 and cultured in medium con-



**FIG. 1.** Differential expression of *Betaig-h3* gene in control BEP2D cells and cells of three heavy ion-induced tumorigenic cell lines ( $^{56}\text{FeT1-T3}$ ) by Northern blot analysis. The blots were hybridized to  $^{32}\text{P}$ -labeled *Betaig-h3* cDNA probes. After stripping, the membranes were rehybridized to a human  $\beta$ -actin probe.

taining 500  $\mu\text{g}/\text{ml}$  of the G418 (Gibco) for 21 days. Colonies were isolated using a cloning ring and maintained in the presence of 300  $\mu\text{g}/\text{ml}$  of G418.

### Tumorigenicity of $^{56}\text{FeT2}$ Cells in Nude Mice after *Betaig-h3* Transfection

*Betaig-h3*- or empty vector-transfected  $^{56}\text{FeT2}$  tumorigenic cells were injected subcutaneously into the left flanks of nude mice. Tumors were palpated and measured with calipers, and tumor volume was calculated as  $[\text{longest diameter} \times (\text{shortest diameter})^2] \times 0.5$ . Control animals were inoculated with either control BEP2D cells or parental  $^{56}\text{FeT2}$  tumorigenic cells. For each cell line, two independent experiments were performed. Animals were killed humanely as soon as tumor nodules grew to around 1 cm in size.

## RESULTS

### *Betaig-h3* is Down-regulated in $^{56}\text{Fe}$ Heavy-Ion-Induced Tumorigenic Cell Lines

We demonstrated previously that loss of *Betaig-h3* expression is causally related to the tumorigenic phenotype of BEP2D cells induced by high-LET  $\alpha$ -particle radiation (6). To determine whether down-regulation of the *Betaig-h3* gene is a frequent event in  $^{56}\text{Fe}$ -ion radiation-induced tumorigenic BEP2D cells, Northern blots were used to check the mRNA levels of this gene in cells of three heavy-ion-induced tumorigenic cell lines ( $^{56}\text{FeT1-T3}$ ). As shown in Fig. 1, expression of *Betaig-h3* gene was down-regulated by six- to eightfold in  $^{56}\text{FeT1-T3}$  cells compared to the parental BEP2D control cells. The result indicates that decreased expression of the *Betaig-h3* gene may be related to the acquisition of malignant phenotype in heavy-ion-irradiated BEP2D cells.

### Transfection of Wild-Type *Betaig-h3* cDNA into $^{56}\text{FeT2}$ Tumorigenic Cells

To determine the significance of *Betaig-h3* down-regulation in tumorigenesis, expression of *Betaig-h3* was examined in the highly tumorigenic  $^{56}\text{FeT2}$  cells using a pRc/CMV2-*Betaig-h3* vector. The empty pRc/CMV2 vector was used as the control. As shown in Fig. 2, the parental  $^{56}\text{FeT2}$  and  $^{56}\text{FeT2}$ -pRc/CMV2 cells expressed very low and sim-

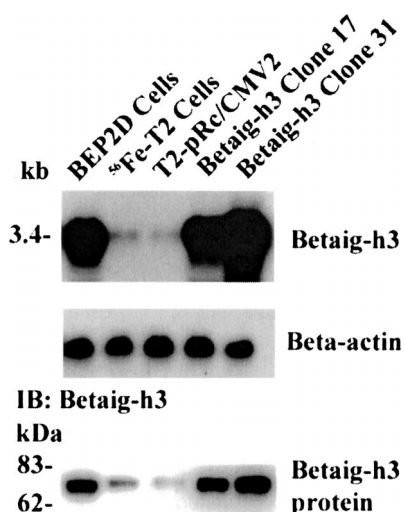


FIG. 2. mRNA and protein levels of *Betaig-h3* gene determined by Northern blotting and immunoblotting (IB) in control BEP2D,  $^{56}\text{FeT2}$  and *Betaig-h3*-transfected tumorigenic cells (clones 17 and 31).

ilar levels of *Betaig-h3* protein relative to control BEP2D cells. After *Betaig-h3* transfection, expression of this gene in clone 17 occurred at a level similar to that seen in control BEP2D cells, whereas clone 31 had a twofold higher level.

#### Suppression of Tumorigenicity in $^{56}\text{FeT2}$ Cells by *Betaig-h3* Transfection

To determine whether ectopic expression of the *Betaig-h3* gene in heavy-ion-induced tumorigenic cells could suppress the tumorigenicity *in vivo*, we injected  $5 \times 10^6$  cells of each of the following cell lines into nude mice: control BEP2D cells,  $^{56}\text{FeT2}$  tumorigenic cells,  $^{56}\text{FeT2-pRc/CMV2}$  cells, and *Betaig-h3*-transfected cells (clones 17 and 31). The tumor volumes were measured weekly. As shown in Table 1, no tumors formed from control BEP2D cells after more than 20 weeks. However, 8/8 mice injected with  $^{56}\text{FeT2}$  cells and 8/8 mice injected with empty vector-transfected  $^{56}\text{FeT2}$  cells developed progressively growing tumors at 4 weeks with average tumor volumes of  $338 \text{ mm}^3$  and  $377 \text{ mm}^3$ , respectively. In contrast, the sites (16/16 mice) injected with *Betaig-h3*-transfected cells (clones 17 and 31) developed significantly smaller tumors than those injected with the parental  $^{56}\text{FeT2}$  cells, with an average tumor volume of  $88 \text{ mm}^3$  ( $P < 0.05$ ). At 2 and 3 weeks,

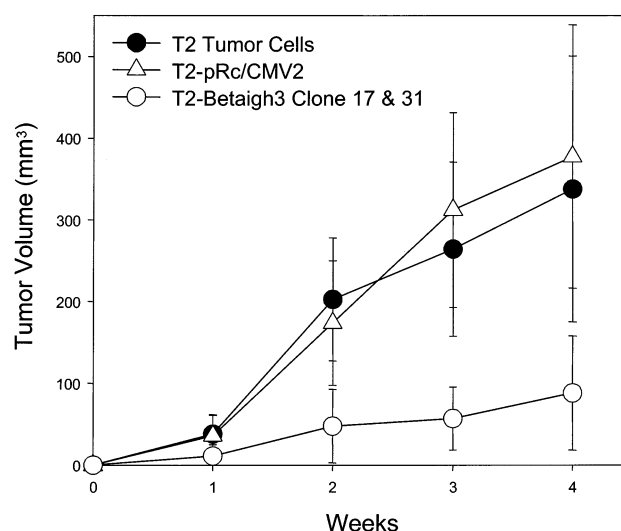


FIG. 3. Inhibition of tumor growth for *Betaig-h3*-transfected cells relative to cells transfected with vector alone and to parental  $^{56}\text{FeT2}$  tumorigenic cells. Volumes are shown as the means  $\pm$  SD of eight independent tumors.

tumor growth (Fig. 3) was suppressed significantly in mice injected with *Betaig-h3*-transfected  $^{56}\text{FeT2}$  tumorigenic cells.

#### Induction of *Betaig-h3* Gene Expression by Exogenous TGF $\beta$ 1 and Biologically Active Levels of TGF $\beta$ 1 in $^{56}\text{FeT1-T3}$ Tumorigenic Cells

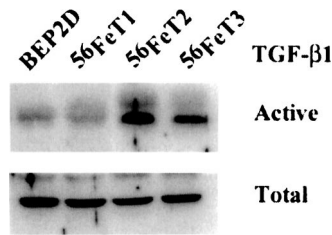
To determine whether down-regulation of the *Betaig-h3* gene results from dysregulation of TGF $\beta$ 1, biologically active levels of TGF $\beta$ 1 were screened using Western blotting. As shown in Fig. 4, nontumorigenic BEP2D cells have a relatively low level of active TGF $\beta$ 1 in the culture medium. However, two of the three tumorigenic cell lines ( $^{56}\text{FeT2}$  and T3) have two- to threefold higher levels of active TGF $\beta$ 1 than BEP2D cells, while the third ( $^{56}\text{FeT1}$ ) has a similar level. To determine whether down-regulation of the *Betaig-h3* gene is due to inactivation of downstream genes of TGF $\beta$ 1, induction of *Betaig-h3* expression was investigated by incubating the control BEP2D cells and malignantly transformed cells with 5 ng/ml TGF $\beta$ 1 for 48 h. As shown in Fig. 5, the protein level of the *Betaig-h3* gene was twofold higher in TGF $\beta$ 1-treated BEP2D cells than in the untreated cells. However, expression of this gene was

TABLE 1  
Suppression of Tumorigenicity by *Betaig-h3* Gene

Cell type	Tumors/total mice	Tumor volume at 4 weeks (mm $^3$ )
BEP2D	0/8	—
$^{56}\text{FeT2}$ tumorigenic	8/8	$337.55 \pm 159.73$
$^{56}\text{FeT2-pRc/CMV2}$	8/8	$377.39 \pm 161.12$
$^{56}\text{FeT2-Betaig-h3}$ clone 17	8/8	$88.02 \pm 69.84^a$
$^{56}\text{FeT2-Betaig-h3}$ clone 31	8/8	

Note. The tumor volumes were measured as described in the Materials and Methods.

<sup>a</sup>  $P < 0.05$  compared with parental tumor cells.



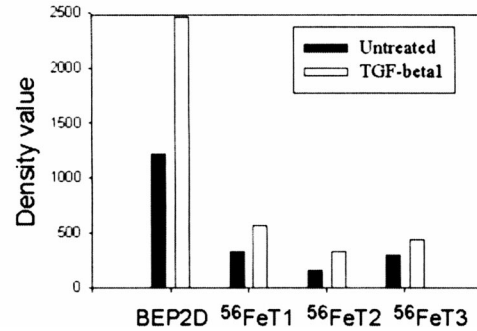
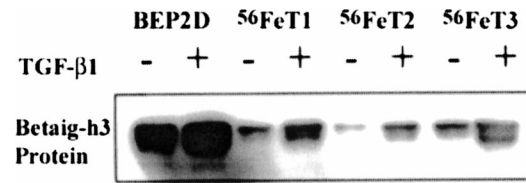
**FIG. 4.** Level of biologically active TGF $\beta$ 1 protein in culture medium determined by immunoblotting. Total TGF $\beta$ 1 in whole cell lysates were used as the control. Human anti-active TGF- $\beta$ 1 (Promega) and anti-TGF- $\beta$ 1 (Santa Cruz) antibodies were used in this study.

slightly induced by exogenous TGF $\beta$ 1 in  $^{56}\text{FeT1-T3}$  cells and was much lower than in control BEP2D cells. In addition, no mutations or deletions were found at the locus of the *Betaig-h3* gene in  $^{56}\text{FeT1-T3}$  cells by using southern blotting and cDNA sequencing techniques (data not shown).

#### DISCUSSION

Of the various components of ionizing radiation in space, high-energy (HZE) heavy ions have been of special concern to radiobiologists and NASA administrators. At the doses and dose rates encountered in space, the late induction of cancer in the astronauts is an important risk to be considered (11). Compared to low-LET radiation, HZE particles are highly effective in inducing gene mutation and neoplastic transformation in various metabolically active cells from yeast to mammals (12). In particular, mutagenic lesions induced by heavy ions appeared to be less repairable and to involve multilocus deletions in different genes, including S1 and HPRT (4, 13). These data provide an explanation for the toxicity of these ions and suggest that increased carcinogenic risk of exposure to HZE heavy ions may be due to an increased likelihood for deletion or alterations of chromosome regions inclusive of tumor suppressor genes. This is supported by studies showing that exposures to HZE heavy ions produce tumorigenic variants of immortalized human mammary (H184B5) and bronchial epithelial (BEP2D) cells (3, 9). Cell fusion experiments indicated that the neoplastic phenotype could be suppressed by fusion with normal cells. The data suggest that tumorigenicity is recessive in nature and highlight the loss of a tumor suppressor gene(s) as a likely mechanism for heavy-ion carcinogenesis.

We previously showed that expression of the *Betaig-h3* gene is frequently deleted in high-LET  $\alpha$ -particle-induced tumorigenic BEP2D cells. The *in vivo* tumorigenicity assay has shown that loss of expression of this gene is causally related to the tumorigenic phenotype of BEP2D cells (6, 8). Because high-LET heavy-ion radiation is as effective as high-LET  $\alpha$  particles in inducing gene mutation and deletion we proposed that *Betaig-h3* is often down-regulated and plays an important role in tumorigenic process induced



**FIG. 5.** Induction of *Betaig-h3* gene expression by incubating control BEP2D cells and  $^{56}\text{FeT1-T3}$  tumorigenic cells with 5 ng/ml TGF $\beta$ 1 for 48 h. Samples containing equal amounts of protein concentrated from the culture medium were loaded and used for immunoblotting analysis.

by heavy-ion radiation. To prove this, the mRNA levels of this gene were screened in three tumorigenic cell lines by using Northern blotting. We found a significant decrease of *Betaig-h3* expression in these cell lines compared with the control cells. In addition, recovery of its expression in heavy-ion-induced tumorigenic cells significantly suppressed their tumorigenicity *in vivo*. Our data provide evidence that down-regulation of *Betaig-h3* gene expression plays a pivotal role in heavy-ion carcinogenesis. We have previously found that expression of the *Betaig-h3* gene is either lost or down-regulated in cells of six human primary lung tumor cell lines (7). Those results, together with our present data, support the hypothesis that loss of *Betaig-h3* expression is involved in the progression of normal human bronchial epithelial cells to malignant tumors.

Tumor development is a multistep process during which accumulation of genetic and epigenetic events determines the transition from a normal to a malignant state (14, 15). This is supported by our data showing that in malignant BEP2D cells induced by high-LET heavy ions, cells progressed toward malignancy in a stepwise fashion and eventually became tumorigenic in nude mice (9). Malignant cells have an enormous complexity of altered gene functions, including activation of growth-promoting genes as well as silencing of genes with tumor growth-suppressing functions, all contributing to uncontrolled tumor growth (15, 16). Recent data from others showed that the *Betaig-h3* gene product can mediate apoptosis in Chinese hamster ovary (CHO) and H1299 human lung cancer cells (17). The apoptotic response to unscheduled proliferation may constitute a general defense mechanism to prevent transformation (18). Therefore, loss of *Betaig-h3* expression may favor the growth selection and clonal expansion of cells

with tumorigenic potential by rendering them resistant to apoptosis.

*Betaig-h3* is a downstream gene of TGF $\beta$  that induces its expression in many cell types (5). TGF $\beta$  is a multi-functional cytokine with both tumor-suppressor and tumor-promoting activities, depending on the stage of carcinogenesis and the responsiveness of the tumor cells. TGF $\beta$  inhibits epithelial cell growth in early tumor stages, whereas in advanced stages, many tumor cells lose their growth-inhibitory response to TGF $\beta$ , which is commonly associated with increased tumor cell invasion and metastasis (19). Based on our observations, the *Betaig-h3* gene may be an important candidate for mediating the tumor-suppressor function of TGF $\beta$  because it is expressed at a normal level in early stages of transformation but is significantly down-regulated in the late stages of transformation (6, 7). This is supported by our present studies indicating that active form of TGF $\beta$ 1 is elevated in two of the three heavy-ion-induced tumorigenic cell lines we examined; however, all these cell lines are resistant to the induction of *Betaig-h3* expression by exogenous TGF $\beta$ 1. The data suggest that defects in the TGF $\beta$ 1 signaling pathway are responsible for the down-regulation of *Betaig-h3* expression, which renders the tumorigenic cells highly invasive and metastatic even though these cells secrete high levels of TGF $\beta$ 1. Although chromosome 5q31, which contains the regions where the *Betaig-h3* gene has been mapped, is often deleted in many types of human cancer (20), we failed to identify any mutations or deletions in the locus of the *Betaig-h3* gene in heavy-ion-transformed cells.

Several studies have shown that Betaig-h3 protein may affect the interaction of cells with their extracellular matrix by regulating the expression of integrin receptor expression (10). Although  $\alpha$ 5 $\beta$ 1 integrin receptor was demonstrated to be differentially expressed in  $\alpha$ -particle-transformed BEP2D cells, this kind of alteration was not identified in heavy-ion-induced tumorigenic cells (data not shown). The data suggest that another yet-unidentified downstream effector(s) mediates the physiological function of the *Betaig-h3* gene. Our present findings provide strong evidence that down-regulation of *Betaig-h3* expression contributes to the neoplastic transformation of BEP2D cells induced by heavy-ion radiation.

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