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SHORT COMMUNICATION

Green Fluorescent Protein Alters the Transcriptional Regulation of Human Mitochondrial Genes After Gamma Irradiation

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Abstract Green fluorescent proteins (GFP), extensively used as reporters in biological and imaging studies, are assumed to be mostly biologically inert. Here, we test the assumption in regard to the transcriptional regulation of 18 mitochondrially encoded genes in GFP expressing human T-cell line (JURKAT cells) exposed to gamma radiation. Using quantitative polymerase chain reaction, we demonstrate that wild type and GFP expressing JURKAT cells have different baseline mitochondrial transcript expression (10 out of the 18 tested genes) and after a single dose of radiation (100 Gy) show a significantly different transcriptional regulation of their mitochondrial genes. While in wild type cells, ten of the tested genes are up-regulated in response to radiation exposure, GFP expressing cells show less transcriptional regulation with a small down-regulation in five genes. Our results indicate that the presence of GFP in the cytoplasm can alter the cellular response to ionizing radiation.

Keywords Green fluorescent protein · JURKAT cells · Mitochondria · Gene expression · *Aequorea victoria* · Gamma radiation

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Abbreviations

GFP	Green fluorescent protein
EmGFP	Emerald green fluorescent protein
FBS	Fetal bovine serum
DMEM	Dulbecco's modified eagle medium
qPCR	Quantitative polymerase chain reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
tRNA	Transfer ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation

Introduction

Green fluorescent protein (GFP), a fluorescent protein originally isolated from jellyfish *Aequorea victoria* [1], is frequently used as a reporter of the expression of genes and the localization their associated proteins [2–4]. This enables real time monitoring of the target gene expression and the associated cellular events in vivo to determine the biological significance of the gene under study [1, 5].

It is assumed that the presence of the introduced fluorescent proteins is biologically inert. However, there are some indications that GFP can affect cell viability [2, 6], impair differentiation or development [7–9], affect subcellular distribution [10], alter gene/protein expression [11, 12], cell functions [3, 4, 13] or may even lead to pathology development in transgenic animals [14–16].

Changes in mitochondrial genome or its transcriptional regulation after ionizing irradiation has been studied previously [17–19], including in cells expressing GFP [20]. To investigate the effect of GFP expression on cell response to

stress, we produced a transgenic cell line from JURKAT cells that stably expresses GFP and applied quantitative polymerase chain reaction (qPCR) to examine the mitochondrial transcript expression in wild type and GFP expressing cells before and after a single exposure to 100 Gy of gamma radiation.

Materials and Methods

Plasmid Construction

The plasmid pcDNA6.2/C-EmGFP/TOPO (Invitrogen, Carlsbad, CA, USA) was digested with *Eco*RI and ligated to produce a construct that expresses EmGFP alone. The construct was verified by sequencing.

Cell Culture and Transfection

JURKAT cells (clone E6-1) were maintained at 37 °C with 5 % CO_2 in DMEM supplemented with 10 % FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were transfected using Amaxa nucleofection according to instructions provided by the manufacturer. Briefly, 1×10^6 cells were resuspended in Nucleofector Solution V, mixed with 2 μg of EmGFP plasmid DNA and transfected using program X-001. To create a polyclonal stable GFP expressing cell line, blasticidin was added to the culture media 2 days after transfection. Cells were maintained in media containing blasticidin at 5 μg/mL for 2 to 3 weeks. Once a stable cell line had been produced, cells were maintained in media supplemented with a lower concentration of blasticidin (3 μg/mL). These cells were found to be of homogenous GFP expression as confirmed by fluorescent microscopy (Fig. 1). We did not see obvious indication for reduced viability in GFP expressing cells.

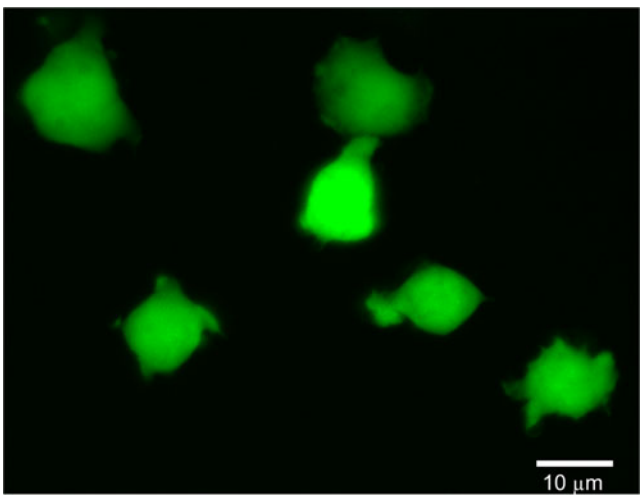

Fig. 1 GFP expressing cells. Fluorescent microscopic image of JURKAT cells with stable GFP expression. Scale bar = 10 μm

Gamma Irradiation

The wild type and GFP expressing cells, at a density of 0.16×10^6 cells/ml, were gamma irradiated at room temperature at 100 Gy using a ^{60}Co irradiator (GammaCell 220). The radiation dose used in this study was within the experimental range, as this level of dose is sometimes needed in cell radiation experiments [21–23] to produce a measurable effect. Furthermore, this particular dose was selected based on our observation that acute changes in mitochondrial gene expression upon this level of irradiation can be sufficiently detected by qPCR (manuscript in submission).

The dose rate of 39.0±0.8 Gy/min was determined using the standard Fricke dosimeter [24]. At this dose rate, the effect of the dose during transit of the GammaCell 220 chamber (5.2±0.4 Gy) was significant and was taken into account when calculating the exposure times. Another set of the samples was kept at room temperature and was not irradiated. Cells were used for total RNA extraction immediately after the irradiation.

RNA Isolation and Reverse Transcription

Total RNA extraction was performed using the PureLink™ RNA mini kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The extracted RNA was further treated with DNase using the PureLink™ DNase Set (Invitrogen, Carlsbad, CA, USA) to ensure the removal of contaminating DNA prior to the qPCR experiments. The concentration of the RNA was determined using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purity of the extracted RNA was assessed spectrophotometrically using the A260/A280 ratio.

First strand cDNA was synthesized using oligo(dT) primers from 6 ng of freshly prepared total RNA using the SuperScript® III First-Strand Synthesis kit, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNA of each sample was equally diluted with DEPC-treated water for the subsequent qPCR assay.

qPCR

qPCR primers of 18, out of a total of 37, mitochondrial genes as well as β-actin (a nuclear-encoded housekeeping gene) were included in this study. These primers were obtained from published literature. The specificity of the complex IV subunit 2 primer set was further confirmed by sequencing (Accession: AF004339) as the original paper did not specify the target subunit [25]. All their sequences are listed in Table 1.

Table 1 PCR primers. The listed primers were used to examine the expression of the following mitochondrially-encoded genes—rRNA: 12S, 16S; tRNA; *MtTL1* mitochondrially encoded tRNA leucine 1 (UUA/G), *MtTV* mitochondrially encoded tRNA(Val), *MtTF* mitochondrially encoded tRNA phenylalanine; mRNA: Complex I = subunits ND1, ND2, ND3, ND4, ND4L, ND5 and ND6; Complex III = subunit CYB; Complex IV = subunits 1, 2 and 3; Complex V = subunits 6 and 8. β-actin, a nuclear-encoded gene, was also investigated

Coding origin	Gene name	Forward primer	Reverse primer	Reference
Mitochondria	12S	CCCAAACTGGGATTAGATACCC	GTTTGCTGAAGATGGCGGTA	[32]
	16S	GCCTGTTTACCAAAAACATCAC	CTCCATAGGGTCTTCTCGTCTT	[32]
	MTTF	CCAAACCCCAAAGACACCC	GAACGGGGATGCTTGCATG	[33]
	MTTV	CTGGAAAGTGCACTTGGACG	GGGTAAATGGTTTGGCTAAGG	[33]
	MTTL1	TATACCCACACCCACCCAAG	GCGATTAGAATGGGTACAAT	[33]
	Complex I subunit ND1	ATGGCCAACCTCCTACTCCT	GCGGTGATGTAGAGGGTGAT	[34]
	Complex I subunit ND2	CATATACCAAATCTCTCCCTC	GTGCGAGATAGTAGTAGGGTC	[35]
	Complex I subunit ND3	TTACGAGTGCGGCTTCGACC	ACTCATAGGCCAGACTTAGG	[35]
	Complex I subunit ND4	CCTGACTCCTACCCCTCACA	ATCGGGTGATGATAGCCAAG	[34]
	Complex I subunit ND4L	TAGTATATCGCTCACACCTC	GTAGTCTAGGCCATATGTG	[35]
	Complex I subunit ND5	ACATCTGTACCCACGCCTTC	TCGATGATGTGGTCTTTGGA	[34]
	Complex I subunit ND6	GGATCCTCCCGAATCAAC	GTAGGATTGGTGCTGTGG	[35]
	Complex III subunit CYB	TGAAACTTCGGCTCACTCCT	AATGTATGGGATGGCGGATA	[36]
	Complex IV subunit 1	GGCCTGACTGGCATTGTATT	TGGCGTAGGTTTGGTCTAGG	[34]
	Complex IV subunit 2	CAGGAAATAGAAACCGTCTGAACTATCCTG	CTGTGGTTTGCTCCACAGATTTCAGTGCAT	[25]
	Complex IV subunit 3	CCCGCTAAATCCCCTAGAAG	GGAAGCCTGTGGCTACAAAA	[34]
	Complex V subunit 6	CACACCTACACCCCTTATCCC	TCATTATGTGTTGTCGTGCAG	[37]
	Complex V subunit 8	ATGGCCCACCATAATTACCC	GCAATGAATGAAGCGAACAG	[34]
Nucleus	β-actin	GTGGGGCGCCCCAGGCACCA	CTCCTTAATGTCACGCACGATTTC	[25]

qPCR was performed using the CFX 384™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Diluted cDNA of 1 μl was added to 4 μl of reaction mixture containing 2.5 μl of SsoFast™ EvaGreen® Supermix (BioRad, Hercules, CA, USA) and 5 pM of each of the forward and reverse primers. Each sample was run in duplicate. A positive control, which was later used as a reference in the PCR quantification, was included in the assay.

The thermal cycling conditions were 98 °C for 30 s, followed by 45 cycles at 98 °C for 5 s, 63 °C for 10 s. At the end of the 45th cycle, the temperature was raised to 72 °C for 10 min to ensure complete extension of the products. A melt curve analysis was performed after the qPCR to confirm the specificity of the results. Non-specific signal was removed from the analysis. The mean Cq value of each sample was calculated using the CFX Manager™ Software (version 1.5) (BioRad, Hercules, CA, USA). The amount of gene amplified after radiation treatment was quantified relative to the reference sample i.e. $2^{\text{Cq (reference sample)} - \text{Cq (target sample)}}$.

Statistical Analysis

Values were presented as mean ± SD. Significance between groups was determined by Student's unpaired *t*-test. A p value of <0.05 was considered statistically significant.

Results

The transcript expression levels of 18 mitochondrial genes revealed significant differences between wild type and GFP expressing cells (i.e. transgenic JURKAT cells homogenously express GFP), at baseline condition as well as after 100 Gy of gamma irradiation.

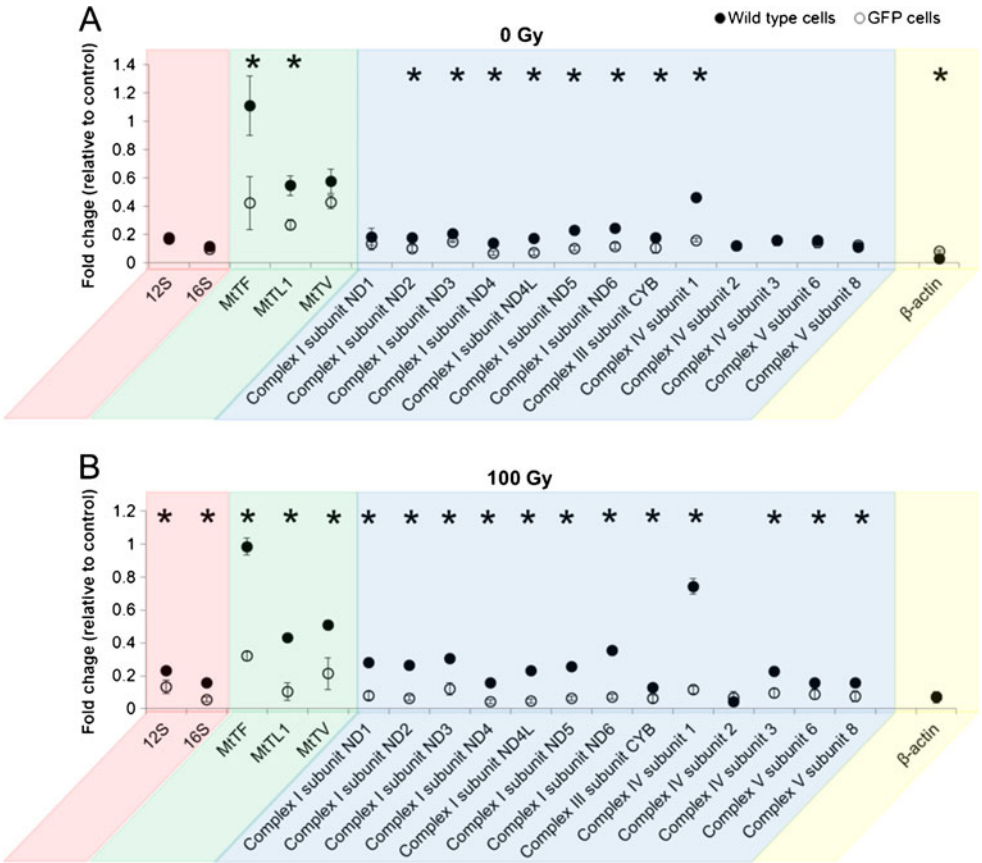

Fig. 2 Comparison on the transcript expression between wild type and GFP expressing cells before and after 100 Gy of gamma irradiation. Wild type JURKAT cells and GFP expressing cells were exposed to (**a**) 0 (*baseline*) or (**b**) 100 Gy of gamma radiation at room temperature. Eighteen mitochondrial as well as β-actin genes were examined using qPCR. Quantification was performed relative to the reference sample: $2^{\text{Cq (reference sample)} - \text{Cq (target sample)}}$. The level of fold change of each tested gene is shown. The *black dots* represent the transcript level of the wild type JURKAT cells, while the *white dots* represent that of the GFP expressing cells. The *red-shaded region* indicates the mitochondrial ribosomal RNAs; *green-shaded region* indicates the mitochondrial transfer RNAs; *blue-shaded region* indicates the mitochondrial messenger RNAs. The *yellow-shaded region* indicates the housekeeping gene (β-actin) tested in this study. Error bar = SD; N=3. Furthermore, the difference in mitochondrial transcript expression between wild type and GFP expressing cells was tested using Student's unpaired *t*-test. * indicates a statistical significant difference in transcript expression between the wild type (*black dot*) and GFP expressing cells (*white dot*)

It was observed that wild type and GFP expressing cells differed in regard to β-actin transcript expression, whereby cell expressing GFP had a higher baseline expression (Fig. 2a, β-actin). Furthermore, β-actin transcript in the wild type JURKAT cells had a statistically significant increase (~ 2.5 fold) in expression upon radiation treatment (Fig. 3a, β-actin). Due to the unstable β-actin transcript expression across samples and treatment conditions, β-actin was, therefore, not used as the reference gene for qPCR quantification and results were quantified as fold change relative to a reference sample (see *qPCR* in Materials and Methods).

When comparing the level of mitochondrial transcript expression between wild type and GFP expressing cells before and after irradiation, ten out of the 18 tested mitochondrial genes showed significant differences between these two cell lines at baseline level (Fig. 2a, asterisks); while 17 tested mitochondrial genes in the wild type cells showed higher mitochondrial transcript expression when compared to that of GFP cells after 100 Gy of gamma irradiation (Fig. 2b, asterisks).

When comparing the level of mitochondrial transcript expression of the same cell line before and after irradiation, 12 out of the 18 tested mitochondrial genes of the wild type cells showed expression changes. Though some of those changes were small, they were statistically significant with ten genes showing an increase while two showing a decrease (Complex III subunit CYB and Complex IV subunit 2) (Fig. 3a, asterisks). In contrast, when GFP was present in the cells, the transcript levels of only five mitochondrial genes were significantly changed showing reduction after gamma irradiation (Fig. 3b, asterisks).

Discussion

The overexpression of a foreign molecule within a cell, such as GFP introduced as a gene reporter, is generally assumed

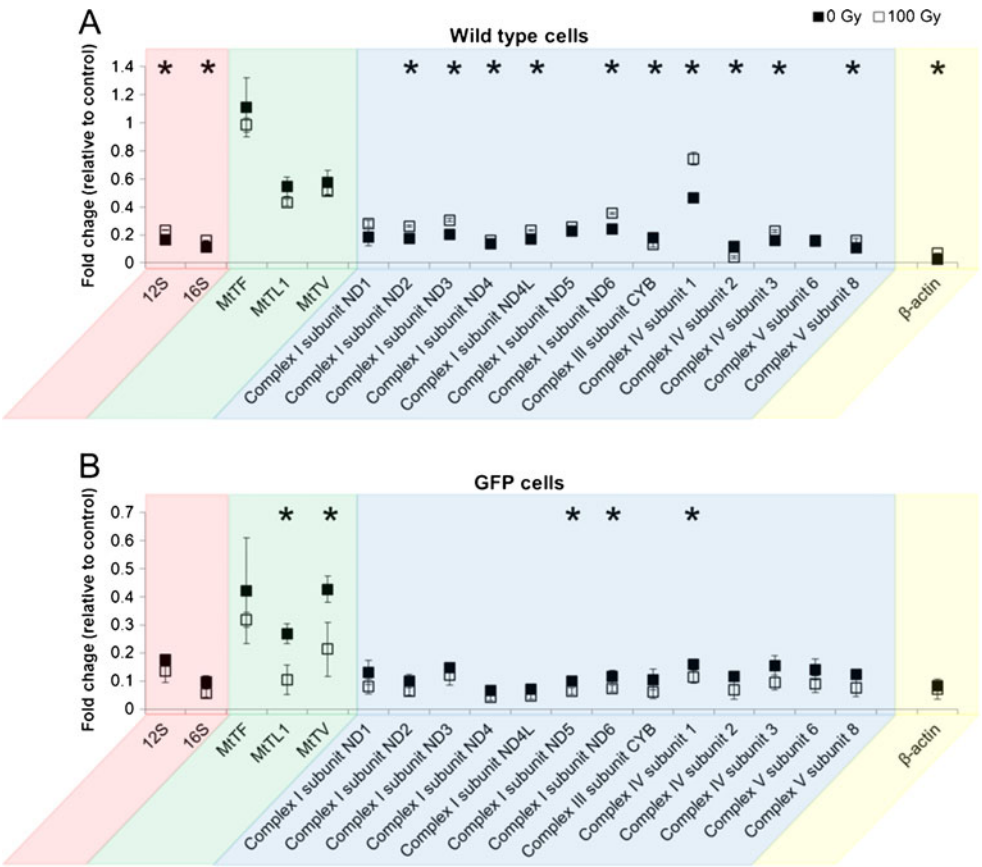

Fig. 3 Comparison on the transcript expression of the same cell type (wild type or GFP expressing cells) before and after 100 Gy of gamma irradiation. Results in Fig. 2 were rearranged to show the effect of radiation on (**a**) wild type JURKAT cells and (**b**) GFP expressing cells. The levels of fold change of 18 mitochondrial as well as β-actin gene transcripts of each cell type before (*black squares*) or after 100 Gy (*white squares*) of irradiation were shown. The *red-shaded region* indicates the mitochondrial ribosomal RNAs; *green-shaded region* indicates the mitochondrial transfer RNAs; *blue-shaded region* indicates the mitochondrial messenger RNAs. The *yellow-shaded region* indicates the housekeeping gene (β-actin) tested in this study. Error bar = SD; *N*=3. Furthermore, the difference in mitochondrial transcript expression of each cell type before and after irradiation was tested using Student's unpaired *t*-test. * indicates a statistical significant difference in transcript expression between the irradiated (*white squares*) and un-irradiation (*black squares*) cells

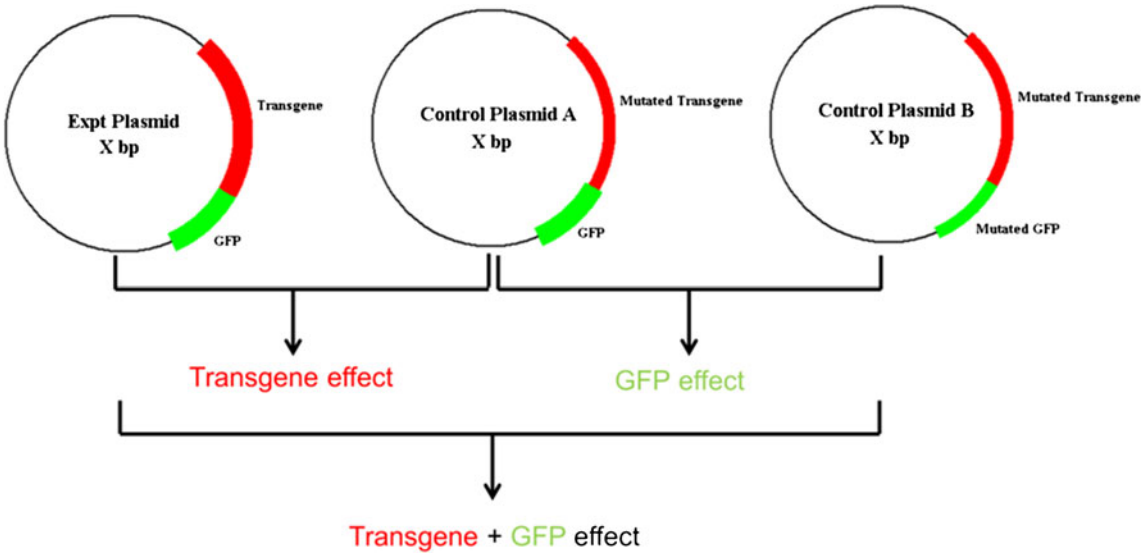

Fig. 4 Recommended controls for transgenic experiment involving GFP. Experimental plasmid (Expt Plasmid) consists of a transgene gene (*thick red insert*) fused with GFP gene (*thick green insert*). Control plasmid A is produced by mutating the transgene (*thin red insert*) while maintaining the GFP gene unchanged. This plasmid can be compared with Expt Plasmid to assess the effect of the transgene. Control plasmid B is produced by mutating both transgene and GFP gene (*thin green insert*). This plasmid can be compared with Control plasmid A to assess the effect of the GFP. Furthermore, Expt plasmid can be directly compared with Control plasmid B to assess the effect of transgene that has been fused with GFP gene. Theoretically, the "transgene effect" should be same as the "transgene + GFP effect" if GFP does not affect the function under investigation. Otherwise, the "GFP effect" (by comparing Control plasmids A and B) should be fully accounted for, in order to assess the function of the transgenes correctly. Please note that all experimental and control plasmids are of identical size (an arbitrary size of X bp)

not to interfere with the cell's normal functions. Mitochondria and cell functional state are tightly linked [26], and even subtle regulation differences in mitochondrial genes is likely to affect overall cellular responses. We, therefore, studied changes in mitochondrial gene expression in cells with GFP overexpression to see whether GFP affects the post-radiation response.

We observed that wild type and GFP expressing cells clearly differ in the baseline expression of the mitochondrial transfer RNAs (Fig. 2a, asterisks, green region) and messenger RNAs of Complex I, III and IV (Fig. 2a, asterisks, blue region). Transfer RNA plays a significant role in the transport for amino acids to the ribosomes while messenger RNAs are the transcript for protein translation. In particular, these RNAs of the mitochondria are essential for the synthesis of the mitochondrial electron transport chain [27, 28], a system for aerobic cell respiration and energy production. Such differences in transcript expression suggest that Complex I, III and IV regulation hence mitochondrial function of the GFP expressing cells could have been altered.

Transfection itself could be affecting cell health [29]. Specifically, GFP has been reported to alter catalytic activity, signal transduction, metabolism and cell stimulation [12]. In our study, we did not find evidence for reduced viability in the JURKAT cells with stably expressing GFP. Nevertheless, GFP appeared to alter transcriptional regulation at baseline as well as after ionizing radiation exposure. We observed that our GFP cells are relatively less responsive to radiation than its wild type parental cells. More importantly, the tested mitochondrial genes of the GFP expressing cells decreased in expression instead of an increase as in the wild type cells after gamma irradiation (Fig. 3b, asterisks).

Currently few studies [30, 31] contain systematic investigations as to whether a reporter protein, such as GFP, affects the cell function under investigation. Our observation, that GFP alters mitochondrial transcript expression in human T-cell line after gamma irradiation, highlights the need to controls for the possible non-specific GFP effects (Fig. 4).

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