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Candidate tumour suppressor Fau regulates apoptosis in human cells: An essential role for Bcl-G

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ABSTRACT

FAU, which encodes a ubiquitin-like protein (termed FUBI) with ribosomal protein S30 as a carboxy-terminal extension, has recently been identified as a pro-apoptotic regulatory gene. This activity may be mediated by Bcl-G (a pro-apoptotic member of the Bcl-2 family) which can be covalently modified by FUBI. *FAU* gene expression has been shown to be down-regulated in human breast, prostate and ovarian tumours, and this down-regulation is strongly associated with poor prognosis in breast cancer. We demonstrate here that ectopic *FAU* expression increases basal apoptosis in human T-cell lines and 293T/17 cells, whereas it has only a transient stimulatory effect on ultraviolet-C (UVC)-induced apoptosis. Conversely, siRNA-mediated silencing of *FAU* gene expression has no effect on basal apoptosis, but attenuates UV-induced apoptosis. Importantly, prior knockdown of Bcl-G expression ablates the stimulation of basal apoptosis by *FAU*, consistent with an essential downstream role for Bcl-G, itself a candidate tumour suppressor, in mediating the apoptosis regulatory role of *FAU*. In 293T/17 cells, Bcl-G knockdown also attenuates UV-induced apoptosis, so that Bcl-G may constitute a common factor in the pathways by which both *FAU* and UV-irradiation induce apoptosis. UV irradiation increases Bcl-G mRNA levels, providing an explanation for the transient nature of the effect of ectopic *FAU* expression on UV-induced apoptosis. Since failure of apoptosis is fundamental to the development of many cancers, the pro-apoptotic activity of the Fau/Bcl-G pathway offers an attractive explanation for the putative tumour suppressor role of *FAU*.

1. Introduction

The retrovirus Finkel–Biskis–Reilly murine sarcoma virus (FBR-MuSV) induces osteosarcomas in susceptible mice and contains the transduced genes c-*fos* and *fox* [1]. *Fox* is an antisense sequence to the cellular gene *FAU* (FBR-MuSV associated ubiquitously expressed gene) and has been shown to increase the tumorigenicity of the virus [1]. In addition, overexpression of *FAU* in Chinese hamster V79 cells has been shown to confer resistance to the carcinogen arsenite [2]. Together, these observations indicate a tumour suppressor role for the *FAU* gene. On the other hand, *FAU* has been reported to transform human osteogenic sarcoma cells to anchorage-independence [3], suggesting that the *FAU* gene may additionally possess oncogenic activity in certain circumstances.

FAU encodes a ubiquitin-like protein, termed FUBI, fused to ribosomal protein S30 as a carboxy-terminal extension [4]. These two products are thought to be cleaved *post*-translationally. FUBI has 37% amino acid sequence identity (57% sequence similarity) to ubiquitin and retains the C-terminal G-G dipeptide motif that participates in isopeptide bond formation between ubiquitin and lysines of target proteins. It lacks, however, internal lysine residues which serve as sites of polyubiquitin chain formation, indicating that the biological function of FUBI is distinct from that of ubiquitin.

FUBI has been shown to interact with several proteins in murine cells. When conjugated to a T-cell receptor α-like protein, it comprises the β-subunit of murine monoclonal non-specific suppressor factor, which exhibits immunomodulatory activity [5]. Interactions with histone 2A (non-covalent modification) [6] and Bcl-G (covalent modification) [7] also occur, and the FUBI/Bcl-G complex can associate with ERKs and inhibit ERK activation by MEK1 [8]. Notably, Bcl-G is a pro-apoptotic member of the Bcl-2 family [9], but the effect of FUBI-mediated modification of Bcl-G on apoptosis was not addressed in these studies.

Using functional expression cloning approaches in murine cells, our laboratory isolated two clones which confer resistance to apoptotic cell death and which contain sequences antisense to *FAU*

Abbreviations: ALAS1, 5-Aminolevulinic acid synthase; ANOVA, analysis of variance; ECL, enhanced chemo-luminescence; ERK, extracellular signal-regulated kinase; FAU, Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)-associated ubiquitously expressed gene; FUBI, FAU ubiquitin-like protein; MCT, Bonferrini's multiple comparison test; MEK, MAPK/ERK kinase; RT-PCR, reverse transcriptase polymerase chain reaction; siRNA, small interfering RNA; z-VAD, benzyloxycarbonyl valylalanylaspartic acid fluoromethyl ketone

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[10]. Consistent with this, overexpression of *FAU* per se was shown to stimulate apoptosis in mouse W7.2c thymoma cells. Since failure of apoptosis is fundamental to the development of many cancers [11,12] regulation of apoptosis may serve as the functional basis of the putative tumour suppressor role for *FAU*. It can be further postulated that FUBI-mediated modification of Bcl-G forms part of the molecular mechanism by which *FAU* regulates apoptosis. Indeed, recent studies in our laboratory have shown that *FAU* gene expression is down-regulated in human breast, prostate and ovarian tumours [13–15] and that siRNA-mediated silencing of *FAU* and *BCL-L14* (which encodes Bcl-G), either alone or in combination, attenuates apoptosis induction in epithelial cell lines derived from such tissues [13,14]. Furthermore, we have presented evidence which suggests that Fau and Bcl-G act in the same apoptosis pathway in human breast and prostate cell lines [13,14].

In the present study, we have tested the hypotheses that i. *FAU* regulates apoptosis in human T-cell lines and HEK 293T/17 cells, and ii. Bcl-G is essential for *FAU* action in the latter cell line.

2. Materials and methods

2.1. Materials

Culture media, supplements, molecular biology reagents and fine biochemicals were from Invitrogen (Paisley, UK) or Sigma-Aldrich Company Ltd (Gillingham, UK). GeneJammer transfection reagent was from Agilent Technologies UK Limited (Stockport UK) and RNAiFect was from QIAGEN Ltd (Crawley, UK). siRNAs, Silencer siRNA labelling kit, TaqMan Universal Master Mix and TaqMan Gene Expression Assays were from Applied Biosystems (Warrington, UK). The CaspaTag fluorescein caspase activity kit was from Intergen (Oxford, UK). Bradford Coomassie Plus Reagent was from Perbio Science UK Ltd (Cramlington, UK). Ready gels, the GS-800 scanner and Quantity one software were from Bio-Rad Laboratories (Hemel Hempstead, UK). Immobilon-P membrane and the ReBlot Plus Kit were from Millipore (Watford, UK) and ECL Western blotting detection reagent and Hyperfilm-ECL were from GE Healthcare (Chalfont St. Giles, UK).

2.2. Cell lines

Human cell lines were employed, comprising the suspension cell lines, Jurkat and CEM-C7 (both T-lymphoblastic leukaemia cells), and the adherent cell line, 293T/17 (embryonic kidney-derived). Subclones of Jurkat and CEM-C7 cells, previously generated in our laboratory [16,17] and which exhibit uniform sensitivity to apoptosis induction were used, whereas 293T/17 cells were obtained from ATCC-LGC Promochem (Teddington, Middlesex, UK) and used directly. Cell lines were routinely cultured in RPMI-1640 medium supplemented with L-glutamine (2 mM), fetal bovine serum (10%) and gentamicin (50 μg/ml).

2.3. Plasmid DNA transfection

pCMV-SPORT6-FAU (IMAGE clone: 5216042; accession BI908605; GI: 16171620) was digested with EcoRI and XhoI and the insert was directionally cloned into pcDNA3 to generate pcDNA3-*Fau*. The orientation of the insert was verified by sequencing (Eurofins MWG Operon, London, UK). GeneJammer transfection reagent was used for transfection of 293T/17 cells, according to the manufacturer's instructions. Briefly, transfections were conducted in 6-well (35 mm in diameter) tissue culture dishes, and employed 2 μg plasmid (pcDNA3-*Fau* or pcDNA3) and 10 μl transfection reagent per well. Jurkat and CEM-C7 cells were transfected by electroporation using a gene pulser with a capacitance extender unit (Bio-Rad Laboratories) in 4 mm gap electroporation cuvettes. Early log phase cells were washed with – and resuspended in – Opti-MEM I at 2×10^7 cells/ml. Cells (0.4 ml) were electroporated at room temperature with 40 μg plasmid at 1050 μF capacitance and either 238 V (CEM-C7 cells) or 292 V (Jurkat cells). After 10 min, cells were added to 10 ml Iscove's Modified Dulbecco's Medium (IMDM) supplemented with L-glutamine (2 mM), fetal bovine serum (20%) and gentamicin (50 μg/ml). Transfection efficiency was assessed by carrying out parallel transfections with the plasmid pEGFP (encodes a green fluorescent protein) and the proportion of cells exhibiting fluorescence was determined by microscopy 24–48 h *post*-transfection. Transfection efficiencies were routinely 40–45% for 293T/17 and CEM-C7 cells and 45–50% for Jurkat cells.

2.4. RNA interference

Silencer predesigned siRNAs to *FAU* (codes 46005 and 10907; herein termed Fau1 and Fau2, respectively) and *BCL-L14* (codes 120721 and 120720; herein termed BclG1 and BclG2, respectively), along with *Silencer* negative control #1 siRNA (code 4611) were used here. RNAiFect was used for the transfection of 293T/17 cells, according to the manufacturer's protocol. Transfections were conducted in 6-well plates in culture medium and contained 30 nM siRNA. Cells were transferred to T25 flasks 24 h *post*-transfection and maintained for 96 h before use. Suspension cells were transfected with siRNA (30 nM final concentration) by electroporation, using a similar protocol to that described for plasmid transfection, except that cells were allowed to incubate at room temperature for 20 min *post*-electroporation before transfer to 10 ml of a 1:1 mix of complete IMDM and routine culture medium. Cells were split 1:1 with routine culture medium at 24 h *post*-transfection and maintained at 37 °C for 96 h before use.

To determine transfection efficiency, parallel transfections were conducted with Cy3-labelled siRNA prepared using Silencer siRNA labelling kit, according to the supplied protocol. The proportion of cells exhibiting fluorescence was determined by microscopy 24 h *post*-transfection. Transfection efficiencies were routinely 50–55% for CEM-C7 and Jurkat cells, and 80–85% for 293T/17 cells.

2.5. Induction of apoptosis

Suspension and trypsinised adherent cells were resuspended at 6×10^5 cells/ml culture medium, and irradiated with ultraviolet light at 254 nm (UV). The doses administered were 20 J/m^2 for Jurkat and CEM-C7 cells and 40 J/m^2 for 293T/17 cells and these doses were verified using a UV (shortwave) intensity meter. Controls were mock-irradiated. Immediately after exposure, cells were transferred to fresh medium then plated (cell density of 2×10^4/cm^2 for adherent cells or 3×10^5/ml for suspension cells). At the indicated times post-UV exposure, cells were harvested for assessment of apoptosis; suspension cells were studied directly, whereas 293T/17 cells were trypsinized and adherent cells were combined with non-adherent cells prior to assessment.

2.6. Assessment of apoptosis

Apoptosis was routinely monitored by assessment of nuclear morphology. Cells were stained with acridine orange (25 μg/ml) and observed by fluorescence microscopy. Cells containing condensed or fragmented chromatin were considered to be apoptotic.

In some experiments, apoptosis was additionally assessed by measuring caspase activation using a commercial kit (CaspaTag fluorescein caspase activity kit), which contains a carboxyfluorescein derivative of benzyloxycarbonyl valylalanylaspartic acid fluoromethyl ketone (z-VAD-fmk), according to the manufacturer's instruction; cells were also stained with Hoechst 33342 to visualise cell nuclei. The proportion of apoptotic cells was determined by fluorescence microscopy and direct counting.

2.7. Cell viability assay

To assess short term cell viability, cell suspensions were stained with 0.1% (w/v) nigrosin blue and counted using a Neubauer chamber (light microscopy). Those cells which excluded the dye were considered to be viable.

2.8. Clonogenic growth assay

The colony forming ability of suspension cell lines was determined by plating cells (*ca.* 10,000) in 3 ml complete IMDM further supplemented with 10% cell-conditioned medium and 0.5% (w/v) Noble agar in 6-well plates. The overlay comprised 1.5 ml complete IMDM containing 10% cell-conditioned medium. Colonies were counted after 3–4 weeks incubation. 293T/17 cells (*ca.* 200 cells) were directly plated in 5 ml routine growth medium supplemented with 10% (v/v) cell conditioned medium in 60 mm diameter culture dishes and incubated for 10 days. Colonies were counted after staining with crystal violet (0.5% (w/v) in methanol).

2.9. Real time RT-PCR

Total RNA was isolated using TRIZOL reagent, digested with RNase-free DNase, then reverse transcribed using random hexamer priming and SuperScript II Reverse Transcriptase, according to the supplied protocols. Real-time PCR was conducted using TaqMan Universal Master Mix and TaqMan Gene Expression Assays (assay codes Hs 00609872_g1 for FAU, Hs 00373302_m1 for BCL2L14 and Hs 00167441_m1 for ALAS1), as recommended by the manufacturer, and was run on an ABI Prism Sequence Detection System model 7000. A standard curve, comprising cDNA prepared from the 22Rv1 human prostate cell line, was included with each assay run to allow relative quantitation. Assays usually contained 0.1–30 ng standard or 5 ng sample cDNA in a final volume of 25 μl. For quantitation of Bcl-G, which is a low abundance mRNA, sample and standard input were 500 ng and 0.6–60 ng cDNA, respectively. For each assay, a standard curve of threshold cycle (C_T) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective C_T values. Bcl-G sample values were corrected for increased sample input, and all data were expressed relative to the endogenous control gene, ALAS1.

2.10. Immunoblotting

Cells were washed twice with PBS and resuspended in ice-cold RIPA buffer containing 1% (v/v) Calbiochem protease inhibitor cocktail set III and 0.2 mM iodoacetamide. After 30 min at 4 °C, samples were centrifuged (10,000 *g*; 10 min; 4 °C) and the protein content of the supernatant was assayed using Bradford Coomassie Plus Reagent. Samples (15 μg protein) were denatured with Laemmli buffer by boiling (10 min), then centrifuged (10,000 *g*; 10 min; 4 °C). Supernatants were electrophoresed (12% Tris–HCl Ready gels), then electrotransferred in Towbin buffer to Immobilon-P membrane. Immunodetection was by a standard protocol supplied with the ECL Western blotting detection reagent. For Bcl-G, primary antibody (code PA1-4209; Affinity BioReagents, Golden, CO) was used at 1:2000 dilution, and secondary antibody (goat anti-rabbit IgG; peroxidase-conjugate; code A0545, Sigma-Aldrich Company Ltd) was used at 1:10000 dilution. Blots were stripped (ReBlot Plus Kit) and reprobed for β-actin, using primary antibody (code A5441; Sigma-Aldrich Company Ltd) at a dilution of 1:10000 and secondary antibody (goat anti-mouse immunoglobulins, peroxidase-conjugate, code P0447; Dako, Ely, UK) at a dilution of 1:5000. Blots were then exposed to Hyperfilm-ECL (preflashed to 0.05 optical density units above background). Images were captured and densitometrically analysed using a GS-800 scanner and Quantity one software (both from Bio-Rad Laboratories).

2.11. Statistical analysis

Data were analysed by Student's *t*-test (unpaired) or, for comparison of more than two groups, by one-way analysis of variance. Post-hoc analysis for the latter was by Bonferroni's multiple comparison test (MCT) or, when comparing data to a single control group, by Dunnett's MCT.

3. Results

3.1. Ectopic expression of FAU

Transient transfection of CEM-C7, Jurkat and 293T/17 cell lines with pcDNA3-*Fau* markedly increased *FAU* mRNA levels: FAU/ALAS1 ratios for pcDNA3-*Fau* versus pcDNA3 transfectants were: 5.41 ± 0.83 versus 1.79 ± 0.03 for CEM-C7, 5.65 ± 1.38 versus 0.93 ± 0.31 for Jurkat, and 16.05 ± 1.93 versus 0.49 ± 0.27 for 293T/17 (all $P<0.01$, $n \geq 3$; Student's *t*-test). In all cell lines, ectopic *FAU* expression was associated with increased apoptosis and decreased short-term cell viability in the absence of UV-irradiation (Fig. 1). In contrast, there was no consistent effect on UV-induced apoptosis, at least at the time points studied in this experiment (Fig. 1). However, in a further study, assay of *pan*-caspase activity in CEM-C7 cells revealed that ectopic *FAU* expression was associated with increases in both basal and UV-induced apoptosis at 4.5 h *post*-UV irradiation (the proportion of fluorescent cells for pcDNA3-*Fau* versus pcDNA3 transfectants were: 56.6 ± 2.4 versus 28.4 ± 1.8% for mock-irradiated cells and 60.2 ± 4.6 versus 37.2 ± 4.5% for UV-irradiated cells; both $P<0.01$, $n = 3$; one-way ANOVA and Bonferroni's MCT), whereas, at 24 h *post*-UV irradiation, basal apoptosis only was increased (data not shown).

In separate experiments, the colony-forming ability of CEM-C7, Jurkat and 293T/17 cells transiently transfected with *FAU* was found to be reduced when compared with cells transfected with vector alone (Fig. 2), confirming the significant pro-apoptotic role of *FAU* in these cell lines, even when *FAU* expression was transient. Furthermore, there was no significant effect of *FAU* on clonogenic survival of UV-irradiated cells (data not shown) in keeping with our observations on short-term cell viability (Fig. 1).

3.2. Silencing of FAU expression

Two different siRNAs (termed Fau1 and Fau2 siRNAs) were employed to examine the effects of *FAU* gene silencing on apoptosis. FAU/ALAS1 transcript ratios for cells transfected with Fau1 and Fau2 versus negative control siRNAs were: 1.14 ± 0.11 and 1.08 ± 0.16 vs 2.17 ± 0.17 for CEM-C7 cells; 0.70 ± 0.05 and 0.64 ± 0.07 vs 1.25 ± 0.02 for Jurkat cells, and 0.23 ± 0.01 and 0.26 ± 0.01 vs 0.51 ± 0.08 for 293T/17 cells (all $P<0.01$, $n \geq 3$; one-way ANOVA and Dunnett's MCT). Thus, *FAU* gene expression was reduced by approximately one half with each siRNA. In all cell lines, this had negligible effects on apoptosis and cell viability in the absence of an apoptotic stimulus, but markedly attenuated UV-induced apoptosis and loss of cell viability (Fig. 3).

3.3. Mediation of FAU pro-apoptotic activity by Bcl-G

Fau, or, more specifically, the FUBI moiety, has been shown to covalently modify a pro-apoptotic member of the Bcl-2 family, Bcl-G [7]. It is possible therefore, that Bcl-G is involved in the pro-apoptotic action of Fau.

To address this postulate, we firstly investigated the effect of siRNA-mediated silencing of Bcl-G on apoptosis in 293T/17 cells. Two different Bcl-G siRNAs were studied, both of which reduced Bcl-G

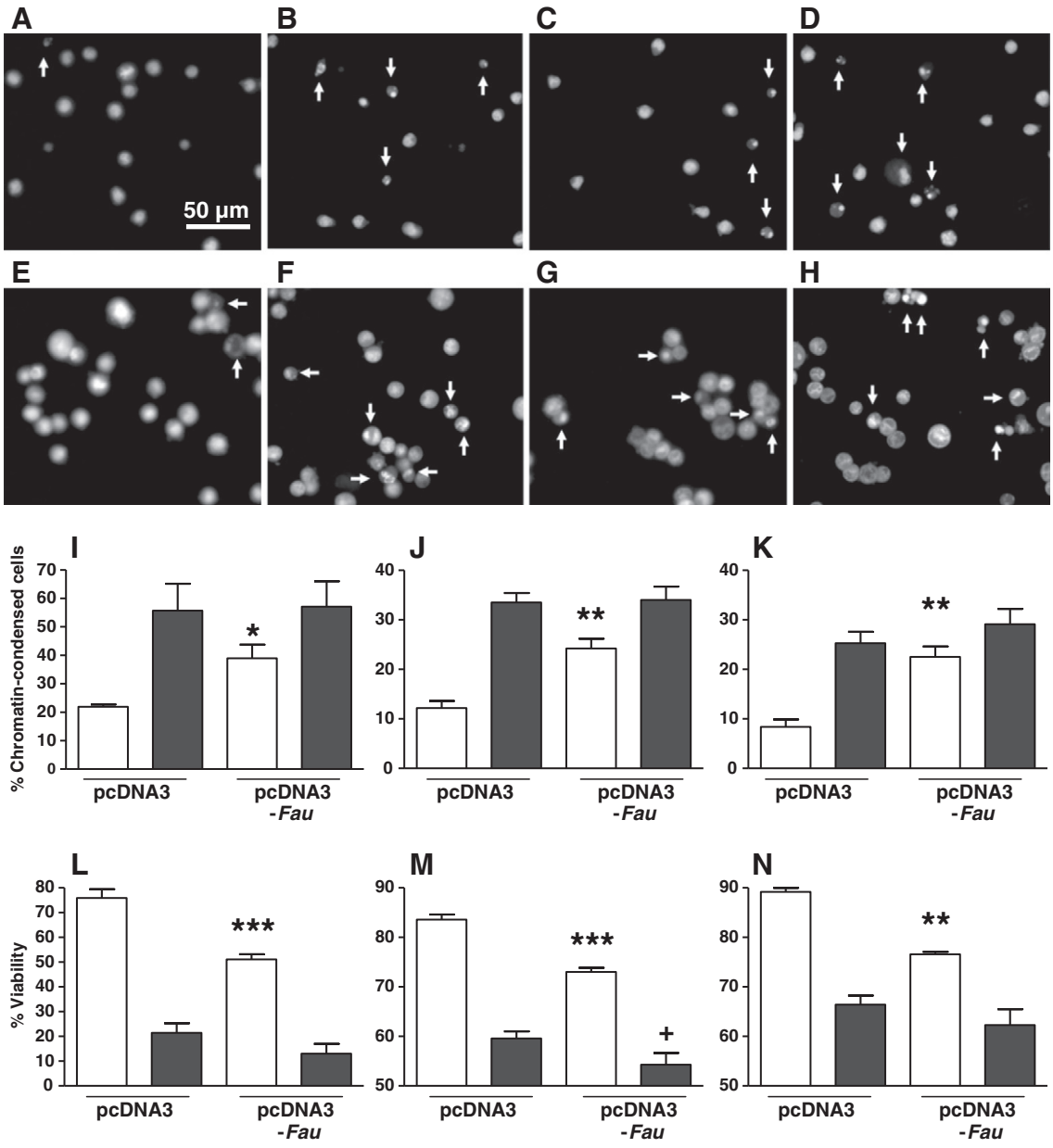

Fig. 1. Transient over-expression of *Fau* in T-cell lines and 293T/17 cells influences apoptosis and cell viability. Cells were transfected with a pcDNA3-*Fau* construct or pcDNA3 alone and, after 24 h, either exposed to UV light or mock-irradiated. The proportion of apoptotic cells was determined by acridine orange staining after a further 24 h (T-cell lines) or 48 h (293T/17 cells), whereas short-term cell viability was determined by dye exclusion at 48 h *post*-UV irradiation. Panels A–H: example images of acridine orange-stained Jurkat (A–D) and 293T/17 (panels E–H) cells; vector/treatment conditions are: pcDNA3/mock-irradiated (panels A and E); pcDNA3/UV-irradiated (panels B and F); pcDNA3-*Fau*/mock-irradiated (panels C and G); and pcDNA3-*Fau*/UV-irradiated (panels D and H); cells showing condensed and/or fragmented chromatin, characteristic of apoptosis, are indicated by arrows; scale bar in panel A also applies to panels B–H. Panels I–K: bar charts showing proportion of apoptotic cells in transfected CEM-C7 (panel I), Jurkat (panel J) and 293T/17 (panel K) cultures; UV-irradiated cells are closed bars and mock-irradiated controls are open bars. Panels L–N: bar charts showing proportion of apoptotic cells in transfected CEM-C7 (panel L), Jurkat (panel M) and 293T/17 (panel N) cultures; UV-irradiated cells are closed bars and mock-irradiated controls are open bars Data(panels I–N) are mean ± SEM; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, pcDNA3-*Fau* versus pcDNA3 (mock-irradiated) and $^{+}P<0.05$ pcDNA3-*Fau* versus pcDNA3 (UV-irradiated) (one-way ANOVA and Bonferroni's MCT); n = 6 (T-cell lines) or n = 4 (293T/17 cells).

mRNA (Fig. 4A) and protein (Fig. 4B) levels. At the functional level, silencing of Bcl-G expression was found to attenuate UV-induced increases in *pan*-caspase activity (Fig. 4C) and cell death (Fig. 4D), similar to the effects of silencing of *FAU* expression (Fig. 3).

Next, the effect of Bcl-G knockdown on *FAU*-mediated stimulation of the basal apoptotic rate was examined. For this experiment, 293T/17 cells were first transfected with one of the two different siRNAs to the *BCL-L14* gene or negative control siRNA, then (after 4 days) transfected with either the pcDNA3-*Fau* construct or pcDNA3 alone. At 24 h after the second transfection, Bcl-G mRNA levels were reduced ($P<0.01$, n = 4; one-way ANOVA and Dunnett's MCT) in cells transfected with BclG1 and BclG2 siRNAs when compared with negative control siRNA, irrespective of whether cells had received pcDNA3-*Fau* or pcDNA3 alone in the second round of transfection (pooled Bcl-G/ALAS1 ratios for NC, BclG1 and BclG2 siRNAs were: 0.0137 ± 0.0015, 0.0074 ± 0.0010 and 0.0071 ± 0.0009, respectively). Similarly, *FAU* mRNA levels were increased ($P<0.001$, n = 4; Student's *t*-test) in cells transfected with the pcDNA3-*Fau* construct when compared with those transfected with pcDNA3 alone, irrespective of whether cells had received negative control, BclG1 or BclG2 siRNA in the first round of transfection (pooled FAU/ALAS1 ratios for pcDNA3 and pcDNA3-*Fau* transfectants were: 0.34 ± 0.04 and 14.59 ± 2.15,

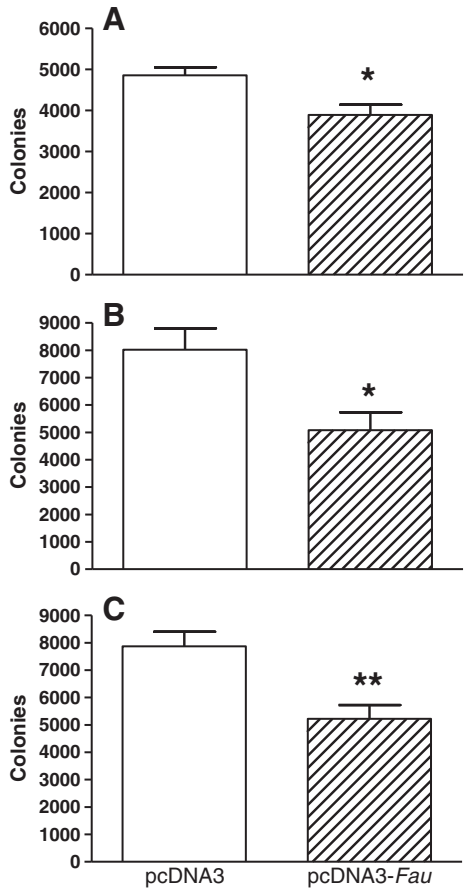

Fig. 2. Transient over-expression of *Fau* reduces the clonogenic activity of T-cell lines and 293T/17 cells. CEM-C7 (panel A), Jurkat (panel B) and 293T/17 (panel C) cells were transfected with a pcDNA3-*Fau* construct or pcDNA3 alone and, after 24 h, aliquots were plated in soft-agar (T-cell lines) or direct onto tissue culture plastic (293T/17 cells). Colonies of T-cell lines were counted by direct observation after 3–4 weeks incubation, whereas colonies of 293T/17 cells were counted following crystal violet staining after 1–2 weeks incubation. Data are mean ± SEM; $^{*}P<0.01$, $^{**}P<0.005$, pcDNA3-*Fau* versus pcDNA3 (Student's *t*-test); n = 10.

respectively). At the functional level, ectopic *FAU* expression clearly increased apoptosis and reduced both short-term viability and clonogenic survival in cells which had been previously transfected with negative control siRNA (Fig. 5 & data not shown), as expected. However, in cells which had been pre-treated with siRNAs to *BCL-L14*, this pro-apoptotic activity of *FAU* was either markedly reduced (in the case of BclG1 siRNA) or abolished (in the case of BclG2 siRNA) (Fig. 5). Similarly, *FAU* was without any significant effect on short-term viability (data not shown) and clonogenic survival (Fig. 5) in cells pre-treated with siRNAs to *BCL2-L14*. Thus, these findings support an essential role for Bcl-G in mediating the stimulation of basal apoptosis by Fau.

3.4. Effect of ectopic FAU expression and siRNA-mediated silencing of FAU on Bcl-G protein levels

Ubiquitin (and several ubiquitin-like proteins) often regulate the proteasomal degradation of target molecules [18–21]. Despite showing high homology with ubiquitin, *FAU*-encoded FUBI lacks internal lysine residues usually associated with polyubiquitin chain formation, thus mechanism(s) involving *FAU*-mediated modulation of Bcl-G protein levels appear unlikely. To examine this postulate, steady state levels of Bcl-G protein (specifically the long isoform of Bcl-G) were determined in T-cell lines and 293T/17 cells after modulation of *FAU* gene expression. Indeed, neither ectopic *FAU* expression nor siRNA-mediated silencing of *FAU* were associated with changes in Bcl-G protein levels in any of the three cell lines (Fig. 6).

3.5. Effect of UV-irradiation on FAU and BCL2-L14 gene expression in HEK 293T/17 cells

One possible explanation for the lack of any sustained effect of ectopic *FAU* gene expression on UV-induced apoptosis, as demonstrated earlier (e.g. see Fig. 1), is that both Fau- and UV-induced apoptosis may in part be mediated through common factor(s). Having shown that Bcl-G is necessary for Fau-mediated stimulation of the basal apoptotic rate, and given that BCL2-L14 is a putative p53-responsive gene [22], the effect of UV irradiation on Bcl-G (and Fau) mRNA levels in 293T/17 cells (which express a functional p53) was examined. Whilst UV-irradiation resulted in a robust induction in Bcl-G gene expression (Fig. 7A), it did not affect steady state Fau mRNA levels (Fig. 7B).

4. Discussion

We show here that ectopic *FAU* expression stimulates the basal apoptotic rate in human T-cell lines and in 293T/17 cells. Conversely, siRNA-mediated down-regulation of endogenous *FAU* expression attenuates UV-induced apoptosis in all three cell lines. These findings are in broad agreement with earlier studies in murine cells [10] and in human breast, prostate and ovarian cell lines [13–15], demonstrating that *FAU* regulates apoptosis in multiple mammalian cell types.

FAU encodes a ubiquitin-like protein, termed FUBI, with ribosomal protein S30 as a carboxy-terminal extension. In mouse cells, FUBI covalently modifies a pro-apoptotic member of the Bcl-2 family, Bcl-G [7]. This prompted us to investigate whether Bcl-G was required for the apoptosis-promoting activity of *FAU* in human cells. Similar to the effects of Fau knockdown, siRNA-mediated silencing of Bcl-G expression attenuated UV-induced apoptosis in 293T/17 cells, consistent with findings in breast and prostate cell lines [13,14]. Crucially, in the present study, prior knockdown of *BCL-L14* (Bcl-G) gene expression was found to abolish the increase in basal apoptotic rate consequent upon ectopic *FAU* expression, despite an increase in apoptosis produced by the Bcl-G siRNAs alone (Fig. 5). This finding supports the hypothesis that Bcl-G plays an essential down-stream role in mediating the pro-apoptotic activity of Fau. Indeed, consistent conclusions have been drawn from studies on the effects of *FAU* and *BCL2-L14* down-regulation in breast and prostate cell lines: Although down-regulation of each gene alone attenuates UV-induced apoptosis, no additional effect is produced when the two genes are down-regulated together [13,14].

While ectopic *FAU* expression resulted in a sustained increase in the basal apoptotic rate in all cell lines, effects on UV-induced apoptosis appeared more transient, being limited to the first few hours after UV irradiation. Thus in CEM-C7 cells, *FAU* enhanced UV-induction of caspase activity at 4.5 h *post*-irradiation but this effect had disappeared by 24 h. One explanation for this finding is that the mechanisms by which *FAU* and UV-irradiation induce apoptosis involve a common factor; several lines of evidence from the present study suggest that this factor is Bcl-G. Firstly, Bcl-G is essential for stimulation of the basal apoptotic rate by Fau; secondly, siRNA-mediated silencing of Bcl-G expression attenuates UV-induced apoptosis, and thirdly, UV irradiation induces *BCL2-L14* (but not *FAU*) gene expression; consistent with the identification of *BCL2-L14* as a putative p53-responsive gene [22]. Thus, when cellular *FAU* gene expression is enhanced, basal apoptosis is increased by a mechanism centring on Bcl-G. However, when the same cell is subsequently exposed to high levels of UV irradiation, Bcl-G expression increases, so that the pro-apoptotic effect of Bcl-G per se are likely to predominate at later time points.

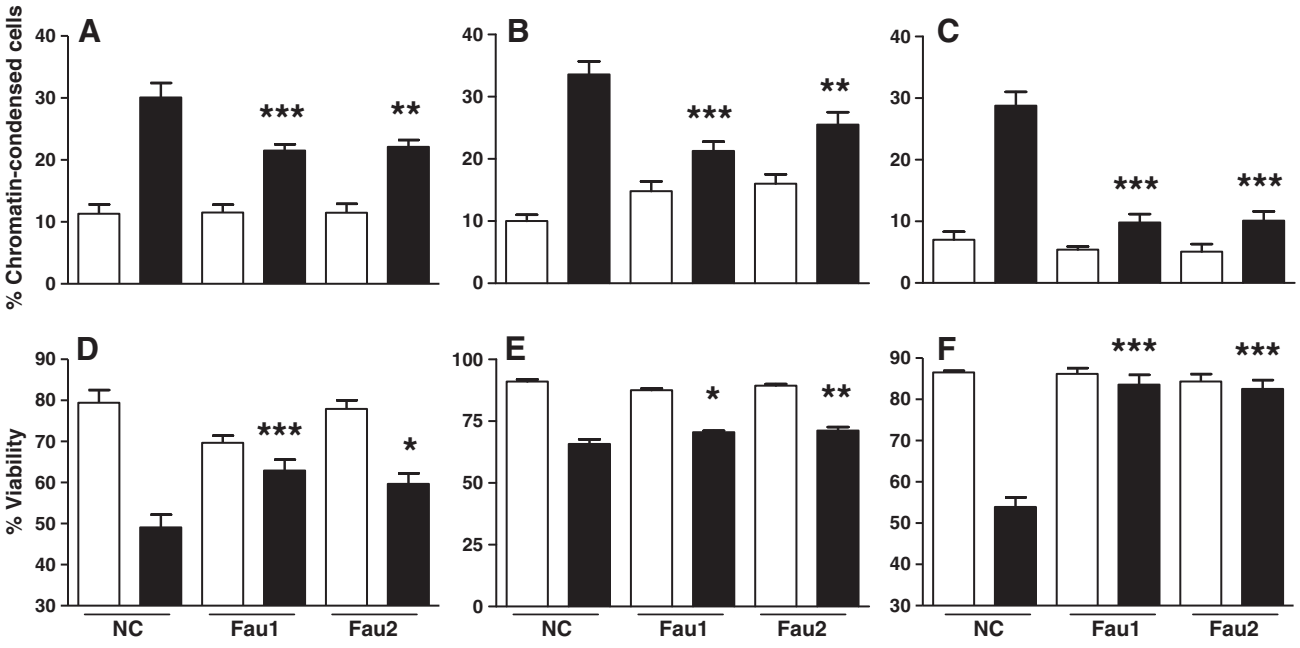

Fig. 3. siRNA-mediated knockdown of *Fau* expression in CEM-C7, Jurkat and 293T/17 attenuates UV-induced apoptosis. CEM-C7 (panels A and D), Jurkat (panels B and E) and 293T/17 (panels C and F) cells were transfected with two different siRNAs to *Fau* (Fau1 and Fau2) or negative control (NC) siRNA and cultured for 96 h. Cells were either exposed to UV light (closed bars) or mock-irradiated (open bars) and, after 48 h, the proportion of apoptotic cells was determined by acridine orange staining (panels A–C) and short-term cell viability was determined by dye exclusion (panels D–F). Data are mean ± SEM; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Fau1 or Fau2 versus NC siRNA (UV-irradiated) (one-way ANOVA and Bonferroni's MCT); n = 8 (CEM-C7 cells), n = 6 (Jurkat cells) or n = 3 (293T/17 cells).

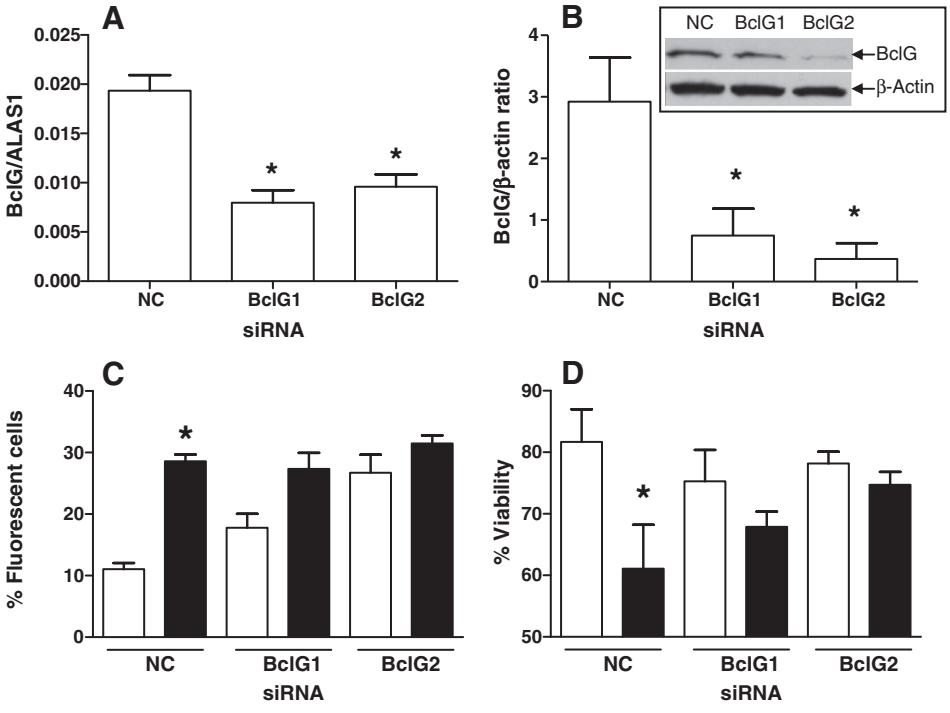

Fig. 4. siRNA-mediated knockdown of Bcl-G attenuates induction of apoptosis by UV irradiation in 293T/17 cells. Cells were transfected with siRNA to *BCL2L14* (BclG1or BclG2 siRNA) or negative control (NC) siRNA. After 96 h, samples were taken for assessment of Bcl-G mRNA and protein levels, and cells were then either exposed to UV (closed bars) or mock irradiated (open bars). Cells were assessed for apoptosis (C; CaspaTag assay) and cell viability (D; vital dye staining) after a further 48 h. A) Bcl-G RNA levels expressed relative to the house-keeping gene ALAS1; $^{*}P<0.01$ versus NC siRNA (one-way ANOVA and Dunnett's MCT); n = 4. B) Bcl-G protein levels expressed relative to β-actin; $^{*}P<0.05$ versus NC siRNA (one-way ANOVA and Dunnett's MCT); n = 4. C) Proportion of apoptotic cells in UV-treated (closed bars) and mock-irradiated cells (open bars); $^{*}P<0.001$ versus mock-irradiated control (one-way ANOVA and Bonferroni's MCT); n = 4. D) Cell viability in UV-treated (closed bars) and mock-irradiated (open bars) cultures; $^{*}P<0.05$, versus mock-irradiated control (one-way ANOVA and Bonferroni's MCT); n = 4.

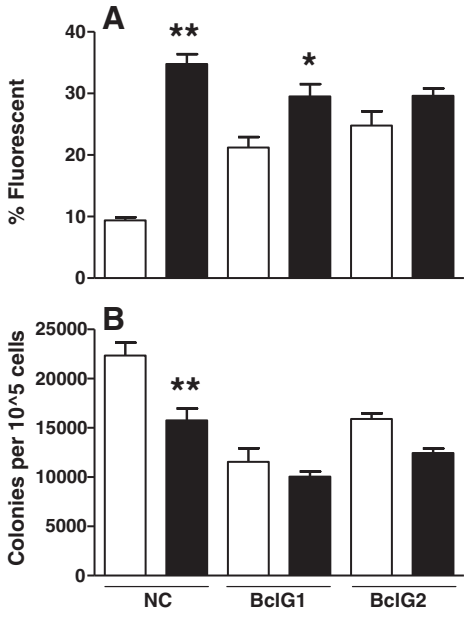

Fig. 5. siRNA-mediated knockdown of Bcl-G attenuates induction of apoptosis by *Fau* in 293T/17 cells. Cells were transfected with two different siRNAs to *BCL2L14* (BclG1 and BclG2) or negative control (NC) siRNA. After 96 h, cells were transfected with a pcDNA3-*Fau* construct (closed bars) or pcDNA3 alone (open bars). After a further 24 h, cells were re-plated for assessment of apoptosis (by CaspaTag at 48 h *post*-plating; panel A) and colony formation (by crystal violet staining after 10 days; panel B). Data are mean ± SEM; $^{*}P<0.01$, $^{**}P<0.001$, pcDNA3-*Fau* versus pcDNA3 (one-way ANOVA and Bonferroni's MCT); n = 4.

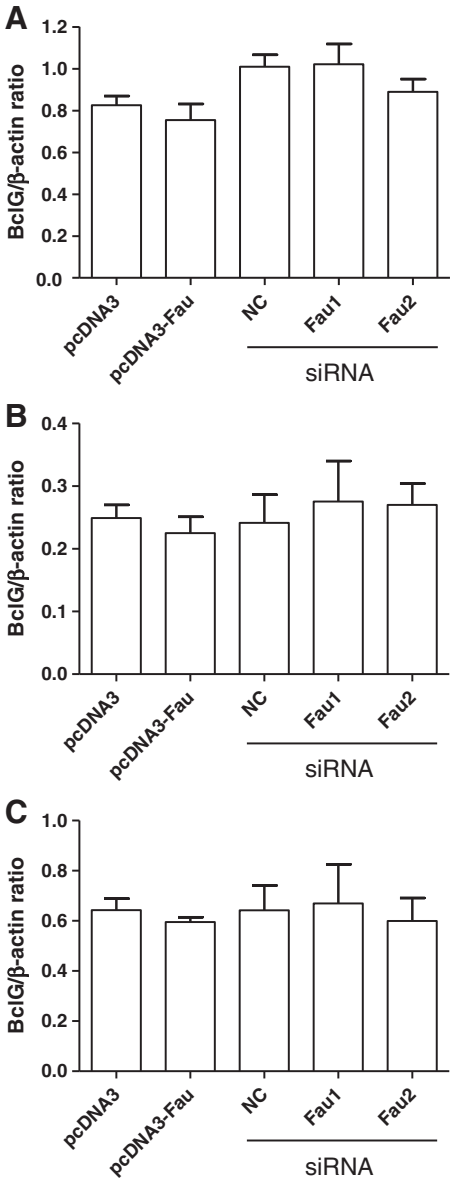

Fig. 6. Effect of ectopic *FAU* expression and siRNA-mediated silencing of *FAU* gene expression on Bcl-G protein levels in CEM-C7 (panel A), Jurkat (panel B) and 293T/17 (panel C) cells. Cells were transfected with pcDNA3, pcDNA3-Fau, a negative control (NC) siRNA or one of two different siRNAs to *FAU* (Fau1 and Fau2). Cells were harvested for Western blot preparation at either 48 h (pcDNA3 and pcDNA3-Fau transfectants) or 96 h (siRNA-transfected cells) *post*-transfection. Blots were immunodetected with an antibody to Bcl-G, stripped, then with an antibody to β-actin; resulting images were analysed densitometrically. Data are expressed as Bcl-G:β-actin ratio; n ≥ 3 cultures.

Ubiquitin is well established as an important regulator of apoptosis [19–21], and, in addition, it is increasingly recognised that a range of ubiquitin-like proteins, in particular SUMO-1, DAP-1 and Fat-10, have roles in apoptosis control [23–26]. Findings from present and previous studies [7,13–15] raise the possibility that FAU-derived FUBI also falls into the latter category.

Ubiquitin controls the levels of many key apoptosis regulatory molecules (including p53, the inhibitor of nuclear factor KB, c-FLIP, inhibitor of apoptosis proteins (IAPs) and Bcl-2 family members) *via* the formation of lysine-48 polyubiquitin chains, facilitating their proteasomal degradation [19–21]. A similar mechanism of action is unlikely for FUBI, since lysine-48 is not conserved in this ubiquitin-like protein. Indeed, with the exception of certain members of the SUMO family, ubiquitin-like proteins are not thought to form chains on target molecules [18]. Consistent with this, cellular Bcl-G protein levels were unaffected by up- or down-regulation of *FAU* gene expression in the present study. However, an increasing body of evidence indicates that modification of proteins with monomers of ubiquitin or ubiquitin-like proteins, rather than inducing degradation, markedly influences their activity, intracellular localisation or inter-molecular interactions [18–21,27]. In this regard, the FUBI/Bcl-G complex has been shown to associate with ERKs and to inhibit ERK activation by MEK1 in murine cells [8]. Nevertheless, the molecular mechanisms by which *FAU* regulates apoptosis remain to be elucidated; possibilities include FUBI-mediated targeting of Bcl-G to the mitochondrion, or modulation of the interaction of Bcl-G with other constituents of the cellular apoptotic machinery, for example.

Although down-regulation of Bcl-G clearly inhibits apoptosis induced both by UV irradiation and by Fau (Figs. 4 and 5), basal apoptosis rates are higher under these conditions. This is a reproducible and specific effect of two independent Bcl-G siRNAs in 293T/17 cells, but it has not been observed in other cell lines [13,14]. This indicates that in certain cell lines, such as 293T/17, Bcl-G can have anti-apoptotic effects, in addition to the pro-apoptotic effects generally associated with BH3-only members of the Bcl-2 family. Whilst such activity has not previously been reported for Bcl-G, anti-apoptotic activity has also previously been reported for the BH3-only protein N-Bak [28].

In summary, present and previous findings are consistent with a pro-apoptotic regulatory role for *FAU*, mediated *via* Bcl-G. This activity is likely to be of clinical importance since *FAU* expression is down-regulated in several tumours, including those that affect the prostate, the ovaries and the breast (where down-regulation of Fau is significantly associated with poor prognosis), and probably constitutes the functional basis of the putative tumour suppressor gene function of *FAU*. More detailed understanding of the FAU/Bcl-G pathway is warranted, since this may facilitate the design of novel cancer chemotherapies.

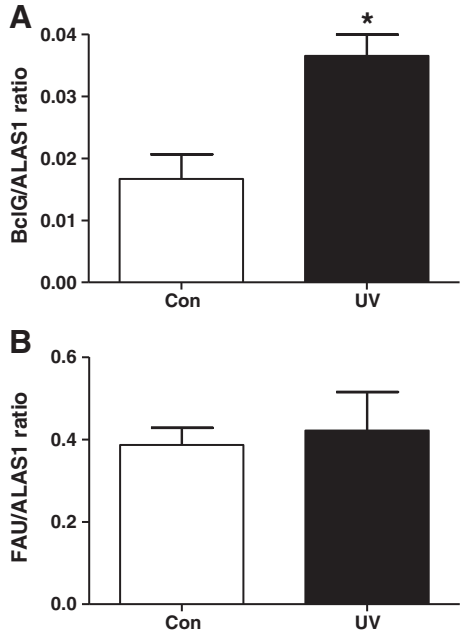

Fig. 7. Effect of UV-irradiation on *BCL2L14* (panel A) and *FAU* (panel B) gene expression in 293T/17 cells. Cells were irradiated with a UV-dose of 40 J/m^2, then after 24 h, RNA was extracted and cDNA was prepared. This was subjected to real-time PCR analysis for determination of Bcl-G, Fau and ALAS1 (endogenous control gene) mRNA levels; data are expressed relative to ALAS1 (*$P = 0.02$, Student's *t*-test; $n = 3$).

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