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Article

The Tardigrade Damage Suppressor Protein Promotes Transcription Factor Activation and Expression of DNA Repair Genes in Human Cells in Response to Hydroxyl Radicals and UV-C Exposure

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Abstract: The Ramazzottius varieornatus tardigrade is an extremotolerant terrestrial invertebrate belonging to the phylum of Tardigrada. At a length of 0.1-1.0 mm, tardigrades are small animals with an exceptional tolerance to extreme conditions such as high pressure, chemicals and irradiation. These properties have been attributed to the recently-discovered Dsup protein. Dsup is a nucleosome-binding protein that prevents DNA damage against X-ray and oxidative stress without impairing cell life, also in Dsup-transfected animal and plant cells. However, the precise "protective" role of this protein is still under study. We performed experiments on human cells and shows that, as compared to control cells, Dsup+ cells are more resistant to UV-C exposure and H2O2. Real-time PCR identified different expression patterns of endogenous genes involved in apoptosis, cell survival and DNA damage repair in Dsup+ cells in response to H2O2 and UV-C. While H2O2 treatment in Dsup+ cells only marginally involved the activation of pathways responsible for DNA repair reinforcing the idea of a direct protective effect of the protein on DNA, in UV-C exposed cells, Dsup efficiently upregulates DNA damage repair genes. In conclusion, our data may help to delineate the different mechanisms by which the Dsup protein operates in response to different insults.

Keywords: Dsup, tardigrade, UV-C, Oxidative stress, DNA repair, transcription factors

1. Introduction

In 2016, the sequencing of the Ramazzottius varieornatus tardigrade genome ended with the discovery of a unique DNA-associated protein with the ability to protect DNA from irradiation stress [1]. This protein, termed Damage suppressor protein (Dsup), is hypothesized to be responsible for the extraordinary characteristics of tardigrades. Also known as water bears, tardigrades are small invertebrates (0.1-1.0 mm in length) that have adapted to live in numerous habitats, such as marine, freshwater and terrestrial environments [2]. In the absence of water, they can enter an anhydrobiotic state to survive harsh conditions. They can resist extreme temperatures [3,4], vacuum, high pressure [5], radiation [6], chemicals and also direct exposure to open space [7]. How Dsup may exert its protective role is still a matter of study. Hashimoto et al. have shown that the protein directly interacts with free DNA [1]. Three years later, Chavez et al., in a purified biochemical system, proved that Dsup is a nucleosome-binding protein enriched (more than 60%) in serine, alanine, glycine and lysine (SAGK) residue [8]. SAGK are disorder-promoting amino acids [9] implied in the coverage of the chromatin and consequently, DNA protection [8]. Similarly to its functions in vivo, Dsup-transfected human cells have higher resistance to X-ray [1] and oxidative stress [8]. Even tobacco plants benefit from Dsup transfection by acquiring protection against damaging stress such as genomutagens or radiation [10]. In plants, Dsup affects the expression of endogenous

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genes involved in DNA damage signalling and repair [10]. This opens the possibility that, in addition to a direct interaction and coverage of DNA, Dsup induces protection by altering the expression levels of endogenous genes critical for cell survival and proliferation.

DNA damage may occur as a consequence of cellular metabolism or as a result of various exogenous factors. All these factors lead to damage to double-stranded DNA molecules, such as single-stranded DNA segments (ssDNA) or double-stranded DNA breaks (DSBs). Reactive oxygen species (ROS) usually cause the generation of DSBs, whereas exposure to non-ionising UV radiation causes biochemical changes such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs), which in turn lead to DNA structure alterations, formation of bends or curves, and the arrest of replication forks during replication [11].

Ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia-mutated Rad-related (ATR) are the major regulators of the DNA damage response. Both ATM and ATR are large kinases phosphorylating Ser or Thr residues followed by Gln. DSBs lead to ATM activation, while ssDNAs mainly involve ATR. ATM activates its downstream kinase checkpoint, kinase 2, and works in coordination with the MRE11–RAD50–NBS1 (MRN) complex [12]. ATR activates downstream kinase Chk1 and Chk2, and works with TopBp1, Claspin and RAD9-RAD1-HUS1 (9-1-1) complex [13]. ATM exerts its action mainly at the G1, S and G2 checkpoints; ATR activation occurs during every S-phase of the cell cycle to repair damaged replication forks and to prevent the premature entry of cells into mitosis [14].

One of the critical upstream regulators of ATM and ATR is the BRCA1 DNA Repair Associated (BRCA1) protein. BRCA1 is one of the main factors in maintaining genome integrity in mammalian cells. It is also involved in repair mechanisms and checkpoint pathways, together with the MRN complex [15].

To prevent ROS, cells can also activate a number of antioxidant enzymes. Catalase (CAT) is one of these, strongly mitigating oxidative stress by destroying cellular hydrogen peroxide to produce water and oxygen [16]. CAT actions are linked to superoxide dismutases (SODs). SODs dismutate superoxide anions to H2O2 that are catalyzed to H2O by CAT, peroxiredoxins (Prxs), or glutathione peroxidases (GPx) [17].

In this article, we investigated the role of Dsup protein in ROS and UV-C protection in transfected human cells. We highlighted that Dsup acts by promoting specific transcription factor activation, and by signalling pathways linked to DNA damage repair and cellular antioxidant activity.

2. Materials and Methods

2.1 Cell Transfection

The HEK293 cell line (mycoplasma-free, verified by N-GARDE Mycolpsma PCR reagent set, Euroclone) was kindly donated by Prof. Sandra Donnini (University of Siena). pCXN2KS-Dsup was a gift from Prof. Kunieda Takekazu (Addgene plasmid #90019; http://n2t.net/addgene.90019; RRID: Addgene_90019). Cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The expression construct was transfected into HEK293 cells using Lipofectamine® 2000 Reagent (Life Technologies), and stably transfected cells were selected by 700 μg/ml G418 (SERVA Electrophoresis GmbH) treatment for 3 weeks. time of submission, please state that they will be provided during review. They must be provided prior to publication.

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2.2 Evaluation of Dsup transcript presence by endpoint and real time reverse transcriptase-PCR

Total RNA was extracted from cell pellets using the SV Total RNA Isolation System (Promega) following manufacturer's instructions, and reverse-transcribed using M-MuLV-RH First Strand cDNA Synthesis Kit (Experteam). Dsup expression was evaluated by endpoint PCR using the following primers: forward 5'-TCCACAGAACCCTCTTCCAC-3' and reverse 5'- GACGATGCCACATCCTTCAC-3' (T annealing: 55°C, 35 cycles, amplicon length: 560 pb). PCR products were visualized in a 2% agarose gel with ethidium bromide.

After 2, 5, 7 and 9 days after stabilized Dsup culture, expression was quantified with real time RT-PCR using GAPDH as reference gene and following conditions reported by Hashimoto et al. [1].

2.3 Cell viability

MTT metabolic assay (Vybrant® MTT Cell Proliferation Assay Kit, Molecular Probes) was used to quantify cell viability. To evaluate Dsup-induced resistance against free radicals, both Dsup+ and untransfected HEK293 were seeded at 100,000 cells/ml density in a 96-well plate. After 24 hr of incubation, cells were treated with 250, 500 and 1000 μM hydrogen peroxide (H2O2) for 4 hr or O/N in complete medium (10% FBS, +/- G418). To evaluate Dsup-induced resistance against radiation, both Dsup+ and untransfected cells were plated at 100,000 cells/ml density in a 96-well plate. Before treatment, complete medium was removed and replaced with 100 μl PBS. Cells were exposed for 5'' or 15'' to UV-C (source 8W lamp, 4 mJ/cm2). After treatment, cells were incubated in complete medium for 24 or 48 hr before subsequent evaluations and then incubated at 37°C for 4 hr with the tetrazolium dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (yellow) which, in healthy cells, is converted by mitochondrial enzymes into an insoluble formazan (purple). Solubilization was carried out with DMSO (50 μl) at 37°C for 10 min. Then, the amount of viable cells was determined by measuring absorbance at 540 nm in a microplate reader (Tecan).

2.4 Cyclobutane pyrimidine dimers (CPDs) evaluation

To measure DNA damage in terms of CPD formation, 100,000 Dsup+ and untransfected HEK293 cells/ml were plated in 6 cm diameter dishes, and exposed for 5'' or 15'' to UV-C (source 8W lamp, 4 mJ/cm2). DNA was extracted using QIAamp DNA micro kit (Qiagen) immediately after exposure (time 0) and after a recovery of 24 or 48 hr. DNA was treated with T4 Endonuclease V enzyme (10 IU/μl) O/N at 37°C and DNA fragments were run in a 1% agarose gel containing ethidium bromide at 90V for 70 min.

2.5 Transcription factor evaluation

Evaluation of transcription factor activity was carried out by ELISA. Both Dsup+ and untransfected HEK293 were seeded at 100,000 cells/ml density in 6 cm diameter dishes. Cells were treated O/N with 250 and 1000 μM H2O2 or exposed to UV-C (source 8W lamp, 4 mJ/cm2) for 5'' (recovery time 24 and 48 hr). To obtain nuclear extracts, pellets were resuspended in 1 ml of cold buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) added with 100 μl protease inhibitor cocktail (Sigma) and 500 mM PMSF, and incubated in ice for 15 min. Then, lysates were centrifuged for 2 min at 17,000 rcf (4°C). Supernatants, containing cytoplasmatic proteins, were stored at -80°C. Pellets were incubated in 50 μl cold complete buffer (provided by the kit) for 30 min at 4°C. Then, samples were centrifuged for 5 min at 17,000 rcf (4°C). Supernatants, containing nuclear extracts, were stored at -80°C. Before assay, protein concentration was evaluated by Bradford (Sigma) and working aliquots of 1 mg/ml were prepared for each

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sample. Final tested concentration was 20 μg/ml. MAPK pathway (ATF2, p-c-Jun, c-Myc, MEF2, STAT1α) (Abcam), AP-1 family (c-Fos, FosB, Fra-1, p-c-Jun, JunB, JunD) and CREB/pCREB (Active Motif) were evaluated following manufacturer's instructions.

2.6 Gene Expression Analysis

RT-qPCR was performed using Rotor-Gene Q (Qiagen) to analyze the expression of endogenous genes in Dsup+ and untransfected cells (100.000 cells/ml) after treatment, with 250 and 1000 μM of H2O2 for 4 hr and O/N, and exposure to UV-C for 5'' or 15'' (recovery time 0, 24 and 48 hours). RNA was extracted using SV Total RNA Isolation System (Promega) and reverse-transcribed using M-MuLV-RH First Strand cDNA Synthesis Kit (Experteam). FastStart Essential DNA Green Master Mix (Roche) was added to each tube together with 300 nM specific primers (available upon request). Annealing temperature was 60°C. A melt curve was added at the end of each amplification to exclude the presence of non-specific products. Samples were normalized to GAPDH and ribosomal RNA 18s and quantification was determined by using the 2-ΔCT method. Each sample was run in triplicate.

2.7 Statistical analysis

GraphPad Prism software version 5 was used for statistical analyses. For qPCR analysis, at least three separate replicates for each assay were performed. Sample differences were assessed by one-way ANOVA test. Survival data and transcription factor activation data were analyzed using paired t-test. For all comparisons, a p value of <0.05 was considered significant.

3. Results

3.1 Cell survival under stress conditions

To examine the effect of Dsup on cell death induced by Reactive Oxygen Species (ROS), we established a HEK293 cell line stably expressing Dsup protein (Dsup+) (Supplementary figure 1A and B) and exposed it, together with untransfected HEK293, to 250, 500 or 1000 μM H2O2 for 4 hours or O/N. In in vivo and in vitro models, these H2O2 dosages have been shown to induce apoptosis. The percentage of cell survival (evaluated by MTT) was greater in Dsup+ cells for all treatments and at each time point (Fig. 1A and 1B). At all concentrations, cell survival was lower than the basal condition (represented as 100% of survival, shown as a dotted line in figures).

Similarly, we exposed Dsup+ and untransfected cells to 5'' of ultraviolet light, and observed a reduction in cell death and an increase in cell survival and growth (even above basal condition) in transfected cells (Fig. 1C) after 24 or 48 hours of recovery. After 15'' of exposure, we obtained similar results with a comparable survival rate (Fig. 1D). We chose an UV-C source, as UV-C is the shortest wavelength of the three forms of UV. The shorter the wavelength, the more harmful the UV radiation. The main target of UV-C irradiation in living cells is nuclear DNA. The formation of DNA lesions such as pyrimidine dimers inhibits DNA replication and causes chromosomal breakage and cell death. To evaluate whether cell protection mediated by Dsup was linked to a reduction in cyclobutane pyrimidine dimers (CPDs) formation, we exposed Dsup+ and untransfected HEK293 to 5'' or 15'' of UV-C and collected cell pellets for DNA extraction immediately or after 24 or 48 hours of recovery. DNA was treated with T4 endonuclease V enzyme, which cleaves DNA at sites of CPD damage. As shown in Figure 1E, at all time points Dsup+ cells did not display CPD formation after 5''. Similar data were obtained after 15'' (not shown).

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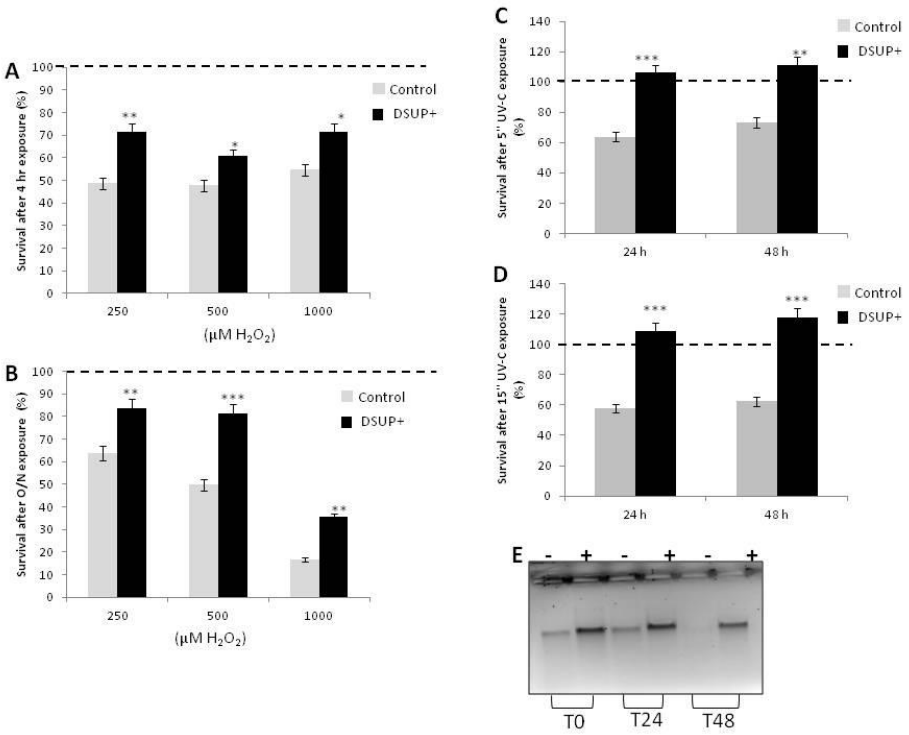

Figure 1. Cell survival after 4 hr (A) or O/N (B) treatment with increasing (250, 500, and 1000 μM) concentration of H2O2. Survival after 5'' (C) or 15'' (D) of UV-C exposure and 24 or 48 hr of recovery. In all figures, 100% represents the basal condition (dotted line), gray bars are untransfected cells (control), and black bars are Dsup+ cells. *p<0.5; **p<0.01; ***p<0.001 by paired t-test. E) Representative gel out of three of cyclobutane pyrimidine dimers (CPDs) formation in Dsup+ and untransfected cells (control) exposed to 5'' of UV-C and treated with T4 endonuclease V enzyme.

3.2 Transcription Factor Activation in response to stress condition

To evaluate whether Dsup expression was able to modulate transcription factor (TF) activation, MAPK pathway (ATF2, p-c-Jun, c-Myc, MEF2, STAT1α), AP-1 family (c-Fos, FosB, Fra-1, p-c-Jun, JunB and JunD), and CREB/pCREB expression were evaluated with ELISA. As shown in Figure 2 panel A, in untrasfected cells treatment with 250 or 1000 μM H2O2 resulted in a up-regulation of pCREB, c-Fos, FosB, Fra-1, and JunD above basal condition (dotted line in figure). In Dsup+ cells, on the other hand, these TFs were down-regulated in comparison to basal condition and were significantly inhibited compared to untransfected HEK293. Only c-Myc was down-regulated relative to control (dotted line) in both cell lines, but in this case we observed a significant up-regulation in Dsup+ cells compared to untransfected cells (Figure 2A). No variation was detected for JunB, p-c-Jun, and STAT1α (not shown) in both cell lines.

Considering that outcomes on survival for 5'' or 15'' were comparable, we selected 5'' UV-C as time of exposure to be shown for transcription factor activation analysis. After irradiation, cells were allowed to recover for 24 or 48 hr (Figure 2B), Untransfected HEK293 showed a markedly activation of Fra-1, JunD, and p-c-Jun, while no differences were observed for FosB and STAT1. In Dsup+ cells, UV-C induced down regulation of FosB, Fra-1, JunD, and in all cases a significant difference between untransfected cells and Dsup+ cells was observed. For p-c-Jun and STAT1 an up-regulation was evident in Dsup+ cells, and the p-c-Jun up-regulation was even significantly greater than that observed in untransfected HEK293. Only c-Myc was down-regulated relative to control (dotted line) in both cell lines, and, again, we found a significant up-regulation in Dsup+ cells compared to untransfected cells (Figure 2B).

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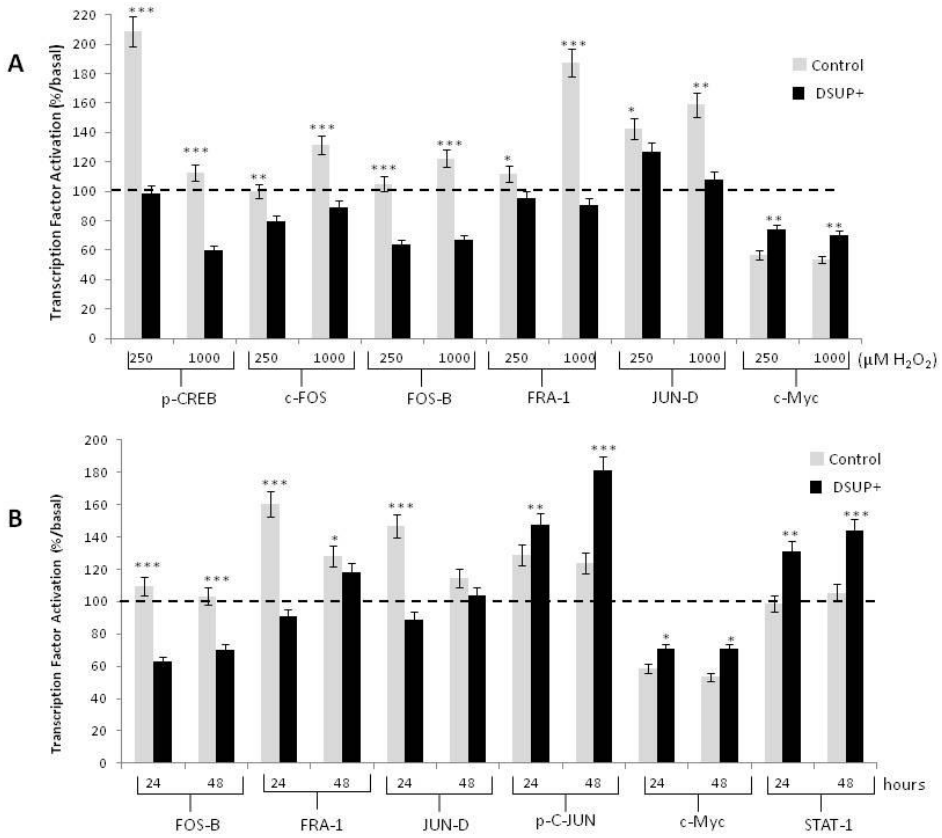

Figure 2. Transcription factor activation in Dsup+ and untransfected HEK293 (control) after 250 or 1000 μM H2O2 O/N (A) or 5'' UV-C (B). Results are reported as percentage over basal condition (100% and dotted lines in figure). MAPK pathway (ATF2, p-c-Jun, c-Myc, MEF2, STAT1α), AP-1 family (c-Fos, FosB, FRA-1, p-c-Jun, JunB, and JunD) and CREB/p-CREB expression were evaluated, but in figures only differentially expressed TF are reported. *p<0.5; **p<0.01; ***p<0.001 by paired t-test.

3.3 Gene expression in response to stress conditions

In order to explore whether Dsup affected the expression of endogenous genes under stress conditions (low to high dose H2O2 and UV-C exposure), RT-qPCR was performed to analyze genes involved in DNA damage response and repair, as well as in cell survival and protection from oxidative stress. As summarized in Tables 1 and 2 and in Supplementary Figures 2 and 3, Dsup-expressing cells showed different transcription patterns for some of the analyzed genes. Specifically, Dsup+ cells treated with H2O2 (Table 1, Supplementary Figure 2) showed an increase in Bcl2 and, at some conditions, CASP8 genes with a reactivation of telomerase. In parallel, some of the genes involved in DNA repair and cell cycle checkpoint (PARP1, BRCA1-2, RAD50, RAD17, and ATM) were up-regulated, while others (ERCC6, XRCC6, and RAD1) were moderately down-regulated. DDB1 was up-regulated after 4h of exposure but down-regulated at a longer time (O/N). SOD1 was markedly up-regulated in response to H2O2.

Table 1. Changes in transcription levels of selected endogenous genes in Dsup+ cells compared to untransfected cells exposed to H2O2 (μM).

Target	Gene ontology	4h		O/N	
		250	1000	250	1000

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hTERT	Telomerase	↑	~	↑	↑
Bcl2	BCL2 Apoptosis Regulator	↑	↑	~	~
CASP3	Caspase 3	~	~	~	~
CASP8	Caspase 8	↑	~	~	~
DFFB	DNA Fragmentation Factor Subunit Beta	~	~	↑	↑
PARP1	Poly [ADP-ribose] polymerase 1	↑	↑	↑	↑
PARP2	Poly [ADP-ribose] polymerase 2	~	~	~	~
BRCA1	BRCA1 DNA Repair Associated	~	~	↑	↑
BRCA2	BRCA2 DNA Repair Associated	~	~	↑	~
RAD50	RAD50 Double Strand Break Repair Protein	~	~	↑	↑
ERCC1	ERCC Excision Repair 1, Endonuclease Non-Catalytic Subunit	~	~	~	~
ERCC6	ERCC Excision Repair 6, Chromatin Remodeling Factor	~	↓	~	~
XRCC6	X-Ray Repair Cross Complementing 6	~	↓	↓	~
DDB1	Damage Specific DNA Binding Protein 1	↑	↑	↓	↓
ATR	ATR Serine/Threonine Kinase	~	~	~	~
RAD1	RAD1 Checkpoint DNA Exonuclease	↓	~	↓	↓
RAD17	RAD17 Checkpoint Clamp Loader Component	↑	~	↑	↑
ATM	ATM Serine/Threonine Kinase	~	~	~	↑
SOD1	Superoxide Dismutase 1	↑	↑	↑	↑
SOD2	Superoxide Dismutase 2	~	↓	~	~
CAT	Catalase	~	~	~	~

In contrast, in Dsup+ cells exposed to UV-C (Table 2, Supplementary Figure 3) we observed a decreased expression of apoptotic genes, and an increase of some of the genes involved with DNA repair (XRCC6, ERCC6, ATR, and BRCA1), while others (BRCA2

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and ERCC1) were down-regulated. A general hypoexpression was also observed for cell cycle checkpoint genes (RAD1, RAD17, and ATM).

Table 2. Changes in transcription levels of selected endogenous genes in Dsup+ cells compared to untransfected cells exposed to UVC

Target	Gene ontology	T0		T24		T48	
		5''	15''	5''	15''	5''	15''
hTERT	Telomerase	~	~	~	~	~	~
Bcl2	BCL2 Apoptosis Regulator	↓	↓	↓	↓	↓	↓
CASP3	Caspase 3	~	↓	~	~	~	~
CASP8	Caspase 8	~	↓	~	~	~	~
DFFB	DNA Fragmentation Factor Subunit Beta	~	↓	~	~	~	↓
PARP1	Poly [ADP-ribose] polymerase 1	~	↓	~	~	↓	↓
PARP2	Poly [ADP-ribose] polymerase 2	~	~	↑	~	↓	~
BRCA1	BRCA1 DNA Repair Associated	↑	~	↑	~	~	~
BRCA2	BRCA2 DNA Repair Associated	~	↓	~	↓	↓	↓
RAD50	RAD50 Double Strand Break Repair Protein	~	~	~	~	~	↓
ERCC1	ERCC Excision Repair 1, Endonuclease Non-Catalytic Subunit	~	↓	~	↓	~	↓
ERCC6	ERCC Excision Repair 6, Chromatin Remodeling Factor	~	~	↑	↑	~	~
XRCC6	X-Ray Repair Cross Complementing 6	↑	~	↑	~	↑	~
DDB1	Damage Specific DNA Binding Protein 1	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**n.a.**	**n.a.**
ATR	ATR Serine/Threonine Kinase	↑	↑	↑	↑	↑	↑
RAD1	RAD1 Checkpoint DNA Exonuclease	~	↓	~	↓	~	↓
RAD17	RAD17 Checkpoint Clamp Loader Component	~	↓	~	↓	~	↓
ATM	ATM Serine/Threonine Kinase	~	↓	~	↓	~	~

Results from expression studies showed that H2O2 and UV-C exposure involved different pathways to respond to cell damage. This phenomenon is depicted in Figure 3

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in which String v.11.0 was used to evaluate protein interaction network of differentially expressed genes after H2O2 (3A) and UV-C (3B) treatments.

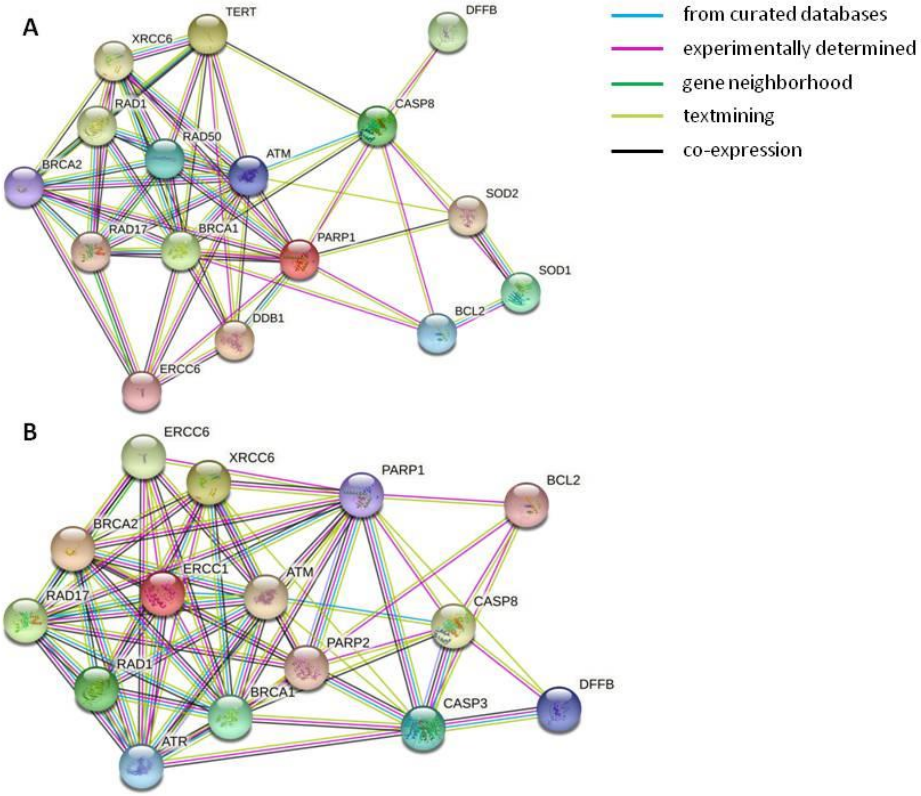

Figure 3. The STRING protein-protein interaction network for differentially expressed genes after H2O2 treatment (A) and UV-C exposure (B). Colored lines between the proteins indicate the various types of interaction evidence: a green line indicates neighborhood evidence; a purple line indicates experimental evidence; a light green line indicates text-mining evidence; a light blue line indicates database evidence; a black line indicates coexpression evidence.

4. Discussion

In this study we investigated the cellular mechanisms responsible for the protection against external insults in mammalian cells expressing the Dsup protein. To this purpose, transcription factor modulation and expression of gene pathways associated with damage response and repair were analyzed in Dsup+ and untransfected HEK293 cells exposed to oxidative stress and UV-C irradiation. In Dsup+ cells, we observed an increase of cellular survival in both oxidative stress and UV-C irradiation conditions, with a significant reduction of CDP formation in the case of UV-C exposure. However, the cellular mechanisms involved in the response to these insults appeared to differ significantly. In the case of oxidative stress, untransfected cells responded to damage-activating pCREB and AP-1 family transcription factors (c-Fos, FosB, Fra-1, JunD). In the Dsup+ cells, the upregulation of these factors was not observed. c-Myc was down-regulated relative to control in both cell lines, but Dsup+ cells showed a significant upregulation in comparison to untransfected HEK293. It is known that H2O2 increases the transcription of AP-1 that, when upregulated, spontaneously concentrates in the nucleus to activate gene expression [18]. Similarly to AP-1, CREB activation is also induced by ROS and is critical for cell survival [19], promoting DNA repair after treatment with hydrogen peroxide. The activation of CREB in response of oxidative stress is dependent upon ATM kinase, which can either switch on or off CREB transcriptional activity, depending on the extent of

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DNA damage [20]. It has been shown that CREB signaling protects human neuronal cells from oxidative DNA damage not only by inducing anti-apoptotic genes and cell cycle arrest, but also increasing the expression of genes responsible for DNA repair. In Dsup+ cells, CREB is downregulated, suggesting that all these mechanisms are likely not activated. In addition, CREB is able to negatively regulate c-Myc and S phase induction [21]. This could explain the upregulation of c-Myc in Dsup+ cells, where CREB is downregulated. The induction of c-Myc may promote the G1/S phase transition and the cell cycle progression. On the other hand, c-Myc may play a role in determining cellular redox balance. It has been reported that c-Myc transcriptionally regulates g-glutamyl-cysteine synthetase (g-GCS), the rate-limiting enzyme catalyzing the biosynthesis of glutathione, the most abundant antioxidant and a major detoxification agent in cells [22].

At the gene expression level, in Dsup+ cells Bcl2 expression increased only after 4 hours of treatment with H2O2, whereas the other caspases did not display any increase, consistently with data from survival. Conversely, Dsup+ cells showed a constant increase over time of PARP1, hTERT, and SOD1 expression. It has been reported that PARP1 recruitment is one of the earliest events in DNA damage response following several types of insults, such as oxidative and metabolic stresses [23]. PARP1 recognizes DNA breaks, and is involved in the early recruitment of factors that facilitate DSB repair [24], including BRCA1 [25], whose expression in our study was upregulated in Dsup+ cells after O/N treatment with H2O2. On the other hand, BRCA2, which acts downstream of BRCA1 and is primarily involved in homologous recombination typically associated with DSB [15], was only weakly upregulated in Dsup+ cells.

hTERT is the catalytic subunit of the telomerase holoenzyme that, in addition to its role in the maintenance of telomeres, exhibits antioxidant activity. hTERT overexpression can decrease the basal cellular ROS levels, but also inhibit endogenous ROS production [26]. Interestingly, it has been shown that c-Myc is able to stimulate hTERT expression, binding a c-Myc E-box within the hTERT proximal promoter [27]. Also in this case, among the mechanisms by which hTERT seems to regulate the intracellular redox status, is the capability of modulating glutathione levels in the cells and conferring survival advantages. SOD1 is a well-characterized, ubiquitously-expressed and highly-conserved enzyme, considered a key regulator of antioxidant response. Overexpression and de novo synthesis of SOD1 mRNA has been reported in H2O2-treated cells [28]. SOD1 catalyzes the dismutation of superoxide radical into oxygen and hydrogen peroxide, which are further eliminated by other enzymes such as catalase [29]. Unexpectedly, catalase expression did not increase in Dsup+ cells after H2O2 treatment. However, it must be considered that in our study we evaluated differences in gene expression, but did not quantify protein expression or activity. Even though the molecular mechanisms regulating the expression of catalase have yet not been completely elucidated, other factors, such as catalase subcellular localization and post-transcriptional regulation, may play a role in the protection of cells from oxidative stress [30]. Of note, the expression of ATM and ATR kinases, two key factors in DNA damage response caused by oxidative stress, did not display significant variations in Dsup+ cells. This suggests that their activity is not required, and Dsup+ cells can survive and proliferate without switching on the mechanisms of DNA repair. Taken together, all these data indicate that H2O2 treatment in Dsup+ cells only marginally involves the pathways responsible for DNA repair, and suggest that the amount of DNA damage may be limited. This is in agreement with what was reported by Chavez and colleagues [8], who showed that Dsup is a nucleosome-binding protein able to preserve chromosomal DNA from hydroxyl radical-mediated cleavage. Thus, it can be hypothesized that, while the DNA is “physically” protected from damages by Dsup, detoxification mechanisms aimed at removing ROS and limiting oxidative stress are activated (SOD1, hTERT, probably g-GCS), allowing cells to survive and grow. On the other hand, after the UV-C exposure, the transcription factors (FosB, Fra-1, JunD, and p-c-JUN were activated in untransfected cells, while Dsup+ cells exhibited and increase of p-c-Jun

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(more evident than in untansfect HEK293) and STAT1. Also in this case, c-Myc was downregulated relative to control in both cell lines, but it was significantly upregulated in Dsup+ compared to untransfected HEK293. In mammalian cells, STAT1 participates in type I interferon (IFN) pathways and activates type I IFN-response genes [31]. It has been shown that type I IFNs are key factors in normal skin growth and maintenance, and are usually downregulated in the majority of skin tumors [32]. UV exposure interferes with STAT1 activation, hindering IFN from exerting its protective effects [31,33]. It can therefore be hypothesized that STAT1 plays an important role in the response to UV damage. c-Myc acts in a similar way to p-c-Jun, a well-characterized regulator of the mammalian UV response, essential for the UV-irradiated cells to avoid growth stall and restart cell cycle [34]. On the other hand, both p-c-Jun and c-Myc can increase p53-dependent apoptosis, exerting a direct regulation on cell destiny after UV exposure, switching the response from cell cycle arrest recovery to apoptosis or vice-versa [35]. At gene expression level, we observed a marked reduction of Bcl2 in Dsup+ cells, in agreement with the experimental observation of an increase of cell survival and proliferation, even above the basal line. We also found a relevant up-regulation of ATR kinase expression, which is considered the main player in UV-induced DNA damage. ATR activation, occurring during the S phase of the cell cycle, allows repairing stalled replication fork and avoiding arrest of replication or premature entry into mitosis [14]. In addition, Dsup+ cells exhibited increased expression in other genes related to ATR rescue pathway: BRCA1, which is recruited during the S/G2 phase and contributes to the UV irradiation response operating in gap repair at photoproduct-stalled replication forks level [36]; XRCC6, which participates in the UV-G2 checkpoint [37]; ERCC6, whose gene represents a potential target for inactivation by UV light and seems to act as a “dosimeter” of DNA damage (though DNA damage exceeds a certain threshold, ERCC6 transcript is depleted and cell death is promoted) [38]. In Dsup+ cells, ATM expression was similar or lower than that in untransfected cells. ATM activation occurs mainly under oxidative stress and double-stranded DNA breaks, while it is less involved in response to UV exposure [14]. In the same way, we observed a decrease in expression of BRCA2, usually involved in response to DSBs [15]. Other factors related to UV-induced CPDs repair, such as RAD1 and RAD17, were down-regulated in Dsup+ cells. This may appear to be quite surprising; however, it is not unusual that the expression of gene responsible for UV damage response apparently remains unchanged or, rather, slightly decreases after UV exposure [39]. Also in this case, a post-transcriptional regulation of these factors cannot be excluded.

All these data seem to suggest that, in contrast to what was observed for H2O2 treatment, DNA is not "physically" protected by Dsup after UV-C irradiation, but rather Dsup activates more efficiently mechanisms of damage repair (STAT1, c-Myc, p-c-Jun; ATR, BCRA1). In this way, cells may remove CPDs and recover faster, mitigating the deleterious effects on cell survival (Figure 4).

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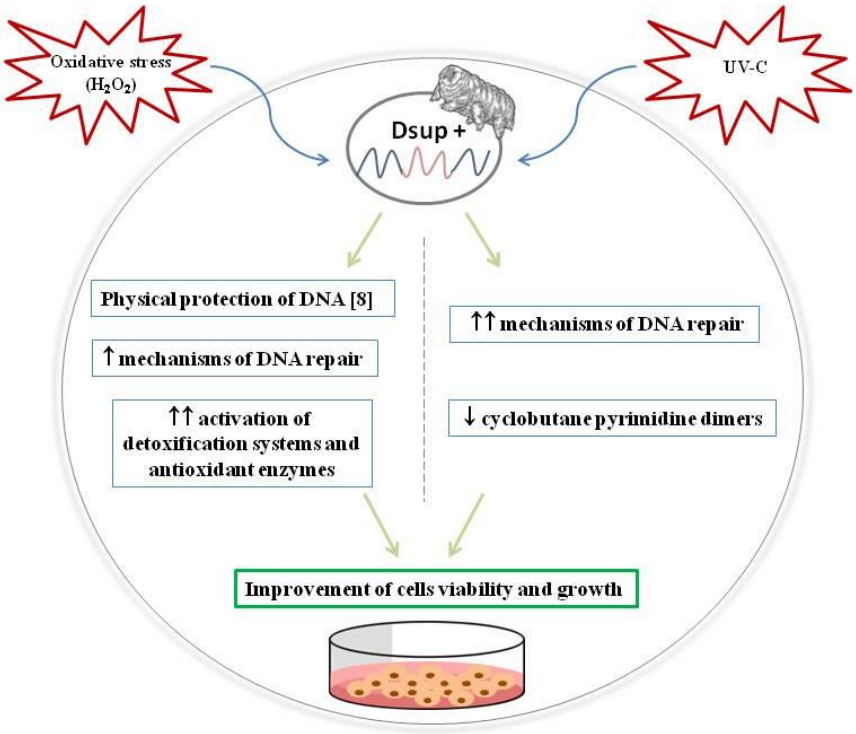

Figure 4. Schematic model of protection mechanisms in mammalian cells expressing Dsup protein (Dsup+) exposed to oxidative stress and UV-C irradiation. After H2O2 treatment, Dsup+ cells mainly activate detoxification system and antioxidant enzymes limiting oxidative stress, while DNA repair mechanisms are moderately turned on, probably due to DNA "physical" protection mediated by Dsup [8]. On the other hand, after UV-C exposure, Dsup+ cells respond activating more efficiently DNA repair genes reducing cyclobutane pyrimidine dimers formation. All these protective mechanisms give Dsup+ cells a greater resistance to external stress, improving their viability and growth respect to untransfected cells. ↑: moderate; ↑↑: high; ↓: low/reduced

It is worth noting that our results are in perfect agreement with those obtained in tobacco plants expressing the Dsup gene. Transfected plants displayed fewer DSBs and generated less response from DSB signaling pathways than the plants without Dsup when exposed to genotoxic stress, while, when UV-C irradiated, exhibited an increased ATR expression and an enhance of DNA damage sensing and DNA repair pathways involving ATR [10].

We are aware that our study has some limitations. In particular, for the gene expression analysis, genes included in the study, although representative of key pathways, are only some of those potentially involved in DNA damage response. Furthermore, many of the corresponding proteins are regulated by phosphorylation/dephosphorylation, so a substantial uniformity in gene expression does not necessarily reflect uniformity in their activity. Nevertheless, our data may help to delineate the different ways in which the Dsup protein operates in response to different insults. Further studies are needed to better characterize how Dsup works in the cell, and we believe that our study may lay the groundwork for a deeper understanding of its activity.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1, Figure S2 and Figure S3

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