Study of the radioprotective properties of the Damage Suppressor (Dsup) protein on a model organism *D. melanogaster* and human cell culture HEK293T

THEME 1132

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Study of the radioprotective properties of the Damage Suppressor (Dsup) protein on a model organism D. melanogaster and human cell culture HEK293T

Theme 1132

E.V. Kravchenko

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Contents

Annotation

The aims of this project are examination of radioprotective properties of a new Damage suppressor (Dsup) protein on a model object *D. melanogaster* and human cell cultures and studying mechanisms of action of this protein. Dsup protein is a new protein discovered in 2016 in the extremophilic organism *Ramazzottius varieornatus* - one of the most radioresistant species of multicellular organisms. The creation of *D. melanogaster* lines and human cell cultures expressing this protein will make it possible to assess the possibility of increasing their radioresistance during irradiation with different types of ionizing radiation and a wide range of methods applicable to these model organisms will allow to begin studying the effects of Dsup on the molecular level and the level of whole organism. To solve these problems, a wide range of molecular biological methods will be used: in particular, the creation of lines of organisms expressing the Dsup protein and GFP-Dsup fusion protein, transcriptome analysis, sequencing, immunostaining of polytene chromosomes, synthesis, isolation and purification of significant quantities of Dsup protein in bacterial cells etc. For a number of tasks which are necessary for implementation of this project, authors already have significant achievements, in particular, *D. melanogaster* lines stably expressing Dsup and a human cell culture HEK239T with transient expression of Dsup have already been created. It should be noted that there is no data about multicellular model organisms expressing the Dsup protein, therefore, the tasks solved during the project are new and important not only for molecular biology and radiobiology, but also for biotechnology, space research and some other disciplines requiring increase the level of radioresistance of organisms.

The requested project budget for 2021-2023 years is 160 kUSD.

Introduction

The objectives of this project are obtaining data on the properties of the unique protein Dsup found in extremophiles Tardigrade and study its effect on radioresistance and other parameters of living organisms. Under the conditions of an increase of background radiation level due to various technogenic components, the problem of cosmic radiation, which impedes the prolonged stay of organisms in space, and the general mechanisms underlying the aging of cells and their damage by ionizing radiation, studying of new mechanisms for increasing radioresistance is one of the most important directions of molecular biology and radiobiology. At the moment, there is a very limited number of experimental data describing the mechanisms of Dsup action (Chavez et al., 2019; Hashimoto et al., 2016), performed either *in vitro* (outside the living organism) or on cell culture, which does not reflect the whole variety of reactions that can be caused by Dsup protein in a multicellular organism with different types of tissues, organ systems and complex interactions between them at different levels of organization (in particular, impact on life expectancy, behavior, specialized metabolic processes, etc.). Therefore, the study of the effect of the Dsup on the model object *D.melanogaster*, which became the basis for studying the processes occurring at different levels of organization of living systems, will allow describing the important properties of this protein and the mechanisms of its interaction with life processes of *D.melanogaster*. Research methods in this project include both modern methods of transcriptome analysis, the creation of hybrid proteins and fluorescence microscopy, and methods requiring a good classical molecular biological base (sequencing, molecular cloning methods, immunostaining, etc.).

During the implementation of the project, it is planned for the first time to evaluate the effect of Dsup protein under normal conditions and after exposure to various types of ionizing radiation on the functioning of a multicellular organism (*D. melanogaster*) at the level of longevity and radioresistance, at the level of transcriptomes, and at the chromosomal level. For a human cell culture, it is planned for the first time to obtain data on the effect of Dsup protein on cells at the transcriptome level and to evaluate changes in the radioresistance of a cell culture expressing Dsup after exposure to protons and heavy ions. The obtained data is expected to make an important contribution to the description of new mechanisms of radioresistance at different levels of organization of living systems and further evaluate the possibility of their use in various fields of biology and medicine.

State of research on problems studied in the project

Extremophilic organisms that have adapted to extreme environmental conditions (high/low temperature, high/low pressure, pH<3, pH>9, high levels of ionizing radiation, salinity, etc.) have been a subject of interest of biologists for many years. The vast majority of extremophilic organisms belong to unicellular organisms, for example, bacteria and archaea. However, among animals there are also species that can survive in extreme environmental conditions - in particular, tardigrades.

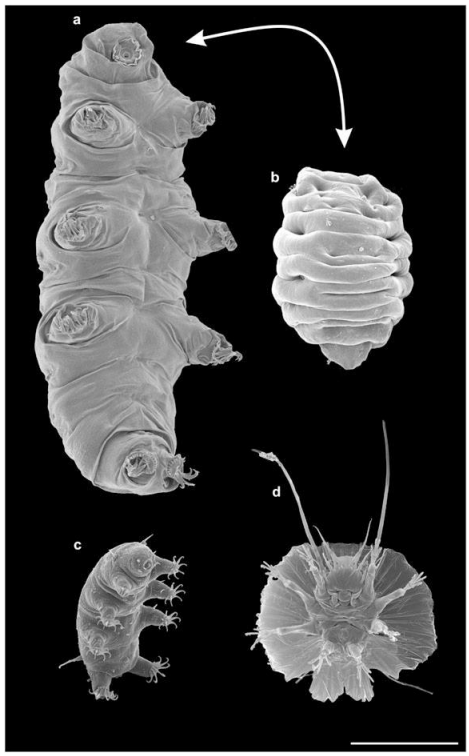

Fig. 1. Scanning electron microscopy of some tardigrade species. a,b *Richtersius coronifer* in hydrated and anhydrobiotic states. c - *Isoechiniscoides sifae*. d – *Actinarctus cf. doryphorus* (ventral view). Scale bar = 100 μm. From book “Water Bears: The Biology of Tardigrades”. Editor: Ralph O. Schill. Springer 2018.

Resistance of the tardigrades to ionizing radiation is of particular interest. Several recent studies on different types of organisms have shown that Tardigrades are among the most radiation-resistant animals on Earth, able to survive after exposure to both rare and dense ionizing radiation at doses of about 5 kGy (Charlotta Nilsson et al., 2010; Horikawa et al., 2012, 2008, 2006; Jönsson and Wojcik, 2017). The molecular mechanisms of such radioresistance are still largely unclear.

The extreme radioresistance of tardigrades made them a model organism for studying the influence of space conditions on living organisms and in low Earth orbit during the FOTONM3 mission, several experiments were conducted with tardigrades (TARDIS (Jönsson et al., 2008), RoTaRad (Persson et al., 2011), TARSE (Rebecchi et al., 2011, 2009)). During the TARDIS (Tardigrades in Space) project, the tardigrades were exposed to space vacuum (10-6 Pa), cosmic radiation (100 mGy) and UV radiation for 10 days. The greatest negative effect on the survival rate of tardigrades compared with the control group was caused by UV irradiation of the full spectrum, while the effects of vacuum and cosmic radiation did not significantly affect survival (Jönsson et al., 2016, 2008).

Despite a significant number of studies on the unique radioresistance of tardigrades, almost all of these data describe various survival parameters without going to the cellular and molecular level, therefore, the mechanisms of radioresistance of tardigrades to ionizing radiation are almost not studied and are of considerable interest.

Molecular mechanisms of tardigrade radioresistance

In the pre-omics techniques period, two experiments were conducted to search for tardigrade genes, which expression changes in response to exposure to ionizing radiation. Beltrán-Pardo et al. (Beltrán-Pardo et al., 2013) showed a significant increase in the amount of Rad51 protein after γ-irradiation (70 Gy) of hydrated *M. cf. tardigradum*,

which indicates the activation of DNA repair systems associated with homologous recombination. Jönsson and Schill (Jönsson and Schill, 2007) demonstrated increased expression of the Hsp70 gene after γ-irradiation (500 Gy) *R. cf. coronifer*. Hsp70 are chaperone proteins that take part in a large number of processes associated with responding to different types of stress and maintaining genomic stability. Homologous variants of these proteins are present in all living organisms.

A breakthrough in research occurred in 2016 when the genome of *Ramazzottius varieornatus* - one of the most radioresistant species of tardigrades was sequenced (Hashimoto et al., 2016). After data analyzing and comparing the proteins of *R. varieornatus* with all the known proteins of other organisms, a unique protein was discovered - Damage suppressor (Dsup), which is present only in tardigrades. On a HEK239 cell culture it was demonstrated that after transfection with a vector containing GFP-Dsup, the fluorescent signal was localized in the nucleus, which indicates the possible participation of Dsup in the protective mechanisms from radiation exposure to DNA. The irradiation of cells transfected with Dsup with γ-quants at the dose of 4 Gy showed the increase in their survival and the decrease in radiation damage at the DNA level (γ-H2AX foci detection, COMET assay) compared with the irradiated untransfected control (Hashimoto et al., 2016). Since irradiation before COMET was carried out on ice and DNA fragmentation was analyzed immediately after irradiation, the repair processes did not significantly contribute to the decrease in the number of DNA breaks, which speaks in favor of the Dsup radioprotective functions, rather than enhancing the repair processes.

The Dsup protein consists of 445 amino acid residues forming the N-terminal and C-terminal regions between which the putative α-helix is located. *In vitro* experiments have shown that Dsup interacts with DNA molecules regardless of their nucleotide sequence (Hashimoto et al., 2016). The cell line expressing Dsup with a deleted C-terminal region did not exhibit increased resistance to X-rays, and the line expressing only the C-terminal region showed nuclear localization of this part of the protein, but with the formation of an abnormal nuclear structure. The binding of a single C-terminal region of the Dsup protein to DNA is likely to affect replication and / or transcription. A similar effect is observed for other proteins that can bind to DNA: for example, the overproduction of histone-like proteins in bacteria causes DNA condensation and loss of cell viability (Spurio et al., 1992). The N-terminal Dsup region and the putative α-helix possibly neutralize these effects in the full-sized Dsup protein (Hashimoto and Kunieda, 2017).

Later studies in 2019 (Chavez et al., 2019) showed that Dsup binds with nucleosomes rather than with protein-free DNA and reduces the level of DNA damage by hydroxyl radicals. Unexpectedly, the Dsup conserved region required for binding to nucleosomes is very similar to the domain that binds to nucleosomes in vertebrate HMGN proteins (RRSARLSA consensus) (Chavez et al., 2019; González-Romero et al., 2015). Orthologous protein was found in another sp. of tardigrades.

Dsup proteins are charged and enriched in serine, alanine, glycine and lysine residues (more than 50%), which can form a disordered secondary structure (Dunker et al., 2001).

It is assumed that direct binding of Dsup to nucleosomes and the formation of a diffuse mass of protein around chromosomal DNA provides protection against hydroxyl radicals.

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Purpose and objectives of the project

The main goals of the project are:

- study mechanisms of Dsup protein action
- assessment of the prospects for using Dsup to increase the radioresistance of multicellular complex organisms

To achieve these goals, it is necessary to solve a number of problems:

1. Optimization and synthesis of DNA sequence encoding a Dsup protein

Dsup protein experiments require a source of this protein. Since further experiments are mainly carried out on the model genetic object *D.melanogaster*, the DNA sequence encoding the Dsup protein (LC050827.1) was optimized taking into account the frequency of occurrence of various codons in the *D.melanogaster* genome for a stable high level of synthesis of this protein. Then the optimized sequence of 1338 bp in length was synthesized and tested for correct synthesis using sequencing. This optimized and tested sequence is used in all further experiments.

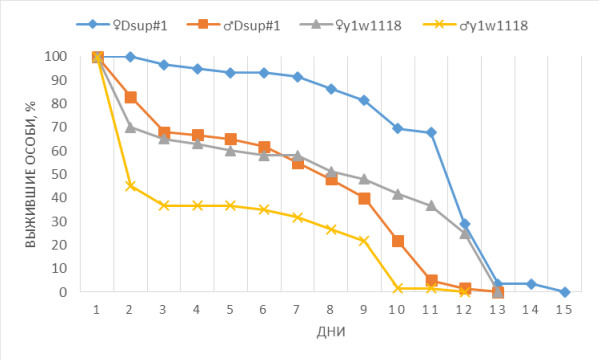
2. Generation of the *D. melanogaster* line stably expressing Dsup

The synthesized sequence encoding the Dsup protein was inserted into the pCaSpeR4 vector (Drosophila Genomic Resources Center, DGRC) under the control of the strong constitutive promoter of the β-actin gene - Act5C. The resulting construction, together with a vector encoding a transposase source (Karess and Rubin, 1984), was introduced via microinjections into *D. melanogaster* embryos at the preblastoderm stage, according to the previously described protocols (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The resulting adults were crossed with the y^1w^{1118} line, and in the next generation, transformants that showed the appearance of eye color (a selective marker in pCaSpeR4) were selected. As a result, 5 independent *D. melanogaster* lines containing this molecular genetic construction at different positions of the genome were obtained. To our surprise, it turned out that for 3 out of 5 lines it was impossible to get a homozygous line (at the moment the lines are in a heterozygous state), and in the 2 homozygous lines that we managed to get, the frequency of elimination of the DNA coding Dsup initially located between the inverted repeats of the P-element in pCaSpeR4 is noticeably increased. It is quite possible that this may be due to the influence of the new Dsup protein that appeared in the genome, which requires further study. Nevertheless, accurate estimates require determination of the exact place of insertion of the construct carrying the Dsup gene into the genome using inverted PCR and sequencing for all the obtained lines.

3. Evaluation of the radioresistance of *D. melanogaster* strains stably expressing Dsup

To assess the effect of the Dsup protein on the radioresistance of *D. melanogaster*, the line Dsup No.1, which is maintained in the homozygous state, was irradiated with γ-quants at the MT-25 accelerator in FLNR JINR at a dose of 1000 Gy, which is close to the values $LD_{50/2}$ (Parashar et al., 2008).

The experiments were conducted for *D. melanogaster* line No.1 expressing Dsup and the initial line y^1w^{1118} (separately for females and males, 60 individuals each). The first obtained data speak in favor of increasing the radioresistance of the Dsup№1 line.



The graphs (Fig. 2) show an increase in survival in the interval up to 10-12 days after irradiation of both males and females of the DsupN°1 line compared to males and females of the control y^1w^{1118} line.

Fig. 2. Survival of females and males of the DsupN°1 and y^1w^{1118} lines after irradiation with γ-rays at a dose of 1000 Gy.

Obviously, obtaining accurate estimates requires a significant increase in the sample, independent repetitions, and demonstration of the observed effect for different lines expressing Dsup. Interestingly, that between females and males of y^1w^{1118} line we observed different radiosensitivity, which in itself is interesting for study and can be explained in the course of the planned transcriptome analysis with study of differentially expressed genes.

4. Generation of a stable cell line HEK293T expressing fusion protein GFP-Dsup and assessment of the radioresistance of this cell line to various types of ionizing radiation

Previously, Hashimoto et al. (Hashimoto et al., 2016) demonstrated nuclear localization of the Dsup-GFP fusion protein, where the N-terminus of GFP was fused to the C-terminus of Dsup. It was shown that the orientation of the target protein and GFP relative to each other can affect protein folding and distort the function of the target protein and its localization. Since the C-terminus of Dsup plays an important role in determining its functions (Hashimoto et al., 2016), we checked the variant when the C-terminus of Dsup is not spatially influenced by GFP and made reverse orientation in the fusion protein: GFP-Dsup, where C-terminus of the GFP was fused to the N-terminus of Dsup. HEK293T cell line was transfected with obtained construct, where it showed a clear nuclear localization (Fig. 3). Thus, we confirmed the results of Hashimoto et al. and had the opportunity to create the HEK293T line stably expressing Dsup, which will allow to evaluate the radioresistance of this cell line to various types of ionizing radiation, in particular, to protons and heavy ions.

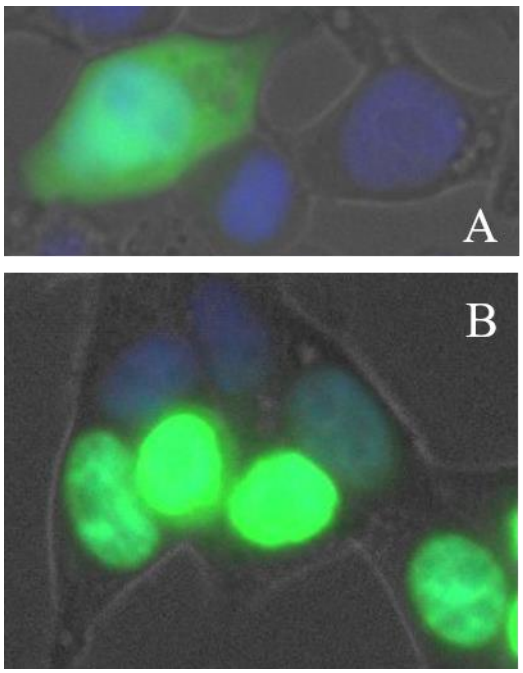


Fig. 3. Photograph of the localization of GFP protein (A) and the GFP-Dsup (B) fusion protein in HEK293T cells. Nuclear DNA stained using DAPI. The complete match of the green fluorescent signal from GFP-Dsup and the blue fluorescent signal from DNA located in the nucleus in Fig. 3B indicates nuclear localization of GFP-Dsup

5. Transcriptome analysis of *D.melanogaster* and the HEK293T cell line expressing Dsup protein under normal conditions and after exposure to ionizing radiation

Now, there is no data on what happens at the level of interaction between genes and, as a consequence, biological processes in the case of the addition of the Dsup protein in cells, which can affect a number of fundamental processes. Apparently, the alleged interactions of Dsup with nucleosomes can affect chromatin compaction and thus affect the level of expression of many genes. An assessment of this effect can be obtained by comparing the transcriptoms of organisms expressing Dsup and control organisms. Also it is interesting to analyze transcriptome differences (response formation) after exposure to ionizing radiation between control and Dsup-expressing organisms. For such evaluations, the microchip hybridization method, which is widely used at the JINR DLNP (Affymetrix), and RNA-seq, which was also carried out at the JINR DLNP, can be used. The study of different radiosensitivity at the level of transcriptomes between males and females of the original $y^{1}w^{1118}$ line, which was identified in the course of already conducted experiments, is of particular interest.

6. Study of GFP-Dsup fusion protein distribution on *D. melanogaster* polytene chromosomes

To understand the mechanisms of the Dsup protein action, it is important to evaluate its distribution on the chromosomes. The optimal method for a quick assessment of this distribution is immunostaining of *D. melanogaster* polytene chromosomes. Immunostaining is planned for *D. melanogaster* lines expressing the GFP-Dsup fusion protein with rabbit polyclonal antibodies to the GFP protein, followed by detection with secondary antibodies labeled with Alexa Fluor 488. In the case of uniform distribution of the Dsup protein on the chromosomes, we can predict a non-specific protective mechanism of action of this protein. In the case of irregular distribution, it is necessary to analyze the regions where Dsup protein binds to identify patterns of binding. It is also important to evaluate the possible change in the degree of compaction of polytene chromosomes in the case of Dsup protein binding to them.

7. Study of the life span of *D.melanogaster* lines expressing Dsup

Because of the alleged ability of the Dsup protein to bind to nucleosomes, it is interesting to evaluate the effect of possible changes in chromatin structure on the functioning of the whole organism, for instance – difference in D.melanogaster life span between natural and Dsup expressing strains. Such studies require a significant number of males and females of the studied lines and take more than 3-4 months per one experiment with a continuous assessment of the number of dead / living individuals.

8. Creation of an expression vector for production of Dsup protein in *E. coli* cells, extraction and purification of Dsup protein for preliminary crystallization experiments

Since the secondary structure of the Dsup protein has not been studied yet, it is interesting to express the Dsup protein in *E. coli* culture, followed by purification and attempted crystallization or analysis using spectrometric methods. Determination of the secondary structure of the protein will allow to make an assumption about mechanisms of its binding to DNA and / or other proteins and to simulate various scenarios of its mechanisms of action.

Human Resources

Among the project participants: 3 candidates of biological sciences with extensive experience in molecular genetics, molecular biology and radiobiology, competent in all the methods listed in the project (JINR and Koltsov Institute of Development Biology RAS), 2 graduate students (Faculty of Bioengineering and Bioinformatics, Moscow State University and Irkutsk State University), staff of DLNP JINR.

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SWOT Analysis

Strength of the project: the authors have already done preliminary experiments; JINR has accelerators that make it possible to obtain declared doses and types of radiation; JINR DLNP has a significant part of the equipment required for the project; some experimental data have already been obtained; in addition to experienced specialists, the team includes graduate students and young scientists

Project weaknesses: high competition in this field of research with research teams from other countries.

6. TIMETABLE

activities on the project Study of the radioprotective properties of the Damage Suppressor (Dsup) protein on a model organism *D. melanogaster* and human cell culture HEK293T

Work stages	Work content
2021	Determination of the localization of constructs containing the Dsup gene in the *D.melanogaster* genome. Conducting irradiation sessions of *D. melanogaster* lines expressing Dsup and initial lines in 3-5 repetitions with γ-rays at the MT-25 accelerator in FLNR JINR at a dose of 1000 Gy with estimation of survival and longevity. Conducting an experiment to evaluate the effect of Dsup protein on the life span of *D.melanogaster* under normal conditions compared to the initial lines. Creation of a molecular genetic construct for expression of the GFP-Dsup fusion protein in *D. melanogaster*. Transcriptomic analysis of the response of *D. melanogaster* lines containing Dsup and the initial line under normal conditions and after exposure to ionizing radiation
2022	Generation of HEK293T cell line stably expressing the fusion protein GFP-Dsup. Carrying on experiments to assess the effect of the Dsup protein on the radioresistance of this cell line with the construction of dose - response curves (protons, heavy ions). Transcriptome analysis of HEK293T cell line stably expressing the fusion protein GFP-Dsup and the original HEK293T line under normal conditions and after exposure to ionizing radiation. Microinjection of construct for expression of a GFP-Dsup fusion protein in *D. melanogaster* embryos, production of lines carrying GFP-Dsup
2023	Evaluation of distribution of GFP-Dsup fusion protein on *D. melanogaster* polytene chromosomes by immunofluorescence analysis. Creation of an expression vector for production of Dsup protein in *E. coli* cells, isolation and purification of Dsup protein for preliminary experiments on its crystallization.

Form №29

Estimation of Budget for Project realization

Study of the radioprotective properties of the Damage Suppressor (Dsup) protein on a model organism *D. melanogaster* and human cell culture HEK293T

№	TASKS	Total value	2021	2022	2023
	Direct costs for the project				
1	Materials (kUSD)	100	40	30	30
2	Equipment (kUSD)	40	40	-	-
3	Travel resources (kUSD)	20	6	7	7
	Total direct cost:	160			

Project Leader E.V. Kravchenko

DLNP Director V.A. Bednyakov

DLNP Chief Engineer Economist G.A. Usova

Form №26

Proposed Time-Schedule and Necessary Resources

for implementation of the Project

Study of the radioprotective properties of the Damage Suppressor (Dsup) protein on a model organism *D. melanogaster* and human cell culture HEK293T

Required equipment,and sources of financial support		Cost (kUSD)	2021	2022	2023
Equipment	1. Homogenizers & Accessories	20	20	-	-
	2. Orbital shaker	1	1	-	-
	3. Rotary laboratory mixer	1	1	-	-
	4. Thermostat	2	2	-	-
	5. Centrifuge	11	11	-	-
	6. Automatic external CO_2 switcher	5	5	-	-
Sources of financial support	JINR budget	40	40	0	0

HEAD OF THE THEME G.V. Mitsyn

THE LEADER OF THE PROJECT E.V. Kravchenko