Annex 3.

Form of opening (renewal) for Project / Sub-project of LRIP

APPROVED

JINR DIRECTOR

____________/________________

"____" ____________________ **202 г.**

PROJECT PROPOSAL FORM

Renewal of a research project within the Topical plan of JINR

1. General information on the research project of the theme

1.1 Theme code - 04-2-1132-2017

1.2 Project code

1.3 Laboratory DLNP

1.4 Scientific field Life science

1.5 Title of the project

Protection against physical and chemical stresses with tardigrade proteins (TARDISS)

1.6 Project leader - Elena Kravchenko

1.7 Project deputy leader - Anna Rzyanina

2. Scientific case and project organization

2.1 Annotation

The aim of this project is to study the properties of a new radioprotective tardigrade protein Damage suppressor (Dsup), to study the mechanisms of its action and to develop schemes for its application in nuclear medicine, ecology, astrobiology, biotechnology and pharmaceutics using various model organisms. The Dsup protein is a new protein discovered in 2016 in the tardigrade *Ramazzottius varieornatus*, one of the most radioresistant species of multicellular organisms known. During the implementation of the first part of the project in 2021-2023, we created *D. melanogaster* lines and human cell culture HEK293 expressing this protein, for which we showed a significant increase in radioresistance during exposure to various types of ionizing radiation. For *D. melanogaster* lines expressing *Dsup*, a transcriptomic analysis was performed, which revealed the effect of the Dsup protein on a number of processes at the cellular and organismal levels. In the course of the experiments to determine the structure of the Dsup protein, the physical dimensions of the Dsup protein molecule were estimated for the first time, some parameters of the DNA-Dsup complex were established, and the existence of a possible secondary structure of the Dsup protein was shown.

The tasks to be solved during the implementation of the project are new and important not only for fundamental molecular biology and radiobiology, but also for applied areas of biotechnology, space research and other disciplines that require an increase in the level of radioresistance of organisms.
The next tasks of this project include: the creation of a regulated scheme for the expression of the gene encoding the Dsup protein for the temporary switch of Dsup-mediated radioprotection only at the required time interval on the model of *D. melanogaster* lines, the assessment of the effect of the Dsup protein on chromatin compaction in cells, the study of the stability and properties of the Dsup protein during exposure to high temperatures and ionizing radiation, modification of synthetic substrates with the Dsup protein for purifying solutions from nucleic acids and for extracting DNA and RNA from various biological fluids.
To solve these problems, we will use a wide range of molecular-biological and biophysical methods: in particular, protein production, purification and chromatography, SAXS, DLS and circular dichroism methods, ATAC sequencing, transcriptomic analysis, methods of molecular cloning and genome editing.
As a result of the project, a model scheme for the inducible synthesis of a radioprotective protein in living cells will be developed, the stability of the Dsup protein and its effect on the spatial organization of DNA in the cell will be assessed, a method will be developed for purifying solutions from nucleic acids and concentrating DNA and RNA from various biological fluids using Dsup protein.

2.2 Scientific case

2.1 Annotation

The main goal of the project is to study the properties and possibilities of practical application of tardigrade stress-protective proteins. The Damage suppressor (Dsup) protein was chosen as the first protein under study. A study of its mechanisms of action will be carried out and possible schemes for application in nuclear medicine, ecology, astrobiology, biotechnology and pharmaceutics using various model organisms will be developed.
There are a number of tasks in broad range of fields concerning protection from ionizing radiation: protection from overall increase in the level of background radiation due to various technogenic components; the problem of cosmic radiation, which prevents living organisms from staying in space for a long time; the need to protect healthy tissues from radiation during radiation therapy of malignant tumors; and a number of common mechanisms, underlying cell aging and their damage by ionizing radiation. Thus, a search for new mechanisms for increasing radioresistance is one of the most important areas of molecular biology and radiobiology.
Members of Tardigrada (tardigrades) are one of the most resistant to various types of stress animals on Earth, in particular they are able to survive after exposure to both rare and dense ionizing radiation at doses of about 5 kGy.
The extreme radioresistance of tardigrades has made them a model organism for studying the effect of space conditions on living organisms, and several experiments with tardigrades were carried out during the FOTONM3 mission (TARDIS (Jönsson et al., 2008), RoTaRad (Persson et al., 2011) , TARSE (Rebecchi et al., 2011, 2009)). During the TARDIS (Tardigrades in Space) project, tardigrades spent 10 days in space vacuum (10-6 Pa), exposure to cosmic radiation (100 mGy) and UV radiation. The greatest negative effect on the survival of tardigrades compared to the control group was caused by full spectrum UV irradiation, while exposure to vacuum and space radiation did not significantly affect the survival (Jönsson et al., 2016, 2008).
In 2016 the genome of *Ramazzottius varieornatus*, one of the most radioresistant tardigrade species, was sequenced (Hashimoto et al., 2016). After comparing *R. varieornatus* proteins with all already known proteins of other organisms, a unique protein, Damage suppressor (Dsup), was found, which is present only in tardigrades. On 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 involvement of Dsup in the protective mechanisms against the effects of

radiation on DNA. Irradiation of cells transfected with Dsup with γ-quanta at a dose of 4 Gy showed an increase in their survival and a decrease in radiation damage at the DNA level (γ-H2AX foci detection, COMET assay) compared with the irradiated non-transfected control (Hashimoto et al., 2016). Since irradiation before COMET was carried out on ice and DNA fragmentation was analyzed immediately after irradiation, repair processes did not significantly contribute to a decrease in the number of breaks in DNA, which speaks in favor of the radioprotective functions of Dsup, rather than its enhancement of repair processes. It is assumed that direct binding of Dsup to nucleosomes and the formation of a diffuse protein mass around chromosomal DNA provides protection from reactive oxygen species.
The objectives of this project are: to create the first regulated expression scheme for the gene encoding the tardigrade protein Dsup to temporarily turn on Dsup-mediated radioprotection only at the right time on the model of *D. melanogaster* lines, to evaluate the effect of the Dsup protein on chromatin compaction in cells, to study the stability and properties of the protein Dsup during exposure to high temperatures and ionizing radiation, to develop synthetic substrates with the Dsup protein for purifying solutions from nucleic acids and concentrating DNA and RNA from various biological fluids.

2.2.1. Obtaining of a regulated pattern of expression of the gene encoding the Dsup protein in a model object *D. melanogaster*

During the previous stage of the project, we demonstrated the possibility of a significant increase in the radioresistance of model organisms using the Dsup protein. However, along with an increase in radioresistance, we also observed a number of side effects associated with nonspecific repression of transcription. In the case of using the Dsup protein to increase the radioresistance of plants, bacteria, and many farm animals, these side effects do not play a significant role. However, for organisms that require fast responses of the nervous system to external stimuli, the preferred radioprotection scheme based on the presence of the Dsup protein in cells will be a regulated expression scheme. This will make it possible to strictly regulate protein synthesis in cells and turn on Dsup-mediated radioprotection only in the required period of time. Inducible gene expression makes it possible to synthesize a protein in response to a certain external stimulus due to the rapid activation of target gene transcription, and after the stimulus is removed, the inducible gene quickly returns to its original, inactive state.

To solve this problem, using the methods of molecular cloning and genome editing, a *D. melanogaster* line expressing Dsup under the control of the promoter of the metallothionein gene will be created and an assessment of the basal level of Dsup transcription and the level of transcription in the case of promoter induction with different concentrations of copper compounds will be carried out. These *D. melanogaster* lines will also be assessed for radioresistance and side effects before, during and after induction of Dsup expression.

2.2.2. Evaluation of the effect of the Dsup protein on chromatin compaction in cells

The Dsup protein is able to nonspecifically bind to nucleic acids due to electrostatic interactions. Therefore, an important task is to evaluate changes in the level of chromatin compaction in Dsup-expressing cells. This will make it possible to better understand the mechanisms of action of this protein in the cell and evaluate its effect on the state of chromatin in the nucleus. For quantification of open chromatin regions, the ATAC (Assay for Transposase-Accessible Chromatin) sequencing method for *D. melanogaster* constitutively expressing Dsup embryos (obtained in the previous stage of the project) and the control line will be used. The obtained data on the degree of compactness/openness of chromatin will be supplemented by the results of transcriptomic analysis, which will allow comparing the state of chromatin with the level of expression of specific genes. As a result of these experiments, the state of chromatin and transcriptome will be simultaneously

described in the presence and absence of the Dsup protein, which will allow not only characterizing the degree of Dsup influence on active chromatin, but also mapping new regulatory elements in the *D. melanogaster* genome.

2.2.3. Study of the stability and properties of the Dsup protein during exposure to high temperatures and ionizing radiation

Purified Dsup protein preparations can potentially be used for pharmacology and medicine as a cryoprotectant, preservative and stabilizer for DNA/RNA-containing drugs and vaccines, as well as a protective agent in radiotherapy and chemotherapy. To evaluate these possibilities of use, it is necessary to study the stability and properties of the Dsup protein during exposure to high temperatures (60-1000C) and ionizing radiation. At the previous stage of the project, we developed a method for obtaining concentrated solutions of pure protein Dsup (~20-30 mg/ml). Such Dsup protein solutions will be irradiated with gamma quanta at doses of 2-5 kGy using the MT-25 setup (FLNR JINR) or heated to 60-1000 C, then the protein structure will be analyzed by SAXS, DLS and circular dichroism methods (FLNP JINR, Moscow Institute of Physics and Technology), the degree of its fragmentation will be assessed (SDS-PAGE, Western blotting) as well as the functional characteristics (Dsup-DNA, Dsup-RNA gel mobility shift analysis, study of Dsup-DNA complex formation by SAXS, DLS and circular dichroism methods (FLNP JINR, MIPT)).

2.2.4. Development of a technique and material for purifying solutions from nucleic acids and concentrating DNA and RNA from various biological fluids using the Dsup protein

The ability to effectively concentrate extracellular or free DNA and purify biological and biotechnological liquids from traces of nucleic acids is necessary for working with human medical samples in diagnostics and medical procedures, in biotechnological and pharmaceutical production of DNA-containing drugs, including vaccines, in environmental monitoring etc. The Dsup protein, which can nonspecifically bind any nucleic acids, is a good candidate for developing a technique for the selective purification of solutions from nucleic acids and the concentration of DNA and RNA from various biological fluids by filtering large volumes of fluids. To obtain a porous selective filtering material, it is planned to modify sorbents and porous membranes with the Dsup protein due to covalent crosslinks (glutaraldehyde) and noncovalent interactions (His-NiNTA). Samples containing nucleic acids will be filtered through modified porous materials, with an assessment of their concentration in solution before and after filtration, followed by desorption of nucleic acids from the carrier material and an assessment of the possibility of amplifying concentrated nucleic acids using polymerase chain reaction (PCR) for further analysis. Thus, a technique will be developed for using materials modified with the Dsup protein to concentrate extracellular DNA from solutions and purify biological fluids from nucleic acids. It is important to note that this work will relate to the direction of the development of functional materials based on proteins of extremophilic organisms, which has been actively developing in recent years, and is the first to use tardigrade proteins for these purposes.

Expected results:

1. Obtaining of a regulated expression scheme for the gene encoding the Dsup protein in the *D. melanogaster* model object for the development of a controlled system for the temporary increase in radioresistance of the whole organism.
2. Evaluation of the effect of the Dsup protein on chromatin compaction in cells to establish the fundamental characteristics of the Dsup protein and map new regulatory elements in the *D. melanogaster* genome.

3. Obtaining data on the stability and properties of the Dsup protein during exposure to high temperatures and ionizing radiation in order to evaluate the use of this protein for pharmacology and medicine, as a cryoprotectant, preservative and stabilizer for vaccines and other DNA/RNA-containing drugs, as well as a protective agent for radio- and chemotherapy.
4. Development of a technique and material for purifying solutions from nucleic acids and concentrating DNA and RNA from various biological fluids using the Dsup protein.

During the implementation of the project, it is planned 2 publications in Russian peer-reviewed journals and 3 publications in foreign journals indexed by WoS (Q1, Q2); defense of master's and PhD works.

Risks: difficulties with the delivery of imported reagents for molecular biological work

2.3 Estimated completion date

2024-2028

Work stages	Work contents
2024	1. Obtaining of a *D. melanogaster* line expressing Dsup under the control of the promoter of the metallothionein gene and assessment of the basal level of Dsup transcription and the transcription level in the case of promoter induction by different concentrations of copper compounds. 2. Production of the Dsup protein in *E. coli* cells, isolation and purification of the Dsup protein. 3. Irradiation of Dsup protein solutions with gamma quanta at doses of 2-5 kGy using the MT-25 setup (FLNR JINR) or heating at 60-1000C, analysis of the protein structure using SAXS, DLS, circular dichroism methods (FLNP JINR, MIPT), assessment of the degree of its fragmentation (SDS-PAGE, Western blotting)
2025	4. Evaluation of radioresistance and the severity of side effects before, during and after the induction of Dsup expression for the *D. melanogaster* line expressing Dsup under the control of the metallothionein gene promoter. 5. Evaluation of the radioresistance of the *D. melanogaster* line expressing Dsup under the control of the metallothionein gene promoter after transcription induction and irradiation with γ-quanta at the MT-25 facility (FLNR JINR) at doses of 500-1000 Gy.
2026	6. ATAC sequencing of *D. melanogaster* embryos expressing Dsup and control lines. Bioinformatic data analysis 7. Transcriptomic analysis of *D. melanogaster* embryos expressing Dsup and control lines. Bioinformatic data analysis
2027	8. Production of the Dsup protein in *E. coli* cells, isolation and purification of the Dsup protein. Isolation of DNA molecules of different sizes. 9. Irradiation of Dsup-DNA complexes with gamma quanta at doses of 2-5 kGy using the MT-25 setup (FLNR JINR) or heating at 60-100^{0}C, Dsup-DNA, Dsup-RNA gel mobility shift analysis, study of Dsup-DNA complex formation by SAXS, DLS and circular dichroism methods (FLNP JINR, MIPT).

2028	10. Modification of sorbents and porous membranes with the Dsup protein due to covalent crosslinks (glutaraldehyde) and non-covalent interactions (His-NiNTA). 11. Filtration of samples containing nucleic acids with assessment of their concentration in solution before and after filtration, subsequent desorption of nucleic acids from the carrier material and assessment of the possibility of amplifying concentrated nucleic acids using polymerase chain reaction (PCR) for further analysis.

2.4 Participating JINR laboratories

DLNP

2.4.1 MICC resource requirements

Computing resources	**Distribution by year**				
	1st year	2nd year	3rd year	4th year	5th year
Data storage (TB) - EOS - Tapes	- 6	- 6	- 6	- 6	- 6
Tier 1 (CPU core hours)	-	-	-	-	-
Tier 2 (CPU core hours)	-	-	-	-	-
SC Govorun (CPU core hours) - CPU - GPU	20000 3000	20000 3000	20000 3000	20000 3000	20000 3000
Clouds (CPU cores)	-	-	-	-	-

2.5. Participating countries, scientific and educational organizations

Organization	**Country**	**City**	**Participants**	**Type of agreement**
-	**-**	**-**	**-**	**-**
-	-	-	-	-

2.6. Key partners -

3. Manpower

3.1. Manpower needs in the first year of implementation

№№ n/a	**Category of personnel**	**JINR staff, amount of FTE**	**JINR Associated Personnel, amount of FTE**

1.	research scientists	3, 2	-
2.	engineers	3	-
3.	specialists	-	-
4.	office workers	-	-
5.	technicians	1	-
	Total:	**7,2**	**-**

3.2. Available manpower
3.2.1. JINR staff

No.	Category of personnel	Full name	Division	Position	Amount of FTE
1.	research scientists	Kravchenko E.V. Rzyanina A.V. Zarubin M.P. Kuldoshina O.A.	DLNP SMGC DLNP OP DLNP SMGC DLNP SMGC	Head of Sector Senior Researcher Junior Researcher Junior Researcher	1 0.2 1 1
2.	engineers	Azorskaya T.O. Tarasov K.A. Yakhnenko A.S.	DLNP SMGC DLNP SMGC DLNP SMGC	Engineer Engineer Engineer	1 1 1
3.	specialists				
4.	technicians	Dubovik Ya.V.	DLNP SMGC	Technician	1
	Total:				**7.2**

3.2.2. JINR associated personnel

No.	Category of personnel	Partner organization	Amount of FTE

1.	research scientists	-	-
2.	engineers	-	-
3.	specialists	-	-
4.	technicians	-	-
	Total:	**-**	**-**

4. Financing
4.1 Total estimated cost of the project/LRIP subproject

250 kUSD

4.2 Extra funding sources
-

Project (LRIP subproject) Leader ___________/____________/

Date of submission of the project (LRIP subproject) to the Chief Scientific Secretary: __________
Date of decision of the laboratory's STC: __________ document number: __________

Year of the project (LRIP subproject) start: _________________

(for extended projects) – Project start year: ________

Proposed schedule and resource request for the Project / LRIP subproject

<table>
<tr><th colspan="3" rowspan="2">Expenditures, resources, funding sources</th><th rowspan="2">Cost (thousands of US dollars)/ Resource requirements</th><th colspan="5">Cost/Resources, distribution by years</th></tr>
<tr><th>1st year</th><th>2nd year</th><th>3rd year</th><th>4th year</th><th>5th year</th></tr>
<tr><td colspan="2" rowspan="8"></td><td>International cooperation</td><td>25</td><td>5</td><td>5</td><td>5</td><td>5</td><td>5</td></tr>
<tr><td>Materials</td><td>225</td><td>45</td><td>45</td><td>45</td><td>45</td><td>45</td></tr>
<tr><td>Equipment, Third-party company services</td><td>-</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Commissioning</td><td>-</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>R&D contracts with other research organizations</td><td>-</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Software purchasing</td><td>-</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Design/construction</td><td>-</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Service costs (planned in case of direct project affiliation)</td><td>-</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="4">Resources required</td><td rowspan="4">Standard hours</td><td>Resources</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>– the amount of FTE,</td><td>40</td><td>7.2</td><td>8.2</td><td>8.2</td><td>8.2</td><td>8.2</td></tr>
<tr><td>– accelerator (MT-24 FLNR)</td><td>400</td><td>80</td><td>80</td><td>80</td><td>80</td><td>80</td></tr>
<tr><td>– reactor,…</td><td></td><td>-</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td rowspan="2">Sources of funding</td><td>JINR Budget</td><td>JINR budget (budget items)</td><td>250</td><td>50</td><td>50</td><td>50</td><td>50</td><td>50</td></tr>
<tr><td>Extra fudning (supplementary estimates)</td><td>-</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
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Project (LRIP subproject) Leader_________/________________/

Laboratory Economist _________/________________/

APPROVAL SHEET FOR PROJECT / LRIP SUBPROJECT

Project PROTECTION AGAINST PHYSICAL AND CHEMICAL STRESSES WITH TARDIGRADE PROTEINS

TARDISS

THEME 04-2-1132-2017

PROJECT LEADER ELENA KRAVCHENKO

AGREED

JINR VICE-DIRECTOR	SIGNATURE	NAME	DATE	
CHIEF SCIENTIFIC SECRETARY	SIGNATURE	NAME	DATE	
CHIEF ENGINEER	SIGNATURE	NAME	DATE	
LABORATORY DIRECTOR	SIGNATURE	NAME	DATE	
CHIEF LABORATORY ENGINEER	SIGNATURE	NAME	DATE	
LABORATORY SCIENTIFIC SECRETARY	SIGNATURE	NAME	DATE	NAMI
THEME / LRIP LEADER	SIGNATURE	NAME	DATE	NAMI
PROJECT / LRIP SUBPROJECT LEADER	SIGNATURE	NAME	DATE	NAMI
				NAMI

APPROVED BY THE PAC	SIGNATURE	NAME	DATE