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A tale of two Dsups: Do tardigrade Damage Suppressor (Dsup) proteins differ in their ability to protect human cells from DNA damage?

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Abstract

Tardigrades are widely recognized for their remarkable survival abilities in the face of extreme conditions. The damage suppressor protein (Dsup) is a unique protein in tardigrades presumed responsible for the unusual resistance of tardigrades to various stressors. Dsup has been shown to decrease lethality and improve proliferation of human cells after exposure to ionizing radiation (IR). Recently, it has been shown that it is able to do this by creating a physical barrier surrounding the DNA, which then protects it from IR-related damage. Thus, Dsup may have a role in certain tardigrades' ability to survive IR, oxidative damage, and UV-C. However, the Dsup protein is not well-conserved even among tardigrades, and previous studies suggest that only the Dsup protein from *Ramazzottius varieornatus* (*Rv*) is able to protect the DNA of bacteria from IR, while the Dsup from *Hypsibius exemplaris* (*He*) is not. Given this uncertainty, we set out to examine whether both *Rv* Dsup and *He* Dsup can protect the DNA of human cells from IR-related damage. We set out to create expression vectors to express both versions of tardigrade Dsup in human cells. We successfully created a human expression vector containing *He* Dsup, but encountered technical difficulties obtaining the *Rv* Dsup expression vector. Once we are able to successfully clone *Rv* Dsup into a vector for human cell expression, we can move

forward with expression in human embryonic kidney cells. These cells will then be exposed to the radiomimetic drug bleomycin to assess the effectiveness of both proteins in protecting human cells against IR-related DNA damage. This research will establish if the divergence in the sequence of the Dsup protein truly results in a divergence in the ability to protect human cells from IR-related DNA damage and provide us with interesting insights into the extreme stress tolerance of tardigrades.

Introduction

Tardigrades, also known as moss piglets or water bears, are microscopic animals belonging to the phylum Tardigrada. Tardigrades are widely known for their ability to survive extreme conditions, for example, ionizing radiation, oxidative stress, intense temperature swings, and even space (Guidetti et al,. 2012). The mechanisms behind their extreme survival is still a major area of investigation. However, their resistance to these stressors varies across tardigrade species. Tardigrades belong to the clade Ecdysozoa and are positioned closely to Arthropoda and Onychophora in phylogeny (Jørgensen et al,. 2019). There are three recognized classes of tardigrades: Eutardigrada, Mesotardigrada, and Heterotardigrada. The most categorized class with at least five known subfamilies is Eutardigrada. Eutardigrada is a terrestrial-dwelling class of tardigrades that are commonly identified by a double set of claws on each leg and a lack of marine lateral appendages (Jørgensen et al,. 2019). Members of the Hysibiidea family have been popular subjects of experiments investigating stress tolerance and development in the last decade, specifically, species from the genus *Ramazzotius* and *Hypsibius* (Fig. 1 a and b, respectively) (Hashimoto et al., 2016).

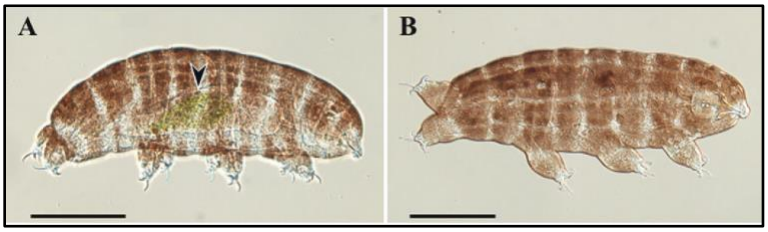

Figure 1a. *Ramazzottius varierornatus* species from the Hysibiidea family. (Cantara et al., 2025)

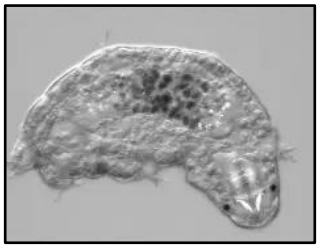

Figure 1b. *Hysibius exemplaris* species from the Hysibiidea family. (Goldstein Lab, UNC Chapel Hill).

We only have information about stress tolerance mechanisms from a handful of tardigrade species (over 1000 described species to-date), and many of these are representatives from Eutardigrada (Hashimoto et al., 2016) (Clark-Hachtel et al., 2024) (Anound et al., 2024). One molecular tool specific to the Hysibiidea family is a tardigrade-unique damage suppressor protein (Dsup) (Fig. 2). This protein is found in both the *Ramazzottius varierornatus* (*Rv*) and *Hysibius exemplaris* (*He*) species. Dsup is thought to be a divergent protein in the Eutardigrada class that got passed down from an unknown ancestor (Jørgensen et al,. 2019). Alignment of the Dsup proteins from *Rv* and *He* (Fig. 3) shows how divergent this protein is at the amino acid level, even within the same family of tardigrades. This divergence in protein sequence might be indicative of a divergence in function.

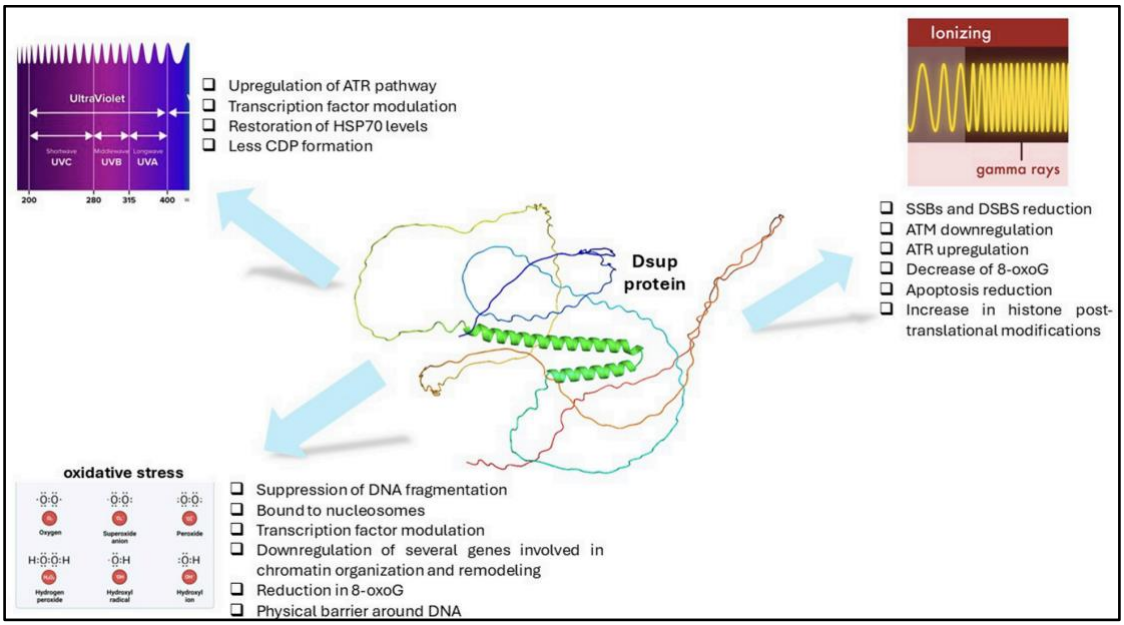

Figure 2. Dsup protein structure: how Dsup reduces DNA damage when exposed to ionizing radiation, ultraviolet radiation, or oxidative stress. (Cantara et al., 2025)

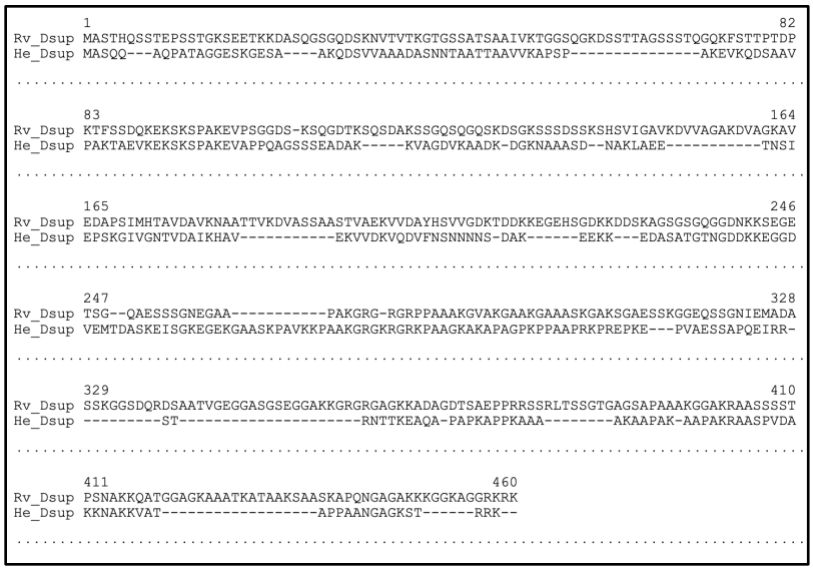

Figure 3. Alignment of *Rv* Dsup and *He* Dsup proteins. *Rv* Dsup (top line) is a longer sequence and contains frequent amino acid differences when aligned with *He* Dsup (bottom line).

From previous studies, it is known that *H. exemplaris* has a remarkable tolerance for ionizing radiation, with *R. varierornatus* being close behind (Hashimoto et al., 2016) (Clark-Hachtel et al., 2024) (Anound et al., 2024). A dose of about 5 gray (Gy) would kill most humans; *H. exemplaris* can survive upwards of 4,000 Gy (Beltrán-Pardo et al., 2015). Upon investigation, Dsup was found to be co-localized with nuclear DNA, suggesting that the protein plays a critical role in protecting DNA from damage (Mínguez-Toral et al., 2020). The mechanisms behind Dsup's function are still not fully understood; however, it is known that Dsup will create a physical barrier between DNA and the damaging event (Zarubin et al., 2024). *Rv* Dsup will bind DNA nucleosomes rather than free DNA and create a tight electromotivated bond around the tardigrade DNA (Zarubin et al., 2024). This barrier protects the DNA from taking a direct hit from ionizing radiation or sustaining IR-related damage. *H. exemplaris* has a structurally similar ortholog protein (Dsup) to the *Rv* Dsup that also binds to DNA in the same manner (Fig. 3) (Chavez et al., 2019). However, in previous studies, it was revealed that only *Rv* Dsup was able to protect bacterial DNA from IR-related damage, while *He* Dsup showed no significant improvement in survival, suggesting that the sequence divergence between these two proteins may result in functional differences (Clark-Hachtel et al., 2024). *R. varierornatus* Dsup was also shown to protect HEK293T cells from UV-C stress, with cells showing upregulation of DNA repair pathways and increased survival (Shaba et al., 2023). This success in bacterial IR and human cell UV-C trials raised questions about whether Dsup could protect eukaryotic cells from IR-related DNA damage. IR and radiomimetic drugs are commonly used as a

non-surgical cancer treatment, and they can cause permanent damage to healthy DNA after prolonged exposure in human cells (Benítez-Bribiesca & Sánchez-Suárez, 1999). There were also questions about whether *Rv* Dsup would still be a superior protector over *He* Dsup in eukaryotic cells, since there has not previously been a direct comparison of the two Dsup proteins in eukaryotic trials. The overall goal of this project is to compare the damage suppressor abilities of *Rv* and *He* Dsup proteins in human cells (HEK293) when exposed to IR-related DNA damage.

The first half of this research involved creating two expression vectors for subsequent transformation and protein expression in human cell lines, with one expression vector being for *Rv* Dsup and the other for *He* Dsup. A successful vector expressing *He* Dsup was created, and its sequence has been confirmed; *Rv* Dsup is still a work in progress.

Methods: Plasmid Creation

GBlock Design

We designed synthetic DNA fragments (GBlock - Integrated DNA technologies) for *Rv* Dsup and *He* Dsup with codon optimization for human cell expression (Table 1). The coding sequences for both Dsup proteins were obtained from a previous study (Kamilari et al., 2019). The Dsup coding sequence is followed by a linker region that connects it to a C-myc tag. The C-myc tag will allow us to confirm adequate expression of the protein in human cells. The GBlock fragments were resuspended to 25 ng/µL in DNAse-free water before use for PCR amplification.

PCR of Insert

*Rv*_Dsup_Hs_i_F1 and *He*_Dsup_Hs_i_F1 forward primers both start with a spacer region, followed by a NHE1 restriction enzyme cut site and 5’ Gblock-binding region (Table 2). *Rv*_Dsup_Hs_i_R1 and *He*_Dsup_Hs_i_R1 reverse primers both start with a spacer region, followed by a KPN1 restriction enzyme cut site and 3’ Gblock-binding region (Table 2). Primers were prepared as 100 uM stock solutions and diluted to 10 uM for the working solutions. *Rv*_Dsup_Hs_i_F1 and *Rv*_Dsup_Hs_i_R1 were used to amplify the insert for the *Rv* Dsup myc pCDNA3.1 plasmid. *He*_Dsup_Hs_i_F1 and *He*_Dsup_Hs_i_R1 were used to amplify the insert for *He* Dsup myc pCDNA3.1 plasmid.

Table 1. *Rv* Dsup (top) codon optimization for *Rv* Gblock and *He* Dsup (bottom) codon optimization for *He* Gblock. Dsup coding sequence (green), linker sequence (purple), myc tag sequence (blue), and stop codon (red).

*Rv*_Dsup_Hs_cod_opt	ATGGCAAGCACACACCAGTCTTCCACTGAGCCCTCCTCAACAGGGAAGTCTGAGGAGACTAAAAAAGACGCCAG CCAGGGATCCGGCCAGGATAGTAAGAACGTGACCGTAACCAAGGGAACCGGTAGCTCAGCTACCTCCGCCGCC ATCGTCAAAACCGGGGGATCCCAGGGTAAGGACAGCAGCACCACAGCCGGCAGCTCATCCACCCAGGGCCAG AAATTCAGCACAACACCTACGGATCCCAAGACTTTTAGCTCAGATCAAAAAGAAAAAAGCAAGAGCCCAGCTAAGG AGGTGCCAAGTGGCGGCGATTCTAAGTCCCAAGGTGACACCAAAAGTCAGAGCGATGCCAAGTCCAGTGGACAG TCCCAGGGTCAGTCCAAGGACAGCGGTAAAAGTAGCAGCGATTCTTCTAAGTCTCACAGTGTCATTGGAGCTGTGA AAGACGTCGTGGCAGGTGCTAAGGATGTTGCTGGCAAAGCCGTTGAAGACGCTCCATCAATAATGCACACAGCCG TTGATGCTGTCAAAAATGCTGCGACCACAGTCAAAGATGTGGCCTCATCAGCAGCAAGTACCGTCGCCGAGAAGG TGGTGGATGCTTACCACAGCGTGGTGGGTGACAAAACTGACGACAAGAAGGAAGGGGAGCACTCTGGAGATAAGA AGGACGATTCTAAAGCCGGATCCGGATCAGGGCAAGGCGGTGACAACAAAAAAAGCGAAGGGGAGACTTCAGGT CAGGCGGAGAGCAGCTCCGGGAATGAAGGCGCAGCCCCTGCCAAAGGTCGCGGCAGGGGAAGACCACCAGC TGCAGCTAAAGGAGTAGCCAAGGGGGCCGCTAAAGGCGCCCGCCGCTTCCAAAGGCGCTAAATCCGGCGCCGAA AGCAGTAAGGGGGGAGAGCAAAGCTCCGGGAACATTGAAATGGCGGACGCGAGCTCCAAAGGCGGGAGTGACC AAAGAGATAGCGCAGCCACAGTGGGCGAGGGCGGCGCTTCCGGTTCAGAAGGTGGCGCGAAAAAAGGAAGAGG CAGAGGCGCAGGCAAGAAGGCCGATGCAGGAGATACTTCCGCTGAACCCCCTAGAAGGTCTAGTAGGCTGACAT CATCTGGAACCGGCGCGGGTAGTGCACCAGCAGCCGCAAAAGGCGGAGCTAAAAGAGCAGCATCTTCATCCTCT ACCCCTAGCAACGCTAAGAAGCAGGCGACTGGAGGCGCAGGCAAAGCCGCCGCTACTAAGGCCACTGCTGCAA AAAGTGCGGCCAGCAAAGCCCCACAGAATGGCGCTGGGGCAAAGAAGAAGGGTGGGAAAGCTGGCGGGCGCA AGCGAAAAGGTGGTGGCGGATCTGAACAGAAGTTGATTTCCGAAGAGGACCTCTAA	Dsup coding Linker C-myc STOP
*He*_Dsup_Hs_Cod_opt	ATGGCATCACAACAGGCTCAGCCGGCCACTGCCGGAGGGGAGAGCAAGGGCGAATCTGCTGCTAAGCAGGACT CCGTGGTCGCAGCCGCAGATGCCTCCAATAACACCGCCGCAACCACTGCCGCGGTTGTCAAGGCTCCAAGTCC TGCGAAAGAAGTGAAACAGGATTCTGCCGCTGTGCCGGCTAAAACAGCGGAGGTGAAGGAAAAATCAAAGTCACC GGCTAAGGAGGTTGCCCCACCACAAGCCGGCAGCAGCTCTGAGGCCGATGCCAAGAAGGTTGCTGGCGACGTC AAGGCCGCTGACAAGGACGGAAAGAACGCCGCGGCATCAGACAATGCTAAGCTGGCAGAGGAAACCAATAGTAT TGAGCCAAGTAAAGGCATAGTGGGTAACACTGTCGACGCAATAAAGCACGCCGTTGAGAAGGTCGTTGACAAAGT CCAGGACGTGTTTAATTCCAATAATAACAACTCTGACGCCAAGGAGGAAAAAAAAGAGGACGCCTCTGCCACTGGC ACTAACGGCGATGATAAGAAAGAGGGCGGAGATGTAGAGATGACAGATGCATCCAAAGAGATCTCAGGAAAAGAG GGGGAGAAGGGGGCCGCCAGCAAACCAGCGGTAAAGAAACCTGCTGCAAAGGGCCGGGGGAAACGAGGCAGA AAGCCTGCCGCCGGCAAAGCCAAAGCTCCCGCCGGCCCCAAGCCACCTGCTGCCCCTCGGAAGCCTCGAGAA CCTAAAGAACCTGTAGCCGAAAGTAGCGCTCCTCAAGAAATACGGCGCAGTACCCGGAACACCACTAAGGAGGC CCAGGCTCCTGCACCTAAAGCTCCTCCAAAGGCCGCTGCAGCCAAGGCTGCCCCTGCAAAAGCAGCTCCAGCT AAAAGGGCTGCCTCTCCCGTAGATGCCAAAAAAAACGCTAAGAAGGTTGCCACAGCCCCCCCTGCCGCCAATGG CGCAGGCAAGAGCACAAGGCGAAAAGGGGGCGGCGGCTCCGAACAGAAGTTGATCTCTGAGGAGGATCTCTAA	Dsup coding Linker C-myc STOP

Table 2. Forward and reverse primer designs for *Rv* Dsup vector and *He* Dsup vector. Spacer region, NHE1 cut site, KPN1 cut site, and Gblock-binding region.

Primer Name	Sequence (coded)	Tm	Fragment Length (bp)	Notes
*Rv*_Dsup_Hs_i_F1	CTTCGCTACGACCACCATGGCAAGCACACACCAGTC	57.6	1409	For amplifying *Rv*_Dsup_myc insert with restriction digest sites
*Rv*_Dsup_Hs_i_R1	CTTCGGTACCTTAGAGGTCCTCTTCGGAAATC	53.1		
*He*_Dsup_Hs_i_F1	CTTCGCTAGCACCACCATGGCATCACAACAGGCTCA	57.2	1058	For amplifying *He*_Dsup_myc insert with restriction digest sites
*He*_Dsup_Hs_i_R1	CTTCGGTACCTTAGAGATCCTCCTCAGAGATC	51.5		

PCR mix (25 µL) using Q5 Taq reaction mix was created with 12.5 µL of Q5 master mix, 1 µL of Gblock template (25 ng/µL), 1.25 µL of forward primer (either *Rv* or *He*, 10 µM), 1.25 µL of reverse primer (either *Rv* or *He*, 10 µM), and 9 µL of DNAse-free water. The recipe

creates one reaction. Once made, the reaction is placed in a thermocycler with the following cycle program: 98°C x 30s, 35 cycles of 98°C x 30s, 55°C x 30s, 72°C x 1.5 min, final extension of 72°C x 5 min. Samples are then kept at 4°C until fragment analysis and gel purification. Add 5 µL of purple loading dye after fully running the PCR.

Run the full volume of PCR sample + dye (30 µl) on 1% agarose gel. Run agarose gel for 35 minutes at 140vV. Purify size-appropriate gel band corresponding to primer fragment length by using Monach© Spin DNA Gel Extraction Kit (T1120S) following the manufacturer's instructions.

PCR Digest

The purified PCR product from above is used for subsequent digestion steps. The digestion reaction for the inserts is composed of 9 µL of purified PCR product, 5 µL of 10x CutSmart Buffer, 1 µL NHE1 enzyme, 1 µL KPN1 enzyme, and 34 µL of DNAse-free water. Alongside the insert, the backbone should also be digested in a separate reaction. The digestion reaction for the backbone can be made with 3.5 µL of pcDNA3.1(+) plasmid DNA, 5 µL of 10x CutSmart Buffer, 1 µL NHE1 enzyme, 1 µL KPN1 enzyme, and 39.5 µL of DNAse-free water. Incubate both the insert and backbone reactions at 37°C for 2 hours. After the incubation period is complete, add 10 µL of purple loading dye and immediately run the full volume of the samples on 1% agarose gel to purify the digested products. Run agarose gel for 35 minutes at 140vV. Purify the gel bands by using Monach© Spin DNA Gel Extraction Kit (T1120S) following the manufacturer's instructions.

Ligation

For a successful ligation, a concentration of 200 ng of total digested plasmid is needed. Typically, that is around 8 µL of the digested backbone, depending on the efficiency of the digestion and the yield after gel purification. The digested insert DNA is added to a 1.5 ml microfuge tube until the volume of backbone + insert is 17 µL. In this case, if using 8 µL of backbone, 9 µL of digested insert is added. 2 µL of 10x ligase buffer is added to the mixture along with 1 µL of T4 DNA ligase. It is important to make a separate control by replacing the digested insert DNA with 9 µL Qiagen Elution Buffer (no-insert control). Mix the tubes well, and incubate at room temperature for 1 hour, then in the fridge overnight.

Transformation

The ligated products are then transformed into JM109 *(E. coli)* bacteria for product amplification and sequence validation. For each ligation, take 1.5 mL microfuge tubes and then divide thawed JM109 cells into 50 µL puddles at the bottom of the tube. Add 1 µL of

ligation into a cell puddle and mix gently. Incubate the tubes on ice for 30 minutes. Immediately heat-shock the tubes in a 42°C bath for 45 seconds. After the 45 seconds of heat-shock, the tubes go back on ice for 2 minutes. Pipette 400 µL of SOC medium into each tube and shake tubes in a 37°C incubator at 230 rpm for 1 hour.

After the hour is up, pellet the cells by centrifuging the tubes at 9000 g for 3 minutes. Discard the supernatant, leaving about ~100 µL of liquid at the bottom along with the pellet. Resuspend the pellet of cells in the remaining supernatant, and then plate all liquid on LB-Ampicillin plates (for selection of cells containing the desired products). Incubate the LB plates with ampicillin added at 37°C overnight.

Miniprep

Ten single colonies from each transformation (except for no-insert control) were selected, individually placed into 5 mL of LB with ampicillin, and allowed to grow overnight in a shaking incubator (230 rpm) at 37°C. 3 mL of bacterial culture was used for subsequent plasmid isolation using Monach© Plasmid Miniprep Kit (T1010S) by following the manufacturer's instructions.

After minipreps were completed, isolated plasmids were digested and run on a 1% agarose gel to check for appropriate insertion length. For each isolated plasmid, 10 µL plasmid DNA, 5 µL of 10x CutSmart Buffer, 1 µL of NHE1 enzyme, 1 µL KPN1 enzyme, and 33 µL of DNAse-free water were combined in a 1.5 microfuge tube. Digest samples at 37°C for 2 hours. Once incubation is complete, add 10 µL of purple loading dye and run samples on 1% agarose gel. If plasmids were confirmed to contain the appropriate-sized insert by digestion and gel electrophoresis, they were prepared for sequencing to confirm the integrity of the ligated product. If necessary, plasmids were diluted to 60 ng/µL in sterile water and sent for sequencing from both the CMV-Forward and BGHR primer sites that flank the insertion site of the pcDNA3.1(+) plasmid. The sequence of the inserted fragment was confirmed via alignment with a hypothetical molecule created at the start of this experiment. Plasmids were only deemed viable to move forward to transfection into human cells if they had no insertions/deletions or non-synonymous single-nucleotide polymorphisms relative to the hypothetical plasmid. We also confirmed that the coding frame was maintained.

Results

Our first steps involved making two expression vectors for *He* Dsup and *Rv* Dsup to be expressed in a HEK293 cell line. We successfully created the plasmid for *He* Dsup myc pCDNA3.1 (Fig. 4a). *Rv* Dsup myc pCDNA3.1 plasmid (Fig. 4b) is still a work in progress.

Myc tags were added to both plasmid sequences to verify protein expression after transformation into human cells. Complications with plasmid creation have stalled the start of human cell line trials; however, efforts to successfully create the plasmid continue. Some technical difficulties with a faulty nanodrop, poor digestion, and faulty LB plates have caused a stall with the *Rv* Dsup plasmid creation. It is also believed that the forward primer had errors at the restriction site, preventing digestion since PCR worked well from these primers, but digestion and ligation failed. We will reorder the primers for *Rv* Dsup plasmid creation in hopes that this will solve this issue. After we successfully obtain plasmids capable of expressing both forms of Dsup, we will proceed with the transformation of the Rv plasmid into human cells.

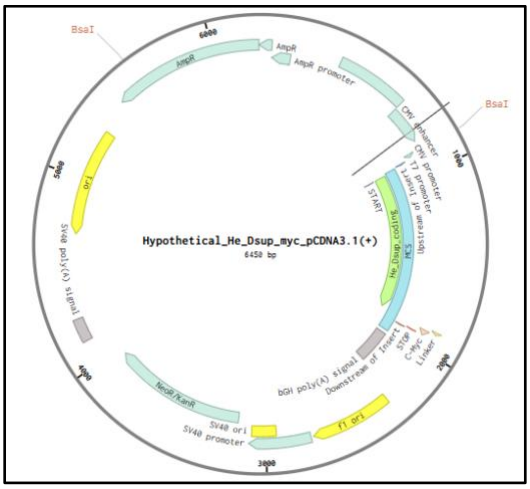

Figure 4a. *He* Dsup myc pCDNA3.1

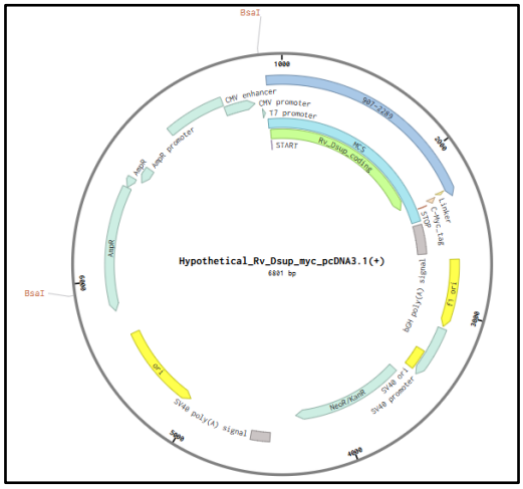

Figure 4b. Hypothetical *Rv* Dsup myc pCDNA3.1

Discussion

We successfully created a plasmid for human cell expression of the *He* Dsup protein. Unfortunately, due to technical difficulties in the plasmid creation process, we were unable to complete the creation of the *Rv* Dsup plasmid for human cell expression. Since the goal of this experiment is to ultimately compare the function of these two potentially divergent proteins to each other, specifically how well they protect the DNA of eukaryotic cells, we chose not to proceed with human cell assays until both expression plasmids are obtained. It is believed that there was an error in the *Rv* forward primer, specifically the restriction digest site. The error would explain why the initial PCR for *Rv* proceeded without issues, but downstream steps like digestion and ligation failed. Reordering the primers would resolve this issue in future attempts to construct the *Rv* vector. After completion of two Dsup vectors, the vectors will be transformed into separate human cell lines and dosed with varying levels of a radiomimetic drug, Bleomycin. Using a radiomimetic drug allows us to mimic the damage that ionizing radiation would cause to DNA. We would be using Bleomycin, which causes single-stranded DNA breaks, cytotoxic double-stranded breaks, and abasic DNA sites (Benítez-Bribiesca & Sánchez-Suárez, 1999). A dose range of 0 µg/ml - 2000 µg/ml will be used based on previous human Bleomycin studies (Benítez-Bribiesca & Sánchez-Suárez, 1999).

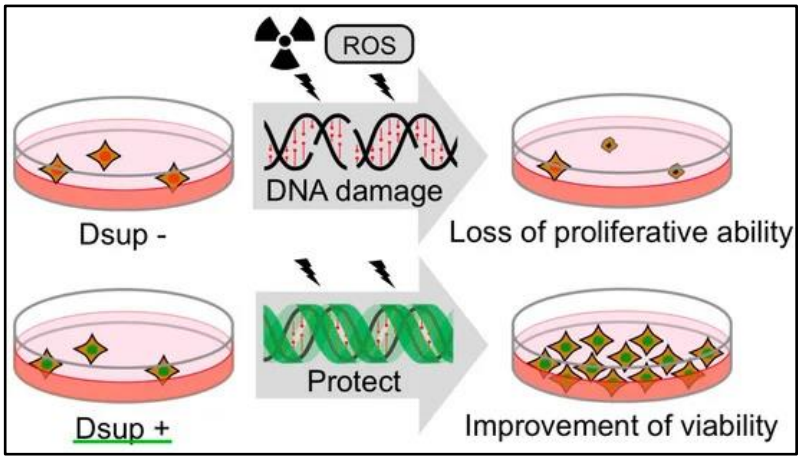

Figure 5. Model of Dsup protecting human DNA from reactive oxidative stress and radiation (Hashimoto and Kunieda, 2017)

Bleomycin will be dissolved in a stock solution and added to the cell culture media. When human peripheral lymphocytes were dosed with Bleomycin, double-stranded DNA breaks peaked at 600 µg/ml and cell apoptosis peaked at 1000 µg/m (Bribiesca & Suárez, 1999). With the addition of our vectors, we anticipate that survival and damage repair will change; having a wide range of doses allows us to see the full picture. The vector cell lines would be compared to a survival curve of control HEK293 cells over seven days. Viability and proliferation of drugged cells will be tested with live/dead staining at the end of seven

days. Daily observation, cell density tests, and observations of proliferation will be carried out. *Rv* Dsup is predicted to protect against damage more than *He* Dsup based on the previous studies in bacteria, and improve survival when compared to our control (Fig. 5) (Clark-Hachtel et al., 2024).

Once completed, this research will establish if the divergence in sequence of the Dsup protein truly results in a divergence in the ability to protect human cells from IR-related DNA damage, and provide us with interesting insights into the extreme stress tolerance of tardigrades.

Acknowledgments

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