Investigating the impact of proteasome inhibition on TRIOBP-1 aggregation in relation to schizophrenia research

Babić, Mario

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SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

Preddiplomski sveučilišni studij

Biotehnologija i istraživanje lijekova

Mario Babić

Ispitivanje utjecaja inhibicije proteasoma na agregaciju TRIOBP-1 u odnosu na istraživanje shizofrenije

Završni rad

Rijeka, 2022

Mentor: doc. dr. sc. Nicholas J. Bradshaw

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Finally, I want to thank my family and I can't emphasize enough how much I value your support at all times especially my grandmother and mother. Without you I am sure I wouldn't be where I am right now.

I want to dedicate this thesis to the memory of my late grandfather.

I know you would be proud.

This undergraduate final thesis was defended on 27th of September 2022

In front of the Committee:

1. Izv. prof. dr. sc. Jelena Ban
2. Izv. prof. dr. sc. Željka Maglica
3. Doc. dr. sc. Nicholas J. Bradshaw

This thesis has 37 pages, 3 tables, 10 figures and 26 citations.

Abstract

Schizophrenia is a chronic mental illness, which a great number of patients around the world struggle with daily. The pathology of schizophrenia is still largely unknown, and the purpose of this thesis is to attempt to solve a piece of the schizophrenia puzzle. The role of TRIO and F-actin-binding protein 1 (TRIOBP-1) is the unsolved problem that we are seeking to resolve and, by doing so, contribute to the broader study of schizophrenia. More specifically, the formation of insoluble TRIOBP-1 aggregates is the main point of interest in my research. TRIOBP-1 is a protein that scientists have previously studied in an attempt to discover its role in the proteinopathy of schizophrenia. What this thesis is seeking to accomplish is expand upon the knowledge revolving around this protein which has already accumulated over the years. Specific data must be obtained on the mechanism of protein aggregation and the involvement of the proteasome in the protein's behaviour. My research involved two TRIOBP-1 constructs: a full-length wild type gene with no alterations and a mutant gene with a deletion between amino acids 333 and 340 as well as a deletion of the N-Terminal section (1-59). The two constructs were used to do a comparison against each other and to test how the two constructs behave when placed in different cell lines. The results showed that the mutant variant of the TRIOBP-1 with the aforementioned deletions did not aggregate into any of the cell lines that it was transfected in which proves that those are the regions responsible for aggregation of the full-length TRIOBP-1 plasmid. The impact of inhibiting the proteasome did not impact the number of insoluble protein aggregates which opened the door for further research on how TRIOBP-1 behaves when a combination of stressors is applied. These findings bring us closer to establishing which exact amino acids are prone to aggregation in TRIOBP-1 and open a new chapter in the investigation of the impact of TRIOBP-1 in the broader study of schizophrenia.

KEY WORDS: TRIOBP-1, Chronic mental illness, Protein aggregation, Stress, Schizophrenia, Proteasome

Sažetak

Shizofrenija je kronična mentalna bolest s kojom se svakodevno bori velik broj oboljelih diljem svijeta. Patologija shizofrenije još je uvelike nepoznata, a svrha ovog rada je pokušati riješiti dio te slagalice. Uloga TRIO i F-aktin-vezujućeg proteina 1 (TRIOBP-1) misterij je koji nastojimo razriješiti i na taj način pridonijeti širem proučavanju shizofrenije. Konretno, formiranje netopivih TRIOBP-1 proteinskih agregata je glavna točka interesa u mom istraživanju. TRIOBP-1 je protein kojeg su znanstvenici prethodno izučavali pokušavajući otkriti njegovu ulogu u proteinopatiji shizofrenije. Ono što ova teza želi postići jest proširiti znanje o ovom proteinu koje se već akumuliralo tijekom godina. Mora se doći do specifičnih podataka o mehanizmu agregacije proteina i uključenosti proteasoma u ponašanje proteina. Moje istraživanje uključivalo je dva konstrukta TRIOBP-1: prvi je full-length wild type gen bez ikakvih alteracija, a drugi mutirani gen sa delecijama između aminokiselina 333-340, kao i delecije N-terminalnog dijela koji odgovara aminokiselinama (1-59). Dva navedena konstrukta su korištena za međusobnu usporedbu te se testiralo kako se ta dva konstrukta ponašaju u različitim staničnim linijama. Rezultati su pokazali da mutirana varijanta TRIOBP-1 s gore navedenim delecijama nije agregirala ni u jednoj staničnoj liniji u koju je transficirana, što dokazuje da su to regije odgovorne za agregaciju TRIOBP-1 plazmida full-length. Utjecaj inhibicije proteasoma nije utjecao na broj netopivih proteinskih agregata što je otvorilo vrata za daljnja istraživanja o tome kako se TRIOBP-1 ponaša kada se primijeni kombinacija stresora. Ova otkrića nas približavaju utvrđivanju koje su točno aminokiseline sklone agregaciji u TRIOBP-1 i otvaraju novo poglavlje u istraživanju utjecaja TRIOBP-1 te u širem kontekstu istraživanja shizofrenije.

KLJUČNE RIJEČI: TRIOBP-1, kronične mentalne bolesti, agregacija proteina, stres, shizofrenija, Proteasom

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1. INTRODUCTION

1. Chronic mental illness

The National Institute of Health states that in the U.S., there are 52.9 million people living with some form of mental illness. This statistic implies that one in five U.S. adults live with a mental illness, whose symptoms vary from severe to mild (1). This is a number that we can expect to grow in the future. An increase in the number of mentally ill patients should not be considered a discouraging thing, as it might indicate an increase in mental health awareness. Taking into consideration that mental health care is becoming progressively available all around the world and seeking mental help is not considered a stigma among the younger generations so much anymore. The World Health Organisation launched an initiative in which they mean to ensure access to mental health care to 100 million more people (2).

Will we ever be able to define a chronic mental illness (CMI)? To define a CMI means to have a thorough understanding of a given mental illness. Unfortunately, not one person or clinician can have a complete grasp on any given mental illness. For that reason, we see a shift in the diagnostics of certain CMI. At the present moment, clinicians prefer the concept of a mental illness spectrum. We can observe the shift in the latest version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (3). The spectrum concept brings a necessary change in a field that is constantly evolving and refining the way that we look at chronic mental illnesses (4).

1.2 Schizophrenia

Schizophrenia is a CMI and a complex illness with three distinct groups of symptoms: positive (novel change in patient behaviour), negative (lack of certain behaviour, previously present in patients) and cognitive (decline in mental processes such as memory or concentration). The symptoms typically surface in early adulthood, and they last for at least half a year to be diagnosed as schizophrenia (3). There is still a great number of researchers all around the world trying to determine out what causes this perplexing, and there is a determined effort being made to gather more information about it. Even if we do not have complete understanding about the disease's aetiology, genetic studies have approximated schizophrenia inheritance at about 79% among twins (5). Another side of the coin is the environment, more specifically traumatic experiences (6) or other risk factors that can influence the onset of schizophrenia. These include the season in which a person is born, the age of their parents, difficulties during childbirth and substance abuse.

1.3 Protein aggregation in neurodegenerative disorders vs CMI

When comparing patients with diagnosed neurodegenerative disorders and CMI, a pattern of shared symptoms can be observed. An example is frontotemporal dementia, caused by the accumulation of TAR DNA binding protein 43 (TDP-43), RNA-Binding protein FUS or a variety of other proteins (7), which shares symptoms (like apathy, and impulsive and repetitive behaviour) with schizophrenia (8). Insoluble protein aggregation is a hallmark of neurodegenerative disorders, such as Alzheimer’s and Parkinson’s disease (9). Protein aggregation (Figure 1.) disrupts protein structure, thus influencing its function. The degree of aggregation is determined by a number of different elements, most of which fall into either the intrinsic (primary, secondary, tertiary, or

quaternary structure) or extrinsic (other factors) categories (such as environment in which the protein is present, processing conditions) (24). Aggregates can either give a new and toxic function to the protein or cause loss of function (10). On the other hand, protein aggregation can be a response of the organism to environmental changes (11). Nevertheless, investigation of protein aggregation can lead to a better understanding of the mechanisms of neurodegenerative disorders and CMI. In normal conditions, cells have systems for clearance of incorrectly folded proteins or protein aggregates like proteasomal degradation or autophagy. These processes are highly regulated; however, any disruption can result in the accumulation of protein in cells and cell toxicity. In patients, dysfunctional proteasome has been correlated with the aging process, neurodegenerative disorders, and schizophrenia (12). There has also been new evidence in support of the notion that there is general proteosomal disruption and the occurrence of general protein aggregation in schizophrenia (25, 26,). *In vivo* and *in vitro* studies have shown that environmental factors like traumatic brain injury and increased oxidative stress can lead to formation of protein aggregates (13,14).

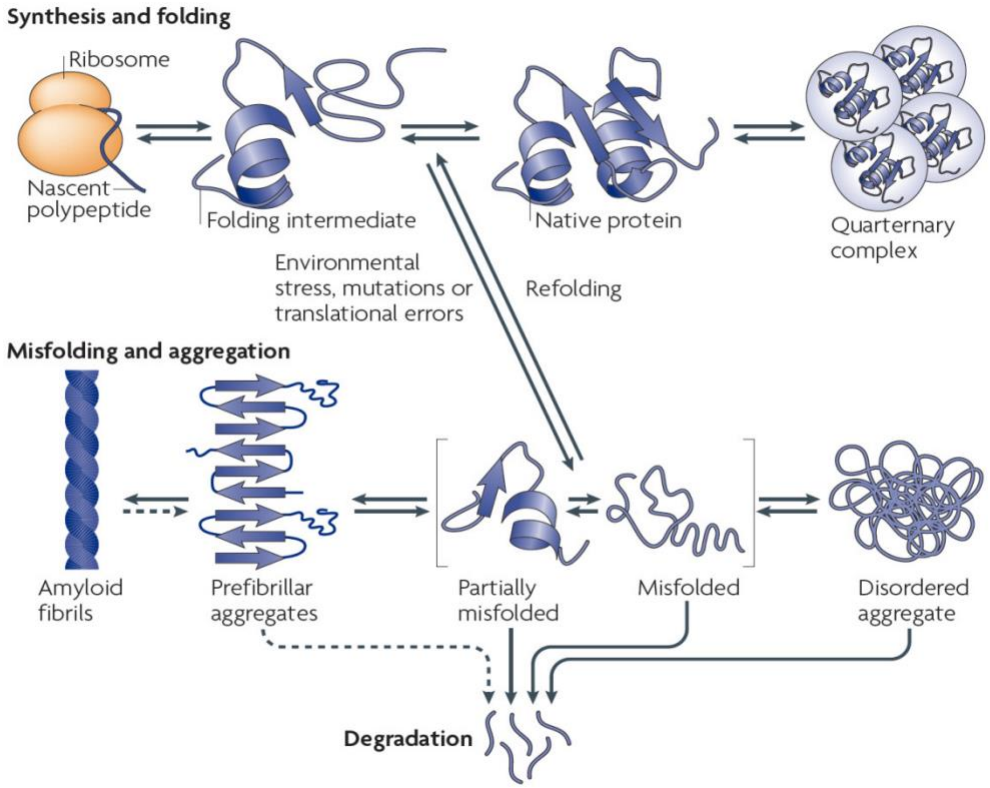

Figure 1: An overview of the aggregation of biological proteins. A protein will fold through a variety of intermediate states on its way to achieving its natural, three-dimensional structure both during and after its production at the ribosome. Proteins may become misfolded as a result of exposure to a variety of stressors, mutations in the generated protein, or translational errors. In the process of preventing the build-up of misfolded proteins, misfolded intermediates have the potential to either be refolded back into their natural form or be destroyed by various cellular proteolysis system. Taken from (Tyedmers et al.,2010)

1.4. Using stress to investigate protein aggregation

Oxidative stress mostly refers to reactive oxygen species, which are the by-products of normal cell metabolism. However, an elevated level of reactive oxygen species can lead to oxidation of proteins in neuronal cells, which has often been described in the pathology of neurodegenerative disorders such as Alzheimer's disease (15,16).

The procedure for conducting a blind stress test was developed by referring to the work of a lab colleague Mihaela Bergman on NPAS3, CRMP1 TRIOBP-1 and DISC1 proteins. To summarize her work, she has

done *in vitro* stress treatment with protein aggregation investigations to develop an experimental methodology. At the start of developing a viable methodology transfected cells were treated with stress stimuli for 18 hours and analysed by fluorescence microscopy. Based on earlier papers, 1000 μM iron (II) chloride, calcium chloride and zinc acetate, 50 μM of sodium arsenide, and 10 μM MG132 proteasome inhibitor were utilized in the tests. Negative controls received cell culture medium without stressors. Most cells exposed to these concentrations died and distorted from oxidative stress shock. Aggregates accumulated when treated with sodium arsenide, but the cell cytoskeleton lost shape, indicating cell death. This incubation duration killed Flag-NPAS3 cells. Most transfected samples had many dead cells. Few cells were treated without obvious oxidative stress damage or protein aggregation. SH-SY5Y cells transfected with CRMP1 Sv and NPAS3, stress-treated with sodium arsenide and iron (II) chloride, displayed oxidative stress damage. Stress factor therapy damaged CRMP1 Sv and NPAS3 cells, preventing aggregation. She adjusted her strategy to optimize the procedure by stressing the cells for 6 hours rather than 18. Compared to prior trials, cell death was reduced. However, stress therapy still demolished the cell count. Zinc acetate caused cells to lose their natural shape by shrinking the nucleus membrane. In certain instances, calcium chloride treatment destroyed the nucleus. SH-SY5Y cells transfected with CRMP1 Sv and NPAS3, treated with zinc acetate and calcium chloride for 6 hours, displayed oxidative stress damage which prevented aggregation. Since the cells showed oxidative stress damage, the technique needed to be modified. Following that she stressed transfected neuroblastoma cells for 3 hours to investigate whether that caused cell damage or not. In most instances, the cells were not harmed, which is necessary for protein aggregations and transfection. The 3-hour stress treatment resulted in the healthiest cells. Neuroblastoma cells have developed aggregates. When stressed with sodium arsenide, NPAS3 translocated to the cytoplasm, suggesting aggregation. SH-SY5Y cells transfected with CRMP1 Sv, NPAS3, TRIOBP-1

and DISC1 were stressed for 3 hours with MG132, sodium arsenide, and iron (II) chloride. Only CRMP1 Sv does not display stress-induced aggregation among the 4 proteins tested.

1.5. Proteins implicated as aggregating in CMI

Due to the overlap of symptoms between the early stages of neurodegenerative disorders and CMI, recent research has suggested a shared impairment in pathology between the two. So far, some proteins encoded by risk factors from genetic studies have been detected as insoluble in patients' brain samples (17) or observed to aggregate as purified proteins (18). If a protein accumulation is determined to be insoluble, we refer to it as an aggregation. At the same time, hypothesis-free assays from patient samples also elucidated novel proteins as aggregating in CMI (19). The following proteins have been implicated as aggregating in CMI: Disrupted in Schizophrenia 1 (DISC1), dysbindin-1, Trio-Binding Protein 1 (TRIOBP-1), and Neuronal PAS Domain Protein 3 (NPAS3).

1.6. TRIOBP-1

TRIOBP-1 or Tara protein in normal cells regulates cytoskeletal organization by binding to actin. The main parts of TRIOBP-1 are three regions: the N-terminal PH domain, central domain, and C-terminal domain (Figure 2.)

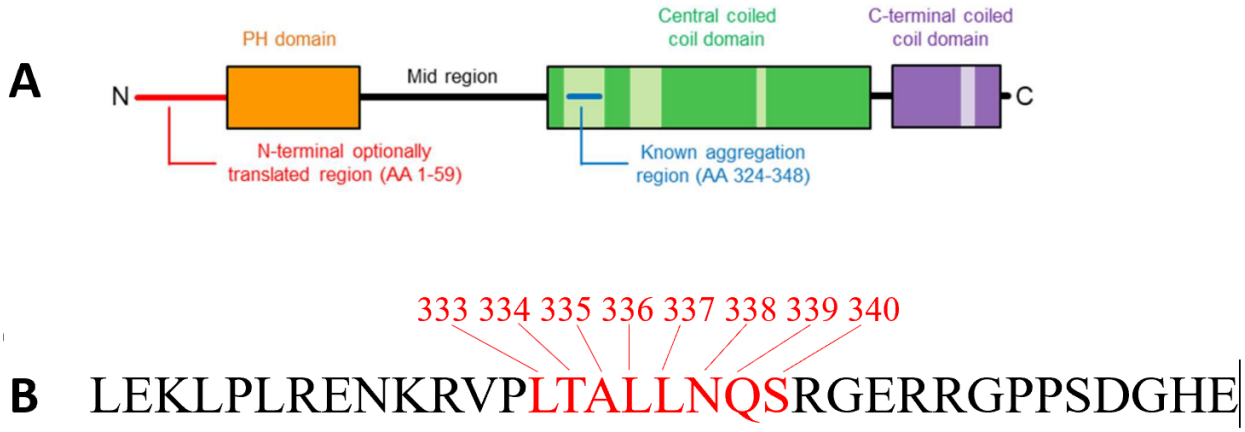

Figure 2. Known TRIOBP-1 structure. (Adapted from (21)). The domain 70 structure of TRIOBP-1, shown in a one-dimensional format. In coiled-coil domains, predicted coils are shown in darker shades, while loops are shown in paler shades. Amino acid (AA) numbers refer to the 652 AA form of TRIOBP-1, which includes the optionally translated region. (B) A section of the primary structure of TRIOBP-1 construct used in this thesis, with the amino acid deletion (333-340).

High expression of the *TRIOBP-1* gene was observed in the brains of patients with diagnosed schizophrenia. Another approach used purified insoluble protein fraction brain samples from patients with schizophrenia for immunization of mice, producing monoclonal antibodies which among other proteins, specifically recognized TRIOBP-1 (20). Visual evidence of TRIOBP-1 aggregation has been provided in human neuroblastoma cells SH-SY5Y, as well as a more detailed investigation of the 8 amino acids Central domain (333-340) and the optionally translated N-terminus (1-59). Removal of both of these regions appears to greatly reduces or abolishes aggregation (21).

2. AIMS OF THE THESIS

The aims of this thesis were to observe how the protein TRIOBP-1 and its mutant counterpart would act *in vitro*. This research was done in order to further our knowledge of TRIOBP-1 and its possible role in schizophrenia and other chronic mental illnesses. If one wishes to expand their understanding of mental diseases as well as our ability to identify and treat them, research on the biological origin of a chronic mental illness is essential. Because of this, in addition to the findings of previous studies on the role of TRIOBP-1 in schizophrenia, I made the decision to focus my study on this matter. The second objective of the study was to acquire a more in-depth understanding of the relationships between various sources of stress and the development of mental disorders. More precisely, the goal of my study was to observe and evaluate the influence of stress factors on the *in vitro* behaviour of TRIOBP-1. The final objective of my work was to concentrate on the proteasome inhibitor, which has showed promise in previous experiments, to assess if inhibiting the proteasome would increase the development of insoluble protein aggregates. The decision was made to base all of the objectives of this thesis on two different TRIOBP-1 variants, which were the wild type full-length TRIOBP-1 (aa 1-652) and a non-aggregating mutant variant of TRIOBP-1 (60-652, Δ333-340).

With all of this in mind, the objectives of this thesis may be broken down into the following categories:

- The first item that had to be determined was whether or not the deletions on the mutant variant had any effect on the tendency of the protein to aggregate when compared to its wild type counterpart.
- Secondly, we were interested in seeing what effect inhibiting the proteasome would have on the cell's ability to degrade newly formed aggregates, and we wanted to do this with both the mutant and the wild type variants.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1. PLASMIDS AND VECTORS

Table 1: List of plasmids. Plasmids in the pdcDNA-Flag vector, with antibiotic resistance to AMP

Vector	Protein encoded	Origin	Publication
pdcDNA-Flag	TRIOBP-1	Nicholas Bradshaw and Carsten Korth, Düsseldorf	Bradshaw et al. (2014) 15
pdcDNA-Flag	TRIOBP-1 (60-652, Δ333-340)	Nicholas Bradshaw, Rijeka	Zaharija et al. (in press)

3.1.2. ANTIBODIES

Table 2. Primary and secondary antibodies, as well as other fluorescent markers, used for Western Blotting and immunocytochemistry.

Name	Supplier	Concentration	Dilution
Anti-Flag M2 – Monoclonal (Mouse)*[1]	Sigma	1 mg/mL	1:2000
Peroxidase Conjugated Affinity Purified Goat anti-Mouse igG	Thermo Fisher Scientific	1 mg/mL	1:2000
Alexa Fluor 555 Goat anti-Mouse IgG	Thermo Fisher Scientific	2 mg/mL	1:1000
Phalloidin-iFlour 488 Reagent	Abcam	2 mg/mL	1:500
DAPI	Sigma	1 mg/mL	1:500

[1] Primary antibody

3.1.3. STRESS FACTOR

Table 3. Stress factors and their concentrations used in stress test and the blind test

Name	Concentration	Solvent
Sodium arsenite	5 µM	dH_20
Iron (II) chloride	30 µM	dH_20
MG132 (Proteasome inhibitor)	10 µM	DMSO

3.2. METHODS

3.2.1 Transforming bacteria

Both of the plasmids that were utilized in this thesis were grown in competent NEB5α bacterial cells, which are an E. coli strain. Plasmids encoding either the wild type or a non-aggregating mutant variant of TRIOBP-1 (60-652, Δ333-340) were introduced into the NEB5α bacteria to induce transformation. In a 1.5 mL Eppendorf tube, 1 µl of plasmid was mixed with 50 µl of thawed NEB5α bacteria, and the solution was then placed on ice for 30 minutes. After the incubation period, the bacteria were subjected to heat shock at 42 °C for exactly 30 seconds. This allowed the transformation to take place. They were then allowed to recover on ice for an additional five minutes. Following the recovery period on ice, 250 µl of LB media (10 g tryptone, 5 g yeast extract, 5 g NaCl, dH2O added up to 1 L, pH adjusted to 7.0) was added to each of the Eppendorf tubes, and afterwards the tubes were placed in a shaking incubator at 37 °C at 250 rpm. The bacteria were then allowed to grow overnight on LB agar plates (1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 1.5 g Agar, dH2O added up to 100 mL) treated with ampicillin at a concentration of 50 micrograms per millilitre. The next day, a single colony from each plate was isolated, transferred to a 15 mL Falcon tube, and cultured in 3 mL of LB medium with ampicillin at a concentration of

50 g/mL. The liquid cultures were kept at 37 °C and 250 rpm in an incubator overnight.

3.2.2. Miniprep (purification of DNA)

After the bacteria had grown in liquid culture for a full 24 hours, they were pelleted by centrifuging them at a speed of 10,000 rpm for ten minutes at a temperature of 4°C. The supernatant was discarded, and the pellet that was produced was retained for the purpose of DNA purification using a commercial kit available from Qiagen called QIAprep Spin Miniprep Kit. Following the completion of the previous step, the pellet was resuspended in 250 µl of P1 buffer before being placed in a microcentrifuge tube. Following this, 250 µl of P2 buffer were added into the tube. After that, the two buffers were thoroughly mixed until the solution became clear. Within five minutes, the reaction was stopped by adding N3 buffer, and after that, the suspension was centrifuged at 13,000 rpm for ten minutes. The QIAgen 2.0 spin column was then loaded with 800 µl of the supernatant, and the mixture was spun for one minute at a speed of 11,000 rpm in a tabletop centrifuge. The supernatant was thrown away after each of the following steps in the process. After that, the column was cleaned by using the PE buffer, and it was spun at a speed of 10,000 rpm for one minute. The supernatant was thrown away, and the tubes were centrifuged once more at 13,000 rpm for an extra minute in order to remove any residual buffer. After placing the spin columns in new, sterile Eppendorf tubes of 1.5 millilitres each and labelling them with the appropriate plasmid, the DNA was eluted by adding the elution EB buffer (0.5 mL 1 M Tris pH 7.4, 200 µL 0.25 M EDTA, dH2O added up to 50 mL). The tubes were then incubated at room temperature for one minute before being spun at 13,000 revolutions per minute for sixty seconds. We were able to determine the amount of pure plasmid DNA present in the samples by using a BioDrop LITE spectrophotometer and setting the absorbance wavelength to 260 nM. The concentrations of the samples were expressed in the form of

micrograms per millilitre, and EB buffer was used as a blank probe to establish a baseline concentration.

3.2.3. Cell lysis

After an overnight period of incubation following the transfection of the HEK293T cells, the cells were washed twice with 1x phosphate-buffered saline (PBS) (80 g NaCl, 2 g KCl, 14.4 g Na2HPO4, 2.4 g KH2PO4, dH2O added up to 1L, pH adjusted to 7.4). Following this washing step, PBS was removed from each well, and afterwards 100 µl of the Cell Lysis Buffer (5 mL 10x PBS, 5 mL 10% Trioton x-100, 1 mL 1M MgCl2, 50 µL DNaseI, 50 µL 100 mM Phenylmethyl-sulphonyl fluoride, dH2O added up to 50 mL) was added. In order to complete the Cell Lysis Buffer, we used DNasel at a concentration of 0.5 µl per 1 mL of the buffer as well as a protease inhibitor cocktail (1x concentration). The plates were then placed on ice for a period of five minutes. After that, the lysed cell suspensions were transferred to accordingly marked Eppendorf tubes with 1.5 mL capacity, and they were incubated on the rotor for a period of thirty minutes. After that, 100 µl of Protein Loading Buffer (6.25 mL 1M Tris pH 6.8, 10 mL glycerol, 20 mL 10% SDS, 3.75 mL dH2O, 5 mg bromophenol blue) and 10 µl of DTT were added to each tube, and the proteins were denatured by inserting them in a thermoblock set to 95°C for five minutes. After that, the samples were prepared for use in SDS-PAGE or for storage at -20°C

3.2.4. Measuring DNA concentrations

The concentration of the samples that were acquired by cultivating bacterial cultures and purifying plasmid DNA was measured using a BioDrop µLITE spectrophotometer, with the absorbance wavelength set to 260 nm. This allowed for an accurate analysis of the samples. It was determined that elution buffer EB would serve best as a blank probe. The

amount of the material that was examined was 1 µl. The concentrations that were obtained by measuring were recorded as micrograms per millilitre.

3.2.5. DNA gel electrophoresis

Electrophoresis on an agarose gel was used to determine whether or not the purified plasmid DNA samples were of the expected quality. The electrophoresis of DNA samples on agarose gels is a procedure that classifies the samples according to their respective sizes. The relative sizes of the samples are determined by electrophoresis. After combining the ingredients necessary to make an agarose gel (0.5 grams of agarose, 50 millilitres of a 1x TAE buffer (50x stock solution; 242 g Tris, 18.61 g EDTA, 57.1 mL acetic acid, dH2O added up to 1L), and 0.5 µl of GelGreen), the mixture was heated in a microwave for one to two minutes, or until the agarose had completely dissolved. When the gel had attained the appropriate consistency, it was loaded with 1x TAE buffer and then transported to the electrophoresis tank, where it remained until it was ready for use. On the microtiter plate, a total of 10 microliters of each sample (a solution consisting of dH2O, DNA loading buffer, and plasmid DNA) and 5 microliters of the marker were deposited (solution of DNA Ladder and DNA Loading buffer).

The electrophoresis was performed at a voltage of 140 V for a period of fifteen to twenty minutes. In order to examine the gel, a BioRad Chemi-Doc MP Imaging System was used.

3.2.6. Cell culture (Transfection and cell splitting)

For my thesis two cell lines were used: HEK239 and SH-SY5Y. HEK293 is a cell line extracted from the kidney of a human embryo. They were used for my first series of experiments because they proliferate rapidly, are

undemanding for maintenance and have a very high transfection rate which enables more protein production. The SH-SY5Y cells are a cloned neuroblastoma cell line derived from a metastatic bone tumour. In contrast to the HEK293 cells SH-SY5Y cells are difficult to sustain and perpetuate cell cycles. Both cell lines were grown in T25 flasks and furthermore they were both grown in similar media. SH-SY5Y cells used D-MEM/F-12 +/+ (ThermoFisher, 10% FCS (foetal calf serum), penicillin and streptomycin solution and 1x MEM non-essential amino acids.) media whereas HEK293 cells used D-MEM +/+ media (ThermoFisher) containing 10% CCS (Cosmic Calf Serum), penicillin and streptomycin solution. Both media are stored at 4°C The cell lines were used exclusively when they reached 80% or higher confluency. At that moment they would be split into several flasks to enable further growth, or they would be arranged in 12 or 24 well plates if used for experiments.

Working in a culture hood requires sterile conditions for this reason, each item that was used in the hood including the workstation was required to be sterilized with 70% ethanol before use. After using the hood, in addition to 70% ethanol to ensure sterile conditions 1% incidine disinfectant was used as well. Furthermore, the hood had a UV light installed which was switched on for 10 minutes before and after working in the hood. Disposable instruments such as serological pipettes that are used in the hood are to be opened in the hood and sterile.

Once the cells are confluent and adhered to the flask surface, Trypsin/EDTA enzyme (Pan Biotech, Trypsin 0.25%/EDTA 0.02%, in PBS w/o: Ca^{2+} and MG^{2+}, w: Phenol red) is used to disassociate the bond between the cells and the flask while keeping the cells vital. When the cells are detached from the flask and D-MEM is added to counteract the trypsin reaction, they are ready to be transferred to additional flasks containing fresh medium. If the cells are used for immunocytochemistry, then a glass coverslip was added to each well of a 12 or 24-well plate

which contained a cell population. All of the flasks and plates were stored in an incubator (Nüve CO_2 incubator (EC 160) at 37°C/5% CO_2).

In order to carry out the transfection procedure, two different sets of solutions had to be prepared: the first contained 0.5 g of plasmid DNA and 100 µL of D-MEM -/- (without serum or antibiotics), and the second contained 2 µL of the transfecting reagent (Metafectene or Metafectene Pro (Biontex)) per well. Both sets of solutions were then mixed together. The solutions were allowed to incubate for five minutes at room temperature. After the incubation, 100 µL of a solution that included the transfecting reagent was added to each tube that contained DNA plasmid, and then the tubes were put in an incubator at 37°C and 5% CO_2 for another 30 minutes. After removing the media containing the serum and antibiotics from the wells, new media containing DMEM -/- was added to the wells. After pipetting the solutions into the wells, the plates were placed in the incubator for a total of 6 hours. Following the incubation, the media were changed out for new media that included serum and antibiotics. Plates were then left to incubate overnight.

3.2.7. SDS and Western Blot (staining)

SDS-PAGE

Samples for SDS-page must first be diluted 1:1 with a 2x protein loading buffer (6.25 1M Tris [pH 6.8], 10 mL glycerol, 20 mL 10% SDS, 3.75 mL dH2O, ~ 5 mg 21 bromophenol blue). The next stage was to add 1M dithiothreitol (DTT) in a volume equal to 10% of the total volume. The samples are heated at 90°C for 5 minutes to further denature the protein. The samples are cooled on ice prior to getting loaded into the gel. The samples are deposited in 10 µl volumes and the marker/ladder in 3.5 µl. The SDS-PAGE is conducted at 180V for 45 minutes. If necessary, the time can be extended.

Transferring to membrane

Membrane transfer is a process in which we transfer the protein from the Gel in SDS-PAGE to an activated PVDF membrane. The membrane is then used in Western Blot. The Transfer is done using a Trans-Blot Turbo™ Transfer System. After the transfer is done the membrane is treated with 20-50ml of Ponceau S staining solution so we can visualise the proteins apart from the membrane.

Western Blot

The Western Blot method is used to analyse the results on the PVDF membrane by binding primary and secondary antibodies onto the samples. The membrane must first be blocked using 5% milk powder in PBS-T. The membrane is then treated with the primary and secondary antibody correspondingly. The membrane is coated with the ECL mixture prior to being sent to the Bio-Rad ChemiDoc Imaging system. When we examine the Blots, we merge the marker images and overlay them with the protein Blot images, and then we utilize the marker ladder to identify the size of our protein band.

3.2.8. Cell staining immunocytochemistry

Fixation and permeabilization

Immunocytochemistry was done on the SH-SY5Y cell line. The cells contained in their respective wells on a 12 or 24 well plate was fixed to glass cover slips used for microscopy with a fixation buffer containing 4% paraformaldehyde (PFA) (Merck KGA) Subsequently a permeabilization buffer (10 mL 10x PBS, 10 mL 10% Triton X-100, dH2O added up to 1 L) was used on the cells which allowed the stains and antibodies to enter the intracellular area.

Staining

The next step was the start of the staining protocol. To begin with all the nonspecific antibody binding sites had to be blocked using a 10% goat serum/PBS solution. When the blocking was concluded we could add the primary antibody. While working on SH-SY5Y cells the primary antibody was deposited into a 1 to 1000 dilution of a 10% goat serum/PBS solution. The practice was to leave on the primary antibody for 3.5 hours and place a damp paper towel so as to avoid the probability that the cells on the cover slip would dry off. The following steps consisted of adding the secondary antibody and the two stains into a 10% goat serum/PBS solution. The secondary antibody and the stains were added in a 1/500 dilution. The solution was applied to the cells and left on for 1 hour concealed in the dark with the use of aluminium foil.

Mounting

The mounting process was done with the use of a modified needle and small forceps to extract the cover slips onto the mounting media which was applied to a glass microscope slide.

3.2.9. Blinded cell stress

In order to conduct the portion of the experiment, which was blinded, 24 Eppendorf tubes had to be prepared. Twelve of them were filled with the selected stressor, which was MG-132, while the other twelve were filled with distilled water. After that, one of my colleagues in the lab was tasked with randomizing all 24 wells. After randomly assigning the tubes, I proceeded to treat all 24 wells with transfected SH-SY5Y using the solution that was included in each of the tubes, while simultaneously labelling the wells with the number that corresponded to the number that was written on the tube. After that, the process was exactly the same as it would have been for any immunochemistry procedure.

3.2.10. Microscopy

The method used for analysing results after immunocytochemistry was immunofluorescent microscopy. The method was done on an Olympus IX83 fluorescent microscope. A Hamamatsu Orca R2 CCD camera and CellSens software were used in the process of capturing and preparing the images for presentation in the thesis. Every experiment that was performed on SH-SY5Y was carried out three times, and each protein that was studied was subjected to fluorescence microscope examination.

4. RESULTS

In the course of my research, I made use of two different variations of TRIOBP-1 to ascertain the circumstances under which the protein aggregates. The TRIOBP-1 (60-652, Δ333-340) is the one which has deletions in the two regions which are thought to be the ones responsible for the aggregation (Figure 3). Additionally, there is a wild type full-length TRIOBP-1 (aa 1-652) variant. After that, these two TRIOBP-1 variations were expressed in two different cell lines, HEK293 and SH-SY5Y cells respectively. The outcomes of expressing them *in vitro* are reported here.

Figure 3. 1) shows the 25 amino acid long "linker" region which is suspected to be the region that is aggregation prone. Prolines are shown in purple while the charged residues are shown as red for positive charge or blue for negative. 2) The same "linker" region but the crossed out amino acid chain represents the deletion from 333 to 340.

4.1. Confirmation of TRIOBP-1 protein expression in HEK293 by Western Blotting

We made the decision to validate that the constructs were successfully expressed in mammalian cells before continuing with our investigation into whether these constructions form aggregates in cell culture. In order to achieve this goal, the human embryonic kidney (HEK293T) cell line was cultured and transfected with both constructs. These were then lysed, followed by Western Blot and detection using an anti-Flag antibody.

Figure 4 shows that there are two distinct protein bands, that can be observed below and the size of the two different structures is equivalent to what we had anticipated it to be. The first is a full-length construct, whereas the second is a mutant form of the protein.

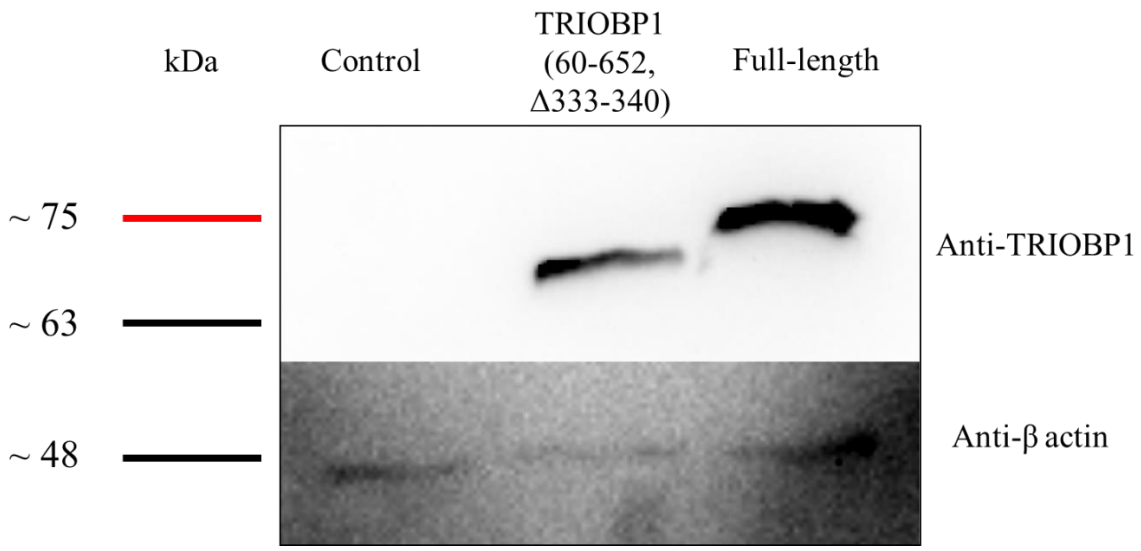

Figure 4. Western Blot analysis of the two protein constructs expressed in HEK293T cells. The two images show a membrane which was obtained by utilizing the western Blot method on the lysates of the cells transfected with the full-length and mutant plasmid respectively.

4.2. Observing TRIOBP-1 protein aggregates in HEK293T cells on a fluorescent microscope

The following stage, which was to view the cells using a fluorescent microscope, was taken after we discovered positive findings in the process of identifying the protein expression using the western Blot analysis.

The HEK239T cell line served as the first subject of the experiment. The monoclonal anti-FLAG M2 antibody was used as the main antibody, while the Alexa Fluor 594 goat anti-mouse IgG antibody was used as the secondary antibody. Regarding the stains, we used phalloidin conjugated with Alexa Fluor 488 for the purpose of staining the cytoplasm, and DAPI was utilized in order to stain the nucleus. The whole procedure was intended to be carried out thrice before one could assert with certainty that the findings are accurate.

Because of the secondary antibody which binds to the anti-FLAG M2 primary antibody, the TRIOBP-1 aggregates glowed with a brilliant red

glow when viewed under the microscope. The DAPI stain caused the nucleus of the cell to shine a vivid shade of blue, whereas the phalloidin stain rendered the actin and the cytoskeleton a bright shade of green colour. The fluorescent microscopy method was carried out with the assistance of cell sense software and used an Olympus IX83 fluorescent microscope.

The results demonstrated the presence of aggregates in the cells which contained the gene variation corresponding to the wild type, however the cells with the mutant construct that had the deletion did not show any aggregation of protein despite the fact that transfection was still visible.

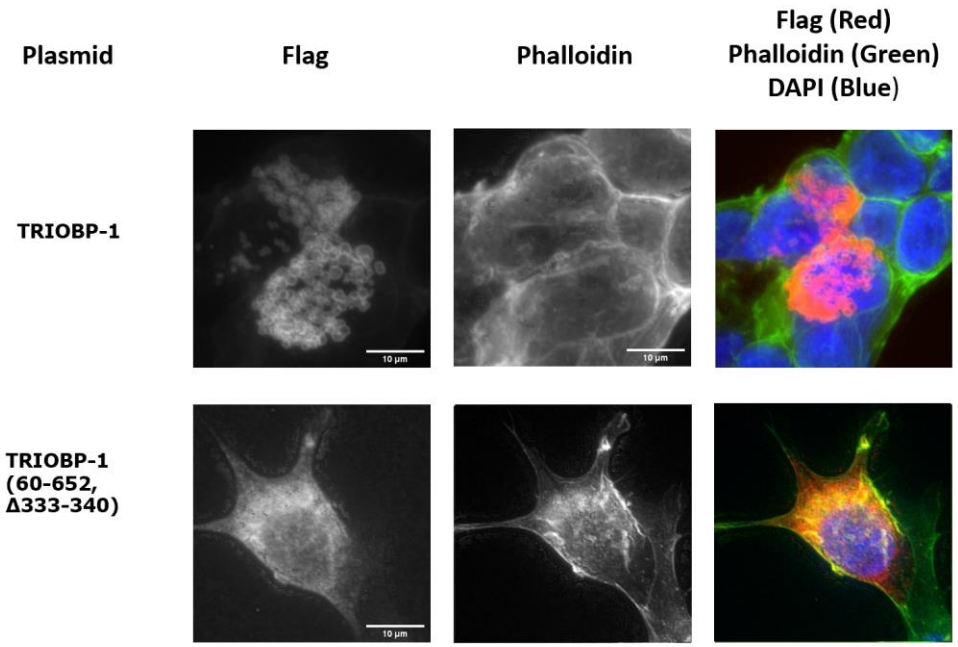

Figure 5. Fluorescent microscopy images of full-length TRIOBP-1 and its mutant counterpart in HEK293T cells. Full-length displays aggregates whereas the construct does not. The images were taken on 60x magnification on the Olympus IX83 fluorescent microscope using CellSense software. The scale bar represents 10 µm.

4.3. Observing TRIOBP-1 protein aggregates in SH-SY5Y cells on a fluorescent microscope

This approach of studying TRIOBP-1 aggregates was done in SH-SY5Y because we want to simulate how the protein will behave in a cell that shares characteristics with human neuronal cells. The SH-SY5Y cells and the HEK293T cells undergo transfection differently due to the fact that for the SH-SY5Y cells, we make use of Metafectene PRO as an assist in transfection rather than the standard Metafectene that is used for the HEK293T cells. Aside from that difference, the procedure for staining the cells and observing them under the fluorescent microscope is identical.

The findings lead to the conclusion that the behaviour of TRIOBP-1 aggregation is exactly the same as the results shown in HEK293T cells (Figure 6).

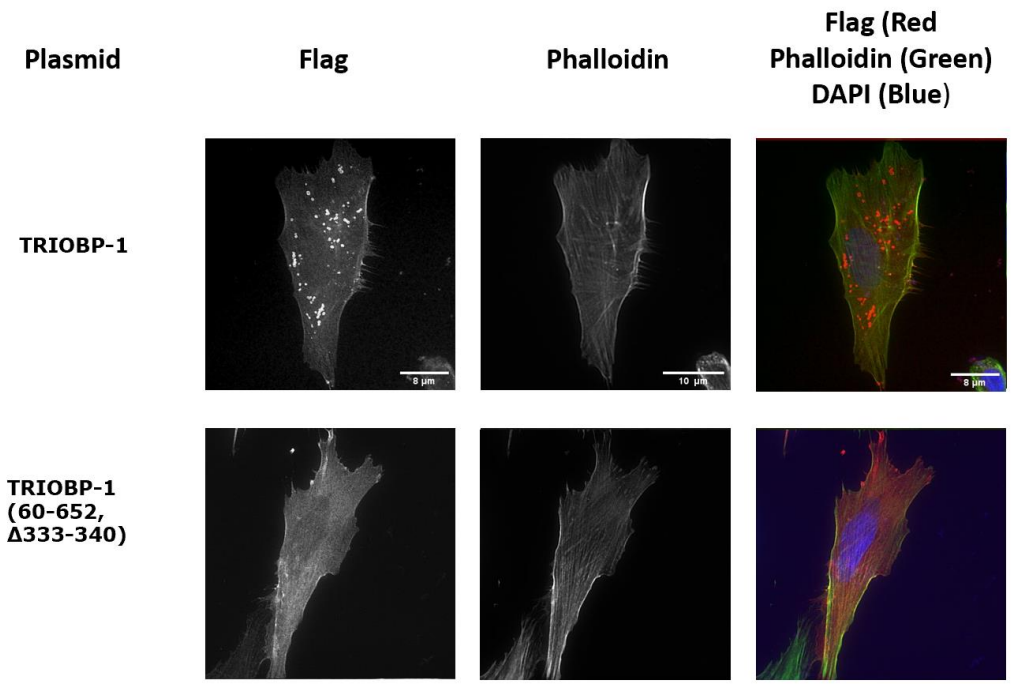

Figure 6: Fluorescent microscopy images of full-length TRIOBP-1 and its mutant counterpart in SH-SY5Y cells. Full-length displays aggregates whereas the construct does not. The images were taken on 60x magnification on the Olympus IX83 fluorescent microscope using CellSense software. The scale bar represents 10 µm.

4.4. How does TRIOBP-1 and its mutant counterpart behave in SH-SY5Y when stress is introduced?

In light of the fact that the studies carried out in HEK293T cells and SH-SY5Y cells had shown fruitful findings and provided some insight into the behaviour of TRIOBP-1, I chose to carry out a further set of tests. In light of the research previously conducted by Mihaela Bergman on the effects of stress factors on NPAS3, I made the decision to use the similar methodology and apply it to my TRIOBP-1 protein constructs.

The stressors that made it into the final list were selected with reference to Mihaela's study as well. The initial stressor was the chemical compound $FeCl_2$. It was reported that being exposed to $FeCl_2$ leads to an increase in the generation of reactive oxygen species (ROS), which in turn leads to an increase in oxidative stress (22). Sodium arsenite was yet another component that contributed to stress and was responsible for the induction of oxidative stress by creating reactive oxygen species. One further trait that sodium arsenite has been shown to have in a number of studies is the ability to change the expression of genes associated to the body's stress response as well as genes involved in the production of antioxidants (23).

Comparing the consequences of each stress factor and observing how the addition of each stress factor to the transfected cells would impact protein aggregation permitted me to determine which stress factor would be the primary focus of my research. This was done for both the wild type and the mutant strains.

MG-132, a cell permeable proteasome inhibitor, was the final stress factor, and the one that I ultimately utilized in the experiment. By attaching to the active site of the proteasome, MG-132 is able to block the proteasome from carrying out its normal role of degrading defective proteins via the process of proteolysis.

After performing initial stress tests, during which we compared the effects of the three stressors that had been shortlisted, the results showed that the proteasome inhibitor might be the best choice if we are trying to induce an increase in protein aggregation. This was discovered as a result of the findings of the first stress test.

MG-132 was the most suitable option for a number of reasons, including the following:

- To begin with, after treating the cells with MG-132, the cells kept their standard structure, and the actin in the cytoskeleton was abundant and easily visible. While the cytoskeleton was disrupted in the cells that had been treated with sodium arsenite and iron (II) chloride, and the overall structure of the cell was damaged in the other two cases (Figure 7).

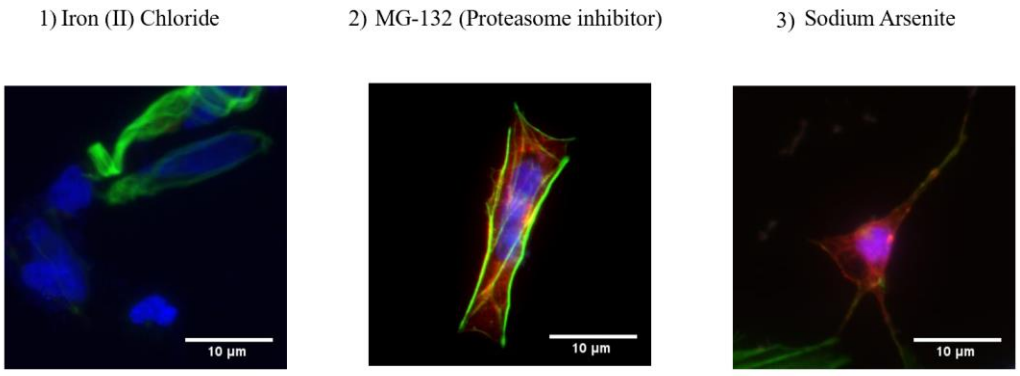

Figure 7: SH-SY5Y transfected with the mutant plasmid cells treated with stressors.

1) Shows cells treated with iron (II) chloride and we can see that the structure of the cell is destroyed. 2) Shows a healthy transfected SH-SY5Y treated with MG-132. 3) depicts a SH-SY5Y which has a compromised cytoskeleton owing to the treatment with sodium arsenite. Images were taken on 60x magnification on the Olympus IX83 fluorescent microscope using CellSense software. The scale bar represents 10 µm.

- The second reason why MG-132 was selected was because, in contrast to the other stressors, it demonstrated an increase in the amount of protein aggregates in the cells that had been transfected with the full-length TRIOBP-1 plasmid (Figure 8)

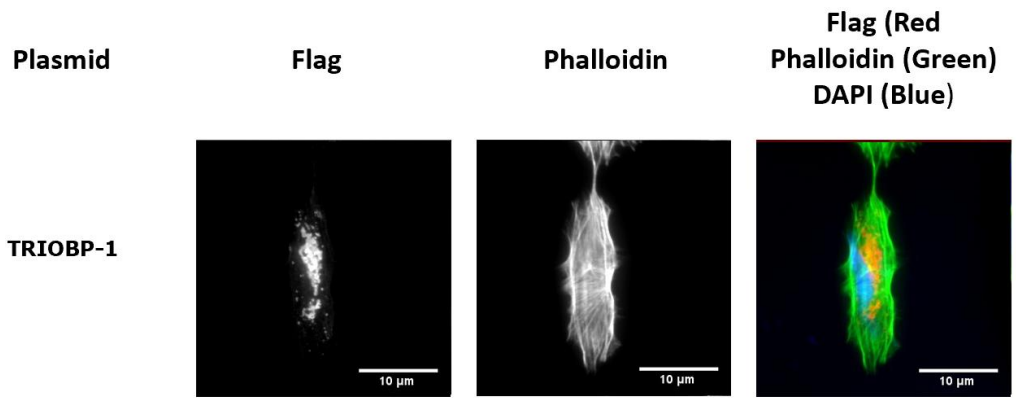

Figure 8: SH-SY5Y cells transfected with full-length TRIOBP-1 treated with MG-132. This figure shows an abundant amount of aggregates in SH-SY5Y cells when treated with MG-132. The images were taken on 60x magnification on the Olympus IX83 fluorescent microscope using CellSense software. The scale bar represents 10 µm.

4.5. Blinded test on SH-SY5Y treated with the proteasome inhibitor

In order to examine the impact that the stressor had on protein aggregation, an experiment that was conducted in the form of a blinded assay was required.

Unexpectedly, the blinded experiment revealed that treating the SH-Y5Y cells did not increase the number of aggregating cells transfected with the full-length plasmid. Also, the number of aggregating cells transfected with the TRIOBP-1 mutant plasmid (60-652, Δ333-340) did not increase statistically significantly. But there was a difference in behaviour of both constructs.

The results of the blinded experiment are shown in (Figure 9). Stressed WT cells showed 8.33 ± 0.8 aggregating cells, while the MT cells showed 3.83 ± 0.3. aggregating cells. The non-stressed WT cells demonstrated 8 ± 0.7 aggregating cells whereas the average number of MT cells equated to 5 ± 1.2 aggregating cells.

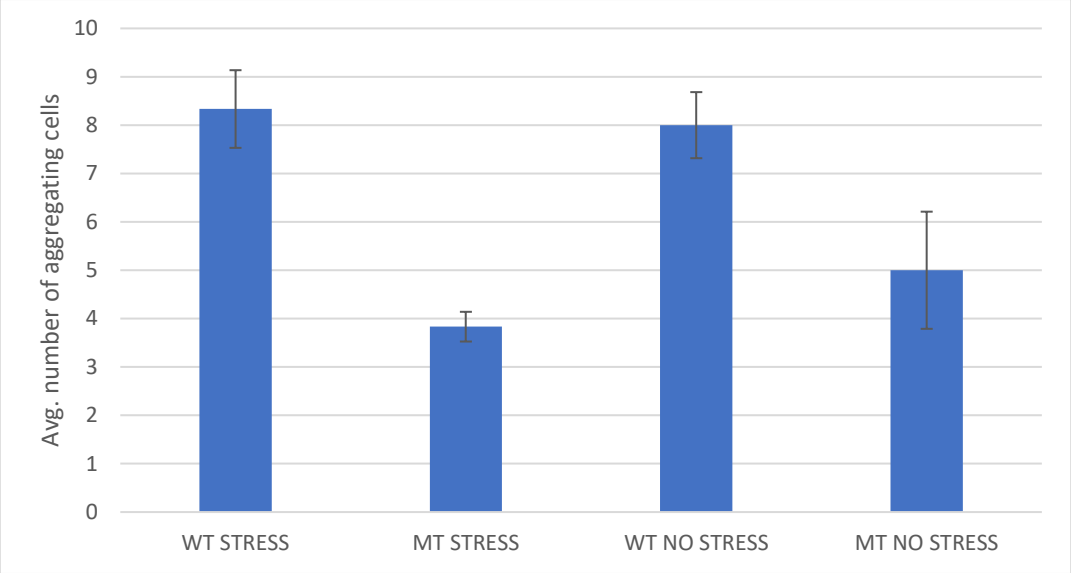

Figure 9: Graph represents the average number of cells which have shown aggregation for each construct and weather or not it was treated with MG-132. The first column represents all cells which were transfected with the full-length plasmid and were not treated with MG-132. The second column represents cells which were transfected with the mutant plasmid, and which were also not treated with MG-132. The third column shows the average number of aggregating cells which were transfected with the full-length plasmid but were treated with MG-132 and finally the fourth column is displaying the average number of aggregating cells on the treated cells transfected with the mutant plasmid.

Since the outcomes of the blinded trial were unexpected, next step was to determine the quantity of aggregates in each transfected cell.

The results are shown in Figure 10. We can observe that the number of protein aggregates in each cell transfected with the full-length plasmid and treated with the proteasome inhibitor did not change when compared with the full-length transfected cells which were not treated with the proteasome inhibitor. The same results can be observed in the case of cells treated with the TRIOBP-1 (60-652, Δ333-340) plasmid.

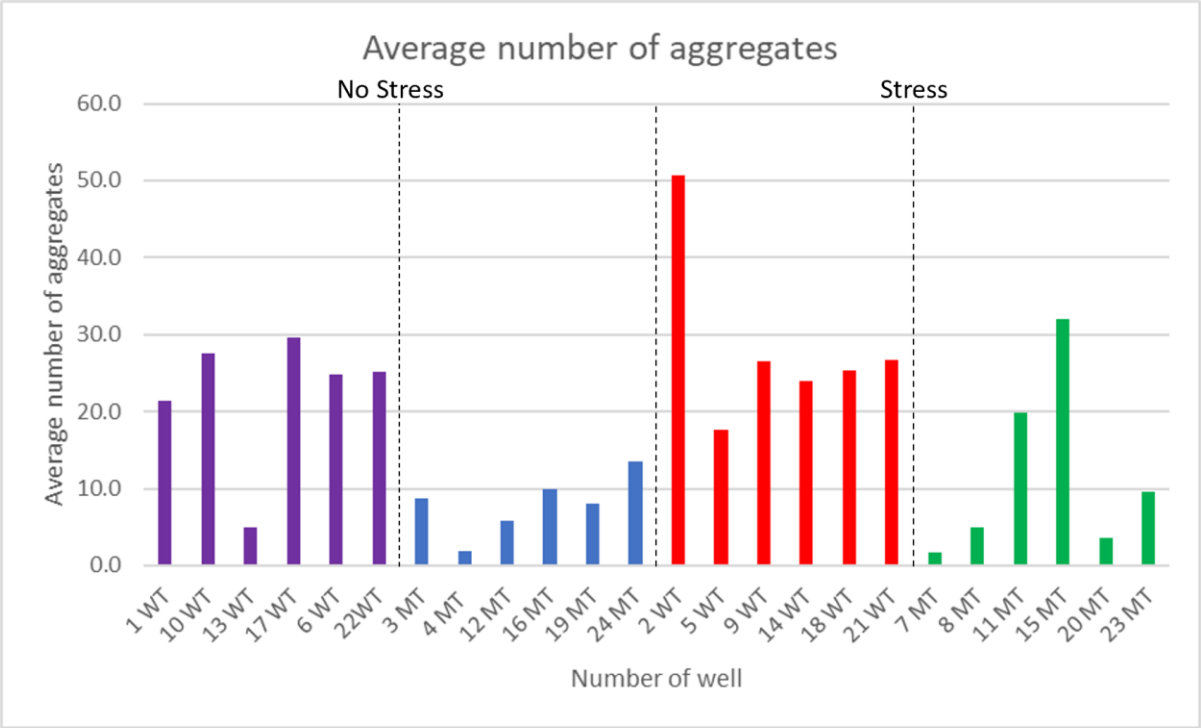

Figure 10. Graph which represents each well in the blinded experiment. On the X-Axis are the wells, whereas the Y-Axis is represented by the average number of aggregates. The set of results in purple represent the wells which contained cells transfected with the full-length construct and were not treated with MG-132. Blue represents the mutant set which also was not treated with MG-132. Red represents full-length with MG-132 and Green represents the mutant plasmid treated with MG132.

5. DISCUSSION

My goal in writing this thesis was to demonstrate that there is a difference between the wild type version of TRIOBP-1 and the mutant variant of this protein.

The two variations that were selected were first expressed *in vitro* using HEK293T cells and SH-SY5Y cells. The selection process was based on prior research. Our findings were validated by a Western Blot analysis, which also demonstrated that the protein that was effectively produced in the cells had the correct size. This was done so that we could be certain that the protein had been correctly transfected. The following step was to determine whether both of the constructed proteins expressed observable protein aggregates in the two different cell lines. The first test, which was performed on the HEK293T cell line, revealed that the full-length construct displayed aggregation, but the mutant construct did not exhibit any aggregated in any of the repetitions of the experiment (Figure 5). This finding was in accordance with what was anticipated. The next step of the procedure in the research was to conduct the same experiment using the SH-SY5Y cell line, and as was to be anticipated, the outcomes were the same (Figure 6).

The surprise findings of the conducted blinded test indicated that the variant TRIOBP-1 (60-652, Δ333-340) appeared to display aggregation in SH-SY5Y cells, just like the full-length wild type construct did (Figure 9). The aggregate like structures visible in the cells transfected with the variant TRIOBP-1 (60-652, Δ333-340) could be the consequence of an overaccumulation of protein in one spot due to overexpression. An overaccumulation of protein in one spot could lead to TRIOBP-1 (60-652, Δ333-340) to form clusters that look like aggregates but are not in fact real aggregates, as are in the case of cells treated with the full-length plasmid. The aggregate-like clusters of TRIOBP-1 (60-652, 333-340) may have been structures that were linked with endocytic vesicles and were

associated with the endocytic recycling compartment. It is plausible that the clusters of TRIOBP-1 (60-652, 333-340) that we are observing are really colocalized endocytic vesicles. To support the validity of this notion, further study would have to be performed.

This discovery came as a total surprise due to the fact that the construct that included the deletions demonstrated that the deletions fully eliminate the tendency of the protein to aggregate. Because the research on TRIOBP-1 demonstrated that two regions, a stretch of eight amino acids (333-340) and an optionally expressed stretch of fifty-nine amino acids at the extreme N-terminus of the protein, are responsible for the tendency of the protein to aggregate, and because both of these regions were removed from the construct variant of the protein, we can assume that the aggregates in this instance are soluble aggregates or other accumulations. Further ultra-centrifuge testing is required to determine whether or not the assertion is correct. This test is based on the idea that aggregated proteins are normally insoluble, and therefore involves purifying the insoluble fraction of a sample, and testing for specific proteins by Western Blotting (21). If the ultracentrifugation test was done to prove that the accumulations found in SH-SY5Y cells we would expect to see bands in the homogenate fraction and no bands in the aggregome fraction proving that the TRIOBP-1 (60-652, Δ333-340) clusters are not insoluble aggregates.

Prior studies have shown that schizophrenia may be associated with a malfunction of the proteasome (25), which most likely has a significant bearing on the proteomic landscape and the cellular function involved in the pathophysiology of this illness. This was one of the primary considerations that went into deciding to make MG-132 the source of the stress. It is possible that the proteasome inhibitor was not functioning correctly, which is something that we could disprove if we used some kind of positive control in the form of an indicator that would show us if the

proteasome inhibitor is performing its function. If this was the case, then the experiment would not have shown the results that were expected.

The results of all of the methods that were used in the research for this thesis supported the fact that there is a distinction between the full-length TRIOBP-1 plasmid and the construct TRIOBP-1 (60-652, 333-340) in the way that they behave *in vitro*, which confirms that the deletions in the construct completely abolish the propensity for aggregation. When the cells were treated with the proteasome inhibitor MG-132, there was no increase in the formation of structures that resembled aggregates in TRIOBP-1 (60-652, 333-340). This lends more credence to the idea that the mutations not only lessen the probability of aggregation but also eliminate it entirely.

In spite of the fact that it has not been established for certain whether or not TRIOBP-1 plays a role in the onset of schizophrenia, we are able to further our understanding of the role it plays as an aggregating protein. Even though the results of my experiment did not result in the anticipated way, which would be that inhibiting proteasomes leads to an increase in the number of aggregates and an increase in the number of cells that have aggregated, there are still possibilities on how the work that I have done could have been done differently or improved using different methods.

Since treating the cells with the proteasome inhibitor did not produce any satisfactory results, more research could be conducted by combining this proteasome inhibitor with a stress factor, such as sodium arsenite. If we treated the cells with an inhibitor of the proteasome, which prevents the cells from carrying out their protein-degrading function, and then introduced a stress factor afterward, we would be able to observe the effect that the stressor has on protein aggregation without the protein being subjected to degradation by the proteasome. Since we already know that the function of the proteasome is impaired in people who suffer

from schizophrenia, I believe that doing an experiment like this might be a potential way to get some understanding about chronic mental illnesses.

In addition, the statistical analysis, in order to be more precise, would have to have a significantly larger collection of data points than I have had in my experiment; yet, doing such a task would be too demanding for a single graduate student.

In most instances, the resolution of the microscope was not high enough to differentiate one kind of aggregate from another. Due to the fact that the cells are observed on a two-dimensional plane, it is difficult to determine whether or not aggregates may be stacked on top of each other, which makes it difficult to acquire an accurate estimate on the total number of aggregates, which was another problem associated with the method by which the data were gathered. When capturing and analysing the pictures that were collected, the usage of a confocal microscope would be required in order to get a higher resolution. This would allow for a more accurate data set to be generated. On a related note, if we are conducting research into the effect that stress has on the aggregation of proteins, I believe that live cell imaging would be a valuable addition to the existing knowledge. This would allow us to determine at what stages of the cell cycle the aggregation of proteins takes place as well as the effect that the introduction of a stressor has on it.

6. CONCLUSION

Since studies have showed that protein aggregation could be a possible cause of chronic mental illnesses, and since TRIOBP-1 is implicated as aggregating in schizophrenia, this thesis aimed to investigate any differences between wild type TRIOBP-1 and a mutant with deletions in the regions that were found to cause aggregation. HEK293T and SH-SY5Y cells were used to express the two variants *in vitro*. In both HEK293T an SH-SY5Y, the full-length construct aggregated, while the mutant construct did not as was expected. The blinded, quantified test revealed that mutant TRIOBP-1 (60-652, Δ333-340) appeared to aggregate in SH-SY5Y cells like the full-length wild type construct. This could be owing to overaccumulation of protein in one area which could lead to the formation of clusters that appear like aggregates but are not. An ultracentrifuge-based insolubility test could confirm the claim. This revelation was a complete surprise because the deletions completely eliminated rather than just supressed the protein's capacity to aggregate. When treated with the proteasome inhibitor MG-132, TRIOBP-1 (60-652, Δ333-340), aggregates did not show an increase in aggregation. This supports the assumption that mutations abolish aggregation completely. All of the methodologies utilized in this thesis research corroborated the fact that the full-length TRIOBP-1 plasmid and the construct TRIOBP-1 (60-652, Δ333-340) behave differently *in vitro*, confirming that the deletions in the construct completely remove the propensity for aggregation. Further research should concentrate on a combination of a stress factor as well as inhibiting the proteasome to observe the behaviour of TRIOBP-1. Despite not knowing if TRIOBP-1 causes schizophrenia, we can better understand its role as an aggregating protein and possibly link it to schizophrenia in the future.

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MARIO BABIĆ

Split, Croatia

+385996388452 mario.babic@uniri.hr

PROFILE

Dedicated Biotechnology Student with the ability to multitask and work well with others. Adept in making key decisions and working with other professionals to achieve goals and solve problems. Well-rounded and professional team player dedicated to continuing academic pursuits at a collegiate level.

EMPLOYMENT HISTORY

- **Student Manager, Last Minute rent a car** ..... May 2018 — Oct 2021
  Split

EDUCATION

- **Split School of Natural Sciences - Chemical Engineering** ..... Sep 2014 — Jul 2018
  High school Split

- **University of Rijeka, Department of Biotechnology** ..... Oct 2018 — Present
  Undergraduate programme "Biotechnology and Drug Research" Rijeka

SKILLS

Ability to Work in a Team ..... ***Expert***
Microsoft Office ..... ***Expert***
Ability to Multitask ..... ***Expert***
Fast Learner ..... ***Expert***
Communication Skills ..... ***Expert***

LANGUAGES

Croatian ..... ***Native speaker***
English ..... ***Highly proficient***
German ***B2***

COURSES

- **Joint ICGEB – ALS Society of Canada Symposium on Inflammation & Proteinopathy in ALS/FTD Spectrum Disorder.** ..... Jul 2022
  Department of Biotechnology, University of Rijeka

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