Investigating aggregation of CRMP1 isoforms in relation to schizophrenia research

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UNIVERSITY OF RIJEKA

DEPARTMENT OF BIOTECHNOLOGY

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Istraživanje agregacije izoformi CRMP1 povezanih sa shizofrenijom

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Thank you for always having my back!

This undergraduate final thesis was defended on 21st of September 2023

In front of the Committee:

1. prof. dr. sc. Milan Mesić
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3. izv. prof. dr. sc. Nicholas J. Bradshaw

This thesis has 36 pages, 2 tables, 10 figures and 41 citations.

Abstract

The majority of chronic mental illnesses (CMI) involve risk factors that contribute to the illness's onset and do not have a single underlying cause. No specific singular cause or mechanism for the development of mental illnesses have been established yet.

Prior studies have revealed the role of protein aggregation as a potential cause of chronic mental illness. CRMP1 is one of the most recently found and least studied proteins whose aggregation is associated with mental illness, particularly schizophrenia. Different versions of the CRMP1 Lv isoform, with deletions of different parts of N-terminal region were explored in this research to determine the influence of N-terminal region on aggregation.

CRMP1 isoforms were expressed in vitro using HEK293 and SH-SY5Y cells and examined using fluorescent microscopy and a quantitative blinded test assay.

While previous studies have suggested CRMP1 Lv to be more prone to aggregation than CRMP1 Sv, in this more relevant and through experiment both CRMP1 Lv and CRMP1 Sv appear to have similar levels of aggregation. Compared to other studies, a relatively small, FLAG

tag is used in this research, which is less likely to obstruct expression and function of protein than GFP fusion proteins used previously, and using human neuroblastoma cells SH-SY5Y provides a more realistic setting than in prior research.

Although the results were not statistically significant, they do suggest that the unique N-terminus region of CRMP1 Lv may be required for its aggregation.

Thus, further research is needed, and it should include examining the effects of proteasome inhibitor and/or introduction reactive oxygen species triggering the cell stress. Co-aggregation with DISC1 should also be investigated further with different isoforms of CRMP1 Lv.

Key words: protein aggregation, CRMP1, mental illness, schizophrenia

Sažetak

Većina mentalnih bolesti ima nekoliko rizičnih čimbenika koji pridonose nastanku bolesti i nemaju jedan temeljni uzrok. Specifični pojedinačni uzroci ili mehanizmi za nastanak mentalnih bolesti još uvijek nisu utvrđeni.

U prethodnim studijama prepoznata je uloga agregacije proteina kao potencijalnog uzroka kroničnih mentalnih bolesti. CRMP1 je jedan od nedavno otkrivenih i najmanje proučavanih proteina čija je agregacija povezana s mentalnim bolestima, posebice shizofrenijom.

Istraženi su različiti izoformi CRMP1 Lv s delecijama različitih dijelova N-terminalne regije kako bi se odredio utjecaj N-terminalne regije na agregaciju. Izoforme CRMP1 eksprimirane su *in vitro* korištenjem HEK293 i SH-SY5Y stanica te su proučavane korištenjem fluorescentne mikroskopije i zaslijepljenog testa.

Dok su prethodne studije sugerirale da je CRMP1 Lv skloniji agregaciji u usporedbi s CRMP1 Sv, u ovom, relevantnijem istraživanju čini se da i CRMP1 Lv i CRMP1 Sv imaju slične razine agregacije. U usporedbi s drugim studijama, relativno mala FLAG oznaka korištena u ovom istraživanju smanjuje vjerojatnost ometanja ekspresije i funkcije proteina, a korištenje stanica ljudskog neuroblastoma SH-SY5Y predstavlja prirodnije i pogodnije uvjete za eksperiment, stoga su i dobiveni rezultati realističniji I relevantniji u usporedbi s dosadašnjim istraživanjima.

Iako rezultati nisu bili statistički značajni, oni sugeriraju da je jedinstvena N-terminalna regija CRMP1 Lv možda potrebna za njegovu agregaciju.

Stoga su potrebna daljnja istraživanja koja bi trebala uključivati ispitivanje učinaka inhibitora proteasoma i/ili uvođenja reaktivnih vrsta kisika radi izazivanja oksidativnog stresa. Daljnji koraci trebali bi uključivati i ispitivanje koagregacije izoforma CRMP1 Lv s DISC1.

Ključne riječi: agregacija proteina, CRMP1, mentalna bolest, shizofrenija

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1. INTRODUCTION

1.1. Mental illness and schizophrenia

Our daily lives are greatly influenced by our mental health, which is a crucial component of our overall well-being. It affects how we react to stress, interact with others, and make prudent decisions. It impacts how we feel, think, and behave.

Mental illnesses are the general term for all diagnosable mental diseases. This includes all significant alterations in behavior, state of mind or thought and distress and/or difficulty engaging in family, social or professional activities [1].

The National Alliance of Mental Illness reports that 22.8% of USA adults experienced mental illness, while 5.5% of USA adults experienced serious mental illness. In the USA, 16.5% of adolescents aged 6 to 17 had experienced a mental health problem. Suicide is third among all causes of death for individuals the ages of 15 to 24 and second among those between ages of 10 and 14. It ranks as the 12th most common cause of mortality overall. According to their psychological autopsy, 90% of those who committed suicide had experienced mental health conditions, and 46% of people who die by suicide had a diagnosed mental health condition [2].

The majority of mental illnesses involve several risk factors that contribute to the illness's onset and do not have a single underlying cause.

Recently, approach to diagnostics and treatment of mental illness is starting to improve as seen in the International Classification of Diseases (ICD). ICD-11 presents movements in a direction of dimensionality which are intended to offer a more in-depth and thorough understanding of mental health conditions, enabling a better evaluation of symptoms and individual variances. But diagnosis still remains primarily based on the evaluation of patients' symptoms, medical history and clinical judgment [3] [4].

Stigma associated with mental illness remains a significant barrier that stops people from seeking medical assistance. Therefore, the fact that globally, more than 70% of people suffering some form of mental illness does not receive any treatment [5] does not come as a surprise. 20-60% of patients with psychiatric disorders are classified as treatment resistant type [6]. No specific singular cause or mechanism for the emergence of mental illnesses were established yet.

Schizophrenia is a complex chronic mental illness. Patients suffer a wide range of symptoms that generally fall into three main categories: cognitive, negative and psychotic. Cognitive symptoms include poor concentration and memory, difficulty expressing thoughts and understanding and slow and disorganized thinking. Apathy, social withdrawal and lack of motivation fall into negative symptoms, while thought disorder, hallucinations and delusion are described as psychotic symptoms. In order to diagnose schizophrenia, symptoms need to persist for at least six months, and they usually occur in early adulthood [74].

The genetic component of schizophrenia was shown by higher concordance rates in monozygotic twins in comparison with dizygotic [8]. Overall, it involves the interplay of multiple genetic and non-genetic determinants.

A combination of factors that include prenatal risk factors, genetics, environment, lifestyle, biochemical imbalance in brain and more cause mental illness. The complex genetics, developmental biology, and late adolescent/early adult onset of schizophrenia are summarized by the two-hit hypothesis of schizophrenia. This theory postulates that early central nervous system (CNS) development is disrupted by genetic or environmental factors, resulting in long-term susceptibility to a "second hit" that triggers the onset of schizophrenia symptoms (Figure 1) [9].

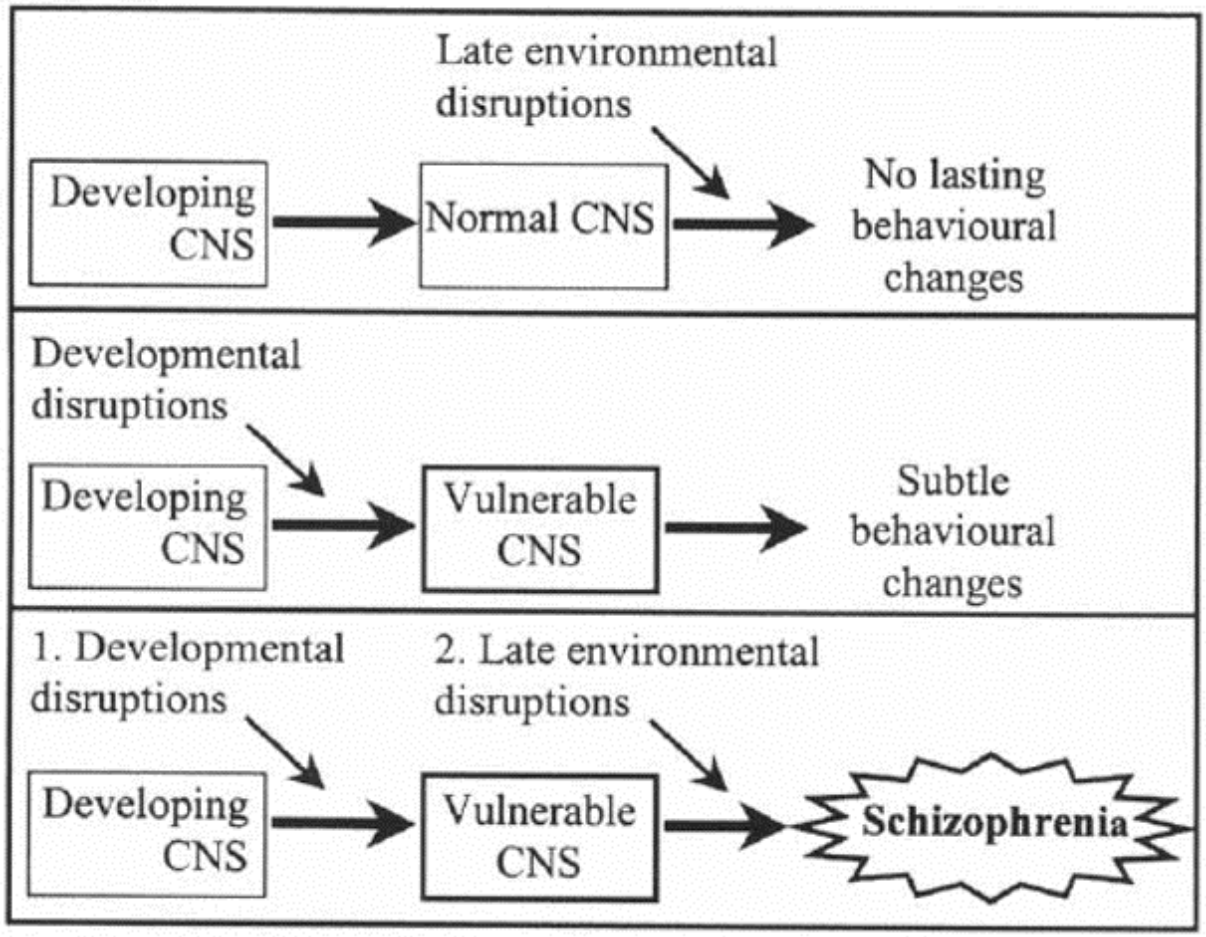

Figure 1 Graphical representation of two-hit hypothesis of development of schizophrenia. Taken from Buusen et al. 2003 [9]

The first hit may come from the environment or the genes, such as stress or an infection during pregnancy. Any additional stressor that manifests later in life, such as drug usage or trauma, can be considered the second hit. According to the two-hit hypothesis, a person's genetic predisposition to vulnerability (the first hit) could make them more susceptible to environmental stressors (the second hit). The theory has drawn more interest as a result of its plausible integration of genetic, developmental, and environmental elements that affect schizophrenia pathogenesis and susceptibility.[10,11]

1.2. Protein aggregation

Proteins are responsible for the development and upkeep of neuronal membranes as well as being the structural support for neurons and involved in the perseverance of the integrity and overall structure of the brain. Some act like catalytic enzymes for a variety of biochemical reactions. Enzymes play a crucial role in neurotransmitter synthesis, energy production and signal transduction. Neurotransmitters are essential in managing mood, behavior, and cognition [12]. Protein synthesis in neurons occurs trough a unique mechanism: neurons have the capacity to synthesize proteins locally in their dendrites and axons. Consequently, protein synthesis and regulation may occur promptly in response to synaptic activity and plasticity [13]. They also control synaptic activity, play direct roles in synaptic transmission and in memory consolidation [14]. During the brain development proteins such as 14-3-3 family are highly expressed and are essential to activities like neurogenesis, neuronal migration and neurite outgrow [15].

Proteins need to uphold their structural integrity in order to be completely functional. Production, folding, transport and degradation of proteins are all typical stages of protein homeostasis (proteostasis). Under normal conditions proteostasis is strictly regulated [16].

When a protein misfolds and cannot be actively corrected or degraded, then it can bind to other misfolded proteins, forming a large misfolded or unfolded protein accumulation called an aggregate. Formation of protein aggregates may lead to either its loss of function or the acquisition of a novel hazardous function due to irreversible alterations [17]. Numerous factors can lead to protein aggregation [16]. Misfolding often makes proteins more likely to aggregate and form insoluble aggregates, as does exposure to oxidative stress. Reactive oxygenic species can damage proteins increasing the risk of aggregation. Proteasome malfunction and some genetic mutation can lead to accumulation of misfolded proteins which are amenable to aggregation. Aging-related mutations like glycation and oxidation are also capable to contribute to the aggregation of proteins [18].

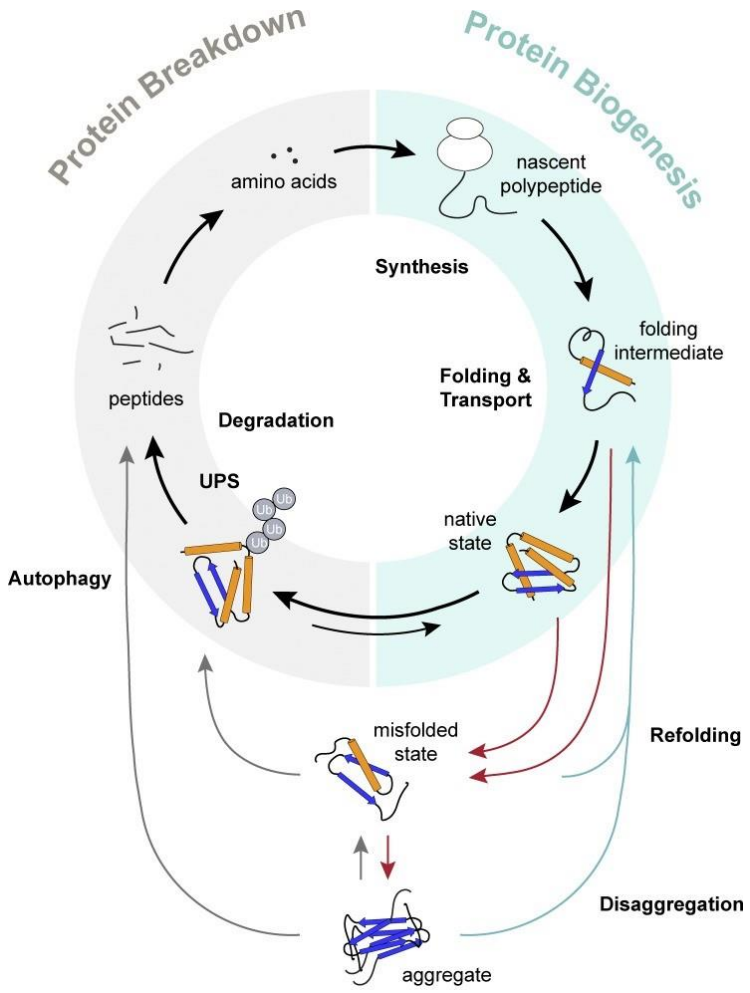

Figure 2 An overview of cellular proteostasis. Proteostasis is a biological mechanism that regulates the production, folding, transport, and degradation of all proteins. To maintain the overall flow, the process depends on regulation by the proteostasis network, which is made up of the translation machinery, molecular chaperones, ubiquitin-proteasome system (UPS) and autophagy. Quality control mechanisms identify nonnative conformations generated by off-pathway events to stop the accumulation of aberrant proteins in the cell. Proteins that have become misfolded and aggregate are either directed to degradation systems or to the folding route through disaggregation and refolding. Taken from Sala AJ et. al., 2017 [19]

Several mechanisms cause protein aggregation as shown in Figure 3. Mechanism 1 shows the tendency of native forms of proteins to reversibly associate. Their surface is self-complementary, thus, it readily self-associates to form small reversible oligomers. More massive aggregates often become irreversible. Mechanism 2 demonstrates the ability of conformationally altered monomer to self-associate. In this case, aggregation will be triggered by inducing stress. Proteins can be modified

by chemical exposure like oxidation or proteolysis. Those monomers usually present with electrical charge disruptions. Their process of oligomerization and aggregation is shown as Mechanism 3. Some protein monomers have low propensity to form minor oligomers due to thermodynamical perplexities. However, if a major enough aggregate succeeds in forming, expansion of the so-called critical nucleus by means of monomer addition is immensely encouraged resulting in rapid emergences of much larger species. This process is marked as Mechanism 4. Environmental alterations such as changes in pH can induce conformational modifications as shown in Mechanism 5 [20].

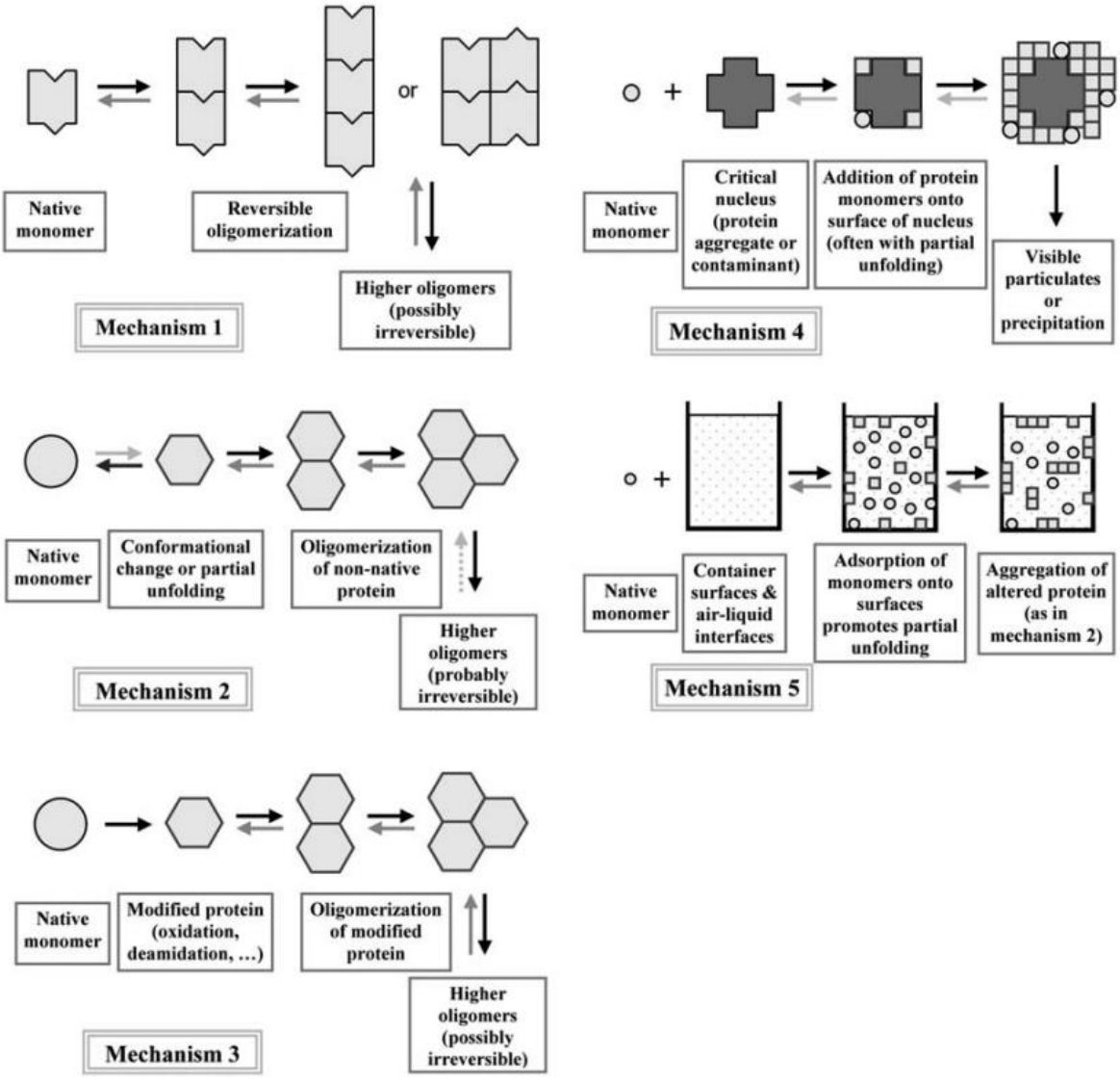

Figure 3:Schematic illustration of five common aggregation mechanisms. Taken from Philo et al. 2009[20]

Post mortem brain tissue samples from schizophrenia patients show an increase in protein insolubility and ubiquitination in the insoluble protein fraction [21]. This suggests that the proteins in examined brain samples are forming insoluble aggregates instead of dissolving properly.

Protein misassemble and aggregation have been observed in patients suffering from mental illness. It is hypothesized that this discovery correlates to the psychiatric symptoms seen in the early stages of many neurodegenerative diseases [22] [23]. It has been suggested that a number of proteins preferentially form aggregates in the brains of certain groups of patients suffering psychiatric disease.

Numerus proteins have been reported to aggregate in schizophrenia including DISC1 (Disrupted in Schizophrenia 1), TRIOBP (TRIO & F-actin Binding Protein), NPAS3 (Neuronal PAS domain containing protein 3) and CRMP1 (Collapsin Response Mediator Protein 1) [22,24–26]. In some instances, multiple proteins aggregate in the same individuals, either through direct interaction of the aggregating proteins (co-aggregation) or parallel aggregation of proteins without physical interaction [27].

1.3. Collapsin Response Mediator Protein 1 (CRMP1)

The CRMP is a group of five cytosolic proteins (CRMP1-5). CRMP proteins are known to be involved in regulation of microtubules. They are highly expressed in both development and in the adult nervous system where they are vital for the development and maturation of neurons [28]. Some patients with mental and neurological disorders have altered CRMP expression [29]. This finding could be in accordance with two-hit hypothesis.

The role of CRMP1 has also been studied in the context of neurodegenerative disorders, specifically Huntington’s disease. Huntington’s disease is known as rare, inherited neurodegenerative

disorder. CRMP1 has found to be a novel suppressor of huntingtin misfolding and neurotoxicity. CRMP1 suppresses huntingtin by aggregating with it. Studies suggest it could perform a protective function in the treatment of Huntington's disease [30,31].

CRMP1 is recognized as a semaphorin 3A signaling protein, which controls neuronal signaling in the developing brain and has the ability to regulate neuronal migration in the cerebral cortex in a reelin-dependent manner [32]. Reelin abnormalities have been linked to aberrant behavioral control and schizophrenia [33].

CRMP1 was also found to be insoluble in brain tissue of some patients suffering schizophrenia, bipolar disorder and major depressive disorder [24]. CRMP1 was consequently listed as one of the proteins thought to aggregate in chronic mental illnesses [3424]. CRMP1 exists in two variants: the long (CRMP Lv) and short (CRMP1 Sv) forms. The two proteins are identical except for their N-terminal region. N-terminus of CRMP1 Lv has 128 unique amino acids, while CRMP1 Sv has 14 (Figure 4). Prior research suggested that CRMP1 Lv could aggregate on its own, while CRMP1 Sv only seemed to aggregate when co-expressed with DISC1 [24,27].

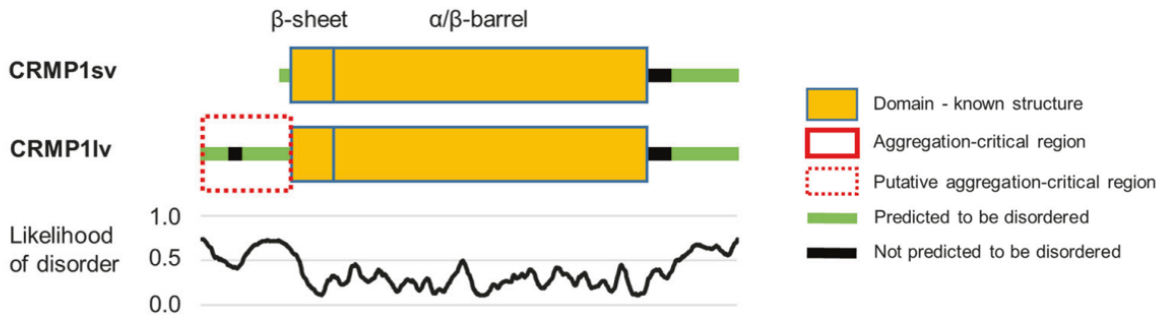

Figure 4 Structure of CRMP1 Sv (Short version) and CRMP1 Lv (Long Version). Taken from Bradshaw and Korth, 2019 [23]

As shown in Figure 4, N-terminal region of CRMP1 Lv appears to be responsible for aggregation, on an account of it not being present in CRMP1 Sv [23].

2. AIMS OF THESIS

This research explores the aggregation properties of different versions of CRMP1. Previous research suggests that CRMP1 Lv is more prone to aggregation in comparison to CRMP1 Sv. These differences likely occur due to the N-terminus of CRMP1 that differs between these variants. The N-terminus of CRMP1 Lv contains 128 unique amino acids, while N-terminus of CRMP1 Sv consists of 14 amino acids. The rest of the proteins 558 amino acids contained in protein structure are identical in both cases.

Thus, the goal is to investigate differences in aggregation of CRMP1 Lv and CRMP1 Sv. Also, versions of CRMP1 Lv that lack part of its N-terminus (encoding amino acids 28-686, 65-686 or 98-686 of CRMP1 Lv) will be examined in order to determine the effect of the specific N-terminus regions of CRMP1 Lv potentially have in aggregation of CRMP1.

This research will therefore test the hypothesis that deletion of parts of N-terminal region of CRMP1 Lv should block or limit the aggregation.

In this thesis we aimed to:

- Reproduce and confirm CRMP1 aggregation in mammalian cells as previously observed
- To determine the differences (if any) in aggregation amongst CRMP1 Lv, CRMP1 Lv (28-686), CRMP1 Lv (65-686) and CRMP1 Lv (98-686) and CRMP1 Sv
- To determine the part of the CRMP1 Lv N-terminus region responsible for aggregation

3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Vectors

Table 1: List of plasmids.

Vector	Protein encoded	Origin	Publication
pdcDNA-FLAG	CRMP1 Lv	Nicholas Bradshaw, Rijeka	Samardžija, Juković et al, *Cells* (2023) 12: 1848
pdcDNA-FLAG	CRMP1 Lv (28-686)	Beti Zaharija & Nicholas Bradshaw, Rijeka	Unpublished
pdcDNA-FLAG	CRMP1 Lv (65-686)	Nicholas Bradshaw, Rijeka	Unpublished
pdcDNA-FLAG	CRMP1 Lv (92-686)	Nicholas Bradshaw, Rijeka	Unpublished
pdcDNA-FLAG	CRMP1 Sv	Nicholas Bradshaw, Rijeka	Samardžija, Juković et at, Cells (2023) 12: 1848

3.1.2. Antibodies

Table 2. List of primary and secondary antibodies 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 Fischer Scientific	1 mg/mL	1:2000
Alexa Fluor 555 Goat anti-Mouse IgG	Thermo Fisher	2 mg/mL	1:1000
Phalloidin-iFlour 488 Reagent	Abcam	2 mg/mL	1:500

3.2. Methods

3.2.1. Transforming bacteria

All plasmids used in this thesis were transformed into the competent NEB5α strain of E. coli and then grown in that line. 50 µL of thawed NEB5α bacteria was mixed with 1µL of plasmid in 1.5 mL Eppendorf tube and placed on ice for 30 minutes. After 30 minutes incubation on ice, samples were exposed to heat shock at 42°C for 30 seconds, which made the transformation possible. Following heat shock, samples were placed on ice for five minutes to allow recovery. After recovery period 250 µL of LB media was added to each tube (LB consists of 10 g of tryptone, 5 g of yeast extract, 5 g NaCl, dH_2O was added up to 1 L, pH was adjusted to 7.0) and allowed to grow on LB agar plates (1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 1.5 g Agar, dH_2O added up to 100 mL) treated with ampicillin 50 µg/mL overnight. The following day a single colony was isolated from each plate and transferred to a 15mL Falcon tube containing 3mL of LB medium containing 50 g/mL ampicillin and kept in shaking incubator set to 250 rpm at 37 °C overnight.

3.2.2. Miniprep (purification of DNA)

After 24 hours in liquid culture, samples were pelleted using 10 000 rpm centrifuge for 10 minutes at a temperature of 4°C. The supernatant was discarded, and pellet was purification using a commercial kit QIAprep Spin Miniprep kit by Qiagen. The pellet was firstly resuspended in 250 µL of P1 buffer and placed in a microcentrifuge tube. Next, 250 µL of P2 buffer was added and both were thoroughly mixed until content became clear. The reaction was stopped after ten minutes by adding 350 µL N3 buffer. The suspension was centrifuged for 10 minutes at 13 000 rpm. The supernatant was transferred to QIAgen 2.0 spin column and centrifuged for 1 minute at 11 000 rpm and the supernatant was thrown away after each of the following steps. The column was cleaned using the PE buffer and centrifuged at 10 000 rpm for one minute, after which the supernatant was discharged, and tube was centrifuged one more time at 13 000 rpm for one extra minute. Spin columns were then placed in labeled, sterile 1.5 mL Eppendorf tubes. The DNA was eluted form the columns using EB buffer (0.5 mL 1 M Tris pH 7.4, 200 µL 0.25 M EDTA, dH_2O added up to 50 mL). The tubes were then incubated for one minute at room temperature and then centrifuged at 13 000 rpm for one minute.

3.2.3. Measuring DNA concentrations

A BioDrop µLITE spectrophotometer with absorbance wavelength 260nm was used to determine the concentration of purified plasmid DNA. Elution buffer EB served as a blank probe.

3.2.4. DNA gel electrophoresis

The purified plasmid DNA samples were examined by electrophoresis on an agarose gel to see if they met the quality expectations. DNA samples are

put through a process called agarose gel electrophoresis, which establishes a size classification for the samples. The agarose gel was prepared (0.5 g of agarose, 50 mL of a 1x TAE buffer (50x stock solution; 242 g Tris, 18.61 g EDTA, 57.1 mL acetic acid, dH_2O added up to 1L), and 0.5 µL of GelGreen). The mixture was then heated in microwave for couple of minutes until agarose have completely dissolved. After the gel cooled down in a proper container it was loaded with 1x TAE buffer and transferred to the electrophoresis tank. A total of 10 µL of each sample (a solution of dH_2O, DNA loading buffer, and plasmid DNA) and 5 µL of the marker (a solution of DNA Ladder and DNA Loading buffer) were applied to the microtiter plate. For the duration of the electrophoresis, a voltage of 140 V was used for 18 minutes. A BioRad Chemi-Doc MP Imaging System was used to evaluate the gel.

3.2.5. Splitting mammalian cells

HEK239 and SH-SY5Y cell lines were employed for my thesis. The HEK293 cell line originates from the kidney of a human embryo. These cells served as the subject of my initial series of tests since they reproduce quickly, have low maintenance requirements and a high rate of transfection, allowing for increased protein output. A metastatic bone tumor gave rise to the cloned neuroblastoma cell line known as SH-SY5Y cells. SH-SY5Y cells are harder to maintain and prolong cell cycles in comparison to HEK293 cells but have more neuronal characteristics.

Both cell lines were grown on the bottom of T25 cell culture flasks. HEK293 were grown in DMEM +/+ media (ThermoFisher, containing 10% Cosmic Calf Serum (CCS), penicillin and streptomycin solution). SH-SY5Y cells were grown in DMEM-F12 +/+ media (ThermoFisher, containing 10% fetal calf serum (FCS), penicillin and streptomycin solution and 1x MEM non-essential amino acids).

Working with mammalian cell cultures requires maximum caution and a sterile environment, which includes sterilization of workstation before and after the use. Also, the hood is equipped with UV light which is also used for 15 minutes before and after each use. All disposable instruments used need to be disinfected before entering the hood and opened once they are inside of it.

When the cells reached confluency, they were split and prepared to be used for further experiments. To disassociate the bonds between cells Trypsin/EDTA enzyme (Pan Biotech, Trypsin 0.25%/EDTA 0.02%, in PBS w/o: Ca^{2+} and MG^{2+}, w: Phenol red) was used. Once cells were disassociated, they were transferred to flasks containing fresh medium. Both fresh medium and Trypsin/EDTA enzyme were preheated to 37°C. The cells used for immunocytochemistry were grown in 24-well plates on top of the glass coverslips inside each of them. All plates and flasks containing cell cultures were stored in an incubator (Nüve CO_2 incubator (EC 160)) at a temperature of 37°C and with a 5% CO_2 concentration.

3.2.6. Transfection

For transfection, media without serum or antibiotics was used. For transfection of HEK293 cells DMEM -/- media (-/- meaning without antibiotics or serum) was used and for SH-SY5Y cells DMEM-F12 -/- media. DNA solutions were prepared using 0.5 µg of plasmid and 100 µL of -/- media. Transfecting reagent solution was made using of 2 µL of Metafectene (Biotex) when transfecting HEK293 cells or Metafectene PRO (Biotex) for SH-SY5Y cells and 100 µL of -/- media. Both solutions were incubated at the room temperature for 5 minutes before mixing the solutions together and incubating them for 30 minutes (37°C/5% CO_2). +/+ media (containing serum and antibiotics) from wells was removed and replaced with -/- media to which were then added prepared solutions containing plasmids and

Metafectene. Cells were then incubated for 6 hours in the -/- media, after which the media as replaced with +/+ media and incubated overnight.

3.2.7 Cell lysis

HEK293T cells were washed two times with 1 x phosphate-buffered saline (PBS) (80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, dH_2O added up to 1L, pH adjusted to 7.4) after the overnight incubation. After the washing process, PBS was removed and 100 µL of the Cell LysisBuffer (5 mL 10x PBS, 5 mL 10% Trioton x-100, 1 mL 1M $MgCl_2$, 50 µL DNaseI, 50 µL 100 mM Phenylmethyl-sulphonyl fluoride, dH_2O added upto 50 mL) was added to each well. DNasel was added to this buffer before use at a concentration of 0.5 µL per 1 mL were used, and the plates were then placed on ice for 5 minutes. The following step was to transfer the lysed cell suspension to previously marked 1.5 mL Eppendorf tubes. Tubes were then incubated on the rotor for 30 minutes. Next, 100 µL of Protein Loading Buffer (6.25 mL 1M Tris pH 6.8, 10 mL glycerol, 20 mL 10% SDS, 3.75 mL dH_2O, 5 mg bromophenol blue) and 10 µL of DTT were added to each of the tubes and the samples were denatured at a temperature of 95°C for 5 minutes, after which they were ready to be used in SDS-PAGE or for storage at -20°C.

3.2.8. SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

Acrylamide running and stacking gels were made using the Mini-PROTEAN Tetra Handcast system (BioRad). 8% acrylamide running gels were handmade using 5.5 mL of dH_2O, 3.2 mL of 30% acrylamide, 3 mL of 1.5M Tris pH 8.8, 120 µL of 10% SDS, 120 µL of 10% APS and 12 µL of TEMED. Stacking gel was prepared using 2.6 mL of dH_2O, 1 mL of 30% acrylamide

mix, 625 µL of 1M Tris pH 6.8, 50 µL of 10% SDS, 50 µL of 10 % APS and 5 µL of TEMED. The stacking gel, because of its lower polyacrylamide concentration is placed on top of the running gel, that allowed ionic gradient to form. Passing through running gel with higher acrylamide concentration and smaller pores, proteins separate according to their molecular weight. Protein samples and my-Budget Prestained Protein Ladder 10kDa-180 kDa marker (Bio-Budget Technologies GmbH, 0.2-0.4 µg/µL) were thawed and loaded onto the gels. The gels were then run for 45 minutes at 180 V.

3.2.9. Transferring to membrane

Following the electrophoresis, proteins were transferred to a Parablot PVDF membrane (Macherey-Nagel, 0.2 µm pore) using the s Trans-Blot Turbo Transfer System (BioRad). To visualize proteins, the membrane was stained using Ponceau S solution (1 g Ponceau S, 4 mL acetic acid, dH_2O added up to 200 mL).

3.2.10. Western Blot

To analyze the results, the membrane was first blocked with 5% milk powder in PBS-T solution for 1 hour on room temperature. Proteins were then detected using primary and secondary antibody and washed three times using the PBS-Tween. To visualize protein bands, Pierce ECL Prime Western Blotting Substrate (ThermoScientific) was used on a ChemiDoc MP Imaging System and Image Lab (BioRad).

3.3. Cell staining and immunocytochemistry

3.3.1. Fixation and permeabilization

Immunocytochemistry was performed on both HEK293 and SH-SY5Y cells. To fix cells to the glass coverslips, Fixation Buffer was used (8 g paraformaldehyde, 20 mL 10x PBS, dH_2O added up to 200 mL, pH adjusted to 7.4) for 15 minutes following permeabilization with Permeabilizing Buffer (10 mL 10x PBS, 10 mL 10% Triton X-100, dH_2O added up to 1 L) for 10 minutes.

3.3.2. Staining

10% goat serum in PBS solution was applied to block all of the nonspecific antibody binding sites. The next step was then adding primary antibody, which was added at a 1 to 1000 dilution into 10% goat serum in PBS solution and left to incubate on room temperature for 3 hours covered with a damp paper under the slip to prevent drying of the slips. Finally secondary antibody was added into 10% goat serum in PBS solution, along with DAPI stain to stain the nucleus and fluorescent phalloidin to stain the actin cytoskeleton, each diluted 500-fold. Cells were then left to incubate in the dark for an hour, and then washed with 1x PBS 3 times.

3.3.3. Mounting

Forceps and a modified needle were used to pluck out the glass coverslips out of wells. Coverslips were then attached to slides using Mounting Medium Fluoroshield (Sigma). The cells were then visualized on an Olympus IX83 fluorescent microscope under 60x magnification.

3.4. Blinded quantitative test

To conduct a blinded quantitative test, 40 Eppendorf tubes were prepared. Five different plasmid samples were loaded into these: 8 tubes per plasmid and prepared for transfection. A colleague in the lab was then asked to randomize tubes assigning them numbers 1 to 40. After blinding the plasmid samples, I proceeded with transfection of the SH-SY5Y cells and continued with immunocytochemistry protocol.

3.5. Microscopy

Microscopy was done on an Olympus IX83 fluorescent microscope. Images were captured using a Hamamatsu Orca R2 CCD camera and CellSens software.

4. RESULTS

4.1. Western blot

A necessary first step is to validate the plasmid expression constructs that will be used. To achieve this, HEK293 cells were cultured and transfected with the five constructs whose structures are shown in Figure 5.

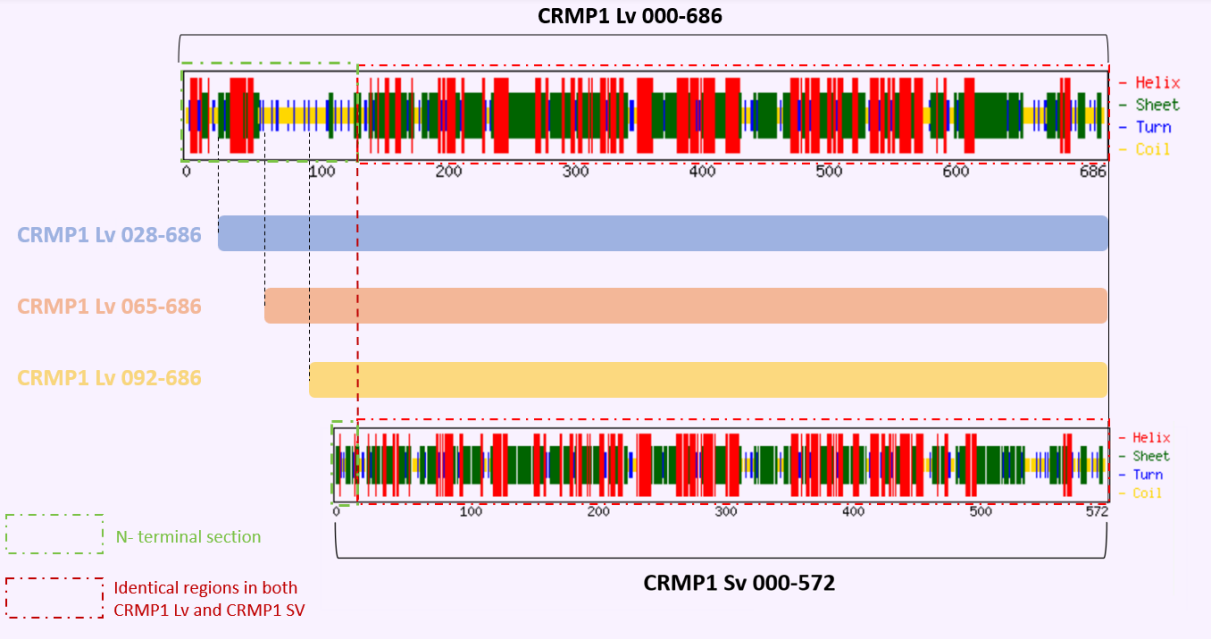

Figure 5 Diagram of CRMP1 secondary structure for CRMP1 Lv and CRMP1 Sv with addition of indicated regions expressed in the thesis. Graphics obtained by program CFSSP: Chou & Fasman Secondary Structure Prediction Server

Cells were then lysed and analyzed using Western Blotting. All proteins were expressed with an N-terminal FLAG tag and were detected utilizing an anti-FLAG antibody.

Figure 6 shows 5 distinct protein band of size equivalent to that anticipated. The first band shows CRMP1 Lv (long version), second CRMP1 Lv (28-686), followed by CRMP1 Lv (65-686), fourth one represents CRMP1 Lv (92-686) and lastly, CRMP1 Sv (short version).

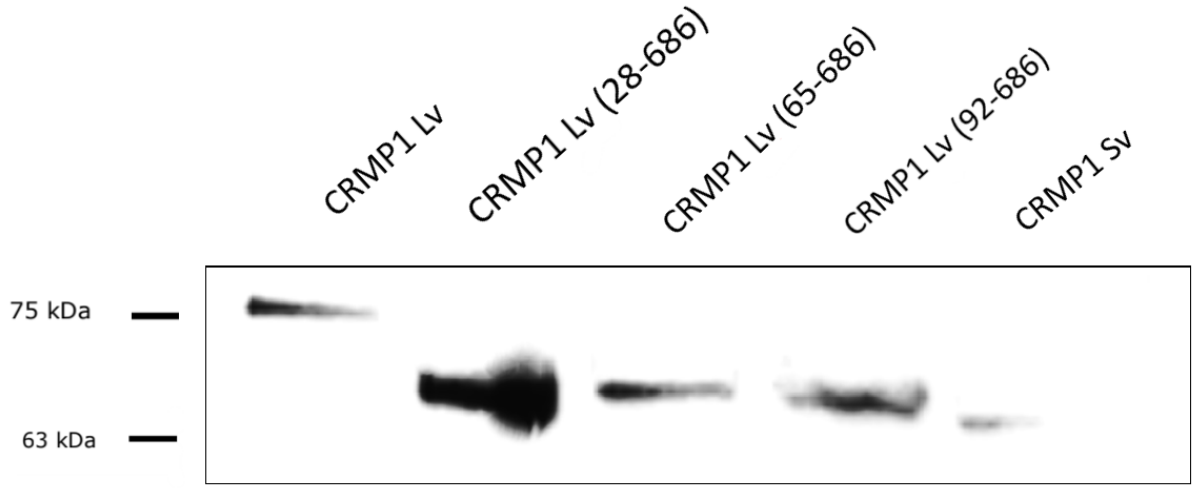

Figure 6 Western blot analysis of CRMP1 Lv, CRMP1 Lv (28-686), CRMP1 Lv (65-686), CRMP1 Lv (92-686), CRMP1 Sv protein constructs expressed in HEK293 cell line. Image presents results obtained using Western Blot method on lysates of HEK293 cells transfected with examined plasmids.

4.2. Fluorescent microscope analysis

Next, each of the constructs was transfected into HEK293 cells and examined by immunofluorescent microscopy, to determine if it formed visible aggregates. Monoclonal anti-FLAG M2 antibody was used as the primary antibody, while Alexa Fluor 594 goat anti-mouse IgG was used as secondary antibody. Bright red signal shows successful transfection, as the secondary antibody has bound to the anti-FLAG M2 primary antibody, while bright blue glow caused by DAPI stain allows the nucleus to be seen.

The results are shown in Figure 7. Transfection was visible, but aggregates were not detected.

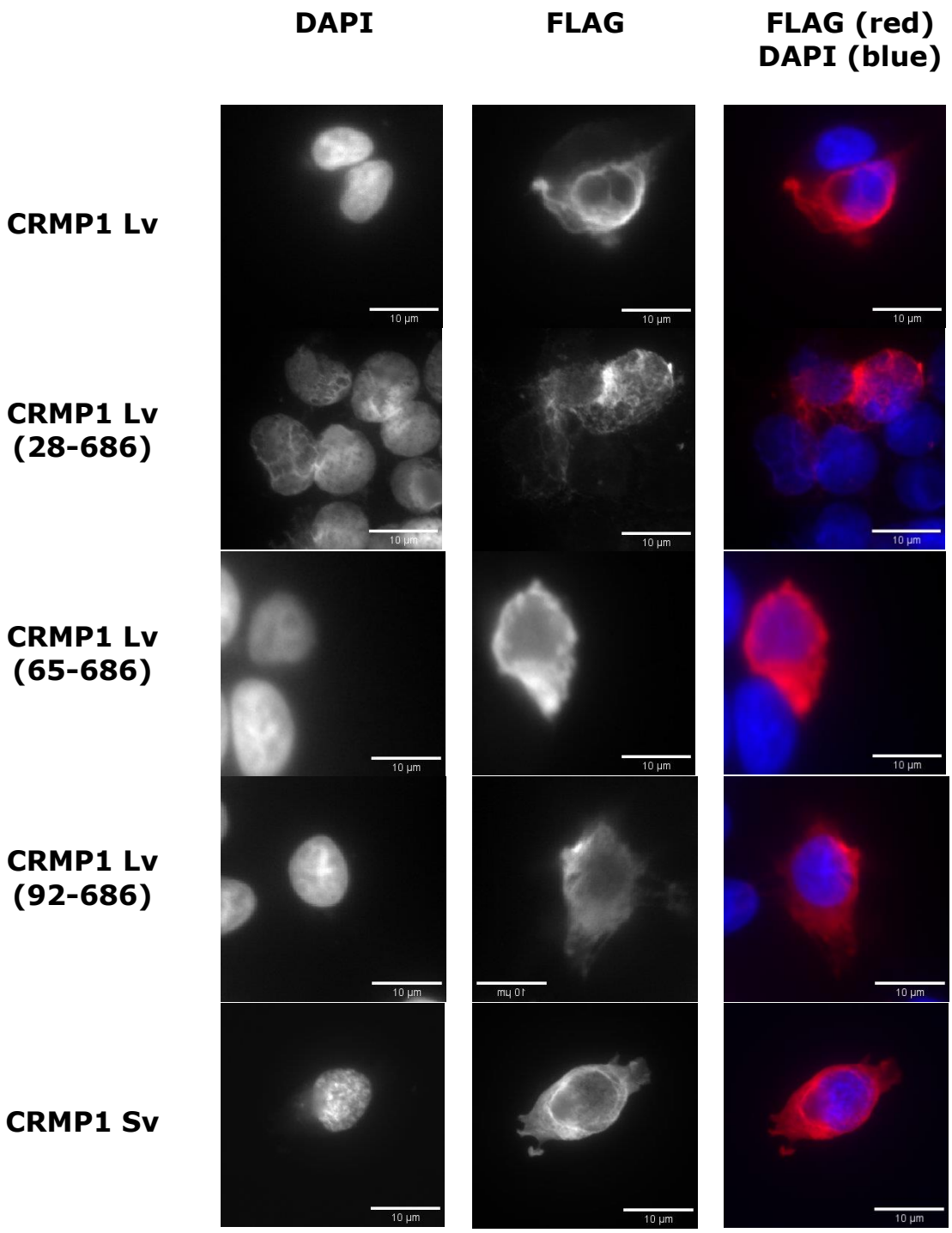

Figure 7 Fluorescent microscopy images of HEK293 cells transfected with CRMP1 Lv, CRMP1 Lv (28-686), CRMP1 Lv (65-686), CRMP1 Lv (92-686), CRMP1 Sv. Results show successful transfections with all of the tested constructs while aggregates were not detected. Images were taken on the Olympus IX83 fluorescent microscope using CellSens software. Magnification was set on 60x. The scale represents 10 µm.

Transfection was also observed in SH-SY5Y cells with monoclonal anti-FLAG M2 antibody as the primary antibody and Alexa Fluor 594 goat anti-mouse IgG was used as secondary antibody. Bright red signal in this case represents successful transfection because the secondary antibody bound to the anti-FLAG M2 primary antibody, while bright blue glow caused by DAPI stain allows the nucleus to be seen and phalloidin stain presents the actin and the cytoskeleton visible in green.

The results are shown in Figure 8. Similar to the result shown in previous example, transfections were visible, but aggregates were not commonly detected.

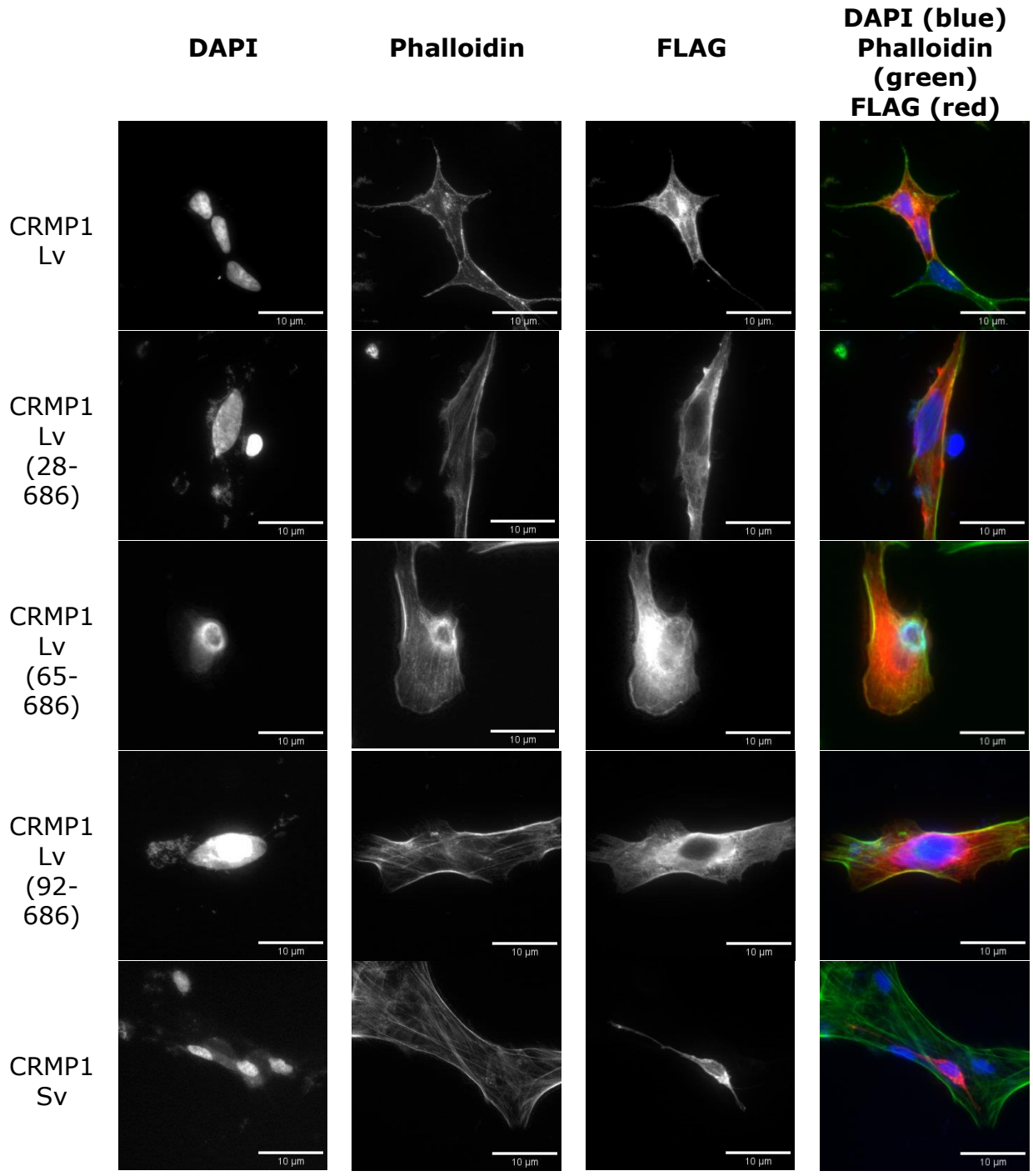

Figure 8 Fluorescent microscopy images of SH-SY5Y cells transfected with CRMP1 Lv, CRMP1 Lv (28-686), CRMP1 Lv (65-686), CRMP1 Lv (92-686), CRMP1 Sv. Results show successful transfections with all of the tested constructs while aggregates were not detected. Images were taken on the Olympus IX83 fluorescent microscope using CellSens software. Magnification was set on 60x. The scale represents 10 µm.

4.3. Quantitative blinded test on SH-SY5Y cells

In order to examine the impact expressed protein and its variants have on SH-SY5Y cells, a blinded test assay was conducted.

After the quantification samples were decoded for statistical analysis. Analysis was performed using GraphPad Prism. Results show an average percentage of mean of aggregating cells, and standard error of mean.

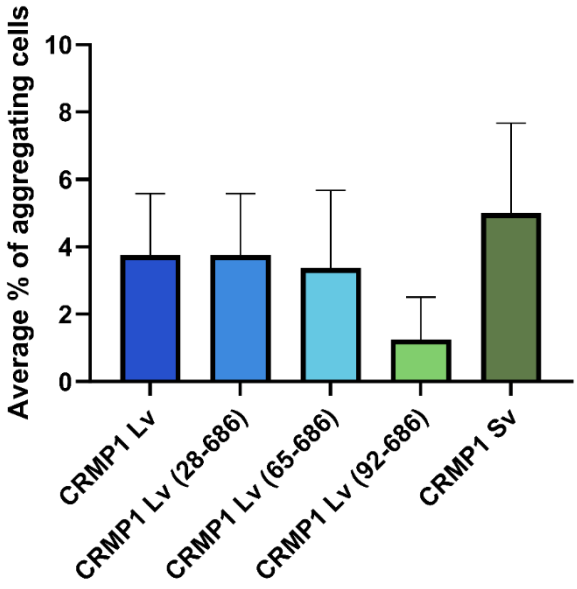

Figure 9 Graph represents the percentage of cells which have shown aggregation-like results. First column represents an average percentage of cells showing aggregation – like results out of the samples transfected with CRMP1 Lv. Second column represents an average percentage of cells showing aggregation – like results out of the samples transfected with CRMP1 Lv (28-686), third column represents an average percentage of cells showing aggregation – like results out of the samples transfected with CRMP1 Lv (65-686), fourth column represents an average percentage of cells showing aggregation – like results out of the samples transfected with CRMP1 Lv (92-686) and fifth column represents an average percentage of cells showing aggregation – like results out of the samples transfected with CRMP1 Sv.

The aggregate-like clusters were detected in 3.75% ± 1.83% of the cells transfected with CRMP1 Lv and CRMP1 Lv (28-686), in 3.36% ± 2.31% of the ones transfected with CRMP1 Lv (65-686), in 1.25% ± 1.25% of cells transfected with CRMP1 Lv (92-686) and in 5.00% ± 2.67% of the cells transfected with CRMP1 Sv (Figure 9).

No results were significant by one-way ANOVA. However, there is a non-significant trend suggesting that CRMP1 Lv (92-686) may aggregate less than the other variants tested.

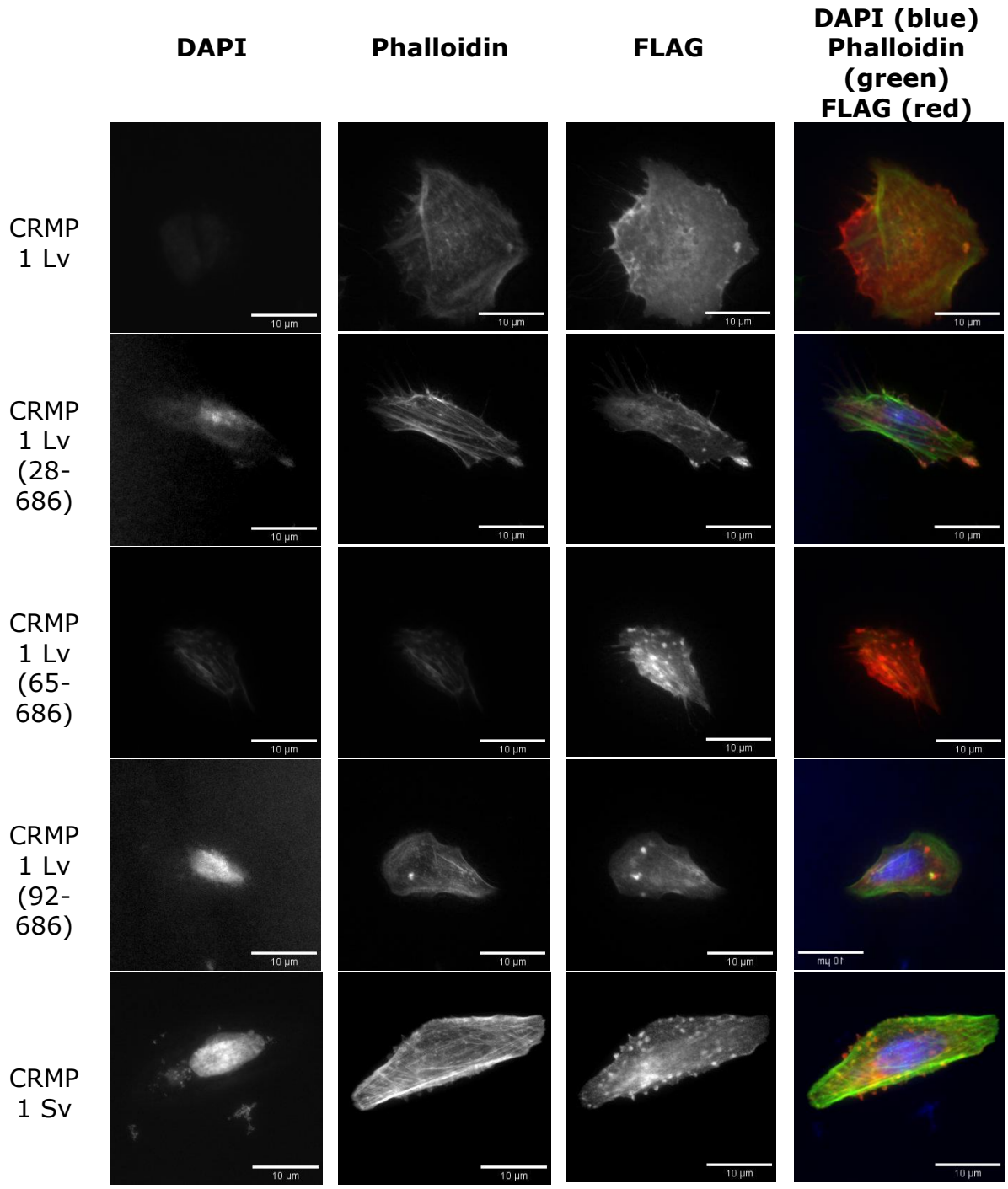

Figure 10 Fluorescent microscopy images of SH-SY5Y cells transfected with CRMP1 Lv, CRMP1 Lv (28-686), CRMP1 Lv (65-686), CRMP1 Lv (92-686), CRMP1 Sv presenting aggregation like clusters. Fluorescent microscopy images of SH-SY5Y cells transfected with CRMP1 Lv, CRMP1 Lv (28-686), CRMP1 Lv (65-686), CRMP1 Lv (92-686), CRMP1 Sv presenting aggregation like clusters. Results show successful transfections with all of the tested constructs while aggregates were not detected. Images were taken on the Olympus IX83 fluorescent microscope using CellSense software. Magnification was set on 60x. The scale represents 10 µm.

5. DISCUSSION

As the result of a hypothesis-free proteomic research, CRMP1 was linked to chronic mental diseases after being identified as a protein that is insoluble in brain samples from individuals with schizophrenia and bipolar disorder [24]. These results also suggest that CRMP1 Lv, which differs only by additional 128 amino acids at the N-terminus, may aggregate more than CRMP1 Sv.

Therefore, my thesis was based on the hypothesis that the N-terminal area of CRMP1 Lv, amino acids 1-128, as the only region present in CRMP1 Lv but absent in CRMP1 Sv was essential for the aggregation of CRMP1 Lv. To examine this hypothesis, experiments were conducted on both CRMP1 Lv and CRMP1 Sv, as well as modified samples CRMP1 Lv (28-686), CRMP1 Lv (65-686) and CRMP1 Lv (92-686) in order to determine which N-terminal area sequence could be responsible for aggregation.

Five variations of CRMP1 protein were selected based on prior research. Constructs were expressed *in vitro* using HEK293 and SH-SY5Y cell lines. To ensure certainty of the successful transfection Western Blot analysis was used on the lysates of transfected HEK293 cells to confirm that the protein was successfully generated in cells and was of the right size.

The next stage of the experiment was to examine whether these proteins showed detectable protein aggregation. Initial test conducted in HEK293 cell line did not demonstrate aggregations of the tested samples in any of the repetitions. The same experiment was carried out as part of following phase, this time utilizing SH-SY5Y cell line, and the findings were the same.

All of the constructs shown successful signs of transfections (Figure 7, Figure 8). There was no visible difference amongst aggregating cells in neither the HEK293 nor SH-SY5Y cell lines.

A blinded test carried out on SH-SY5Y cells with 8 samples of each of the five examined variants. All constructs show low levels of aggregation (1.25% - 5.00% of the cells) (Figure 9).

Given that is commonly reported to aggregate almost exclusively while co-aggregating with other proteins [24,27] these results are in line with the expectations for CRMP1 Sv.

The results regarding CRMP1 Lv aggregation came as a surprise considering that prior research suggested that the long version could aggregate alone [24,27]. CRMP1 Sv was previously reported to co-aggregate with DISC1 in cell lines in the same study that reported CRMP1 Lv aggregating. In that study, both CRMP1 Sv and CRMP1 Lv as well as DISC1 were all GFP-tagged which may lead to heightened aggregation [35]. Recent studies confirmed that CRMP1 when fused with GFP tags presents increased aggregation, CRMP1 Lv more frequently than CRMP1 Sv. The same study confirmed co-aggregation of both CRMP1 Lv and CRMP1 Sv with DISC1 but also shown evidence of CRMP1 Lv and CRMP1 Sv to co-aggregate with each other [27]. The connection between CRMP1 variants and DISC1 co-aggregation and its association with mental illness require further study.

Bader et al. expressed CRMP1 Lv and CRMP1 Sv coupled with GFP for their research. GFP is a large fusion protein approximately 27 kDa. Thus, the size of the tag might have had an impact on the proteins affinity to aggregate. In their original research they also used CAD cell line which is obtained from a mouse catecholaminergic neuronal tumor.

In this research, CRMP1 proteins were expressed in plasmids with considerably smaller FLAG tag. FLAG tag size is roughly 1kDa therefore 27 times smaller than GFP fusion protein. Hence, the FLAG tags used in this research are less likely to obstruct CRMP1 expression and function. This system is closer to the ideal one, which would involve the expression of proteins in neuronal cells without any tags. Also, for this experiment the human neuroblastoma cell line SH-SY5Y was utilized rather than the CAD cells. It is important to emphasize that being a human origin cell line, SH-SY5Y is therefore a better model than mouse origin cell line CAD. As a result, the method used in this thesis offers a point of view that is more appropriate.

While there were no significant differences, it is notable that CRMP1 Lv (92-686) appears to exhibit less aggregation than any of the other constructs. If these findings are accurate, it may imply that some specific region of CRMP Lv (probably 65-91) is critical for aggregation and that Lv and Sv may aggregate by different mechanisms. One other possibility is that CRMP1 (92-686) aggregates are more toxic to cells than other CRMP1 variants, meaning that fewer cells with these aggregates survive and are visible in the experiments. Although the differences presented between the CRMP1 Lv constructs are not statistically significant, they are worth examining further. This research helps us to learn more about the aggregating properties of CRMP1 and lays the foundation for future research.

Regarding the future research, as demonstrated in previous studies [24,27] use of EGFP fusion protein seems to exaggerate CRMP1 aggregation, so it should be considered to use EGFP as a fusion protein. Other possibilities include inducing further aggregation of CRMP1 constructs and comparing them. Notably, prior research connected oxidative stress to the development of psychiatric disorders, with a compromised antioxidant system is shown to be presented in psychiatric disorder [36,37]. It is suggested that oxidative stress is one of the factors that contribute to protein aggregation in mental illness [38]. Thus, introducing additional stress factors like sodium arsenate or hydrogen peroxide should also be considered in the further research. These approaches are commonly used to study TDP-43 aggregation in ALS research [39].

An alternative approach is that proteasome dysfunction was found present in the postmortem cortex of individuals with schizophrenia, therefore linking protein degradation to differential expression of ubiquitin proteasome system components [40]. Treating cells with proteasome inhibitor like MG123 should thus also be considered for the further research to prevent aggregating cells being degraded by proteasome.

Previous research also presented strong evidence of CRMP both Lv and Sv co-aggregation with DISC1 [24,27] and co-aggregation of CRMP1 Lv

and CRMP1 Sv [27]. Co-transfecting variations of CRMP1 Lv with DISC1 is suggested as a next step to see if differences become apparent when CRMP1 is co-aggregating.

Ensuring that cells produce recombinant proteins in controllable and quantifiable manner can be of great help in further research. One of the methods that could be utilized for further research is CRISPR/Cas9-mediated knock-in [41].

6. CONCLUSION

While previous studies have suggested CRMP1 Lv to be more prone to aggregation than CRMP1 Sv, in this more relevant and through experiment where they were (unlike in previous research) expressed alone, both CRMP1 Lv and CRMP1 Sv appear to have similar levels of aggregation. The relatively small FLAG tag used in this research is less likely to obstruct expression and function of protein than GFP fusion proteins used previously. Finally, combined with human neuroblastoma cell line being used presents more relevant results in comparison to prior research.

Different isoforms of CRMP1 Lv with deletions of different parts of N-terminal region CRMP1 Lv (28-686), CRMP1 Lv (65-686) and CRMP1 Lv (92-686) were explored to determine the influence of N-terminal region on aggregation. CRMP1 isoforms were expressed in vitro using HEK293 and SH-SY5Y cells. Although the results did not show statistically significant differences, they do suggest that that the unique N-terminus region of CRMP1 Lv may be required for its aggregation.

Aggregate-like clusters could also appear due to overaccumulation of protein in singular areas which could lead to formation of aggregate-like clusters which should in future experiments be ruled out utilizing ultracentrifuge-based insolubility test to confirm whether these are indeed aggregates.

Nevertheless, differences noticed amongst constructs lay the foundation for future research. Future studies should focus on further research of CRMP1 isoforms, with focus on both N-terminal region of CRMP1 Lv, but also the role of N-terminal of CRMP1 Sv and C-terminal regions potentially have regarding the aggregation. To maintain more stable aggregates EGFP tag should be put in use regarding its aggregation enhancing properties. Due to the nature of mental illness, especially schizophrenia, using proteasome inhibitors like MG123 or exposure to oxidative stress should also be considered to enhance aggregation. Co-aggregation with DISC1 should also

be investigated further with different isoforms of CRMP1 Lv, and to ensure that cells produce recombinant proteins in controllable and quantifiable manner can be of great help in further research.

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President — **Oct 2022 - Present**
Student's council of the University of Rijeka, Rijeka
I represent students at the University of Rijeka in numerous University bodies and committees and in Croatian student Council, and also coordinate work of 10 offices for students with physical disabilities, science, student standard, social activities, cultural activities, international cooperation, IT, PR and publishing, ombudsmen and office for cooperation with associations.

National coordinator of the "UNIRI stvari" project — **2021 - Present**
I am one of the initiators of the **UNIRI stvari** project whose goal is:

- reducing economic inequality and raising the level of awareness and the level of menstrual knowledge among menstruating and non-menstruating people in order to remove the stigma around the concept of menstruation and eliminate menstrual shame;
- provision of free menstrual supplies at the University of Rijeka, as well as other institutions of higher education in Croatia;
- forming "safe places" to collect free menstrual supplies on a monthly basis for people who, due to socio-economic or other reasons, are unable to afford the supplies they change on a regular schedule or use alternative means instead of hygiene supplies.

Member of working groups for drafting laws at the Ministry of Science and Education
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- Law on the student council and student organizations
- Law on student work
- Law on student nutrition

International officer — **Present**
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Member of the University senate — **Present**
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Delegate in the Board of the European Student's Union — **Present**
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Member of the Committee for the Internal audit — **Nov 2021 - Present**
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- Carnival group Kampus (2022.)
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Member of the national team ARDF (**Amateur radio direction finding**) 2012.-2018.
Rotary Award of Excellence
Award of the Rotary Club of Varaždin in 2017 for achievements in high school education and activities in the local community.
Award of the Croatian Society of Energy Engineers
Award for success at the state competition in the basics of electrical engineering and measurements in electrical engineering