# Characterising Cell Lines For Investigating Protein Aggregation In Mental Illness

**Polašek, Lora**

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Lora Polašek

# KARAKTERIZACIJA STANIČNIH LINIJA ZA ISTRAŽIVANJE AGREGACIJE PROTEINA KOD MENTALNIH BOLESTI

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1. .
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**Abstract**

Mental health is an essential part to of our overall well-being and human rights, ranging from complete wellbeing to severe emotional distress. Several risk factors contribute to these diseases, including psychological and biological components such as genetics and emotional abilities, which can often be altered by changes in brain structure and function. Common mental health disorders, including schizophrenia, bipolar disorder, and major depressive disorder, cause significant disturbances in behaviour, cognition, and emotional control, resulting in distress and functional impairment.

Proteins are essential for cellular functions and must fold correctly so they can perform their functions. However, proteins can misfold and aggregate, especially under certain conditions, resulting in toxic aggregates. Normally, cellular quality control systems, such as the ubiquitin-proteasome system and autophagy, manage these aggregates. When these mechanisms fail, protein misfolding diseases, often known as proteinopathies, develop, including neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's. These disorders are characterized by toxic protein aggregation which leads to neuronal dysfunction and cell death, linking proteinopathies to cognitive decline and psychiatric symptoms.

The correlation between mental illness and proteinopathies is becoming increasingly recognised. Proteins such as DISC1 and TRIOBP-1 have been observed to aggregate in mental illnesses, suggesting that they play important roles in the pathophysiology of these conditions.

This thesis focused on providing an insight into the behaviour of TRIOBP-1 and DISC1 in neuronal cells, allowing us to investigate the behaviour of these proteins and their potential connection to mental diseases. The primary aim was to determine the most optimal dose of doxycycline and period of treatment for protein expression,

followed by the detection of protein aggregation by Western blotting, fluorescent microscopy, and insolubility assays. By demonstrating these proteins' aggregation tendencies, trying to contribute to the understanding of how protein misfolding may connect to neurodevelopmental diseases.

**Sažetak**

Mentalno zdravlje je ključni dio općeg blagostanja i ljudskih prava, kreće se od potpunog blagostanja do teške emocionalne patnje. Nekoliko čimbenika rizika doprinosi ovim bolestima, uključujući psihološke i biološke komponente poput genetike i emocionalnih sposobnosti, koje se često mogu promijeniti zbog promjena u strukturi i funkciji mozga. Uobičajeni poremećaji mentalnog zdravlja, uključujući shizofreniju, bipolarni poremećaj i veliki depresivni poremećaj, uzrokuju značajne poremećaje u ponašanju, kogniciji i emocionalnoj kontroli, što rezultira stresom i funkcionalnim oštećenjima.

Proteini su ključni za stanične funkcije i moraju se pravilno saviti kako bi mogli obavljati svoje funkcije. Međutim, proteini se mogu nepravilno saviti i agregirati, osobito u određenim uvjetima, što rezultira stvaranjem toksičnih agregata. Normalno, stanični sustavi kontrole kvalitete, poput sustava ubikvitin-proteasoma i autofagije, upravljaju ovim agregatima. Kada ti mehanizmi zakažu, razvijaju se bolesti uzrokovane nepravilnim savijanjem proteina, poznate kao proteinopatije, uključujući neurodegenerativne poremećaje poput Alzheimerove, Parkinsonove i Huntingtonove bolesti. Ovi poremećaji karakterizirani su toksičnim agregacijama proteina koje dovode do disfunkcije neurona i stanične smrti, povezujući proteinopatije s kognitivnim padom i psihijatrijskim simptomima.

Povezanost između mentalnih bolesti i proteinopatija sve se više prepoznaje. Primijećeno je da proteini poput DISC1 i TRIOBP-1 agregiraju u mentalnim bolestima, što sugerira da oni imaju važnu ulogu u patofiziologiji ovih stanja.

Ovaj se rad usredotočio na pružanje uvida u ponašanje TRIOBP-1 i DISC1 u neuronskim stanicama, što nam omogućuje da istražimo ponašanje tih proteina i njihovu potencijalnu povezanost s mentalnim bolestima. Primarni cilj bio je utvrditi optimalnu dozu doksiciklina i razdoblje tretmana za ekspresiju proteina, nakon čega slijedi

detekcija agregacije proteina pomoću Western blottinga, fluorescentne mikroskopije i ispitivanja netopivosti. Dokazivanjem sklonosti agregaciji ovih proteina, pokušava doprinijeti razumijevanju kako nepravilno savijanje proteina može biti povezano s neurodegenerativnim bolestima.

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# 1 Introduction

## 1.1 What is mental illness?

Mental health is a substantial part of our general health, well-being, and basic human rights. It falls along a wide spectrum, with experiences ranging from an optimal state of well-being to exhausting states of emotional pain and suffering. Mental health conditions are highly prevalent in many countries. This can cause distress and problems in daily life, affecting work, school, and relationships. Approximately one in eight people in the world live with some kind of mental disorder (1). Even though most people are resilient, there are people who are exposed to unfortunate circumstances, such as violence, poverty, inequality, or disability, and that group of individuals is at higher risk. Protective and risk factors include individual psychological and biological elements, such as emotional abilities and genetic predispositions. Many of these factors are shaped by alterations in brain structure and function (2). Mental health disorders are characterized by serious impairments in an individual's behaviour, cognition, and emotional regulation.

There are many different types of mental health disorders that are usually associated with distress or significant disturbances in important areas of functioning. These include illnesses such as schizophrenia (SZ), major depression (MDD), obsessive-compulsive disorder, bipolar disorder (BD), and post-traumatic stress disorder. SZ involves positive symptoms like hallucinations, negative symptoms like lack of motivation, and cognitive difficulties (3), impacting daily life and increasing suicide risk (4). BD alternates between manic and depressive episodes, with genetic and environmental factors influencing its development, similar to schizophrenia (3)(5). MDD causes persistent sadness, lack of energy, and can lead to self-harm or suicide (3), affecting 4.4% of the global population (6).

Due to their lifelong recurrence, some can be categorized as chronic (chronic mental illnesses). This can lead to disabilities and premature

death. The prevalence of mental disorders varies according to age and sex. The most common ones in both males and females are depressive disorders and anxiety disorders. SZ is the mental illness which causes the most impairment, and affects approximately 1 in 200 adults (5).

## 1.2 Protein aggregation

Proteins are biomacromolecules crucial for regulating many cellular functions, including the synthesis of structural components. The core concept of protein folding is the ability to combine various amino acids with distinctive functional groups to create three-dimensional structures that serve a variety of biological and chemical purposes. In living organisms, proteins are created by assembling long chains of amino acids on the ribosomes. These newly formed polypeptide chains must then undergo a complex folding process to form compact three-dimensional structures, known as the 'native state,' which refers to a set of closely related shapes. Only proteins that fold correctly can perform their biological functions under cellular conditions, as proper folding is crucial for processes such as intracellular trafficking, cellular signalling, and regulation of cell growth and differentiation. However, they remain stable under physiological conditions. Even slight changes in the environment can destabilize proteins, causing them to misfold and aggregate (7).

Protein folding into functional shapes is an essential biological process. Under normal conditions, aggregated proteins are degraded or removed by cell quality control systems such as the ubiquitin-proteasome system and autophagy. However, these systems can be overwhelmed and impaired, leading to the generation of toxic protein aggregates. This disrupts the protein balance (proteostasis) in cells and leads them to interact via different mechanisms in the cell to generate toxicity. Protein-misfolding diseases include neurodegenerative, localized, and systemic amyloidosis (7).

### 1.2.1 Proteinopathy

Proteinopathies, also known as protein conformational disorders or protein misfolding diseases, are a group of diseases in which certain proteins become structurally compromised, reducing the ability of the body's tissues, cells, and organs to function in general (8).

Proteinopathies most commonly refer to a group of neurodegenerative diseases characterized by the abnormal accumulation of misfolded proteins in the brain. Accumulation of these misfolded proteins can create aggregates that form toxic structures, causing neuronal dysfunction and cell death. The three most prevalent proteinopathies are Huntington's, Parkinson's, and Alzheimer's diseases (9).

The connection between proteinopathies and mental illness is increasingly being recognized because many neurodegenerative diseases exhibit psychiatric symptoms alongside cognitive decline (10)(11). For example, people with Alzheimer’s disease experience anxiety, depression, and psychosis, whereas those with Parkinson’s disease often report similar mood disorders. One of the mechanisms connecting mental illness and proteinopathies could be neuroinflammation because misfolded proteins can trigger inflammatory responses in the brain, which can then negatively contribute to mood disorders. Cognitive decline is associated with neurodegeneration, which can lead to secondary mental health issues due to the loss of independence and quality of life (11)(9). Another option is that smaller or less toxic misfolded proteins can accumulate in brains of patients with mental illnesses, potentially appearing earlier than in typical neurodegenerative stages (10).

### 1.2.2 Methods to study protein aggregation

There are methods for analysing size and shape of protein aggregates (for example: dynamic light scattering and atomic force microscopy), secondary structure (for example: circular dichroism) and more in-depth analysis at the level of atoms (cryo-electron microscopy). Also, there are now many

protein aggregation prediction tools, based on protein sequence and known data about protein structure (12)(13). All these methods can provide a lot of valuable information about the protein itself, but not how the protein behaves in the cell. Protein aggregation in cells can be investigated visually, for example with fluorescent microscopy if the protein has a fluorescent tag. In that case, a plasmid containing the gene for a protein must be transfected in large amounts into a cell system, leading to an over-expressed protein. Other approaches include experiments with other, non-fluorescent tags, or completely removing the tag, and using antibodies specific to the endogenous protein. An alternative, more precise, approach for investigating the human form of genes are cells with controlled gene expression (14). An example of this is The Tetracycline-controlled Transcriptional Activation system, developed in 1992. These systems use a fusion protein (tTA) that binds to a gene's promoter, requiring the presence of tetracycline or its derivatives for gene expression, so called "Tet-On" system. This system is favoured for its ability to rapidly and reversibly control gene expression in cell models, though it can suffer from leakiness and potential cell toxicity at high tetracycline concentrations (15). The retroviruses were used to add the DISC1 or TRIOBP-1 gene into the genome of SH-SY5Y cells, along with the tTA protein. The cell lines were generated by Dr. Svenja Trossbach and Dr. Nicholas Bradshaw at the Heinrich Heine University, Düsseldorf.

Protein aggregates in these cells can be visualised, for example after staining with specific antibodies and detecting it under a fluorescent microscope. Another approach is to isolate insoluble protein fractions from cell lysates and check if DISC1 and TRIOBP-1 are present in this fraction by Western blot. The protocol for isolating insoluble proteins starts with homogenizing samples from cells in a specific buffer, followed by rounds of centrifugation and different buffers until only the most insoluble protein fraction is left in the pellet. Insoluble proteins usually imply, but does not necessarily guarantee, that they are also aggregating.

## 1.3 Proteins already described as aggregating in mental illness

Proteins can be connected to aggregation in mental illnesses if they were found in patients with diagnosed mental illness using:

a) genetic methods (like DISC1),
b) methods for isolating insoluble proteins followed by proteomic methods (like TRIOBP-1).

Besides DISC1 and TRIOBP-1 (16) (17), there are other proteins which have been described as aggregating in mental illnesses: dysbindin, NPAS3, CRMP1 and EHD3 (10).

### 1.3.1 Disrupted in Schizophrenia 1 (DISC1)

Disrupted in Schizophrenia 1 (DISC1) is a scaffolding protein expressed in brains and other tissues (18). In the literature, DISC1 is referred to as a scaffold protein, because it interacts with over 200 proteins. Due to the abundance of interaction partners, DISC1 is involved in many processes associated with brain development and diseases. The processes and DISC1 interaction partners which facilitate the mentioned processes are listed in Table 1 (19)(20).

**Table 1.** Examples of DISC1 functions and interaction partners

| **Function** | **Example interacting proteins** |
|---|---|
| Brain functions | |
| Neurogenesis | GSK3β, DIXDC1, LIS1, NDE1 |
| Migration of neurons | LIS1, NDEL1, DIXDC1, amyloid precursor protein |
| Neuronal integration and maturation | NDEL1, FEZ1, Girdin, NKCC1, phosphodiesterase 4 |
| Neurosignaling | PSD-95, Kalirin-7, TNIK, serine racemase, TRIO |
| Subcellular level | |
| Nucelus – transcription regulation | ATF4, ATF5 |

| | |
|---|---|
| Centrosome – organization of microtubules | NDE1, NDEL1, LIS1, PCM1, CAMDI, PCNT |
| Mitochondria | Mitofilin |
| Motor proteins | kinesin1, FEZ1 |

However, the mechanism by which this happens and how DISC1 causes mental illness are unresolved because the structure of DISC1 has to be further researched. Using the Expression of Soluble Proteins by Random Incremental Truncation (ESPRIT) technique, four structured regions were identified in the DISC1 protein, named D, I, S, and C. Each region has unique structural properties, influencing their roles in oligomerization. The D and I regions form α-helical dimers, with region I showing a strong tendency to aggregate, while the S region forms a stable tetramer. Region C, located at the end of DISC1, plays a major role in oligomerization and overall protein stability (21). New results showed how the combination of the D and I regions, plus the linker region between them is necessary for DISC1 aggregation (22).


**Figure 1: Domain structure of DISC1 with main domains marker (D, I, S and C)**

DISC1 in mammalian cells was observed to aggregate when overexpressed (16) or when dopamine was added (23). Interestingly, DISC1 aggregates were seen to travel between cells (24) (25) (26). When the human DISC1 gene was expressed in a rat, there were insoluble aggregates of DISC1 and that rat was sensitive to amphetamine, had motor defects and deficits in attention and long-term memory (27). Moreover, when the human DISC1 gene was expressed in *Drosophila*, it was associated with disturbance in sleep homeostasis (28), impaired memory and neurodevelopmental defects

(29), altered social dynamics, decreased ability to climb vertical surfaces and effects on the redox system (30).

### 1.3.2 Trio and F-actin Binding Protein 1 (TRIOBP-1)

Trio and F-actin Binding Protein isoform 1 (TRIOBP-1) is a member of the protein family encoded by the *TRIOBP* gene (31). Besides TRIOBP-1, there are other alternatively spliced isoforms, including TRIOBP-4, TRIOBP-5, and TRIOBP-6. TRIOBP-4/5 mutations are linked to autosomal recessive nonsyndromic hearing loss (32). TRIOBP-1 is the smallest among them, consisting of 652 amino acids in its full length form, while a shorter 597 amino acid isoform is also expressed in humans. In normal human cells functioning as a universally expressed major regulator of actin, TRIOBP-1 binds itself to F-actin and stops it from depolymerising (33). It is involved in cellular migration and interactions between the cell membrane, extracellular matrix and cytoskeleton, the production of stress fibres in the heart and neuronal differentiation and migration (31).

TRIOBP-1 has a complex structure that is crucial for its ability to regulate the actin cytoskeleton and other various cell processes. We can breakdown its structure to 3 main domains: The Pleckstrin homology (PH) domain, Central Coiled-Coil (CCC) domain and C-terminal Coiled-Coil domain (34)(35). The PH domain seems to be folded and compact, it is located near the N-terminus, and it is thought to improve protein-protein interactions, while its exact function is not yet fully understood. The PH domain also contains two extended unstructured loops that may make it prone to aggregation. The C-terminal half of TRIOBP-1 contains two major coiled-coil domains. The CCC domain is bigger and responsible for oligomerisation, which allows TRIOBP-1 to form more complex structures like hexamers. It is important for protein functions and interactions with F-actin. It is suspected that this domain is responsible for aggregation (35). The C-terminal Coiled-Coil Domain is smaller and contributes to the structural integrity of TRIOBP-1. So far it is known that two distinct regions within the TRIOBP-1 protein that can independently induce aggregation: the 59 amino

acids at the N-terminus and a region of 8 amino acids (333-340) in the central part of the protein. An artificial isoform of TRIOBP-1 that lacks both of previously mentioned regions showed reduced potential for aggregation (17)(36).


**Figure 2: Domain structure of TRIOBP-1 with main domains**

Besides aggregating in mammalian cell culture, TRIOBP-1 was also seen to aggregate in rat primary neurons and inhibits neurite development in neuron-like cell culture models (17).

As was previously mentioned, TRIOBP-1 binds to actin and regulates its polymerization. Additionally, TRIOBP-1 was seen to co-localize with other actin associated proteins, actinin and myosin II (33). Moreover, TRIOPB-1 was seen to interact with Nuclear Distribution Element-Like 1 (NDEL1), associated with microtubules (34). In mammalian cells, TRIOBP-1 was seen to co-aggregate with DISC1 and NDE1. While DISC1 is previously described, Nuclear Distribution element 1 (NDE1) plays an important role in the organization of the microtubules and in neurodevelopment. On the other hand, TRIOBP-1 did not co-aggregate with other interaction proteins; HECTD3, TRF1 and PLK1, which are responsible for its phosphorylation, expression levels and localization during prophase and metaphase (37).

## 1.4 DISC1 and TRIOBP-1 in mental illness

DISC1 is a key candidate for the pathology of major mental illnesses, particularly major depressive disorder and schizophrenia. DISC1 was linked to mental disease thorough examination of a unique family with severe mental illness. It was identified that a chromosomal translocation event strongly connected with this severe mental illness (38)(39). Also, there is a mutation in the DISC1 gene, observed in another family with schizophrenia (40). DISC1 was seen as insoluble in brain samples from

patients with schizophrenia, bipolar disorder and major depressive disorder (16)(24).

Previous studies had identified TRIOBP-1 insoluble aggregates specifically in the brains of schizophrenia patients. The researchers used a high stringency protein purification protocol to detect the aggregated, insoluble form of TRIOBP-1 in post-mortem brain samples. This protocol was able to isolate the most insoluble protein aggregates, unlike a lower stringency protocol that would also detect the normal, soluble TRIOBP-1 bound to actin (17). Insoluble TRIOBP-1 is also considerably more prevalent in the brains of patients with major depressive disorder compared to healthy controls (41). This suggests that TRIOBP-1 aggregation, like DISC1, may be a common pathological feature underlying multiple mental illnesses, rather than being specific to one condition(41).

# 2 Aims of the thesis

The concept behind this project is using SH-SY5Y neuroblastoma cells with inducible DISC1 or TRIOBP-1 protein expression, which should be more effective for studying aggregation compared to plasmid-based overexpression. In theory, these cells can be optimized to show minimal aggregation initially, with aggregation triggered later by stress factors or another stimulus.

The system I used was "Tet-On", which in theory allows controlled gene expression by binding a fusion protein (tTA) to gene's promoter in presence of doxycycline, a derivate of tetracycline. The gene of interest (DISC1 or TRIOBP-1) was transfected in the genome of SH-SY5Y Tet-On cells via retroviruses, with tTA protein.

The specific aims are to:

1. test which dose of doxycycline and time period of treatment can induce expression of DISC1 and TRIOBP-1 in these cells
2. if the protein is expressing, check if the aggregates are present (visually or by insolubility assay)

# 3 Materials and methods

## 3.1 Cell maintenance

Mammalian cell line SH-SY5Y, derived from human neuroblastoma (ACC 209, Deutsche Sammlung von Mikroorganismen und Zellkulturen) is used in this project, with inserted DISC1 (23) or TRIOBP-1 gene (Trossbach, Bradshaw & Korth, unpublished).

The cells were cultivated in t25 flasks, with cells adhering to the bottom surface, in a Nüve $CO_2$ incubator at 37°C with a 5% $CO_2$ atmosphere. To ensure a sterile environment within the incubator and hood, all equipment and surfaces were sprayed with 70% ethanol, 0.05% incidin, 70% isopropanol and DNA-Erase spray (Lookout) before use.

The media for them was DMEM-F12 (Thermo Fisher Scientific), with added 1x MEM non-essential amino acids w/o L-Glutamine (PAN Biotech), 10% Fetal Bovine Serum (Sigma-Aldrich) and antibiotics Penicillin-Streptomycin (PAN Biotech).

When cells reached a confluency of 60-75%, they were split into a new flask or to experimental plates. Splitting starts with preheating the trypsin solution (PAN Biotech) and the DMEM-F12 medium to 37°C. I removed the old media from the t25 flask by aspiration and added 1 ml of trypsin per 1 t25 flask which was then incubated for either 5 min in the incubator at 37°C or for 10 min at room temperature in the hood. During the incubation, trypsin breaks down the proteins that enable cells to adhere to the flask. After incubation, the flask was gently tapped on the side to make sure all cells detached. 4 mL of the DMEM-F12 media was added to the flask for neutralising trypsin and cells were transferred to a new flask or to the plate, with fresh media. For setting up a new flask, I used a minimum of 100 µL for SH-SY5Y-DISC1 or 500 µL for SH-SY5Y-TRIOBP-1. For setting up experimental plates, I used from 600 to 800 µl of SH-SY5Y-DISC1 or from 500 to 600 µl of SH-SY5Y-TRIOBP-1 for 6-well plates, from 300 to 400 µl

of SH-SY5Y-DISC1 or from 200 to 300 µl of SH-SY5Y-TRIOBP-1 for 12-well plates. If the experiment was being set up for microscopy, glass coverslips were placed in the wells of the plates before adding media and cells.

## 3.2 Cell freezing and thawing

For freezing, the cells were detached from flask the same way as in the splitting protocol and transferred to 15 ml Falcon tube. The cells were gently centrifuged at 1000 rpm for 5 minutes, after which the media was removed. The cell pellet was resuspended in 1 ml FBS per flask and aliquoted into cryotubes. To ensure safe freezing of cells, the DMSO was added to equal 10% of total volume. As a slow freezing is better for cells, the tubes were firstly cooled at 4°C for minimum of 2 hours, then frozen at -20°C overnight and then stored at -80°C. For thawing of cells, I established a new protocol, which gave a better cell survival rate. Room temperature media was added to a 15 ml Falcon tube. The cells from -80°C were quickly thawed, added to the media in Falcon tube and resuspended. Then they were centrifuge for 5 minutes, at room temperature at around 400-1000 rpm. In the meantime, media was warmed to 37°C and added to a t25 flask. After the centrifuge is done, all media was removed, with it the DMSO from freezing as well. 1 ml of warm media was used to gently resuspend the cells in the pellet, and they were transferred to the t25 flask containing the rest of warm media. The cells were then incubated in the incubator at 37°C, as usual.

## 3.3 Treatment with doxycycline

For initiating gene transcription, the cells were treated with specific amounts of doxycycline. Doxycycline (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO, high purity, Applichem) in a stock solution with a concentration 1 mg/mL. When cells were confluent enough for an experiment, the media was removed from the wells with cells, and 1 ml fresh media was added. Afterwards, to each well was added 0.1, 0.25, 0.5, 1 or 2.5 µL of doxycycline solution, to ensure the following working

solutions: 0.2, 0.5, 1, 2 or 5 µg/mL. The cells were incubated with doxycycline for 24 hours initially, later 48 hours, after which they were either lysed for insoluble protein fraction purification or fixed for immunocytochemistry. Control wells were cells without treatment and cells treated only with 1 µL of DMSO.

## 3.4 Insoluble protein fraction purification

Using a previously published protocol (40), the insoluble protein fraction from the cells were purified. After removing the media from the wells, the cells were washed two times with 1xPBS (Phosphate-Buffered Saline; concentrations: 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mm disodium hydrogen phosphate, and 1.8 mM potassium dihydrogen phosphate). After that, the cells were lysed using 200 µL of lysis solution (which included 50 mM HEPES pH 7.5, 250 mM sucrose, 5 mM magnesium chloride, 100 mM potassium acetate, 2 mM PMSF, 1xProtease Inhibitor Cocktail, and 1% Triton-X100) and incubated on ice for five minutes. Following the transfer of the lysed cells from the wells to ultracentrifuge tubes (Science Services), in each tube added 2.5 µL of Triton-X-100. Before centrifugation, 60 µL of the sample was collected as a control (referred to later as "lysates"). The samples were centrifuged at 20,000 x g for 20 minutes at 4°C (S140-AT fixed-angle rotor in Thermo Scientific Sorvall MTX 150 Micro-Ultracentrifuge). The supernatant was carefully removed, and the pellet was lysed once again by adding previous buffers. After another centrifugation at 20,000 x g for 20 minutes at 4°C, the supernatant was removed, and the pellet was resuspended in 200 µL of buffer A1 (50mM HEPES pH 7.5, 1,6M sucrose, 100mM potassium acetate, 1%Triton-X-100, 1mM PMSF), followed by a centrifugation at 130,000 x g for 45 minutes at 4°C. This step was also repeated. After the second centrifugation, the supernatant was discarded, and this time the pellet was resuspended in 200 µL of buffer B1 (50mM HEPES, pH 7.5, 1M sodium chloride, 20mM magnesium chloride; 30Mm Ca2+, 2 U/mL DNaseI, 1xProtease Inhibitor Cocktail) and incubated overnight at 4°C. The next day, the samples were

centrifuged at 130,000 x g for 45 minutes at 4°C. The supernatant was removed, and previous step was repeated, but without adding DNaseI and incubation. Another round of centrifugation at 130,000 x g for 45 minutes at 4°C followed. The pellet was then dissolved in 200 µL of buffer C1 (50mM HEPES, pH 7.5, 0.5% sarkosyl), with the help of an insulin syringe and a 0.4 mm needle. The samples were then incubated on ice for approximately 1 hour on a shaking tray and later centrifugated at 112,000 x g for 45 minutes at 4°C. The previous step was repeated, needle and syringes used, but the incubation on ice was skipped and the centrifugation was at 112,000 x g for 45 minutes at 4°C. Finally, the pellet that emerged was made of insoluble proteins. That final pellet and previously saved lysates were prepared for Western blot by diluting in 2x protein loading buffer and dithiothreitol (DTT) was added to make 10% of the total volume. The proteins were then denaturated at 95°C for 5 minutes and stored at -20°C.

## 3.5 SDS-PAGE and Western blot

Proteins from cell lysates were separated by their molecular weight on handmade acrylamide gels and run on the BioRad SDS-PAGE system. Gels were poured in 1.5mm glass plates and the solution was made from: acrylamide mix (acrylamide and N,N,N,N-Metylenbisacrylamide, 8-10% of total volume), Tris-Cl 1.5 M pH 8.8, 1% sodium dodecyl sulfate, 1% ammonium persulfate and N,N,N’,N’-Tetramethylethylenediamine (TEMED). The stacking gel (5% acrylamide, Tris-Cl 1.0 M, pH 6.8, 11% sodium dodecyl sulfate, 1% ammonium persulfate and TEMED) was poured on top. Samples and the marker my-Budget Prestained Protein Ladder 10 kDa-180 kDa (Bio-Budget Technologies GmbH, 0.2-0.4 µg/µL) were loaded onto the gel. The electrophoresis was run at 180 V for 45 minutes in 1x SDS-PAGE running buffer (25 mM Tris-Cl, 250 mM glycine, 0.1% SDS). After electrophoresis, the proteins were transferred onto a Parablot PVDF membrane (Macherey-Nagel, 0.2 µm pore) in BioRad Transblot Turbo system. The transfer was checked with Ponceau S staining to visualise the proteins on the membrane. After this, the membrane was blocked for 1

hour in a 5% milk powder/PBS-Tween solution. Proteins were detected using an appropriate primary antibody (anti-DISC1, Invitrogen or anti-TRIOBP-1, Atlas) diluted 1:1000 in PBS-Tween for 4 hours at room temperature or overnight at +4°C. After incubation, the membrane was washed with 1x PBS-Tween (3 times over course of 30 minutes) and secondary antibody (goat anti-rabbit antibody conjugated with HRP, diluted 1:10000 in PBS-Tween) was added on the membrane, followed by incubation at room temperature for 1 hour. The membrane was washed once again with 1x PBS-Tween and the Pierce ECL Prime Western Blotting Substrate (Thermo Scientific) or was used for visualisation. The instrument used for imaging membranes was a ChemiDoc MP Imaging System with Image Lab (BioRad) software.

## 3.6 Immunocytochemistry and fluorescent microscopy

Cells grown on glass coverslips were washed with 1x PBS per well, fixed with fixation buffer (4% paraformaldehyde in 1xPBS) for 15 minutes and permeabilized with permeabilizing buffer (1% Triton X-100 in 1xPBS) for 10 minutes at room temperature. Afterwards, the cells were washed with 1xPBS three times, and blocked in 10% goat serum in 1xPBS solution for a minimum of 45 minutes on a shaking tray at room temperature. After blocking, the cells were stained with primary antibody (anti-DISC1, Invitrogen), diluted 1:1000 in 10% goat serum in 1xPBS, for a minimum of 4 hours at room temperature. Next, the cells were washed 3 times with 1xPBS over 15 minutes and stained with a mixture of secondary antibody (goat anti-rabbit, Thermo Fischer), DAPI (Sigma-Aldrich) and acti-stain 488 Phalloidin (Cytoskeleton), each diluted 1:500 in 10% goat serum in 1xPBS solution. The plate was incubated for 1 hour in the dark at room temperature and once again the cells were washed with 1x PBS 3 times in 15 minutes and once with distilled water. The cells were then attached to the glass slides with commercial Mounting Medium Flouroshield (Sigma). The coverslips were analysed under 40x magnification on an Olympus IX83

fluorescent microscope with Hamamatsu Orca R2 CCD camera and CellSens software. The images for thesis were prepared using ImageJ software.

# 4 Results

## 4.1 Testing doxycycline toxicity

Before the experiments progressed, both DISC1 and TRIOBP-1 cells were visually checked under light microscope after treatment with various levels of doxycycline, to test for doxycycline toxicity. The confluence remained the same across all tested concentrations (0.2, 0.5, 1, 2 and 5 µg/mL), with no obvious cell death.

## 4.2 Testing DISC1 expression using Western blotting

To start off, to test if expression of DISC1 gene in SH-SY5Y-DISC1 cells can by induced, they were treated with doxycycline in the following range of concentrations: 0.2, 0.5, 1, 2 and 5 µg/mL. The treatment was done initially for 24 hours, followed by cell lysis and Western blot. The results are shown in Figure 3.

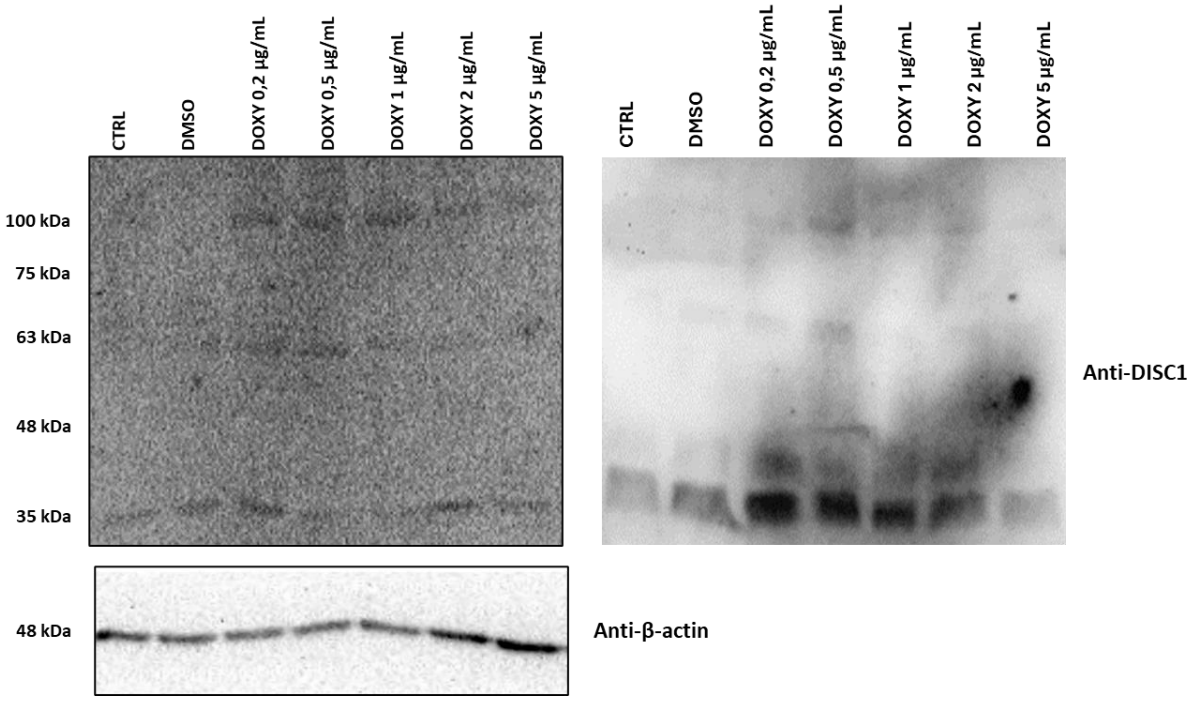



**Figure 3: SH-SY5Y-DISC1 cells show low levels of the majority of DISC1 variants, except for the variant at 35 kDa after 24 hours treatment with doxycycline.** Cells SH-SY5Y-DISC1 were treated for 24 hours with different concentration of doxycycline

(doxy). DISC1 was confirmed by Western blot, stained for both DISC1 and β-actin. The samples were re-run again and stained for DISC1 only (image on the right).

When SH-SY5Y cells are treated for 24 hours with doxycycline, there is no significant difference in DISC1 band intensity. There are multiple DISC1 bands, with the most prominent being the one at 35 kDa across all lanes and weak bands showing up at 100 and 75 kDa for lanes treated with doxycycline only.

In an effort to induce higher DISC1 expression, cells were treated with doxycycline for a longer period (48 hours), again followed with cells lysis and Western blot. The results are shown in Figure 4.

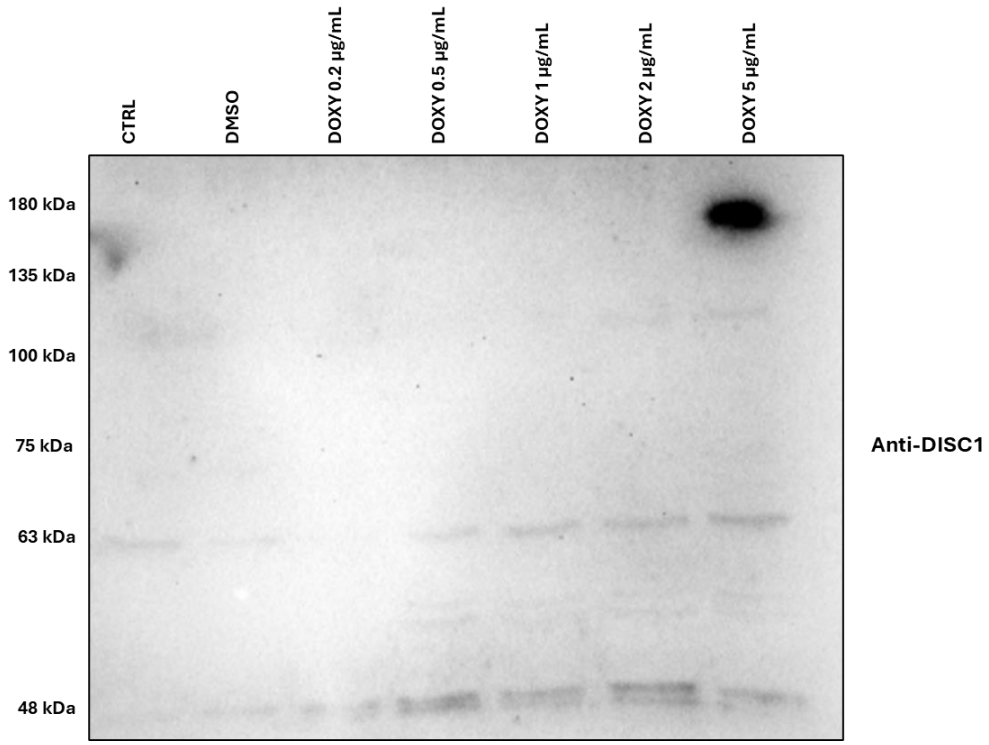



**Figure 4: SH-SY5Y-DISC1 cells show low levels of the majority of DISC1 variants, except for the variant at 35 kDa after 48-hours treatment with doxycycline.** Cells SH-SY5Y-DISC1 after 48-hours treatment with different concentration of doxycycline (doxy). DISC1 was confirmed by Western blot, stained for both DISC1 and β-actin.

When the treatment with doxycycline was prolonged to 48 hours, the more intense higher band of DISC1 start to show up across all lanes. The band at 48 kDa is the most prominent one across all lanes, while the weak band

between 100 and 135 kDa is showing up only after treatment with doxycycline.

## 4.3 Testing DISC1 aggregation by insolubility assay and Western blot

To check if the induced DISC1 is prone to aggregation, after doxycycline treatment the cells were analysed with the insolubility assay to purify insoluble protein fraction and the presence of DISC1 was investigated with Western blot in whole cell lysates and in newly purified protein insoluble fraction.

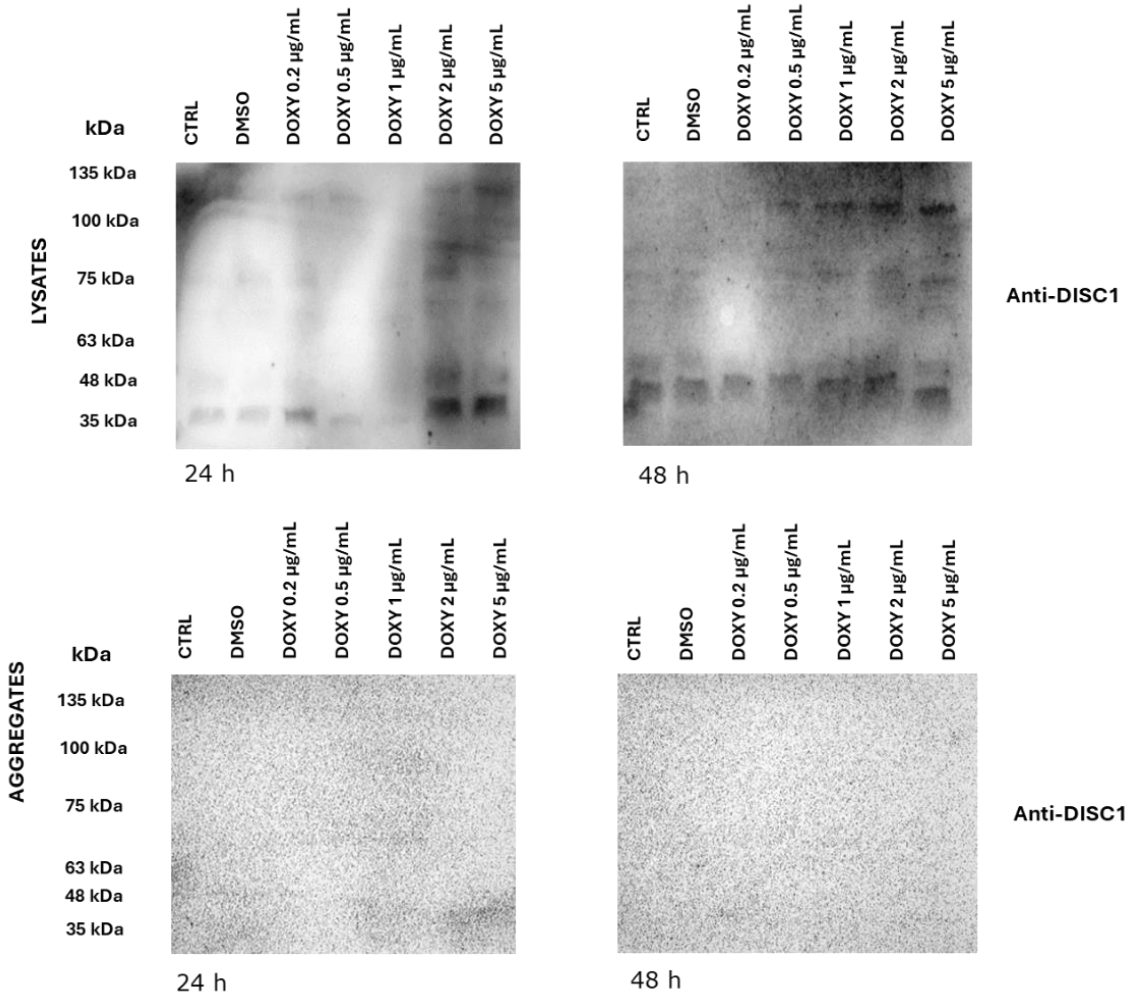


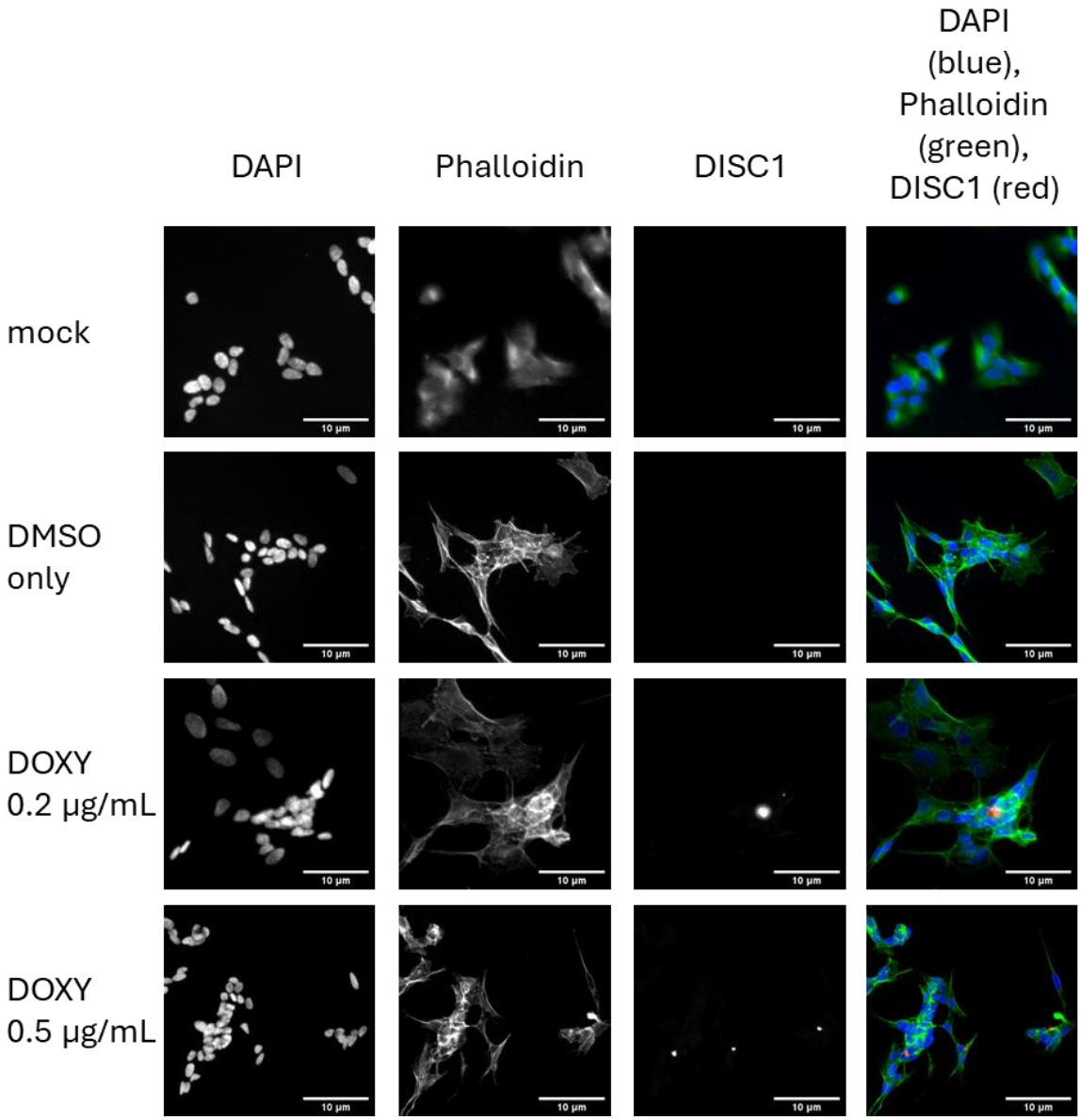

**Figure 5: No aggregation of DISC1 was observed after 24 and 48 hours treatment with doxycycline.** Cells SH-SY5Y-DISC1 after 24 and 48 hours treatment with different concentration of doxycycline. Lysates represent whole protein fraction per

sample, while aggregates represent insoluble/aggregating protein fraction purified by insolubility assay. Expression of DISC1 was confirmed by Western blot.

DISC1 band at 35 kDa is prominent across all lanes in lysates, including untreated cells and cells treated with only DMSO. Higher bands at 75 and 100 kDa were observed in treated cells only.

## 4.4 Testing DISC1 expression by fluorescent microscopy

To provide more visual data about DISC1 expression in SH-SY5Y-DISC1 cells, the cells were seeded in wells with microscopy cover slips and once again treated with doxycycline (the same concentration range as previously). The treatment was done for 48 hours, followed by



immunocytochemistry and analysis on fluorescent microscope. The results are shown in Figure 3.

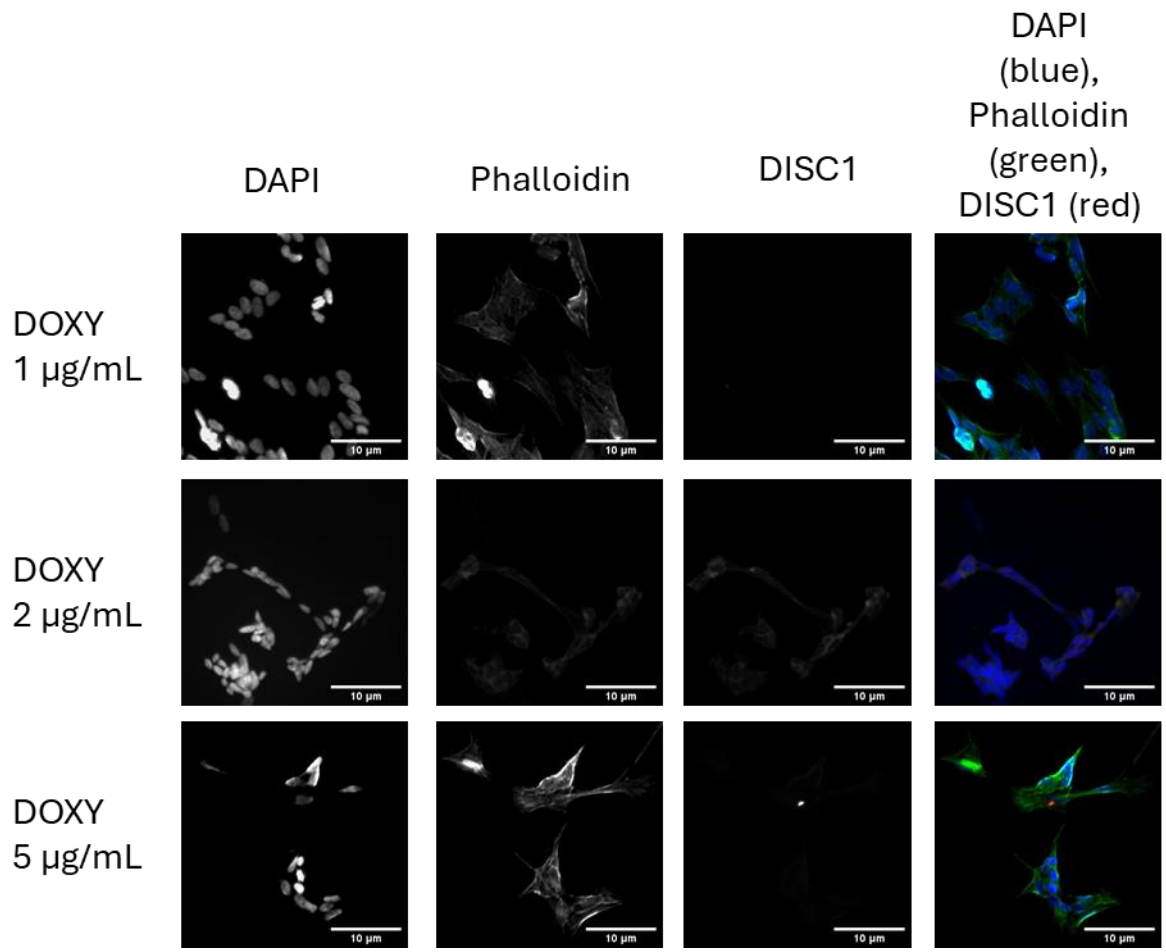



**Figure 6: DISC1 aggregates in wide range of doxycycline concentration after 48-hours treatment in SH-SY5Y-DISC1 cells**. DISC1 aggregates can be seen in after treatment with 0.2, 0.5 and 5 µg/mL doxycycline, while no DISC1 signal was detected after 1 and 2 µg/mL doxycycline. DISC1 expression was confirmed by immunocytochemistry. The images were taken at 40 x and the scale bar represents 10 µm.

When analysed with the fluorescent microscope, the presence of DISC1 aggregates differs depending on the doxycycline concentration. When treated with 0.2, 0.5 or 5 µg/mL doxycycline, there are at least two cells in the imaging field with large DISC1 aggregates. However, after 1 and 2 µg/mL doxycycline there is no DISC1 signal to be detected in all observed cells.

## 4.5 Testing TRIOBP-1 expression by Western blot

Similarly, to DISC1, TRIOBP-1 expression was analysed in SH-SY5Y-TRIOBP-1 after treatment with doxycycline. The cells were treated with a range of concentrations, the same as for DISC1, for 48 hours, after which they were lysed and TRIOBP-1 presence was investigated by Western blot.

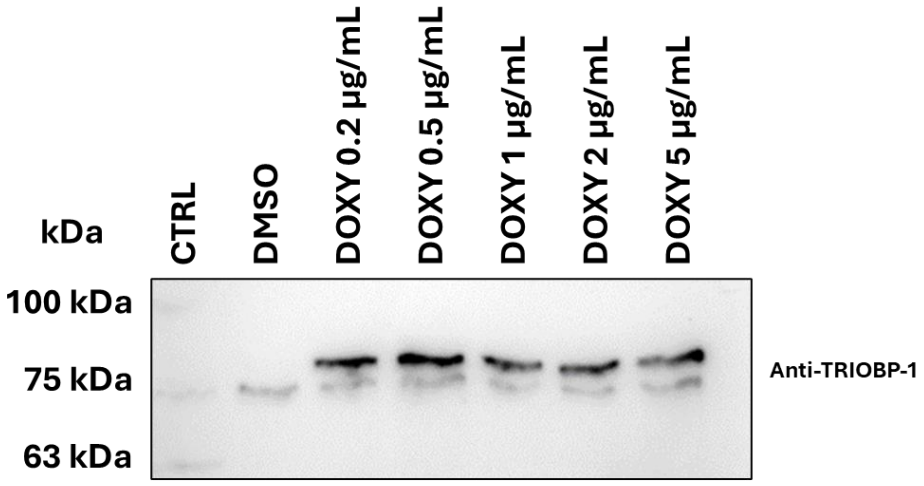



**Figure 7: TRIOBP-1 maintains a similar level of expression in SH-SY5Y cells, regardless of doxycycline amounts added.** Cells SH-SY5Y-TRIOBP-1 after 48-hours treatment with different concentration of doxycycline. Expression of TRIOBP-1 was confirmed by Western blot. The intense TRIOBP-1 band, slightly larger than 75 kDa, is present in cells treated with doxycycline, while the weaker band at 75 kDa is present across all samples.

After doxycycline treatment for 48 hrs, there was no significant difference in TRIOBP-1 band intensity in Western blot. The most intense TRIOBP-1 band, the one a little above 75 kDa, can be observed only after the treatment with doxycycline, while the less intense band, at 75 kDa, is seen in controls and after the treatment.

## 4.6 Testing TRIOBP-1 aggregation by insolubility assay and Western blot

After inducibility of TRIOBP-1 expression by doxycycline treatment of SH-SY5Y-TRIOBP-1 cells was confirmed in Western blot, the cells were seeded in larger 6-wells plate, grown until 70% confluency and then lysed for the insolubility assay described in Materials and methods. Briefly, by insolubility assay, during rounds of different buffers and ultracentrifugation, the insoluble protein fraction was purified and compared to the whole cell lysate in Western blot analysis.

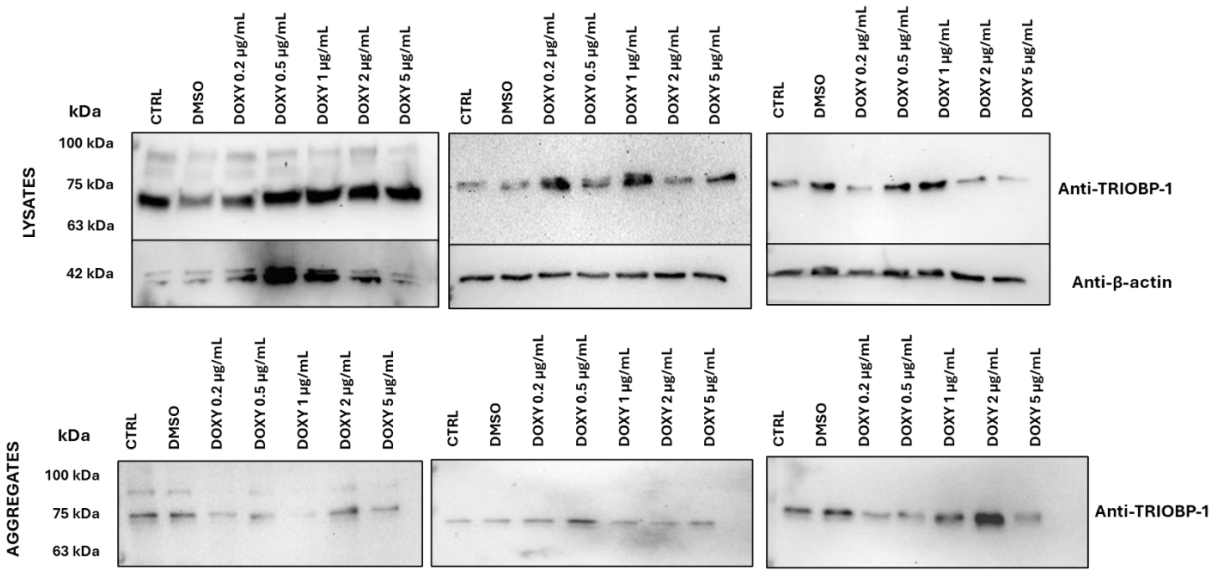


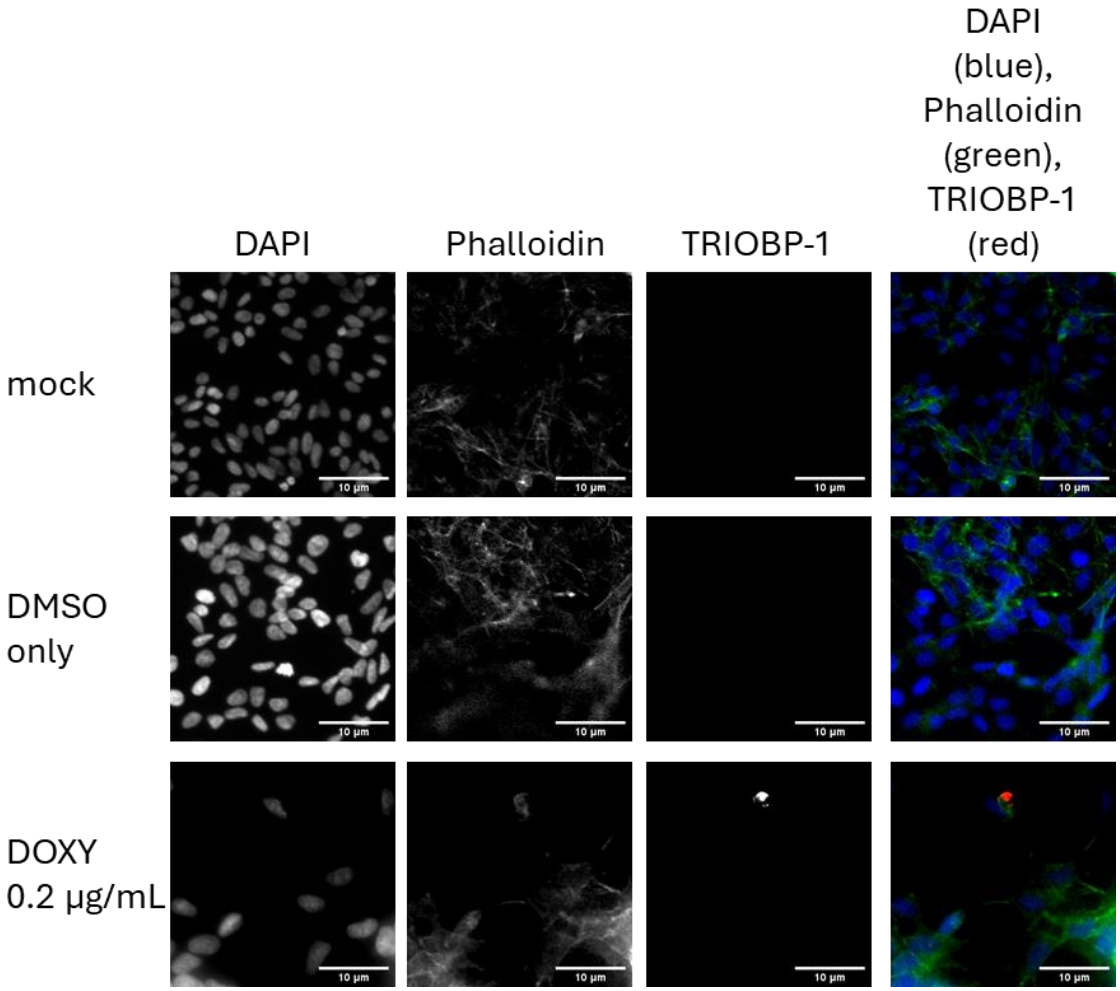

**Figure 8: Aggregation of TRIOBP-1 varies depending on concentration of doxycycline.** Cells SH-SY5Y-TRIOBP-1 after 48-hours treatment with different concentration of doxycycline. Lysates represent whole protein fraction per sample, while aggregates represent insoluble/aggregating protein fraction purified by insolubility assay. Expression of TRIOBP-1 was confirmed by Western blot. The TRIOBP-1 band at 75 kDa is prominent across all lanes, including untreated cells and cells treated with only DMSO. The experiment was repeated three times, as shown.

In the lysate fraction containing all proteins present in SH-SY5Y-TRIOBP-1 cells, there is a little variation between treated cells and controls and among the treatment groups. Treatment with 1 µg/mL gave one of the most intense bands at 75kDa in all three experiments. In one instance the higher

bands of TRIOBP-1 (close to 100 kDa) were detected. The bands are also present in control groups, mock transfected and cells treated with DMSO only. In the insoluble protein fraction, TRIOBP-1 bands at 75 kDa remained consistent, with higher TRIOBP-1 bands visible only in the first experiment. The exception can be seen in the third experiment, where after 2 µg/mL, the band for TRIOPB-1 at 75 kDa is significantly more intense than the rest of the bands.

## 4.7 Testing TRIOBP-1 expression by fluorescent microscopy

To provide more insight into TRIOBP-1 expression after doxycycline treatment of SH-SY5Y-TRIOBP-1 cells, the cells were seeded on cover slips, treated with doxycycline, and analysed with immunocytochemistry and fluorescent microscopy.



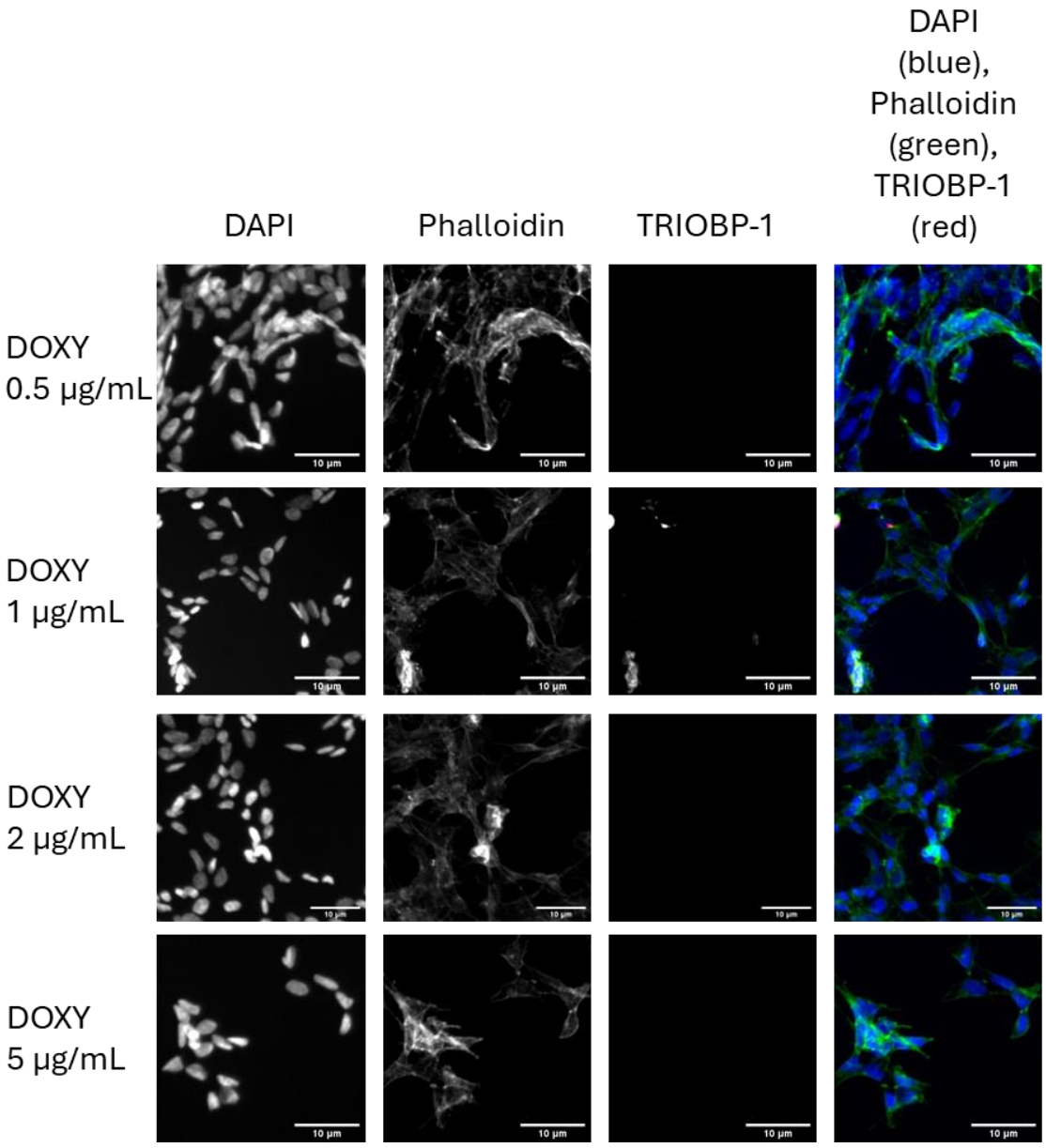



**Figure 9: TRIOBP-1 expression could not be detected visually in SH-SY5Y-TRIOBP-1 cells, after a wide range of doxycycline concentration and 48-hours treatment.** The signal at 0.2 µg/mL treatment is an artefact of staining, not a real signal. TRIOBP-1 expression was investigated by immunocytochemistry. The images were taken at 40 x and the scale bar represents 10 µm.

When TRIOBP-1 expression after doxycycline treatment was investigated with immunocytochemistry, there was no detectable TRIOBP-1 signal. There was a red signal showing up in the cell after 0.2 and 1 µg/mL

doxycycline treatment, however it did not overlap with the cell body, and it was most likely an artefact of staining.

# 5 Discussion

To reflect on aims of this thesis I used cells with inducible expression of DISC1 or TRIOBP-1 proteins to study protein aggregation more effectively than with plasmid-based methods. Using the "Tet-On" approach, I attempted to control the expression of proteins with doxycycline, allowing for initial low aggregation and subsequent triggering by stress stimuli. SH-SY5Y Tet-On cells have been engineered genetically to express DISC1 or TRIOBP-1 in response to doxycycline induction. The aims were to establish the optimal doxycycline dose and treatment duration for protein expression, as well as to detect protein aggregation visually or using an insolubility assay.

In SH-SY5Y-DISC1 cells after 24-hour treatment with doxycycline, there is a DISC1 band showing up at 100 or 135 kDa only in samples treated with doxycycline. The expected size of induced protein based on the gene that was inserted is around 95 kDa. While the observed DISC1 band is higher, probably due to post-translational modifications, for example phosphorylation. Also, it falls in a band range typically seen in humans (19). Hence, there is evidence that these cells could be a system for inducible protein expression. Moreover, there is a constant band at 35 kDa, present in control and treatment samples, which probably represents endogenous DISC1. In humans DISC1 is usually seen in higher band range (65 to 210 kDa), however some reported lower DISC1 bands in the human brain (16) (50 kDa and lower). When the treatment was prolonged to 48 hours, another band at 63 kDa shows up across all samples, probably another variant of endogenous DISC1, also previously observed in literature (19). After the insolubility assay, there was no DISC1 signal in purified insoluble protein fraction after 24 nor 48 hours, hence no DISC1 aggregation was confirmed. The DISC1 signal in whole cell lysates was similar to previous Western blots.

The band of induced DISC1 is less prominent than endogenous DISC1, hence this system needs to be optimized. To start with, the treatment, sample collection and Western blot analysis need to be repeated by someone else, just to rule out technical issues with the experiment itself. During this project, only one anti-DISC1 antibody was used so other antibodies can be tested, if possible, with different epitopes. Antibodies with different epitopes can ensure the detected signal is specific to protein of interest, ensuring the reliability of experimental results. Also if we are looking at shorter variants of DISC1 then this means that only parts of the full protein will be present in them. By using antibodies against different epitopes, we may see DISC1 species that are not visible using this one. Then, a broader range of doxycycline concentration and different time points can be tested. Also, since cells usually remove the proteins which aren't useful to them by proteasomal degradation or apoptosis, those systems can be inhibited and then the cells analysed. As for analysis of cells via fluorescent microscopy, the signal for DISC1 was really low and hard to detect. The staining needs to be optimized (higher concentrations of the antibody used or trying out different DISC1 and secondary antibodies). Another option includes setting up a positive control well with transfected DISC1-eGFP plasmid, which is then overexpressed in these cells. In theory, this would give out a positive signal with anti-DISC1 antibody and would be visible on the green channel as well, since it has a GFP which is able to fluoresce by itself. In terms of the insolubility assay, using a large quantity of cells should increase the total insoluble DISC1 present.

As for SH-SY5Y-TRIOBP-1 cells, the band a bit higher than 75 kDa is present in treated cells only and matches the previously observed size of full-length TRIOBP-1 in cells (42) and expected size for the inserted gene (72 kDa) so the inducible expression of protein TRIOBP-1 with doxycycline treatment for 48 hrs is possible in these cells. Also, there is a band a bit lower present in all cells, which was observed in literature as endogenous TRIOBP-1 in SH-SY5Y (35) but with great confidence we can conclude that

this slightly lower band is 597 amino acid form of TRIOBP-1 that lacks the 59 – amino acid optionally translated N – terminal region. However, it is worth noting when the level of cells was upscaled for the insolubility assay, the band at 75 kDa was present in all tested cells, with probably a high expression of endogenous TRIOBP-1 and induced variant. In the insoluble protein fraction, levels of aggregating endogenous and induced TRIOBP-1 are similar. When analysed with immunocytochemistry and fluorescent microscopy, the TRIOBP-1 signal was hard to detect so this protocol requires more optimization. Similarly, to the immunocytochemistry of SH-SY5Y-DISC1, the range of concentrations of primary and secondary level should be tested in these cells. It would also benefit from setting up a control well with induced TRIOBP-1 just to confirm if the TRIOBP-1 antibody is working. Nevertheless, compared with cell line SH-SY5Y-DISC1, the cell line SH-SY5Y-TRIOBP-1 could be more useful in future since it requires less optimization. Both DISC1 and TRIOBP-1 cells might have a problem with being out-competed by wild type cells. That could be fixed by introducing antibiotics to the cell culture media, that the DISC1 and/or TRIOBP-1 cells are immune to, but which will kill the wild type cells.

Once the system for either of the mentioned cell lines is optimized and expression of proteins is controllable, the first step would be to check for aggregation of these proteins (via fluorescent microscopy, insolubility assay or some novel methods). Moreover, it would be interesting to check how environmental factors (such as temperature, oxidative stress, etc.) affect aggregation of these proteins. One of the ideas for my project when it was starting was to test different amounts of the most prescribed drugs for mental illness (clozapine, haloperidol, citalopram) and check if and how they are affecting DISC1 or TRIOBP-1 in SH-SY5Y cells. Clozapine was used in preventing impaired object recognition in DISC1 L100P mutant mice (43), while haloperidol protects neurite lesions via reducing excessive binding between dopamine 2 receptor and DISC1 (44). Also, DISC1 rs6675281-rs1000731 SNP was connected in citalopram efficacy by genetic

analysis of patients with diagnosed and treated major depressive disorder (45). Since TRIOPB-1 is less researched than DISC1 in terms of mental illnesses, there is not any direct research connecting TRIOBP-1 with these drugs. However, TRIOBP-1 was initially found as insoluble in brain samples from patients with diagnosed schizophrenia (17), investigating the effect of antipsychotic drugs, clozapine and haloperidol, is definitely interesting. Recently TRIOBP-1 was observed in brain samples from patients with diagnosed major depressive disorder (42), so investigating the effect of citalopram, an antidepressant from the group of selective serotonin reuptake inhibitors, on TRIOBP-1 is definitely worth pursuing.

# 6 Conclusion

Characterization of cell lines expressing the proteins DISC1 and TRIOBP-1, with regards to their aggregation in mental illness, was the main goal of this thesis. This thesis focused on improving the study of protein aggregation by using cells with inducible expression of DISC1 or TRIOBP-1 proteins, as well as the "Tet-On" method for controlled protein expression. The main goal was to find the most suitable doxycycline dose and treatment period for protein expression and to detect protein aggregation using Western blotting, fluorescent microscopy, and insolubility assays.

The induction of DISC1 and TRIOBP-1 expression by doxycycline treatment was successful without significant toxicity, which indicates that it is safe to use for the following experiments.

Western blotting showed that DISC1 was successfully expressed in response to doxycycline, with bands appearing at 100 and 135 kDa in treated samples. However, no aggregation of DISC1 was detected in the insolubility assays hence no DISC1 aggregation was confirmed. TRIOBP-1 was successfully expressed with a band at approximately 75 kDa, showing that the gene transfer and induction methods were effective. The observed aggregation of TRIOBP-1 in insolubility assays was consistent across samples, but further optimization is needed to better differentiate between endogenous and induced TRIOBP-1.

Fluorescent microscopy results for both DISC1 and TRIOBP-1 results were deficient, showing low signal intensity. This indicates the need for protocol optimization.

In general, the results demonstrated the advantage of the cell lines and methods developed to study protein aggregation. However, further refinements are required for complete clarification of DISC1 and TRIOBP-1 aggregation.

Adjustments to the experimental setup and increased cell quantities might improve detection and provide clearer insights into DISC1 and TRIOBP-1 role in neurodegenerative conditions.

Further research for both proteins involve refining experimental protocols to enhance protein detection and aggregation analysis. More environmental factors and antipsychotic drugs should be involved in further studies on how they impact DISC1 and TRIOBP-1 aggregation. That can contribute to understanding their roles in mental health and the development of potential therapeutic strategies.

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# Lora Polašek

**Datum rođenja:** 21/06/1999 | **Državljanstvo:** hrvatsko | **Spol:** Žensko | **Telefonski broj:** (+385) 953461911 (Mobilni telefon) | **E-adresa:** lora.polasek@gmail.com |

**Adresa:** Slavka Krautzeka 84, 51000, Rijeka, Hrvatska (Kućna)

## RADNO ISKUSTVO

28/10/2019 – 30/11/2019 Rijeka, Hrvatska
**PRODAVAČICA** SPORTINA DOO

– pružanje općih savijeta kupcima
– ponovno punjenje robe i zaliha
– održavanje trgovine

07/06/2022 – 30/06/2022 Rijeka, Hrvatska
**PROMOTORICA PROIZVODA** MAVI KREATIVNI TIM D.O.O.

– promocija proizvoda
– pružanje općih savjeta o proizvodu

18/05/2023 – 30/07/2023 RIJEKA, Hrvatska
**PRODAVAČICA** PET NETWORK INTERNATIONAL DOO

-promocija proizvoda
-pružanje općih savjeta o proizvodu
-održavanje trgovine

## OBRAZOVANJE I OSPOSOBLJAVANJE

01/10/2018 – TRENUTAČNO Rijeka, Hrvatska
**PREDDIPLOMSKI SVEUČILIŠNI STUDIJ "BIOTEHNOLOGIJA I ISTRAŽIVANJE LIJEKOVA"** ODJEL ZA BIOTEHNOLOGIJU

**Adresa** Radmile Matejčić 2 , 51000, Rijeka, Hrvatska

## JEZIČNE VJEŠTINE

Materinski jezik/jezici: **HRVATSKI**

Drugi jezici:

| | RAZUMIJEVANJE | | GOVOR | | PISANJE |
|---|---|---|---|---|---|
| | Slušanje | Čitanje | Govorna produkcija | Govorna interakcija | |
| **ENGLESKI** | C2 | C2 | C2 | C2 | C2 |
| **NJEMAČKI** | A2 | A2 | A2 | A2 | A2 |

*Razine: A1 i A2: temeljni korisnik; B1 i B2: samostalni korisnik; C1 i C2: iskusni korisnik*

## DIGITALNE VJEŠTINE

Komunikacijski programi (Skype Zoom TeamViewer) | Vjesto koristenje Microsoft Office alata | Windows | Odlino poznavanje rada na drutvenim mreama (npr Facebook ili Instagram) | Molekularni dizajn (Avogadro, PyMOL, UCSF Chimera, VDM, Marvin, Gamess) | poznavanje programa PyMOL Avogadro ChemAxon Marvin VMD GAMESS MacMOLPit

## KONFERENCIJE I SEMINARI

02/12/2021 – 05/12/2021 Virtualna konferencija

### Regional Conference of the Third Edition of Darwin

Dio organizacijskog tima za Regionalnu konferenciju Darwin koja se održavala putem platforme Zoom kao dio Internacionalne konferencije na kojoj je sudjelovalo oko dvadesetak zemalja.

**Poveznica** https://thedarwin.in/

## VOLONTIRANJE

01/11/2022 – 26/11/2022 Rijeka

### Kuglice dobrih želja

Projekt humanitarnog karaktera u suradnji s Gradom Rijekom.

**Poveznica** http://www.usbri.uniri.hr/kuglice-dobrih-zelja/

## VOZAČKA DOZVOLA

**Vozačka dozvola:** AM

**Vozačka dozvola:** B

## OBRAZOVANJE

20/10/2023 – 29/07/2024

### Eksperimentalni završni rad

Provođenje eksperimentalnog završnog rada na 3. godini preddiplomskog studija Biotehnologija i istraživanje lijekova pod vodstvom izv.prof.dr.sc. Nicholas J. Bradshaw na temu "Karakterizacija staničnih linija za istraživanje agregacije proteina kod mentalnih bolesti. ".

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