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SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

Diplomski sveučilišni studij

Biotehnologija u medicini

Tina Fartek

Agregacija EHD3 ovisi o više kritičnih regija

Diplomski rad

Rijeka, 2022.

Mentor: doc.dr.sc Nicholas J. Bradshaw

Acknowledgements

I would like to give my warmest thanks to my mentor Nicholas J. Bradshaw for your help in conducting and writing this thesis. Your time and knowledge are very appreciated. No matter how many questions I had and how many hurdles throughout this process we came up against, you always provided directions. So again, thank you!

I would also like to enormously thank Bobana Samardžija and Beti Zaharija for your assistance in the lab. Throughout many hours in the lab, you were always helpful and provided solutions even if you had hundred other things going on. Not just that, but for your positive attitude and fun times in the lab. You were not just PhD students, but also a friend when I needed you. Also, special thanks for always cheering me on when transferring my gel to membrane!

To my colleagues in the lab: Anja Hart, Mario Babić and Mihaela Bergman. We all spent most of our time in the lab, so thank you for all the laughter, endless coffees, and fried chicken in Formula every day. Without you the atmosphere in the lab would not be the same.

Lastly, I want to express my gratitude to my family, friends and my significant other for believing in me and supporting me. Your support means everything to me.

Master's thesis was defended on 19th of September 2022

In front of the Committee:

1. Izv. prof. dr. sc. Rozi Andretić Waldowski

2. Doc. dr. sc. Christian A. Reynolds

3. Doc. dr. sc. Nicholas J. Bradshaw

This thesis has 45 pages, 2 tables, 11 figures and 41 citations.

Abstract

Schizophrenia is a debilitating chronic mental illness that affects people's ability to perform daily activities. Schizophrenia is treated with antipsychotics and psychotherapy which only target the symptoms, however there is little known about its molecular background. There are several biological theories of schizophrenia, but the most influential is the dopamine hypothesis, which states that there is an imbalance in dopamine transmission. Other theories suggest neurodevelopmental changes, such as brain maturation and altered neurotransmission, as key factors in development of schizophrenia. Along with genetic and environmental factors, which contribute to development of schizophrenia, protein aggregation was introduced as a possible non-genetic cause. Protein aggregation occurs when proteins misfold due to errors in folding or trafficking machinery. One newly discovered protein to aggregate in brains of schizophrenia patients is EH-domain containing 3 (EHD3). EHD3 is believed to be one of the main regulators of endocytic recycling involved, among other functions, in endosome trafficking and promotion of dopaminergic transmission. Previous research showed that full length EHD3 consistently aggregates in SH-SY5Y cells, while a truncated form EHD3 1-399, with its linker region deleted, does not. In contrast, EHD3 1-434, which contains both the linker region and an EH domain, aggregates. Linker region was therefore suspected to be a critical region for EHD3 aggregation. In this thesis, we aimed to determine whether the aggregation occurs within 400-434 amino acids of EHD3 by fluorescent microscopy and ultracentrifugation assay. Ultracentrifugation data surprisingly revealed that EHD3 1-434 does not appear in the aggregome of HEK293 cells, as hypothesized, but it still formed aggregate-like structures in both SH-SY5Y and HEK293 cells. Since EHD3 1-434 probably does not aggregate, we suspected involvement of the EH domain in EHD3 aggregation. Novel fragments EHD3 1-404 and EHD3 400-535 were therefore cloned and expressed in both cell lines. However, they did

not show any signs of aggregation. Co-aggregation of full length EHD3 with non-aggregating EHD3 1-399 was also investigated. Fluorescent microscopy results showed that full length EHD3 induces EHD3 1-399 to aggregation in neuroblastoma cells. Future research is needed to determine the critical region of EHD3 aggregation by generating novel constructs that include small parts of linker region or the EH domain.

Key words: schizophrenia, protein aggregation, EHD3

Sažetak

Shizofrenija je kronična mentalna bolest koja utječe na sposobnost izvršavanja redovnih dnevnih aktivnosti. Tipične linije liječenja shizofrenije su antipsihotici i psihoterapija koji ciljaju samo simptome, no malo se zna o njenim molekularnim uzrocima. Između nekoliko razrađenih bioloških teorija shizofrenije, najviše se ističe dopaminska hipoteza koja počiva na nedostatku ravnoteže u prijenosu dopamina. Druge teorije predlažu neurorazvojne promjene, poput maturacije mozga i promijenjene neurotransmisije, kao glavne čimbenike u razvoju shizofrenije. Uz genetske i okolišne čimbenike koji doprinose razvoju shizofrenije, agregacija proteina predložena je kao moguć ne-genetski uzrok. Agregacija proteina proizlazi iz nepravilnog smatanja proteina uzrokovanog greškama u mehanizmima za smatanje i prijenos. Jedan od novootkrivenih proteina koji agregira u mozgu pacijenata sa shizofrenijom je *EH-domain containing 3* (EHD3). Kao jedan od glavnih regulatora endocitoznog recikliranja, smatra se da je uključen i u endosomalni prijenos i pospješivanje dopaminske transmisije. Prethodna istraživanja pokazuju da EHD3 konzistentno agregira u stanicama neuroblastoma, osim u obliku EHD3 1-399, kada mu je linker regija izbrisana. Nasuprot tome, EHD3 1-434, koji sadrži i linker regiju i EH domenu, agregira. Posljedično, linker regija je pretpostavljena kao moguća kritična regija za EHD3 agregaciju. U ovom radu, težili smo odrediti događa li se agregacija unutar 400-434 aminokiselina EHD3, koristeći se fluorescentnom mikroskopijom i ultracentrifugiranjem. Iznenađujuće, rezultati ultracentrifugiranja pokazuju da se EHD3 1-434 ne nalazi u agregomu HEK293 stanica kao što se pretpostavljalo, ali formira strukture slične agregatima u SH-SY5Y i HEK293 stanicama. Kako EHD3 1-434 ne agregira, sumnjali smo na ulogu EH domene u agregaciji EHD3. Novi fragmenti EHD3 1-404 i EHD3 400-535 stoga su klonirani i eksprimirani u obje stanične linije, no nisu pokazali nikakve znakove agregacije. Također, ispitala se i ko-agregacija „full length" EHD3 s EHD3 1-399 koji obično ne agregira.

Rezultati fluorescentne mikroskopije pokazali su da „full length" EHD3 inducira EHD3 1-399 na agregaciju u stanicama neuroblastoma. Potrebna su daljnja istraživanja kako bi se odredila kritična regija EHD3 agregacije putem kloniranja novih fragmenata koji uključuju male dijelove linker regije ili EH domene.

Ključne riječi: shizofrenija, agregacija proteina, EHD3

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1. Introduction

1.1. Schizophrenia

The adjective "chronic" can be defined as "continuing indefinitely, perpetual, constant", and it is often used to describe progression of an illness (1). The chronic nature of some mental illnesses is one of the most distressing features that impair a person's daily life; including their thoughts, emotions and healthy functioning (2). Chronic mental illnesses (CMI) include schizophrenia, bipolar disorder, and major depressive disorder.

According to the latest Global Burden of Disease Study (2019), schizophrenia affects 20 million people worldwide, roughly 1% of the population. The onset of schizophrenia usually begins with gradual cognitive and social decline in early adolescence, progressing to the first psychotic episode in late adolescence or the twenties (3,4). Symptoms of schizophrenia can be classified into three categories: cognitive impairment, positive and negative symptoms. Cognitive impairment in schizophrenia is characterized by decline in concentration, psychomotor functioning, memory and executive functions (5). Positive symptoms alter thoughts, functioning and experiences through hallucinations, delusions, disorganized speech, and behavior. On the other hand, negative symptoms include loss of motivation and enjoyment in activities, social and emotional withdrawal, as well as difficulty with day to day functioning (6).

The diagnosis of schizophrenia is based on the symptoms patients express and the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5). The specific DSM-5 criteria for schizophrenia includes "experiencing two (or more) of the following symptoms: delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behavior, negative symptoms. Each must be present for a significant portion of time during a 1-month period and at least one must be delusions, hallucinations or disorganized speech" (7). Current

treatments for schizophrenia are limited to antipsychotics, psychotherapy and, less often, brain stimulation. Antipsychotics can be divided into two groups: typical and atypical antipsychotics, with the former causing aversive extrapyramidal symptoms (EPS) (8). Both of them effectively reduce positive symptoms, but the negative and cognitive symptoms remain the same (9). Blockade of the dopamine D_2 receptor is the main mechanism of action of all typical antipsychotics, but what sets them apart is the degree to which they block the D_2 receptor. It is known that 60% receptor occupancy is needed for the antipsychotic effect, while 80% occupancy relates to the EPS (10). Pharmacotherapy is almost always accompanied by psychotherapy, primarily cognitive behavioral therapy (CBT). CBT helps in managing schizophrenia symptoms and relapse prevention, as well as it reduces chances of hospitalization (11).

1.2. Biological theories of schizophrenia

Schizophrenia is a CMI with complex genetic and neurobiological background. One of the first hypotheses of etiology of schizophrenia was the dopamine hypothesis. The dopamine hypothesis suggests "hyperactive dopamine transmission in the mesolimbic areas and hypoactive dopamine transmission in the prefrontal cortex in schizophrenia patients". Dopamine dysregulation can also be found in other brain regions as well, such as the amygdala and hippocampus (12). The prefrontal cortex and amygdala are a part of the limbic system, sometimes known as "emotional brain" and they are important for emotional processing (13). Research suggests that the D_1 receptors are decreased in the prefrontal cortex, while D_2 receptors are found to be increased in the frontal cortex of schizophrenia patients. Dopamine antagonists stimulate D_2 receptors, which is also required for the development of psychosis. This hyperactivation in D_2 receptors could contribute to the failure of antipsychotic treatments (12).

Another hypothesis suggests that schizophrenia is a neurodevelopmental illness whose “pathology is already set at birth and only expresses itself as psychosis later" (14). Ventricular enlargement, aberrant cortical and subcortical structures, and altered neurotransmission of the dopaminergic, glutamatergic, and serotonergic systems appear to be established during gestation. It is hypothesized that the proteins produced by genes involved in schizophrenia may share a common neurodevelopmental pathway that could influence these brain abnormalities (15).

The generation of free radicals from oxygen-related processes has also been linked to accelerated cell aging in schizophrenia. A rise in damaged macromolecules and the failure of protein maintenance systems were indicated as sign of cell aging. When compared to healthy control individuals, schizophrenia patients exhibit higher levels of oxidative cellular damage markers, as well as a higher prevalence of reduced telomere length (16,17). Interestingly, ageing cells have reduced capacity to degrade misfolded or aggregate proteins (18). Cognitive changes seen in schizophrenia patients usually occur over a shorter period than typical aging related changes. Moreover, the deterioration in cognitive skills usually starts at the time of onset of the illness (17).

When researching the molecular mechanisms underlying the pathophysiology of schizophrenia, it is important to pay attention to its neurodevelopmental pathways. The first symptoms of schizophrenia usually appear post adolescence, suggesting an important role for changes in brain maturation such as synaptic pruning, maturation of dopaminergic neurons and full development of myelination (19,20). Synaptic changes were often reported in the pyramidal neurons of the cortex and hippocampus in brains of schizophrenia patients. They are thought to be one of the critical changes for development of schizophrenia (20).

It has been conclusively shown that there are both genetic and environmental components to the development of schizophrenia. Although our understanding of the genetics related to schizophrenia has advanced, the molecular mechanisms of the disease are still poorly understood and are likely to be diverse. While there are many hypothesized theories, we suggest a possible non-genetic cause as an addition to genetic and environmental components: protein aggregation.

1.3. Protein aggregation

Proteins are synthesized on ribosomes through a process called translation. However, protein synthesis is not equivalent to the production of a functional protein. Proteins must fold into their specific three-dimensional conformations, which are a result of interactions between the side chains of their constituent amino acids (21). Sometimes there is a malfunction in the folding machinery which leads to misfolding of proteins. Cells have therefore evolved various mechanisms as quality control systems, to remove misfolded or aggregated proteins (Figure 1). Protein misfolding occurs for a variety of reasons, including transcriptional or translational defects that result in amino acid misincorporation, failure of the folding and chaperone machinery, and faults in post-translational modifications or protein trafficking (Figure 1) (22). Under normal cellular conditions, the hydrophobic residues of proteins are fixed in their native structure. However, misfolded proteins can expose their hydrophobic residues at the surface, and are therefore more likely to trigger protein aggregation (18). Protein aggregation is not uncommon, with recent study on human cells suggesting that approximately 30% of cellular proteins are wastefully synthesized. This strongly implies that even under normal cellular conditions, aggregation is an inevitable destination for many proteins. Accumulation of aggregated protein can disrupt protein homeostasis and lead to extensive cell and tissue damage (23).

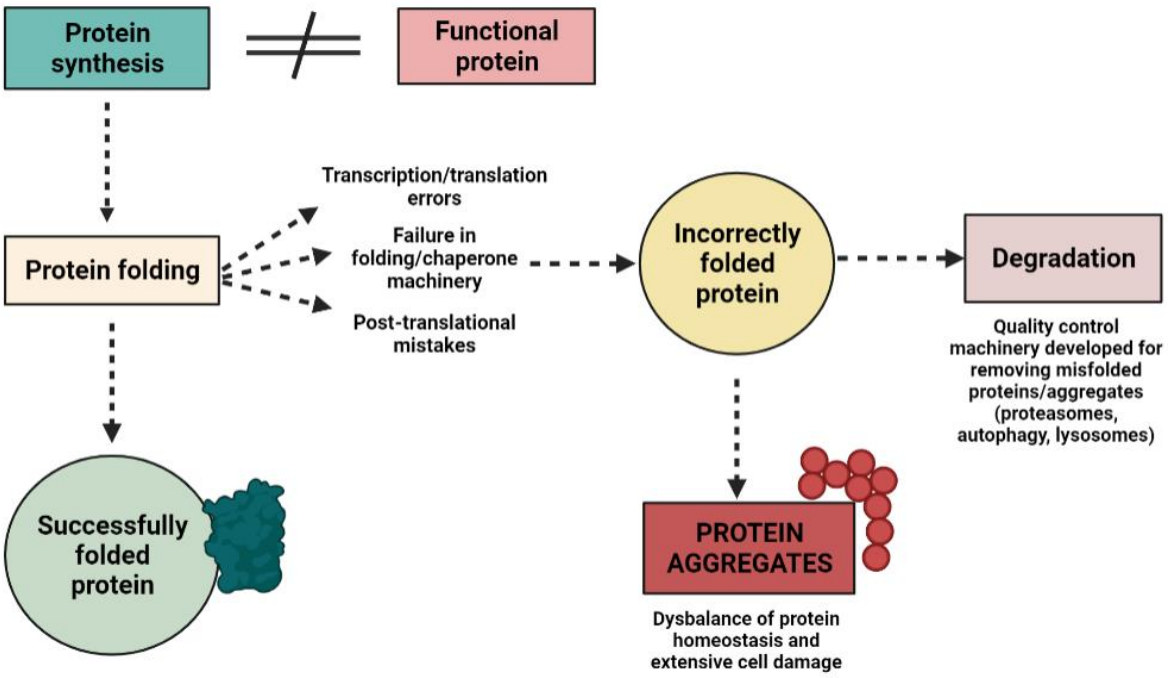

Figure 1. Mechanism of protein folding. Protein folding ensures the synthesis of a functional protein. Due to errors in folding machinery, it can lead to misfolding or protein aggregation. Figure made in BioRender.

The ubiquitin-proteosome system is one of the most important quality control systems for the degradation of intracellular proteins. It induces the ubiquitination of the target protein and its transport to the proteasome. The proteasome is a large complex consisting of two parts: the core particle, which has proteolytic activity, and the regulatory particle that guides proteins to the core and determines substrate specificity. Proteasomes have a role in neurodevelopmental processes, dendrite maintenance, and bioenergy balance, all known to be aberrant in schizophrenia. Furthermore, dopaminergic signaling, which is suggested to be dysregulated in schizophrenia, affects the activity, expression, and location of the proteasome (24,25).

The most prominent structural motif inherent in functional proteins is the α -helix. When a protein becomes toxic, it often goes through conformational changes and acquires a β sheet structure. This specific transition is a feature of amyloid deposits. The abnormal conformation exposes hydrophobic amino acid residues and promotes protein aggregation (26). Amyloid β deposits are a characteristic of Alzheimer disease, but this is not the only observed case of aggregation in neurodegenerative diseases. Other examples include α-synuclein aggregation in Lewy bodies in Parkinson disease, and huntingtin aggregation in Huntington's disease (27). Research from neurodegenerative diseases encouraged other researchers to follow a similar path and explore protein aggregation in CMI.

Five proteins have been studied and published so far in relation to protein aggregation in CMI: DISC1, Dysbindin 1, TRIOBP-1, CRMP1 and NPAS3. Recently, novel insoluble proteins with potential for aggregation were identified and are now being researched. These were identified in research using two sets of brains from the Stanley Medical Research Institute (Kensington, MD, USA) and from the Lieber Institute for Brain Development (Baltimore, MD, USA). The sets consisted of control and brain samples of patients with schizophrenia, major depressive disorder, or bipolar disorder. Insoluble protein fractions (predicted to contain protein aggregates) were purified from these samples and analyzed by mass spectrometry. These samples were then compared between the patients and control groups to identify proteins that may be insoluble in patients (Marreiros, Korth, et al., unpublished). Next, some of the protein samples were expressed in human neuroblastoma cells to confirm their aggregation. One of the proteins that showed aggregation was Eps15 homology domain-containing protein 3 (EHD3) (Dashi & Bradshaw, unpublished).

1.4. Eps15 homology domain-containing protein 3 (EHD3)

The mammalian EHD protein family contains four proteins: EHD1, EHD2, EHD3 and EHD4. Those four proteins are encoded by the *EHD1-4* genes respectively. They share a high degree of amino acid identity (70-86%), but they exhibit distinctive subcellular localizations and regulate different functions (28). EHD3 is predominantly expressed in the heart, brain, placenta, liver, kidneys, and ovaries. Structurally, EHD3 contains a helical domain (encoded in two parts), a conserved G domain (ATP binding domain), a linker region and an EH domain bound to the C-terminus of the protein (Figure 2) (29). The N-terminal region directs protein-protein interactions via the Asn-Pro-Phe (NPF) motifs of the targeted proteins, while the central domain, consisting of coiled coil structures, enables dimerization and oligomerization (30). The G-domain binds ATP and catalyzes its hydrolysis. EHDs can promote vesiculation through their intrinsic ATPase activity and induce membrane bending (31). The EH domain is characterized by two connected calcium-bound EF-hand motifs. Research showed that the EH domain specializes in protein-protein interactions that bind tightly to NPF motifs. Binding of NPF residues is possible through a conserved hydrophobic pocket within the EH domain (32,33). All three residues of NPF motif are needed for a successful identification by EH domain. In addition, this domain is associated with regulation of protein transport, membrane trafficking and endocytosis as well (34,35).

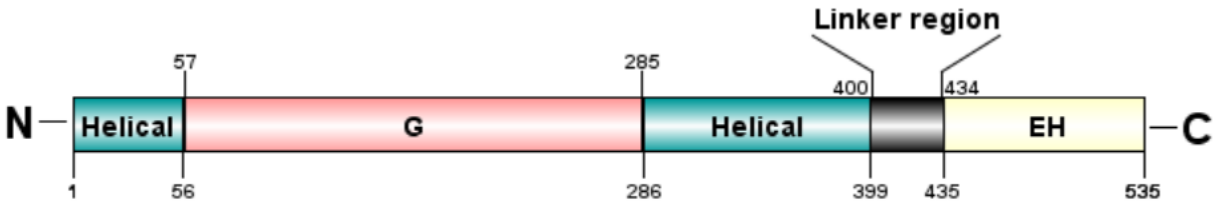

Figure 2. EHD3 protein structure. Structurally, all EHD proteins have the same domains; the two-part helical domain, ATP/GTP binding domain, linker region connecting to the EH domain and C-terminal domain. Figure made in Domain Graph (DOG).

While its biological functions are not well established, it is suggested that EHD3 plays a role in stabilization of tubular recycling endosome (TRE) and therefore regulates endocytic recycling (28). Endocytic trafficking is needed for cellular homeostasis, but it also influences cell shape, cytokinesis, and cell migration. Primary endocytic vesicles fuse to form early endosomes in which sorting of internalized receptor occurs (36). Endosomes are important for internalization, sorting and transport of the cargos to their destinations; either degradation or recycling back to the plasma membrane (37). Another suggested role for EHD3 is regulation of endosome to Golgi transport. When EHD3 is absent, carrier vesicles derived from early endosomes would not reach the recycling endosome. Also in EHD3-depleted cells, there is impaired transport of cathepsin D to lysosomes (33).

EHD1 and EHD3 are thought to be necessary for formation of ciliary vesicles and other events involved in ciliogenesis. They functionally overlap in cilium formation, probably due to their ability to heterodimerize. They are also the first reported membrane-associated proteins whose function affects mother centriole reorganization. One possibility is that the EHD proteins recruit components of the ubiquitylation machinery to the mother centriole (38).

Research shows that the EHD3 protein does not associate with actin, however it seems to be connected to microtubules. Upon treatment with a microtubule disruptive agent, nocodazole, most of the EHD3 in cells was seen to be concentrated in punctate forms. Truncated EHD3, missing its EH domain, displays complete nuclear localization. It is known that EHD3 contains a nuclear localization signal (NLS) located between 315-331 amino acids which could make this deleted form of EHD3 available for the nuclear transport system. This highlights the possibility that EHDs commute between the cytoplasm and the nucleus (30).

Interestingly, EHD3 has been shown to promote dopaminergic transmission by internalizing D_1 dopamine receptors, which are highly expressed in the brain. Disruption of D_1 receptors is linked to cognitive impairment and negative symptoms of schizophrenia (39). Recently, EHD3 was found to be differentially expressed in neurons of the prefrontal cortex of patients with schizophrenia. EHD3 was significantly lower in these neurons and analysis showed statistical significance (40).

Recent research confirmed that EHD3 can clearly aggregate in human neuroblastoma cells (Dashi and Bradshaw, unpublished). Its aggregates are seen to be mostly concentrated in the cytoplasm, near the nucleus. Our research also showed that EHD3 aggregates independently and does not co-aggregate with other proteins implicated in CMI such as TRIOBP-1 or Dysbindin 1A (Fartek & Bradshaw, unpublished). Due to its inability to form aggregates when the linker region is depleted (EHD3 1-399), it was therefore hypothesized that the critical region for EHD3 aggregation is in fact the linker region (Bertosa & Bradshaw, unpublished).

2. Aims of the thesis

The linker region appeared to be a region of interest in determining the critical domain of EHD3 aggregation. In this research, we further investigated the role of the linker region, but other regions as well in EHD3 aggregation. To specify, we tested the hypothesis that aggregation of EHD3 requires its amino acids 400-434. Structurally and functionally, it is important to determine and narrow down the critical region for its aggregation. This way we would get a better understanding of the mechanism of EHD3 aggregation. Furthermore, by targeting this critical region, we could generate aggregation resistant EHD3 and study its effects in mammalian cells and transgenic animals.

In this thesis we aimed to:

1. Replicate and confirm previous results: non-aggregation of EHD3 1-399 and aggregation of EHD3 1-434 in SH-SY5Y cells.
2. Clone new fragments of EHD3 that start at the N-terminus, but terminate between amino acids 399 and 434, and express them in mammalian cells, to narrow down the aggregation region.
3. Determine whether full length EHD3, EHD3 1-399 and EHD3 1-434 exist in the aggregome (purified insoluble protein fraction) of HEK293 cells using an ultracentrifugation method that tests protein insolubility.
4. Investigate co-aggregation of full length EHD3 with EHD3 1-399, as well as EHD3 1-434 and EHD3 1-399 to determine if non-aggregating form of EHD3 can be recruited to aggregation by EHD3 that can form aggregates.

3. Materials and methods

3.1. Size markers

- My-Budget 1kb DNA Ladder - Bio Budget Technologies GmbH
  The marker has 13 blunt-ended fragments in the range from 250 bp (base pairs) to 10 kbp (kilobase pairs). It is used for gel electrophoresis.
- My budget 100 bp+1.5 kb DNA Ladder – Bio Budget Technologies GmbH
  The marker has 10 blunt-ended fragments in the range from 100 bp to 1000 bp, with an additional fragment at 1500 bp. It is used for gel electrophoresis.
- My-budget Prestained Protein Ladder 10-180 kDa -Bio Budget Technologies GmbH
  The marker has proteins with size ranges between 10kDa-180kDa. It is used in Western Blots.

3.2. Antibodies and fluorescent markers

Table 1. Primary and secondary antibodies, as well as other fluorescent markers, used for Western blotting and immunocytochemistry.

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

[1] Primary antibody

3.3. Plasmids and vectors

Table 2. List of expression and entry vectors.

Vector	Encoded protein	Source
pdcDNA-FlagMyc	empty	Prof. Dr. F. Van Roy & Dr. B. Janssens, Ghent University
pDEST-CMV-N-EGFP	empty	Robin Ketteler, University College London, UK
pENTR1A	EHD3 (1-404 amino acids)	Novel to this thesis
pENTR1A	EHD3 (400-535 amino acids)	Novel to this thesis
pdcDNA-FlagMyc	Full length EHD3	Giovanna Dashi & Nicholas Bradshaw
pdcDNA-Flag	EHD3 (1-399 amino acids)	Tina Fartek & Nicholas Bradshaw
pdcDNA-Flag	EHD3 (1-434 amino acids)	Tina Fartek & Nicholas Bradshaw
pdcDNA-Flag	EHD3 (1-404 amino acids)	Novel to this thesis
pdcDNA-Flag	EHD3 (400-535 amino acids)	Novel to this thesis
pDEST-CMV-N-EGFP	Full length EHD3	Beti Zaharija & Nicholas Bradshaw
pDEST-CMV-N-EGFP	Control	Beti Zaharija & Nicholas Bradshaw
pDEST-CMV-N-EGFP	EHD3 (1-399 amino acids)	Novel to this thesis

3.4. Polymerase chain reaction (PCR)

Two rounds of PCR were done. The first round was done to clone the gene fragment, and the second was done to add additional restriction sites. This approach is called nested PCR and it is used to improve sensitivity and specificity. Components for the first PCR reaction included: 11.9 µL ultra-pure (u.p.) H_2O, 4 µL 5x GC Phusion Buffer, 0.4 µL 10 mM dNTPs, 1 µL of forward (EHD3 1-SalIF, EHD3 400-SalIF) and 1 µL of reverse primer (EHD3 404-XbaR, EHD3 535-XbaR), 1 µL template DNA (Full length EHD3), 0.5 µL DMSO and 0.2 µL Phusion DNA Polymerase. Each component was added in an Eppendorf tube for each EHD3 fragment. After the first round of PCR, generated EHD3 fragments were checked with DNA electrophoresis on agarose gel. Second round of PCR consisted of: 11.9 µL u.p. H_2O, 4 µL 5x GC Phusion Buffer, 0.4

µL 10 mM dNTPs, 1 µL of forward (Ext BamKpn F) and reverse primer (ExtDel XbaKpn R), 1 µL template DNA (1st PCR products), 0.5 µL DMSO and 0.2 µL Phusion DNA Polymerase. Fragments from second round of PCR were run on the agarose gel, visualized under a UV-lamp and the correct band was excised.

3.5. Purification of PCR products

Purification was done using a my-Budget gel extraction kit (Bio-Budget). Firstly, 650 µL of gel solubilizer was added into Eppendorf tubes and heated at 50 °C on the heat block until the gel dissolved. At the same time, dH_2O was placed on the heat block to incubate. After the gel dissolved, 50 µL of binding optimizer was added into Eppendorf tubes. The solution was then transferred from Eppendorf tube to a column and centrifuged for 1 minute at 11,000 rpm (revolutions per minute). Flow through was discarded. 700 µL of washing buffer was added to a column and centrifuged for 1 minute at 11,000 rpm. Flow through was discarded and the washing step was repeated. Without adding anything, columns were centrifuged for 2 minutes at 13,000 rpm and the flow through was discarded. Columns were placed into new Eppendorf tubes, and 30 µL of dH_2O was added as an elution buffer and centrifuged for 2 minutes at 11,000 rpm.

3.6. Ligation

Purified PCR products (30 µL of insert, 4 µL FD Green Buffer, 2 µL Sal I, 2 µL Xba I) and vector (20 µL of vector, 2.5 µL FD Green Buffer, 1 µL Sal I, 1 µL Xba I, 1 µL FastAP) were digested overnight at 37 °C. The next day, they were purified, and their concentrations were measured. Depending on their concentrations, needed volumes of the insert, vector and u.p. H_2O for ligation were calculated. 1 µL of Ligation Buffer and 0.5 µL of T4 DNA Ligase (NEB) was added to the reactions as well. Reactions underwent an 18h Ligation program. After ligation, they were transformed into bacteria, purified, and sent for sequencing.

3.7. LR clonase reaction

Entry vectors were transferred by Gateway LR Clonase II Enzyme mix (Thermo Fisher Scientific) into destinations vector to generate a final expression vector. 100 ng entry vector, 150 ng destination vector, 9 µL TE buffer and 1 µL LR clonase were added into an Eppendorf tube. This mixture was then incubated at 25°C for 1 hour. After 1 hour, 1 µL of Proteinase K (Thermo Fisher Scientific) was added and the samples were incubated at 37°C for 5 minutes. Samples were then prepared for sequencing. If the sequencing results were correct, 5 µL of sample was used to transform into bacteria.

3.8. Transformation of bacteria

1 µL of plasmid and 50 µL of NEB5-alpha bacterial cells were pipetted into an Eppendorf tube. The suspension was incubated for 30 minutes on ice. After incubation, heat shock was performed at 42 °C for 30 seconds and the Eppendorf tubes were put immediately on ice for further five-minute incubation. Transformed bacteria were spread on the LB Agar plate containing appropriate antibiotic and incubated overnight at 37 °C.

3.9. Bacterial liquid culture and plasmid DNA purification

After overnight incubation, 3 mL of LB media and 3 µL of antibiotic (100 µg/mL) were added in a Falcon tube. One single colony was picked and scooped out with a pipette tip. The whole pipette tip was then dropped into the Falcon tube which was then put in a shaking incubator at 37 °C and 250 rpm overnight.

The next day, the cultures were first centrifuged for 20 minutes at 4 °C and 3700 rpm. Plasmid DNA purification was done using QIAprep Spin Miniprep Kit. After centrifugation, the liquid in Falcon tubes was thrown away and the bacterial cells were resuspended with 250 µL of Buffer P1. The mixture was then transferred to an Eppendorf tube. To lyse the bacterial cells, 250 µL of Buffer P2 was added and the tubes were inverted until the suspension turned

blue. Immediately after the suspension turned blue, 350 µL of Buffer N3 was added to neutralize the reaction and the tubes were inverted until the suspension turned colorless. Eppendorf tubes were then centrifuged for 10 minutes at 13,000 rpm. After 10 minutes, the supernatant was added to a QIAprep 2.0 spin column and centrifuged for 60 seconds. Spin column was then washed with 750 µL of Buffer PE and centrifuged for 60 seconds. To completely remove wash buffer, it was centrifuged for additional 60 seconds. Column was placed in 1.5 mL Eppendorf tube and 50 µL of previously heated TE buffer (0.5 mL 1M Tris pH 7.4, 200 µL 0.25 M EDTA, dH_2O up to 50 mL) was added. TE buffer was left to incubate for 1 minute and then centrifuged for 1 minute at 13,000 rpm.

3.10. Analysis of plasmid DNA concentration

The concentration of purified plasmid DNA was measured with a BioDrop µLite spectrophotometer. Absorbance wavelength was set to µLite 0.5 mm. Distilled water was used as a standard. 1 µL of samples were added and measured in ng/mL.

3.11. Agarose gel electrophoresis

Agarose gel was made by mixing 0.5 g of agarose and 50 mL of TAE Buffer 1x. The mixture was heated until the agarose dissolved and left to cool down. Then, 2 µL of Gel Green Stain was added to the mixture. The gel was poured into a tray and left to harden. When it solidified, the gel was put into an electrophoresis tank filled with TAE Buffer 1x. Marker was already pre-made, and the samples were made with 7 µL of dH_2O, 1 µL of Green Buffer and 2 µL of DNA. Electrophoresis was run for 18 minutes at 140 V.

3.12. Passaging mammalian cells

In this thesis, two lines of mammalian cells were used: HEK293 and SH-SY5Y cells. Both cell lines are adherent, which means they can grow on the bottom of T25 cell culture flask. HEK293 are human embryonic kidney cells that

require DMEM media (500 mL DMEM, 50 mL FCS, 5 mL non-essential amino acids, 5 mL penicillin/streptinomycin) for growth. SH-SY5Y cells are human neuroblastoma cells that require DMEM-F12 media (500 mL DMEM, 50 mL FCS, 5 mL 100X non-essential amino acids, 5 mL 1x penicillin/streptinomycin). The HEK293 cell line was used for Western blotting, aggregome purification method and immunocytochemistry. SH-SY5Y cells were only used for immunocytochemistry.

While working with mammalian cells, the environment must be sterile to avoid contamination. This is attained by spraying everything with 70% ethanol before placing it in a sterile hood. Trypsin and media were preheated before use at 37 °C. Media from the flask was removed and 1 mL of trypsin was added to break the cells from the surface. The flask was then incubated at 37 °C for approximately 5 minutes. After incubation, the flask was hit firmly to loosen the cells and 4 mL of media was added to the flask to neutralize the trypsin reaction. According to the confluency of cells, an appropriate amount of media and cells were added to a new flask. In general, for 70-100 % confluency, 250 µL of HEK293 cells were diluted to 5 mL of DMEM. On the other hand, 1 mL of SH-SY5Y cells were diluted to 5 mL of DMEM-F12. The T25 flask was then put in an incubator at 37 °C and left until the cells were ready to be split again. Splitting cells into wells required different amounts. Depending on the experiment (Western Blot/Ultracentrifugation), 500-700 µL of cells were split into a 6 well plate diluted to 3 mL of media. 12/24 well plates with coverslips were used for Immunocytochemistry where 70-250 µL of cells were split and diluted to 1 mL of media. The well plates were put back in an incubator overnight so they could be used for transfection.

3.13. Transfection

For transfection, DMEM or DMEM-F12 media without antibiotics or serum was used (-/- media). Depending on the experiment, Metafectene PRO was used

for immunocytochemistry and Metafectene was used for Western blot/aggregome purification method.

Firstly, two solutions were prepared. DNA solution consisted of 0.5 µg of plasmid mixed with 100 µL of -/- media. The second solution consisted of 2 µL of Metafectene PRO and 100 µL of -/- media per well. Solutions were further incubated at 37 °C for approximately 5 minutes. Meanwhile, media from plates was removed and 500 µL of -/- media was added per well. This media was then removed again and 300 µL of -/- media was added per well. DNA and Metafectene PRO solutions were then mixed and incubated at 37 °C for 30 minutes. After 30 minutes, solution was put into the correct well and the 12/24 well plate was put back into the incubator for 6 hours. After 6 hours, the media from wells was removed and 1 mL of +/+ media (media with serum and antibiotics) was added per well. When transfecting EHD3 400-535 for Western blotting, 1 µL of proteasome inhibitor MG132 per 1 mL of +/+ media was added immediately after removing the -/- media.

The protocol for 6-well plates is the same, except the amounts used are tripled. 6-well plates were mainly used for transfection of HEK293 cells which were going to be used for Western blot or the aggregome purification method.

3.14. Cell lysis

Media was removed from the cells and carefully washed twice with PBS (80g NaCl, 2g KCl, 14.4g Na_2HPO_4, 2.4g KH_2PO_4, dH_2O up to 1L, pH 7.4). PBS was then removed and 200 µL of cell lysis buffer (50 mL 10X PBS, 500 µL Triton X-100, 1 mL 1M $MgCl_2$, dH_2O up to 50 mL) was added and left to incubate for 5 minutes. The wells were washed with the added lysis buffer to collect all the cells and transferred to an Eppendorf tube. The tube was incubated on ice for 1 hour with periodic vortexing. Samples were either stored at -20 °C or prepared for Western Blot.

3.15. Western Blot

3.15.1. Acrylamide gels

10% gel was used for proteins of 30-60 kDa, and 15% gel was used for proteins of 5-15 kDa. 10% gels were prepared by mixing 4.8 mL of dH_2O, 3.9 mL 30% acrylamide, 3 mL 1.5M Tris pH 8.8, 120 µL 10% SDS, 120 µL 10% APS and 12 µL TEMED. 15% gels were prepared by mixing the same ingredients, but the volume of dH_2O was 2.7 mL and volume of 30% acrylamide was 6 mL. The running gel was poured in between the glass plates leaving 1 cm empty at the top. The empty part was filled with distilled water and the gel was left to solidify. After the gel hardened, distilled water was poured out and a stacking gel was prepared. The stacking gel contained 2.6 mL of dH_2O, 1 mL 30% acrylamide mix, 625 µL 1M Tris pH 6.8, 50 µL 10% SDS, 50 µL 10% APS and 5 µL TEMED. Stacking gel was poured on top of the running gel, a well comb was added, and the gel left to set.

3.15.2. SDS-PAGE

Lysates were diluted with 200 µL of protein loading buffer (6.25 mL 1M Tris, 10 mL glycerol, 20 mL 10% SDS, 3.75 mL dH_2O, 5 mg bromophenol blue). Then, 20 µL of 1M DTT was added, and the samples were heated at 95 °C for 5 minutes to denature their proteins. After 5 minutes, they were put on ice to cool down. The gels were placed in the SDS-PAGE tank which was filled with running buffer (30 g Tris, 144g glycine, 10g SDS, dH_2O up to 1L). 2 µL of protein marker and 20 µL of samples were loaded into wells. SDS-PAGE was run for approximately 45 minutes at 180V.

3.15.3. Transferring to membrane

The gels were taken out from the tank and the glass plates were removed with a green wedge. The gel was transferred to a plastic tray containing transfer buffer 1x (5.8 g Tris, 2.9g glycine, 4 mL 10% SDS, 200 mL methanol, dH_2O up to 1L). The buffer was poured out and replaced with fresh transfer buffer

1x and left to incubate on the shaker for 10 minutes. Meanwhile, 8x6 cm of PVDF membrane was cut and put in a plastic tray containing methanol to activate it. The membrane was then placed in a tray containing transfer buffer 1x and left to incubate on the shaker for 5 minutes. Then, blotting paper was cut in half. One half of the blotting paper was put in the transfer buffer to wet it and then placed on a cassette. Then, a membrane was placed on top of it and on top of the membrane a gel was placed. The remaining half of the blotting paper wetted in transfer buffer was put on the gel. A roller was used to remove any air bubbles. The cassette was locked and placed in the Transblot system. The gels were transferred for 45 minutes at 25 V.

After the transfer completed, the membrane was put in a plastic tray containing dH_2O. The water was removed and replaced with fresh dH_2O again. The dH_2O was poured out and Ponceau S staining solution (1g Ponceau S, 4 mL acetic acid, dH_2O up to 200 mL) was added and removed after 30 seconds. The membranes were washed with dH_2O until red color washed off and the water was clear. After that, membrane was washed once with PBS-T (50 mL 10x PBS, 450 mL dH_2O, 250 mL Tween 20).

3.15.4. Antibody staining

The membranes were blocked in a solution containing 2.5 g milk powder and 50 mL PBS-T per membrane for 1 hour. After blocking, the membranes were quickly washed two times with PBS-T. Membranes were then incubated in a solution containing 6 mL of PBS-T, 0.3 g of milk powder, 60 µL of 2% sodium azide and 3 µL of either anti-FLAG or anti-EGFP primary antibody overnight. The next day, primary antibody was poured off and the membranes were washed once with PBS-T. They were then washed 3 times with PBS-T, each time 10 minutes on the shaker. The membranes were incubated in a solution containing 6 mL PBS-T and 0.6 µL Goat-anti-Mouse (GAM) secondary antibody for 1 hour. Secondary antibody was removed, and the membranes were washed with PBS-T, each time 10 minutes on the shaker.

3.16. Immunocytochemistry

3.16.1. Fixation and permeabolization

HEK293 or SH-SY5Y cells were washed once with PBS and fixed with approximately 500 µL of fixation buffer (8g paraformaldehyde, 20 mL 10x PBS, dH_2O up to 200 mL, pH 7.4) per well for 15 minutes. After 15 minutes, fixation buffer was removed and permeabolization buffer (10 mL 10x PBS, 500 µL Triton X-100, dH2O up to 50 mL) was added for 10 minutes. It was then removed, and the cells were carefully washed 3 times with PBS.

3.16.2. Cell staining

Coverslips were blocked with 200 µL of 10% goat serum/PBS or 3% BSA/PBS for 1 hour. The blocking media was removed, and 100 µL of primary antibody solution was added. This solution contained 10% goat serum/PBS or 3% BSA/PBS and 1:1000 Flag M2 primary antibody. The coverslips were incubated for 3 hours. After 3 hours, the cells were washed three times with PBS over 15 minutes. 100 µL of secondary antibody mix containing Goat Anti Mouse RED 555, DAPI, 10% goat serum/PBS or 3% BSA/PBS and fluorescently labelled phalloidin was added to the wells and incubated for 1 hour in the dark. After 1 hour, the cells were washed 3 times with PBS over 15 minutes and once with miliQH_2O. The coverslips were attached to the slides with commercial mounting medium. Cells were visualized under an Olympus IX83 fluorescent microscope with CellSens software.

3.17. Aggregome purification method

Media was removed from wells containing HEK293 cells and washed twice with PBS. The cells were lysed with 200 µL of Lysis Buffer (50 mM HEPES pH 7.5, 250 mM sucrose, 5mM $MgCl_2$, 100 mM KAc, 2 mM PMSF, 1x PI). The lysis buffer was left for 5 minutes, and the cells were then transferred to ultracentrifuge (UC) tubes using a cell scrapper. 2.5 µL of Triton X-100 was added per tube. 60 µL of homogenate was transferred from UC tube to an

Eppendorf tube before ultracentrifugation and stored at -20 °C. The samples in UC tubes were spun at 20 000 g for 20 minutes at 4 °C. The supernatant was removed, and lysis buffer and Triton X-100 were added to the pellet. The samples were spun at 20 000 g for 20 minutes at 4 °C. The supernatant was removed, and the pellet was resuspended with 200 µL of buffer A1 (50 mM HEPES pH 7.5, 1.6M sucrose, 100 mM KAc, 1% Triton X-100, 1mM PMSF). The samples were centrifuged at 130 000 g and 4°C for 45 minutes. The supernatant was removed and 200 µL of buffer A1 was added. The samples were again centrifuged at 130 000g and 4°C for 45 minutes. The supernatant was removed, and the pellet was resuspended with 200 µL of buffer B1 (50 mM HEPES pH 7.5, 1M NaCl, 20 mM $MgCl_2$, 30 Mm Ca^{2+}, 100 U/mL DNAse, 1x PI). The UC tubes were then incubated for 30 minutes at 37 °C and then overnight at 4°C.

The next day, the samples were centrifuged at 130 000g and 4°C for 45 minutes. Supernatant was removed and the pellet was resuspended with 200 µL of buffer B1 again, but without DNAse. The samples were centrifuged at 130 000g and 4°C for 45 minutes. Supernatant was removed, and the pellet was dissolved in 200 µL of buffer C1 (50 mM HEPES pH 7.5, 0.5% Sarcosyl) with insulin syringe and needle 0.4 mm. The samples were incubated on ice for 1 hour on a shaking tray. After 1 hour, they were centrifuged at 112 000g and 4 °C for 45 minutes. Then, the supernatant was removed, and the former step was repeated. The samples were after 1 hour centrifuged at 112 000g and 4 °C for 45 minutes. Supernatant was removed and the pellet was resuspended in 20 µL of loading buffer and 2 µL of DTT. 60 µL of loading buffer and 6 µL of DTT were added to the homogenates. The samples were heat shocked at 95°C for 5 minutes and stored at -20°C or Western blotted.

4.Results

4.1. Verifying EHD3 plasmids by Western blotting

Western blotting is used to identify proteins of interest by using specific antibodies to detect them. This was done to confirm that each plasmid is producing a protein of the expected size. In this thesis, we used anti-FLAG primary antibody and GAM secondary antibody to identify EHD3 plasmids with a FLAG tag, or anti-GFP primary antibody to identify EHD3 plasmids containing EGFP tag. All plasmids, except for EHD3 400-535, were run on a 10% acrylamide gel. EHD3 400-535 was run on a 15% gel. Due to the small size of EHD3 400-535, proteasome inhibitor MG132 was used to prevent its degradation in the cell. The numbers refer to amino acids of the protein. Plasmids were expressed in HEK293 cells and lysed to prepare for Western blotting. The structure of each EHD3 plasmid used in this thesis can be seen in Figure 3. Membranes were visualized with ECL Prime Kit and a ChemiDoc system. All plasmids appeared, therefore, to function as expected.

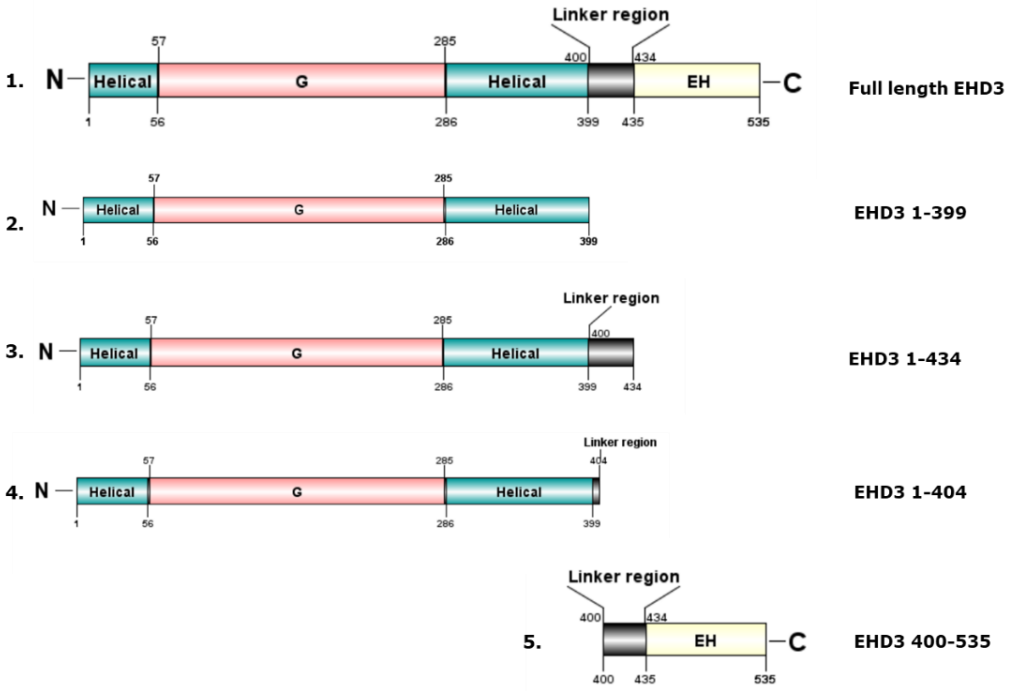

Figure 3. Structural representation of all EHD3 fragments used in this thesis. Numbers refer to amino acids of the protein.

Sizes of almost all the EHD3 plasmids with FLAG tag corresponded to the specific band visible on the membrane. The approximate calculated sizes are for full length EHD3 59 kDa, EHD3 1-399 44 kDa, EHD3 1-434 48 kDa and of EHD3 1-404 44 kDa (Figure 4A). EHD3 400-535 was the only plasmid with a band higher than expected (Figure 4B). However, this is probably due to its post translational modifications in the cell such as phosphorylation or glycosylation.

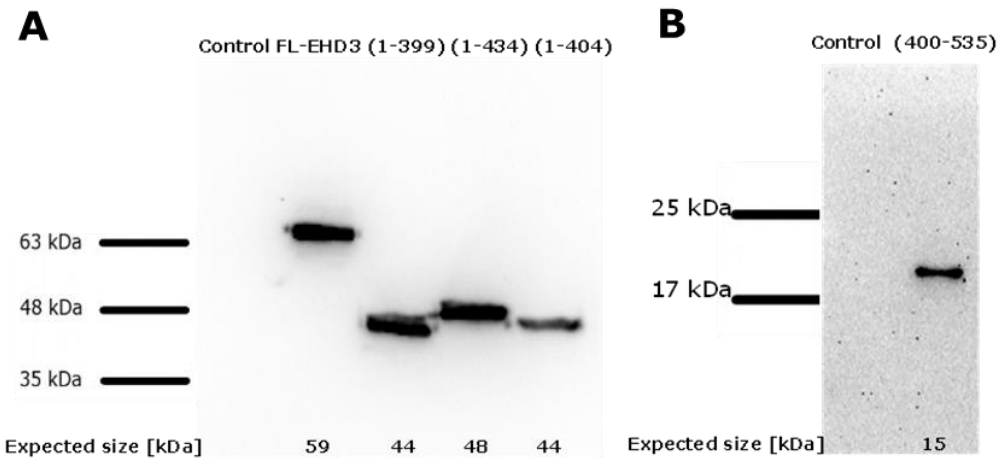

Figure 4. Western blot of EHD3 plasmids containing FLAG tag. EHD3 plasmids were detected with anti-FLAG primary antibody and GAM secondary antibody. Control represents mock transfected HEK293 cells. Size of the protein was compared to Prestained Protein Ladder 10-180 kDa. FL-EHD3 refers to full length EHD3.

The expected sizes of EHD3 species fused to a GFP tag also corresponded to their specific bands on the membrane. Full length EHD3 has a calculated size of 88 kDa, EGFP control 30 kDa and EHD3 1-399 74 kDa (Figure 5). When calculating the sizes of these plasmids, the size of EGFP tag, which is approximately 30 kDa, was also included. The mock transfected control in this membrane is also empty.

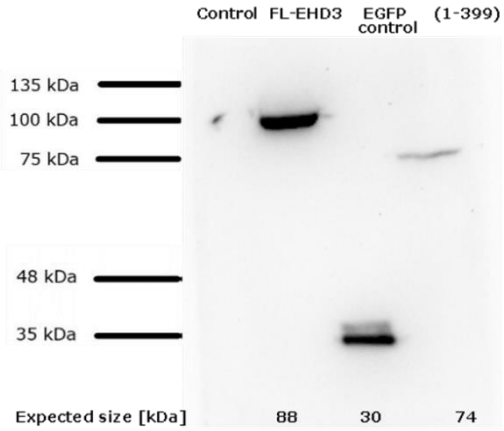

Figure 5. Western blot of EHD3 plasmids containing GFP tag. EHD3 plasmids were detected with anti-GFP primary antibody and GAM secondary antibody. Control represents mock transfected HEK293 cells. Size of the protein was compared to Prestained Protein Ladder 10-180 kDa. FL-EHD3 refers to full length EHD3.

4.2. Testing protein insolubility by ultracentrifugation assay

Previously, fluorescent microscopy was used to investigate aggregation of full length EHD3, EHD3 1-399 and EHD3 1-434. The results suggested that EHD3 1-434 aggregated, while EHD3 1-399 did not. Ultracentrifugation was used as an additional method to confirm aggregation. This is based on the fact that aggregated proteins tend to show greater insolubility than correctly folded/functional proteins. Insolubility of three EHD3 plasmids was investigated; full length EHD3, EHD3 1-399 and EHD3 1-434. The goal was to obtain two fractions from each plasmid, the first fraction being the whole cells homogenate (representing all the proteins from the original cells) and the second fraction being the aggregome (containing only insoluble proteins, including any that are aggregating). The homogenate fraction was not purified in any way, while the aggregome fraction was by ultracentrifugation. The plasmids were grown in HEK293 cells, transfected, and lysed before ultracentrifugation. HEK293 cells were used to generate a larger amount of protein since they are easier to transfect than SH-SY5Y cells and are very efficient for protein production.

The ultracentrifugation assay showed that full length EHD3 appears in the aggregome of HEK293 cells, as expected. This protein previously seen to

consistently aggregate by fluorescent microscopy. Two bands of the same size are visible, one appearing in homogenate section and the other one in aggregome section (Figure 6). The sizes of two bands correspond to the approximate calculated size of full length EHD3, which is 59 kDa. This further validates aggregation of EHD3. EHD3 1-399 is a non-aggregating form which seems to not be present in the aggregome of HEK293 cells after the ultracentrifugation, as expected. Only the band in the homogenate section is visible and it corresponds to the size of EHD3 1-399, which is 44 kDa (Figure 6). EHD3 1-434 was previously indicated to aggregate in SH-SY5Y cells, however ultracentrifugation shows that this fragment is not present in the aggregome of HEK293 cells. There is only one visible band that correlates with the size of EHD3 1-434, which is 48 kDa (Figure 6). This suggests that, for EHD3 1-434, the clusters of this protein seen in SH-SY5Y cells previously, do not represent aggregates. Therefore, it is likely that the linker region itself is not sufficient and critical for EHD3 aggregation. No bands are visible in the mock-transfected control. The obtained results were confirmed in three independent experiments.

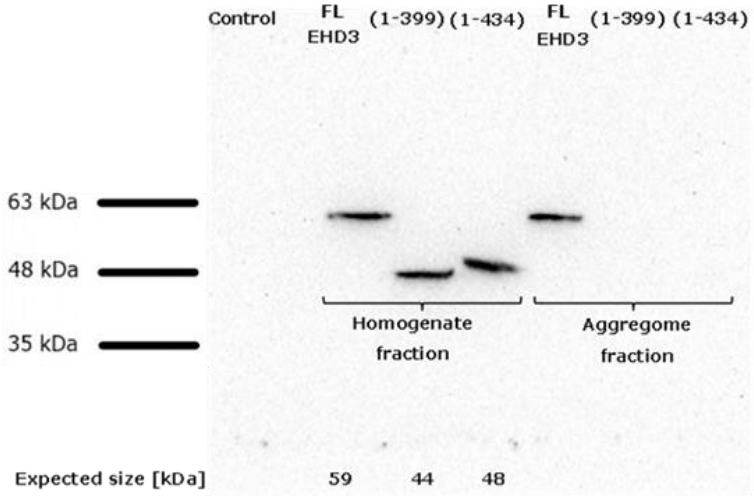

Figure 6. Ultracentrifugation assay shows insolubility of only full length EHD3 in HEK293 cells. EHD3 plasmids were detected with anti-FLAG primary antibody and GAM secondary antibody. Control represents mock transfected HEK293 cells. Size of the protein was compared to Prestained Protein Ladder 10-180 kDa. FL EHD3 refers to full length EHD3. Plasmids in the aggregome fraction underwent ultracentrifugation, while the ones in homogenate fraction did not. The obtained results were confirmed in three independent experiments.

4.3. Expressing EHD3 fragments in SH-SY5Y cells and HEK293 cells by fluorescent microscopy

Immunocytochemistry was used to replicate previous research results, as well as express novel EHD3 fragments in SH-SY5Y cells. To further investigate results of ultracentrifugation assay, we also expressed plasmids in HEK293 cells. This was done to confirm that the results from ultracentrifugation assay were not affected by using a different cell line, HEK293. Plasmids were transfected in both cell lines, and stained with anti-FLAG primary antibody, GAM 555 secondary antibody and Phalloidin. FLAG-tag emits red fluorescence under the microscope, while the GFP tag emits green fluorescence. Phalloidin was used to visualize actin and was only used in cases where plasmids had a FLAG tag. DAPI was used to stain the nucleus blue.

4.3.1. Replicating and confirming previous research results

Due to the unexpected ultracentrifugation results, we attempted to replicate and confirm previous research. As can be seen in Figure 7, full length EHD3 shows clear aggregation in neuroblastoma cells. Its aggregates are amorphous, and they appear mainly in the cytoplasm of the cell, as expected. In contrast, EHD3 1-399 does not show clear aggregation. It forms filamentous structures that are not an obvious sign of aggregation. These red strands of EHD3 1-399 are localized in the cytoplasm of the cell, but also inside the nucleus. EHD3 1-399 is a fragment without a linker region, which is what we suspected to be a crucial region for EHD3 aggregation (Figure 3). Moreover, EHD3 1-434 looks like it is aggregating in neuroblastoma cells, however this does not correlate with the results of ultracentrifugation assay. This fragment shows more amorphous aggregates and a stronger red signal than the full length EHD3. The aggregates appear only in the cytoplasm of the cell. These results agree with those obtained in previous research (Bertosa & Bradshaw, unpublished).

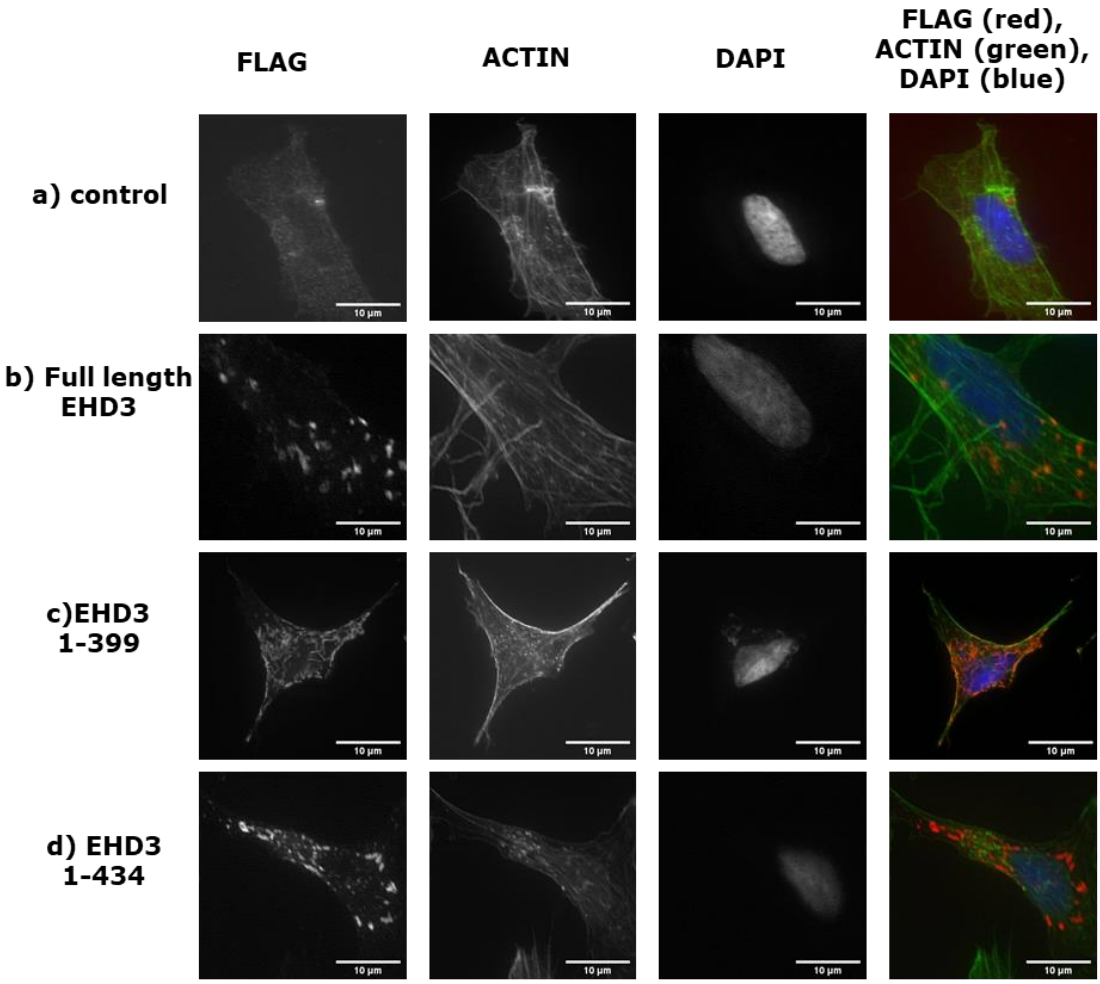

Figure 7. EHD3 1-434, but not EHD3 1-399, aggregates in SH-SY5Y cells. Proteins were stained with anti-FLAG primary antibody and GAM 555 secondary antibody (red signal). Phalloidin was used to stain actin (green signal), while DAPI was used to stain the nucleus (blue signal). Images were obtained under 60X magnification using a fluorescent microscope and Cell Sens software. The scale bar corresponds with 10 µm. Control represents mock transfected SH-SY5Y cells. Obtained images are a result of three independent experiments.

4.3.2. Expressing EHD3 plasmids in HEK293 cells by fluorescent microscopy

To further understand the ultracentrifugation assay data, we expressed the same plasmids in HEK293 cells. This was done to see whether the apparent differences in results from the ultracentrifugation assay using HEK293 cells, and microscopy results using SH-SY5Y, occur just from a change in a cell line. Specifically, whether the use of HEK293 cells instead of SH-SY5Y cells for ultracentrifugation altered protein expression and affected aggregation. According to figure 8, full length EHD3 clearly aggregates in HEK293 cells,

forming amorphous red aggregates in the cytoplasm of the cell. EHD3 1-399 does not show clear signs of aggregation in HEK293 cells. It forms red strands in the cytoplasm and the nucleus, which is not an indication of protein aggregation. EHD3 1-399 also did not appear in the aggregome of HEK293 cells (Figure 6). On the other hand, EHD3 1-434 did not appear in the aggregome of HEK293 cells (Figure 6) but the images of fluorescence microscopy show that it forms aggregate-like structures in the cytoplasm and the nucleus of the cell. All three plasmids therefore exhibit the same expression pattern in HEK293 and SH-SY5Y cells.

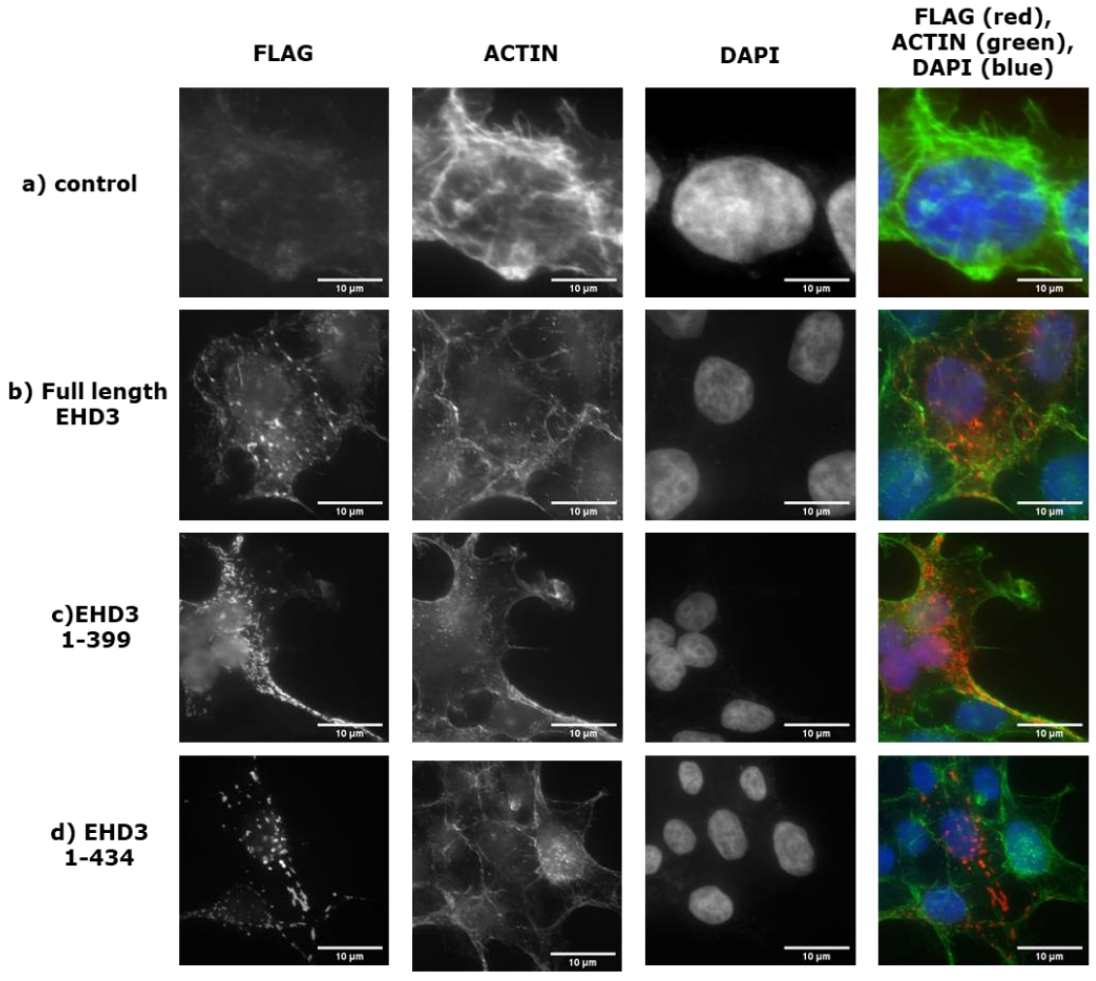

Figure 8. HEK293 cells do not alter EHD3 expression. Proteins were stained with anti-FLAG primary antibody and GAM 555 secondary antibody (red signal). Phalloidin was used to stain actin (green signal), while DAPI was used to stain the nucleus (blue signal). Images were obtained under 60X magnification using a fluorescent microscope and Cell Sens software. The scale bar corresponds with 10 µm. Control represents mock transfected SH-SY5Y cells. Obtained images are a result of three independent experiments.

4.3.3. Expressing novel EHD3 fragments in SH-SY5Y cells by fluorescent microscopy

As a result of previous data, novel plasmids that began at amino acid 1 of EHD3 but terminated at amino acids between 400-434 were cloned. EHD3 1-404 was the first cloned EHD3 fragment and was expressed in neuroblastoma cells. This fragment includes a small part of the linker region, containing 4 amino acids (Figure 3). Just like EHD3 1-399 (Figure 7c), EHD3 1-404 shows filamentous expression rather than amorphous aggregates. This again is not an indication of protein aggregation. The red strands are localized to the cytoplasm and the nucleus of the cell (Figure 9b).

Since either the EH domain and/or linker region are required for EHD3 aggregation, we decided to clone an EHD3 fragment 400-535. This fragment includes both the linker region and the EH domain (Figure 3). EHD3 400-535 was expressed in SH-SY5Y cells and shows no signs of aggregation. This is indicated with a clear red signal, without amorphous aggregates or fibrillar strands (Figure 9c). Overlapping of red signal of EHD3 400-535 and green signal of Phalloidin (actin) creates a yellow signal. This shows that EHD3 400-535 binds tightly to actin. Actin could be an important factor for regulating protein aggregation and therefore determining whether the protein aggregates or not.

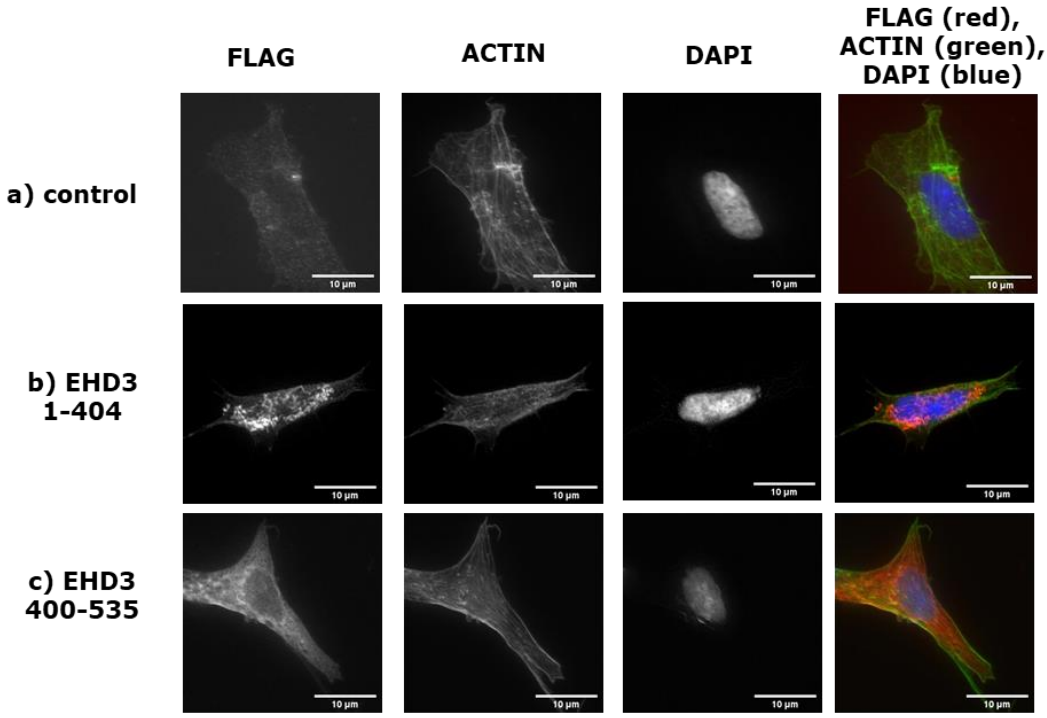

Figure 9. Novel EHD3 fragments do not aggregate in SH-SY5Y cells Proteins were stained with anti-FLAG primary antibody and GAM 555 secondary antibody (red signal). Phalloidin was used to stain actin (green signal), while DAPI was used to stain the nucleus (blue signal). Images were obtained under 60X magnification using a fluorescent microscope and Cell Sens software. The scale bar corresponds with 10 µm. Obtained images are a result of three independent experiments. Control represents mock transfected SH-SY5Y cells.

4.4. Full length EHD3 induces non-aggregating EHD3 1-399 to aggregation in SH-SY5Y cells

Usually, aggregates can spread between cells and therefore affect other proteins. One misfolded molecule can initiate and amplify aggregation of another. This way aggregation is easily spread among cells. To further investigate this phenomenon, full length EHD3 and EHD3 1-399 were co-expressed together, as were EHD3 1-399 and EHD3 1-434. This was done to see whether the aggregating form of EHD3 can induce non-aggregating EHD3 1-399 to aggregation. Proteins were transfected into neuroblastoma cell line, stained with anti-FLAG primary antibody and GAM 555 secondary antibody. Plasmids containing EGFP tag exhibit fluorescence on their own.

Firstly, several EHD3 plasmids were expressed in SH-SY5Y cells in isolation (Figure 10). This was done to ensure that their expression was usual. They served as controls for the co-aggregation experiment. Fluorescence microscopy showed that full length EHD3 with GFP tag forms aggregates in the cytoplasm of the cell, as expected (Figure 10c). Moreover, EHD3 1-399 fused to GFP tag also seems to form aggregate-like structures (Figure 10d). This fragment, when fused to the FLAG tag, forms fibrillar strands within the cytoplasm and nucleus of the cell (Figure 10e). Since the GFP tag is quite big (30 kDa), it can make proteins more prone to aggregation, which is the case with EHD3 1-399. EHD3 1-434 forms clear red aggregates in the cytoplasm and the nucleus (Figure 10f).

Secondly, EHD3 1-399 and EHD3 1-434 were expressed with GFP alone (GFP control) to see whether they behave as usual in the presence of GFP tag (Figure 11a, 11b). Co-transfection of EHD3 fused to GFP tag and EHD3 1-399 shows clear co-aggregation visible as yellow aggregates (Figure 11c). Yellow signal is a result of overlapped red and green aggregates of both plasmids. In other words, EHD3 fused to GFP tag induces normally non-aggregating EHD3 1-399 to aggregation. Moreover, it is also shown that signals of EHD3 1-434 and EHD3 1-399 overlap and give yellow aggregates. While that would usually mean these two plasmids co-aggregate, due to confusing results whether EHD3 1-434 aggregates or not, we cannot say they form aggregates together, but simply clustering or interacting without aggregation (Figure 11d). EHD3 is continuously shown to aggregate on its own, but previous research did not confirm its co-aggregation with other proteins such as Dysbindin 1A or TRIOBP-1. These results give us a better understanding of the mechanism of EHD3 aggregation, knowing that full length EHD3 can induce non-aggregating EHD3 1-399 to aggregation.

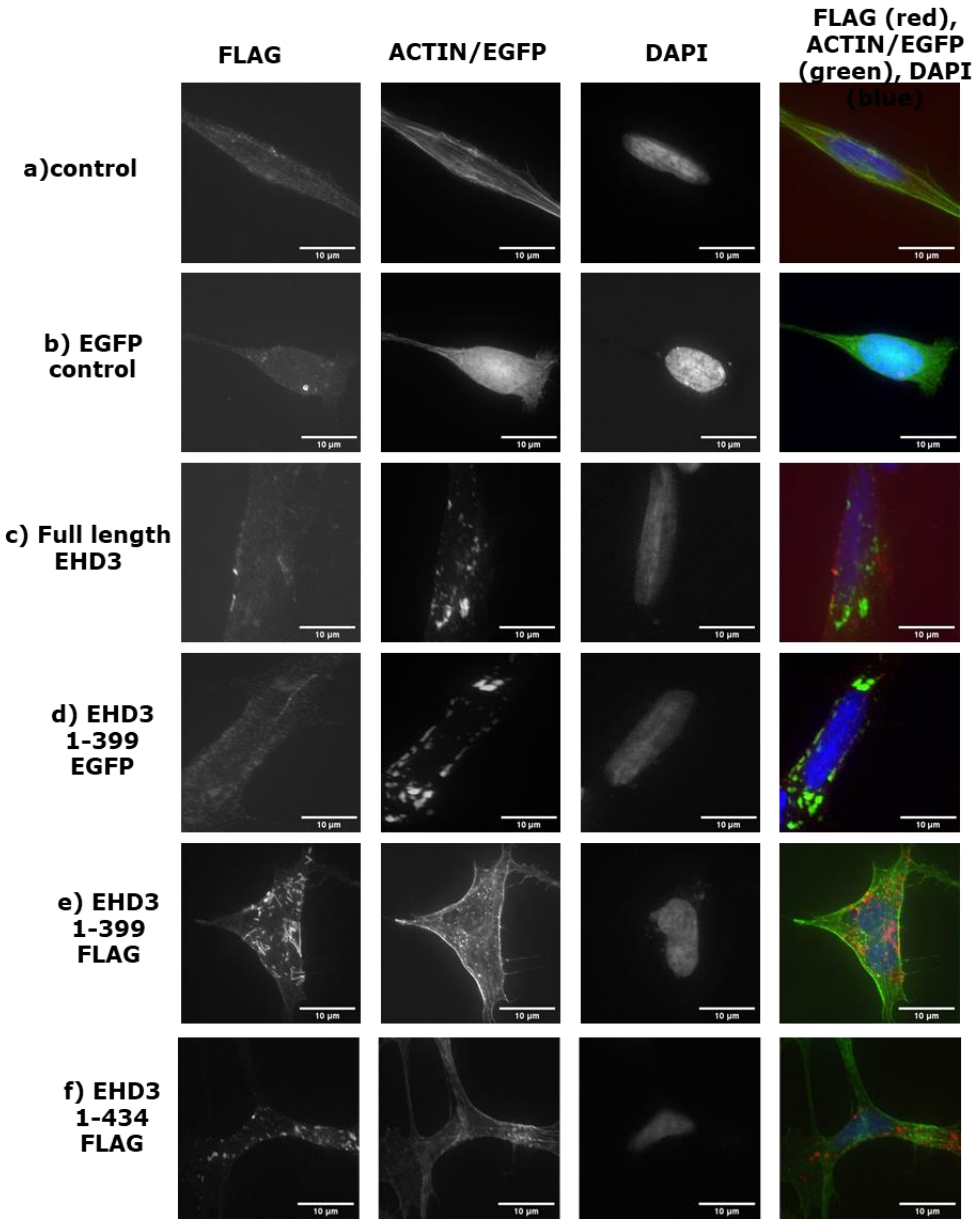

Figure 10. EHD3 plasmids expressed in SH-SY5Y cells as controls for co-aggregation experiment. Proteins were stained with anti-FLAG primary antibody and GAM 555 secondary antibody (red signal). Phalloidin was used to stain actin (green signal), while DAPI was used to stain the nucleus (blue signal). Images were obtained under 60X magnification using a fluorescent microscope and Cell Sens software. The scale bar corresponds with 10 µm. Obtained images are a result of three independent experiments. Control represents mock transfected SH-SY5Y cells, while EGFP control represents expression of EGFP tag.

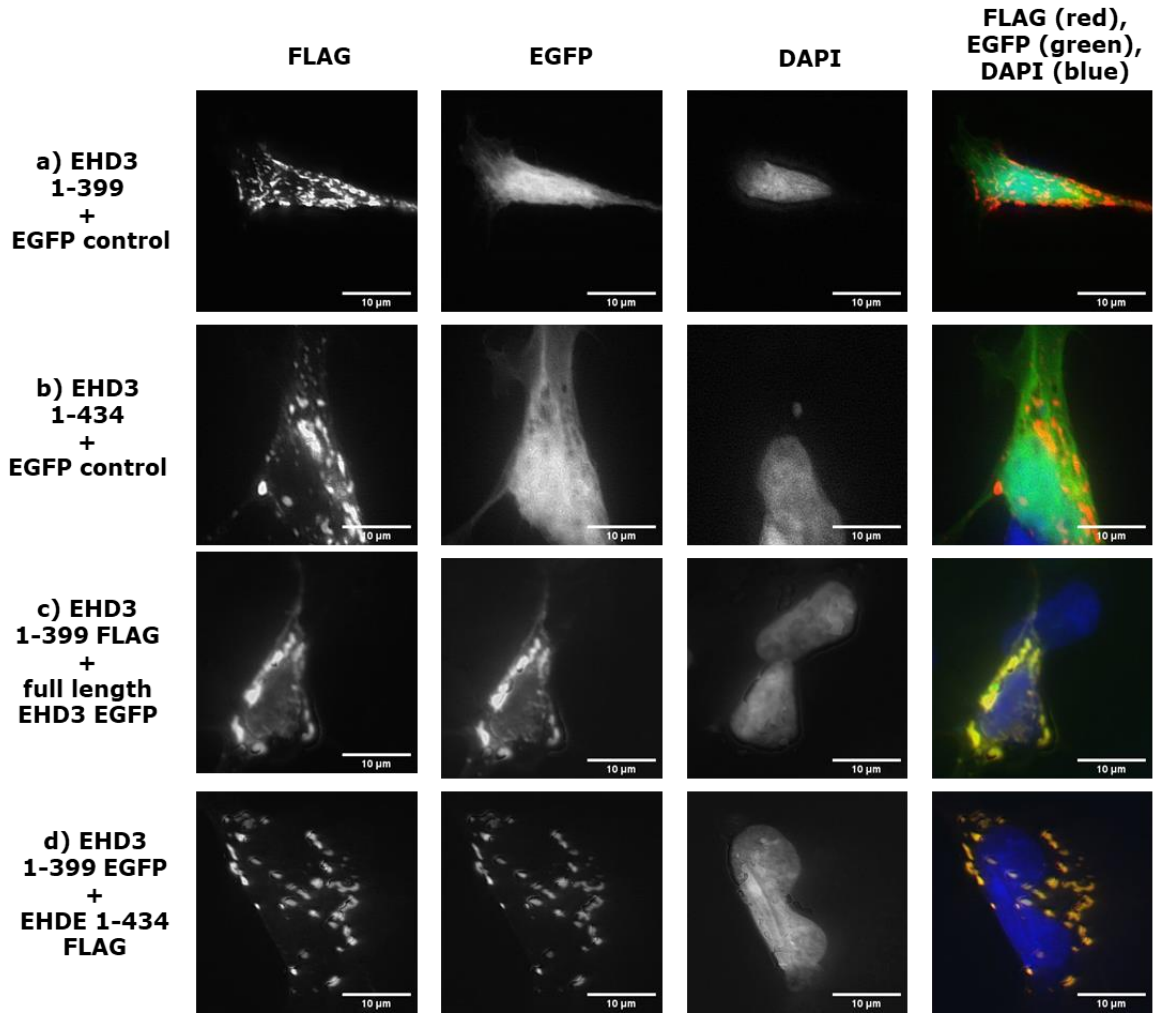

Figure 11. Full length EHD3 induces non-aggregating EHD3 1-399 to aggregation in SH-SY5Y cells. Plasmids with FLAG tag were stained with anti-FLAG primary antibody and GAM 555 secondary antibody (red signal). Plasmids with EGFP tag exhibit green fluorescence on their own. DAPI was used to stain the nucleus (blue signal). Images were obtained under 60X magnification using a fluorescent microscope and Cell Sens software. The scale bar corresponds with 10 µm. Obtained images are a result of three independent experiments.

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5. Discussion

EHD3 is a trafficking protein that belongs to the EHD family. For a long time, molecular functions of EHD3 were only associated with endocytic recycling and endosome transport. Only recently has data from mass spectrometry expanded our views on EHD3. Data revealed that EHD3 is insoluble in post-mortem brains of schizophrenia patients and therefore expected to be aggregating. This discovery is particularly interesting due to protein aggregation being a recent focus of research in CMI, as it may be a possible cause of CMI in addition to genetic and environmental factors.

5.1. The linker region was suspected to be crucial for EHD3 aggregation

Research shows that full length EHD3 continuously aggregates in neuroblastoma cells. The next logical step was to identify a critical region for its aggregation. Identifying the critical region is important because it could influence the generation of an aggregation resistant form of EHD3. This would lead to more experiments on how the aggregation resistant form of EHD3 affects the mammalian cells or transgenic animals. Moreover, it would give us a better understanding on mechanism of EHD3 aggregation, which could in the future be used in the production of novel drugs targeting this region and terminating EHD3 aggregation. Previous research from our laboratory proposed the linker region as a possible critical domain for EHD3 aggregation (Bertosa & Bradshaw, unpublished). It was shown that different fragments of EHD3 have distinctive expression in neuroblastoma cells. EHD3 1-399, which is missing a linker region and EH domain, exhibited filamentous expression in the cytoplasm and nucleus of the cell. The filamentous expression is not an indication of protein aggregation. On the other hand, EHD3 1-434, which is missing an EH domain, clearly forms aggregate-like structures in neuroblastoma cells, displaying amorphous aggregates in the cytoplasm and nucleus of the cell. We tried to narrow down the proposed linker region and

explore whether the aggregation occurs within amino acids 400-434. The idea was to generate five EHD3 constructs; EHD3 1-404, EHD3 1-411, EHD3 1-420, EHD3 1-426, and EHD3 400-535 which would narrow down the linker region, but also give insight into how EH domains affect aggregation in neuroblastoma cells. However, we only managed to complete cloning of two fragments, EHD3 1-404 and EHD3 400-535.

5.2. EHD3 1-434 is not present in aggregome of HEK293 cells after ultracentrifugation assay, suggesting the EH domain to be an aggregation critical region, and not the linker

To validate fluorescent microscopy results with another approach, protein insolubility of full length EHD3, EHD3 1-399 and EHD3 1-434 was tested via ultracentrifugation. Since both full length EHD3 and EHD3 1-434 appear to aggregate in SH-SY5Y cells based on microscopy, we suspected there would be a band visible in both the homogenate and aggregome fraction. Moreover, since EHD3 1-399 does not aggregate in SH-SY5Y cells, we expected only a band visible in the homogenate fraction. Surprisingly, EHD3 1-434 displayed a band in the homogenate fraction, but not the aggregome. The other two EHD3 fragments gave the results expected, with full length EHD3 displaying a band in both fractions and EHD3 1-399 only in homogenate one. Since the ultracentrifugation was done using HEK293 cells, we hypothesized that maybe EHD3 1-434 behaves differently in this cell line. All three EHD3 plasmids were then expressed in HEK293 cells and visualized with fluorescent microscopy. Expression of these plasmids in HEK293 cells was consistent with their expression in SH-SY5Y cells. This raises the question, whether the EHD3 1-434 aggregate-like structures, that are consistently seen in SH-SY5Y and HEK293 cells, are indeed aggregates or are they a consequence of an over-accumulation of protein in one spot due to overexpression. An over-accumulation of protein in one spot could lead to EHD3 1-434 to form clusters that look like aggregates but are not in fact real aggregates. This would

explain why EHD3 1-434 does not appear in the aggregome of HEK293 cells after ultracentrifugation. The only way to establish whether EHD3 1-434 is forming clusters or aggregates, is to analyze the protein with physical and analytical methods. A similar phenotype of EHD3 1-434 expression was observed in nocodazole treated HeLa cells transfected with GFP-EHD3 (30). GFP-EHD3 was seen to accumulate in large punctate structures, similar to the clusters we observed in EHD3 1-434. These punctate structures are affiliated with endocytic vesicles involved with endocytic recycling compartment (30). It is possible that the EHD3 1-434 clusters we are seeing are instead colocalized endocytic vesicles. Interestingly, in the same research EHD3 localized not only to punctate vesicular structures but also tubular structures distributed through the cytoplasm. This tubular expression is very similar to the stranded expression we have seen of EHD3 1-399 in SH-SY5Y and HEK293 cells. However, further experiments are needed to establish these connections.

5.3. More than one EHD3 region influences EHD3 aggregation

The results obtained in this thesis by fluorescent microscopy are consistent with previous results from our lab. In other words, EHD3 1-399 showed filamentous, stranded expression when expressed in SH-SY5Y cells in the cytoplasm and nucleus of the cell. As previously mentioned, this kind of stranded expression is not considered a sign of protein aggregation and probably represents binding to a component of a cytoskeleton. It is possible that depletion of the linker region and EH domain led to formation of a cross β sheet conformation, which is usually a characteristic of amyloid-like fibrils. This conformation could contribute to fibrillization of EHD3 1-399. On the other hand, EHD3 1-434 showed clear aggregation-like structures in neuroblastoma cell in the cytoplasm and nucleus. Novel fragments of EHD3 were also expressed in neuroblastoma cells. EHD3 1-404 seemed to have a similar expression to EHD3 1-399, displaying a fibrillar-like expression throughout the

cytoplasm and nucleus of the cell. Moreover, we suspected that EHD3 400-535 would aggregate since it does contain the linker region. However, EHD3 400-535 showed a diffused signal throughout the cytoplasm and the nucleus, without aggregates or fibrillar-like expression. In other words, EHD3 400-535 does not aggregate, evidently it binds to actin, and displays nuclear localization. Binding of actin may be an important factor for regulating whether a protein will aggregate or not. It is possible that the EH domain is responsible for binding of a protein to actin. Non-aggregation of EHD3 400-535 suggests there could be more than one critical region for EHD3 aggregation, possibly the linker region and the EH domain. This refutes our hypothesis that the critical region for aggregation occurs within 400-434 amino acids. However, further research is needed to investigate this. Research should include generating EHD3 constructs, we failed to do so in this thesis, and C-terminal parts of EHD3 such as EHD3 435-535 to see whether the EH domain is really involved in EHD3 mechanism of aggregation.

5.4. Expression of EHD3 1-399 is altered in the presence of full length EHD3

Full length EHD3 and EHD3 1-399, as well as EHD3 1-399 and EHD3 1-434 were co-expressed in SH-SY5Y cells. This was done to determine whether the aggregating form of EHD3 can induce the non-aggregating EHD3 1-399 to aggregation. A protein can be considered as a recruiter protein if it is able to induce the non-aggregating protein to form aggregates when co-expressed. Usually when two proteins co-aggregate, they exhibit a yellow signal which is an indication of overlapping of their aggregates. Both EHD3 1-434 and full length EHD3 seem to induce EHD3 1-399 to aggregation, however the accumulations that we see of EHD3 1-434 and EHD3 1-399 are likely not aggregates. Firstly, expressed EHD3 1-399 with a FLAG tag does not aggregate in SH-SY5Y cells, but when expressed with GFP tag it does aggregate. Since GFP is quite big (approximately 30 kDa) and very stable, it

can make a protein more prone to aggregation which is what we are seeing with EHD3 1-399. This means that the GFP tag is not suitable for experiments involving EHD3 1-399 since it changes the usual expression of EHD3 1-399. In contrast, the FLAG tag is only a few amino acids long and is therefore unlikely to have an effect. Secondly, giving that EHD3 1-434 is most likely not forming aggregates but instead clusters, we cannot say that it induces EHD3 1-399 to aggregation. These two EHD3 fragments probably strongly interact with each other which is why their aggregates overlap, but they are not co-aggregating. Future experiments should involve switching tags of mentioned EHD3 plasmids. This would include co-expressing full length EHD3-FLAG with EHD3 1-399-EGFP, and EHD3 1-399-FLAG with EHD3 1-434-EGFP. Other experiments should also include co-expressing full length EHD3 with EHD3 1-434 to see whether the expression of EHD3 1-434 changes.

5.5. Future research

The mechanism of aggregation of EHD3 turned out to be more complex than we suspected. While there are still many unanswered questions about EHD3 aggregation, these results gave us an insight into what the next step is in researching the mechanism of EHD3 aggregation. Firstly, the critical region for EHD3 aggregation should be determined and narrowed. Based on a finding that most likely the EH domain is responsible for EHD3 aggregation, although not sufficient by aggregation itself, constructs including the EH domain should be generated. The following constructs should be made and expressed in SH-SY5Y cells: EHD3 435-535 which includes the whole EH domain and it should be narrowed down to see the effect of the EH domain on EHD3 aggregation. If EHD3 435-535 construct aggregates, the EH domain probably does have a role in EHD3 aggregation. EHD3 1-411, EHD3 1-420 and EHD3 1-426 could still be generated as planned to investigate aggregation of EHD3 1-434. Since we can only speculate whether EHD3 1-434 aggregates or not, this construct should be also sent to validation by physical/analytical methods. Even though

it was shown that insoluble EHD3 is present in the brains of schizophrenia patients, it should be verified whether EHD3 aggregates alone or interacts with other proteins as well. So far, EHD3 was found not to co-aggregate with DISC1, Dysbindin 1A or TRIOBP-1 (41). The aim is to generate an aggregation resistant EHD3 to study its effects in mammalian cells and transgenic animals such as *D. melanogaster,* as has already been done for other proteins. The effect of the aggregating EHD3 protein and non-aggregating form of EHD3 on behavior of transgenic animals could be then compared and analyzed. Researchers should also focus on investigating biological functions of EHD3 to get a better understanding of its role in a physiological setting. This is very important because it could lead to the emergence of new hypotheses and discoveries of EHD3 role in schizophrenia pathophysiology. Investigating EHD3 aggregation is only a small fraction of the whole picture in the protein aggregation aspect, but it is equally important to understand protein aggregation better. Understanding biological functions, mechanism of aggregation and its effects in mammalian cells or transgenic animals will lead to a better understanding of EHD3 implications in CMI.

6. Conclusion

Protein aggregation has emerged as a possible non-genetic cause of CMI, an addition to genetic and environmental factors. Aggregation occurs when proteins misfold due to errors in folding or trafficking machinery. EH-domain containing 3 (EHD3) protein is one of the 5 proteins found to form aggregates in brains of schizophrenia patients. In physiological conditions, EHD3 is known to be a key regulator of endocytic recycling, as well as being involved in internalization of D1 dopamine receptors for which expression is decreased in schizophrenia. The linker region was suspected to be a critical region for EHD3 aggregation and was therefore investigated in this thesis. Different EHD3 fragments were generated and expressed in SH-SY5Y and HEK293 cells. All EHD3 plasmids had the same expression pattern in both cell lines. Protein insolubility was also tested via ultracentrifugation as a second approach. This method determined that EHD3 1-434, which aggregates in SH-SY5Y cells, does not appear in the aggregome of HEK293 cells. This is an unexpected finding which needs to be further analyzed but suggests that it is in fact not the linker region, but the EH domain, that is responsible for aggregation. However, there might be more than one critical region for EHD3 aggregation, due to non-aggregation of EHD3 400-535 which contains both the linker region and EH domain. Co-aggregation experiments showed that the full length EHD3 induces non-aggregating EHD3 1-399 to aggregation. EHD3 1-399 can therefore be recruited to aggregation by full length EHD3 and change its expression. Future research should focus on generating novel EHD3 fragments to determine the critical region for EHD3 aggregation and researching EHD3 molecular functions. Due to its biological functions and consistent aggregation in mammalian cells, EHD3 might be strongly implicated in the pathology of schizophrenia.

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Financial support

This thesis was financially supported by a grant from the Croatian Science Foundation (HRZZ: Hrvatska zaklada za znanost): IP-2018-01-9424, "Istraživanje shizofrenije kroz ekspresiju netopivih proteina".

The experimental work has been conducted on equipment financed by the European Regional Development Fund (ERDF), within the project "Research Infrastructure for Campus-based Laboratories at the University of Rijeka" (#RC.2.2.06-0001), and by an equipment subsidy from the Alexander von Humboldt Foundation (Bonn, Germany).

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- competition "Tetragon" - oral and written assessment of high school students, assistance in conduction of the experiment