Interactions between schizophrenia-associated aggregation of EHD3 and the cytoskeleton

Montan, Andrea

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Andrea Montan

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Rijeka, 2024

Mentor: Dr. Nicholas J. Bradshaw, PhD

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Andrea Montan

Interakcije između agregacije EHD3 proteina povezane sa shizofrenijom i citoskeleta

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Abstract

Schizophrenia is a serious mental illness that is characterized by altered behaviour and a diminished ability to perceive reality. In addition to genetic and environmental risk factors, protein aggregation is also considered to be a potential risk factor in the development of schizophrenia. Protein aggregation and misfolding happens when cellular systems are disturbed by internal and external stresses. One of the proteins that has been found to aggregate in the brains of schizophrenia patients is EH-domain containing 3 (EHD3). This belongs to the C-terminal Eps15 homology domain (EHD) protein family, together with the EHD1, EHD2 and EHD4 proteins. EHD3 is involved in the regulation of endosome to Golgi transport, recycling, and in promotion of dopaminergic transmission. Previous research in our lab showed that full length EHD3 aggregated in SH-SY5Y cells, while two truncated versions, containing amino acids 1-399 and 1-434 did not. Also, the EH domain alone (amino acids 435-535), when expressed individually did not aggregate. The conclusion was that more than one region influences EHD3 aggregation. The EH domain is necessary for the initialization of EHD3 aggregation, but other structural regions interact and recruit other molecules that make up the aggregates. In my thesis, we wanted to test the association of EHD3 with actin and how it affects the cytoskeleton. We also wanted to determine how other proteins from EHD family behave inside cells and whether they also form aggregates. Using ultracentrifuge assay, I saw that full length EHD3 co-segregates and is connected with actin inside cells, which I also confirmed using fluorescent microscopy. In ultracentrifuge assay, EHD3 fragments 1-399 and 1-434 did not show association with actin, while in fluorescent microscopy they showed association with actin cytoskeleton. Further research is needed in this field. In blinded, quantified, microscopy assay, EHD3 showed aggregate-like structures in most of the cells, while EHD1 showed this pattern in approximately half of cells. EHD2 and EHD4 showed low signs of potential aggregation. From this we can conclude that, while EHD3 is the only EHD protein confirmed to aggregate, data for EHD1 show its potential for aggregation. Understanding of aggregation of EHD proteins processes and physiological functions may facilitate a better understanding of the pathology and possible causes of schizophrenia development.

Sažetak

Shizofrenija je ozbiljna mentalna bolest koju karakterizira promjena ponašanja i smanjena sposobnost percepcije stvarnosti. Osim genetskih i okolišnih rizičnih čimbenika, agregacija proteina također se smatra potencijalnim čimbenikom za razvoj shizofrenije. Agregacija i pogrešno slaganje proteina događa se kada su stanični sustavi i procesi poremećeni unutarnjim i vanjskim stresovima. EHD3 je jedan od proteina za koji je utvrđeno da agregira u mozgu pacijenata sa shizofrenijom. Taj protein pripada obitelji proteina C-terminalne homološke domene Eps15 (EHD), zajedno s proteinima EHD1, EHD2 i EHD4. EHD3 je uključen u regulaciju transporta endosoma u Golgijevo tijelo, recikliranje te promicanje prijenosa dopaminergika. Prethodna istraživanja u našem laboratoriju dokazala su da EHD3 agregira u SH-SY5Y stanicama, dok njegove dvije skraćene verzije (s aminokiselinama 1-399 i 1-434) ne agregiraju. Također, kada se EH domena (aminokiseline 435-535) ekspresirala pojedinačno u stanici, nije pokazala znakove agregacije. Donesen je zaključak da je više regija uključeno u proces agregacije EHD3 proteina. EH domena neophodna je za inicijalizaciju agregacije, ali druge strukturne regije međusobno djeluje i regrutiraju druge molekule koje čine agregate. U ovom smo radu željeli testirati povezanost EHD3 proteina s aktinom te utvrditi kako utječe na citoskelet stanica. Također, promatrali smo kako se drugi proteini iz EHD obitelji ponašaju unutar stanica i tvore li agregate. Koristeći ultracentrifugu, otkrila sam da EHD3 protein ko-agregira te je povezan s aktinom unutar stanica. Isto sam utvrdila i uz pomoć fluorescentne mikroskopije. Kod testa ultracentrifuge, EHD3 fragmenti 1-399 i 1-434 nisu pokazali povezanost a aktinom dok su kod fluorescentne mikroskopije pokazali povezanost s aktinskim citoskeletom. U ovom su području potrebna daljnja istraživanja. U slijepom, kvantificiranom testu mikroskopije, EHD3 protein pokazao je strukture slične agregatima u većini stanica dok je EHD1 pokazao taj obrazac kod polovice stanica. S druge strane, EHD2 i EHD4 proteini pokazali su male znakove potencijalne agregacije. Iz ovih rezultata možemo zaključiti da, iako je EHD3 jedini EHD protein za koji je potvrđeno da agregira, podaci za EHD1 pokazuju njegov potencijal za agregaciju. Razumijevanje procesa agregacije i fizioloških funkcija EHD proteina može olakšati bolje razumijevanje patologije te omogućiti bolje razumijevanje uzroka razvoja shizofrenije.

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1. Introduction

1.1. Schizophrenia

A mental illness is characterized as a clinically significant disorder in an individual's emotional regulation, thinking and behaviour. World Health Organisation (WHO) data shows that 1 in every 8 people in the world lived with some type of mental illness in 2022 (1). Mental illness includes various diseases that vary in severity, ranging from mild, moderate to severe. To describe these illnesses, two categories can be used: Any Mental Illness (AMI) and Serious Mental Illness (SMI). The AMI category contains all recognized mental illnesses, while SMI is a more severe subcategory of AMI (2). SMIs interfere with and/or limit major life activities and make everyday life difficult. This category of illnesses includes schizophrenia, major depression disorder and bipolar disorder (2).

Schizophrenia is a chronic, serious mental illness that affects cognitive and behavioural human functions. Although schizophrenia is not as frequent as other SMIs, it is the most chronic and disabling disease (3). It is associated with impairments in all areas of life, including social, family, educational and work functioning. It affects approximately 24 million people or 1 in 300 people (0.32%) worldwide (4). The outcome of the disease can vary from complete recovery to chronic need of care and therapy, and these patients, on average, have a lifespan 10 to 20 years below that of the general worldwide population (1). Schizophrenia is an illness with a heterogeneous combination of symptoms that can be divided in 3 categories- 'positive', 'negative' and 'cognitive' (5). Positive symptoms include recurrent psychosis, which is characterized by loss of contact with reality. Psychosis consists of delusions, hallucinations, disorganized behaviour and speech. Negative symptoms refer to a lack of ability to function normally, lack of energy and emotions, social withdrawal, affective flattening and anhedonia (the inability to feel pleasure). Patients with cognitive symptoms have trouble with focusing, memory and other cognitive dysfunctions (5).

Schizophrenia affects men and women equally, although symptoms in men usually appear in the early to mid-20s, while in women it is in the late 20s (6). The prodromal phase, a phase before manifestation of the first psychotic episode is probably the onset of the schizophrenia, begins in the early adolescent years. These patients are typically not referred to treatment until psychosis presents in early adulthood (5). The peak age of schizophrenia onset is 20.5 years with a median age at onset of 25 year

(interquartile range 21–35) (7). The prevalence of schizophrenia does not vary much across countries.

Approximately 8% of schizophrenia patients are diagnosed before age 18 and almost 18% of them experience their first symptoms before age 18 (7). Today, the early identification of disease is viewed as clinically and scientifically very important. There is still no diagnostic test to confirm the presence of this illness, so identification is based on patient medical history and mental state examination. Also, diagnosis of schizophrenia is made based on criteria, which are documented in the Diagnostic and Statistical Manual of Mental Disorder (DSM) or the International Statistical Classification of Diseases and Related Health Problems (ICD). These criteria involve observation of positive, negative and cognitive symptoms of illness along with their duration and effect on the patient's life (5). Based on DSM-5 criteria, at least two symptoms must be observed, in a period of one month or longer and one of the symptoms must be hallucinations, disorganized speech or delusions (8). Also, when diagnosing, the duration and severity of symptoms and presence of some other somatic illness should be considered (8).

There is a range of effective care options for schizophrenia patients, from medication, cognitive-behavioural therapy, psychoeducation and psychosocial rehabilitation. The goal of schizophrenia treatment is to alleviate positive symptoms and decrease the chance of relapse. With the help of appropriate therapy, at least one in three people will be able to fully recover, but more often, schizophrenia requires lifelong treatment, which can help to manage this illness (4). Medications are the first line of defence in the battle with schizophrenia and the most prescribed ones are antipsychotics. The main goal of antipsychotic treatment is to manage the signs and ease the symptoms at the lowest possible dose. Also, some patients are prescribed antidepressants and anti-anxiety drugs (9). The main mechanism of action of all typical antipsychotics is to block the dopamine receptors pathway. Different types of antipsychotics have been developed and can be classified into `typical´ or first-generation antipsychotics and `atypical´ or second-generation antipsychotics (5). First-generation antipsychotics act by non-selectively blocking dopamine receptors in the brain, which leads to a range of frequent side effects. This group includes drugs like chlorpromazine, haloperidol, fluphenazine and perphenazine. Second-generation antipsychotics have a much higher ability to block serotonin receptors (5-HT_{2A}) than dopamine receptors. They are designed to have fewer side effects and they are therefore preferred to first generation ones. This group includes drugs like olanzapine, asenapine, clozapine, brexpiprazole, and iloperidone (9). In schizophrenia treatment, single-target drugs like first generation antipsychotics have limited efficiency so scientists are working on the development of novel pharmacologic agents that act simultaneously on several molecular targets (10). These new targets include glutamatergic, serotonergic, cholinergic

and phosphodiesterase systems. Other methods of treatment of schizophrenia are still in development: more personal, mechanism-based procedures have the greatest chance of generating novel drugs with a larger range and greater efficacy (5). Clinical diagnosis does not tell us which drug will be the most effective for each patient and not all patients can be treated with existing drugs. Therefore, there is a need for better understanding the biology of schizophrenia to treat it better and to help all patients with this illness.

1.2. Genetics and biological theories of schizophrenia

A few different hypotheses have been proposed to explain the neuropathology of schizophrenia. Their focus is on genetic, environmental, neurochemical and neurodevelopmental effects. Development in preclinical and clinical studies with imaging methods leading to improvements of these hypotheses (11). Studies of brain function in individuals have been crucial for understanding links between brain biology, genetic risk and clinical state of a patient, and they encouraged further research in the field of schizophrenia. Based on many genetic epidemiological studies, there is a significant but not exclusive role of genetic factors to the aetiology of schizophrenia (10). Twin studies have provided evidence that both environmental and genetic factors contribute to development of schizophrenia. Studies show that the concordance rate of schizophrenia is 33% in monozygotic and 7% in dizygotic twins (12). In the last few years, large-scale genomic studies have been conducted, and we now have a better picture of how risk alleles of different types contribute to the development of schizophrenia. Genome-wide association studies (GWAS) have proved that schizophrenia is polygenic because they identified over 300 distinct genetic loci containing common alleles of small effect and many hundreds of `en masse´ effects loci (10). Also, GWAS identified 11 rare copy number variants (CNVs) that individually give a high risk of the illness. Most of the risk variants have been discovered in non-coding regions of the genome: miRNAs and lncRNAs. It has been proven that a combination of different pathogenic mechanisms such as aberrant DNA methylation, aberrant polyadenylation of pre-mRNAs, mis-splicing and altered histone code play roles in the development of schizophrenia (11).

The most recent whole exome sequencing studies have shown inherited, rare and de novo single nucleotide deletion/insertion variants in schizophrenia. One of the best-known risk factors for schizophrenia is the microdeletion in chromosomal region 22q11.2, which increases the risk of schizophrenia development by up to 30-40%. The major histocompatibility complex (MHC) locus on 6th chromosome, which contains genes encoding

proteins that are essential for adaptive immunity, is also linked to the development of schizophrenia (11). It has been proven that some genes associated with schizophrenia are those that encode for the protein disrupted in schizophrenia 1 (DISC1), dystrobrevin-binding protein 1 (dysbindin or DTNBP1) and for neuregulin 1 and 3, while the gene encoding synaptosomal-associated protein SNAP25 could also, potentially contribute to the development of this illness (11).

The neurodevelopmental hypothesis is the most dominant hypothesis for understanding the environmental risk factors connected to schizophrenia. This hypothesis primarily concerns that events in early neurodevelopment effect risk of schizophrenia. These risks include maternal infections, maternal stress, pregnancy and birth complications and nutritional deficiencies (11). Also, socio-economic factors, childhood adversity, epilepsy, head injury, severe infections and others have been proven to be associated with schizophrenia. Currently, the most used approaches for testing environmental risk factors are experimental animal studies. These include studies of pre-natal maternal inflammation; stress factors and they observe their impact on neurobiological and behavioural variables. There is always a need for new, more complex and better studies that will combine both pre- and post-natal environmental factors with genetics and potentially provide new evidence for risk factors which affect schizophrenia development (10).

The neurochemical abnormality hypothesis explains the development of schizophrenia as an imbalance in four mains neurotransmitter pathways: dopamine, glutamate, serotonin and GABA (12). Post-mortem studies of patients have produced evidence that shows each of these neurotransmitter systems being altered in schizophrenia (5). In the dopamine hypothesis, positive symptoms are explained through excessive activation and increased density of D2 receptors via the mesolimbic pathway (11). Low concentrations of dopamine in the nigrostriatal pathway are hypothesised to cause motor symptoms because of its effect on the extrapyramidal system. On the other hand, negative symptoms are explained because of low mesocortical dopamine levels in the mesocortical pathway (12). In the glutamate theory, reinforcement of positive and negative symptoms by an NMDA receptor antagonist insinuates the potential role of glutaminergic hypoactivity in the development of schizophrenia. Also, serotonergic hyperactivity, which overactivates 5-HT_{2A} receptors on glutamate neurons in the cerebral cortex plays a role in this illness. Increased levels of serotonin results in the release of glutamate in the VTA and can potentially activate the mesolimbic pathway and consequently cause excess dopamine in the ventral cavity (11). According to the cannabinoid hypothesis, increased activation of the endocannabinoid system through CB1 receptors on GABAergic interneurons leads to a hyperdopaminergic and hypoglutamatergic status, which can potentially lead to the development of schizophrenia (11). Schizophrenia is a very complex illness so despite

advances in our knowledge of the genetics of schizophrenia, the disease's exact molecular pathways still remains poorly understood. In addition to genetic and environmental risk factors, protein aggregation could be one more non-genetic cause of development of schizophrenia.

1.3. Protein aggregation

Each protein is synthesized on ribosomes from a sequence of nucleotides contained in mRNA. This process is called translation, and it completes the flow of genetic information within the cell. For that polypeptide to be useful, it must fold into correct three-dimensional conformation, while multiple polypeptide chains must gather into a functional complex. Afterwards, many proteins undergo different modifications such as cleavage and creating covalent bonds with carbohydrates and lipids. These modifications are important for correct localization of proteins within the cell (13). The proper protein assembly is monitored by other type of proteins, called chaperones. Chaperones catalyze protein folding by assisting the self-assembly process, which is determined solely by the amino acid sequence of a protein. Chaperones bind to and stabilize unfolded or partially folded polypeptides that are intermediates in a folding process. They prevent incorrect folding or aggregating into insoluble complexes (13). Various external and endogenous stresses cause disruption of several different mechanisms, which leads to misfolding and aggregation of proteins. Protein aggregates might accumulate in a cell due to an imbalance in the autophagy processes.

Autophagy is a process of degradation of damaged proteins that occurs naturally in the cells. Also, misfolding can occur due to an error during transcription or translation, because of failure in the chaperone machinery, disruption to the ER under stress or because of an error during post-translational modifications (14). Proteins that fail to fold into their normal configuration and are in a misfolded state lose their normal function, they disrupt protein homeostasis, contribute to lysosome dysfunction and can also become toxic to the cell. All proteins have the potential to misfold under certain conditions because they share the same structural feature called the polypeptide backbone. Even under normal cellular conditions, aggregation is inevitable for most proteins (15).

Since misfolded proteins and protein aggregates interfere with normal functioning of cells and accelerate cell aging, mechanisms have been developed to protect cells from them. Cells have developed four main pathways for eliminating protein aggregates (Figure 1) (16). Probably, the most important pathway is the ubiquitin-proteasome pathway (UPP). In order to be identified by the UPP, aggregates must be marked with ubiquitin. The UPP system then induces the ubiquitination of the target protein and its transport to the proteasome. The proteasome has proteolytic activity and has a role in dendrite maintenance, neurodevelopmental

processes and energy balance, which are all most often aberrant in schizophrenia (17). While the UPP degrades soluble misfolded proteins and aggregates, the autophagy-lysosome pathway (ALP) degrades insoluble ones. A second pathway for eliminating protein aggregates is refolding and depolymerizing the aggregates. This process is carried out by a type of molecular chaperone - the heat shock protein family. They are bringing together aggregates and then separate them into smaller aggregates that are easier to degrade. Another pathway for eliminating aggregates is asymmetric cell division (ACD), which is possible only for dividing cells, and therefore not neurons. ACD can produce a healthier cell with fewer protein aggregates but at the cost of other daughter cell with more aggregates and consequently shorter life (16). The last pathway is discarding protein aggregates by secreting them through a process called exocytosis. In this process, aggregates are secreted in exophers- vesicles that will later be cleared by macrophages.

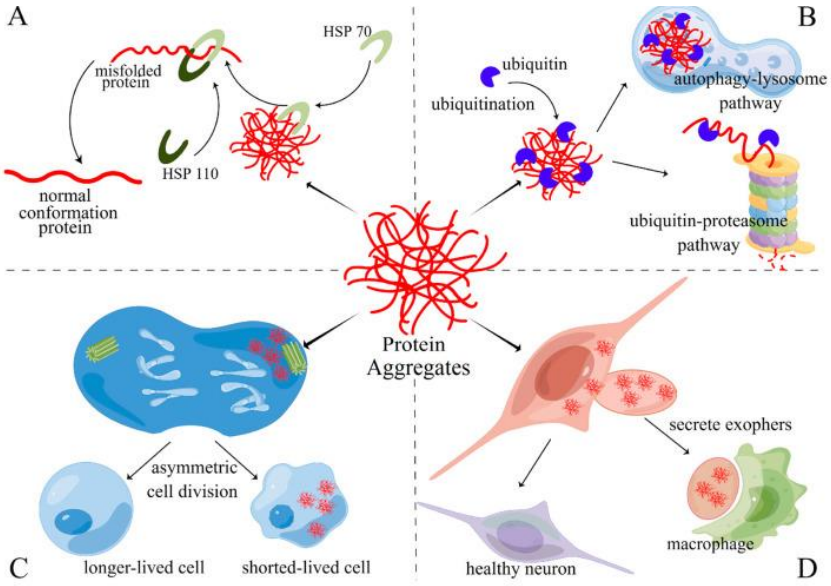


Figure 1. Four types of pathways for eliminating protein aggregates. A) HSPs family for refolding and depolymerizing, B) UPPs and ALPs C) asymmetric cell division (ACD) D) secreting through exophers. Figure from: Wen JH et al. Cellular Protein Aggregates: Formation, Biological Effects, and Ways of Elimination. International Journal of Molecular Sciences. 2023 May;24(10):8593

The best-known diseases caused by protein aggregation are neurodegenerative diseases such as Alzheimer's disease, Parkinson's, Huntington's disease and Alexander's disease. They were all found to be linked with protein aggregation and have been intensively studied (16). In each disease, specific proteins aggregate in different locations in the brain, but what they have in common is that aggregates cause dysfunction,

atrophy and apoptosis in the corresponding parts of the brain. The hallmark of these pathologies is accumulation of amyloid, tau and α-synuclein proteins. Proteins with such a significant role in pathogenesis have not been discovered so far in a case of mental disorders but research is ongoing (18).

Until now, few proteins have shown the possibility of aggregating in the brain of patients with mental disorders. These aggregating proteins might be significant in the development of disorders such as schizophrenia. In the brains of schizophrenia patients, some proteins that have been found to aggregate are DISC1, TRIOBP, CRMP1, NPAS3 and dysbindin-1 (18)(19)(20)(21). Aggregates were first found and identified in brain samples and then confirmed in cells and/or in animal models. Aggregation of these proteins have been mainly investigated using immunofluorescent microscopy to see if a protein forms large clusters that look like aggregates and with insolubility assays, as protein aggregates are expected to be insoluble. A conclusion is made when in both methods, aggregation is present.

More, using the two approaches described above, Eps15 homology domain-containing protein 3 (EHD3) and glutamate decarboxylase 2 (GAD2) had been found to be insoluble in human brain samples, then later shown to form aggregates in neuroblastoma cells as well (31). GAD2 was found in a subset of SMIs patients and results were confirmed with insolubility assays and immunofluorescent microscopy, where a high tendency of GAD2 to aggregate was observed (21). In the same way, from patient's brain samples and later in neuroblastoma cells, the aggregation of EHD3 protein has been proven. For a deeper understanding of the pathogenesis of schizophrenia, more research is needed in the field of protein aggregates. Therefore, this thesis will focus on the potential aggregation of EHD3 protein. New discoveries in this field could also help in the search for new therapeutic targets for treating mental illnesses (19)(21).

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

Eps15 homology domain-containing protein 3 (EHD3) is a protein encoded by the EHD3 gene. It belongs to the C-terminal Eps15 homology domain (EHD) protein family, together with EHD1, EHD2 and EHD4 proteins (22).
The EHD protein family is a group of four endocytic regulatory proteins that are related to the superfamily of large GTPases. These proteins are involved in the recycling of different types of receptors from the early endosome to the endocytic recycling compartment, and they also participate in the transport from the endosomes to the Golgi (22)(23). All four of the EHD

proteins contain an N-terminal helical region, a nucleotide-binding motif (also called a G-domain), then a central helical domain, linker region and an EH domain at the C-terminus end of the protein (Figure 2). Even though they share a high degree of amino acid identity, each EHD protein has different cellular localization and different functions. The EH domain is evolutionarily conserved in species, ranging from yeast to Homo sapiens. This domain has a stretch of approximately 100 amino acids, structured as two calcium-bound EF-hand motifs and a short antiparallel β -sheet. The EH domain contains a positively charged surface that binds to other endocytic regulators containing the Asn-Pro-Phe (NPF) motif (23)(24). Other functions of this domain are to regulate protein transport, membrane trafficking and endocytosis.

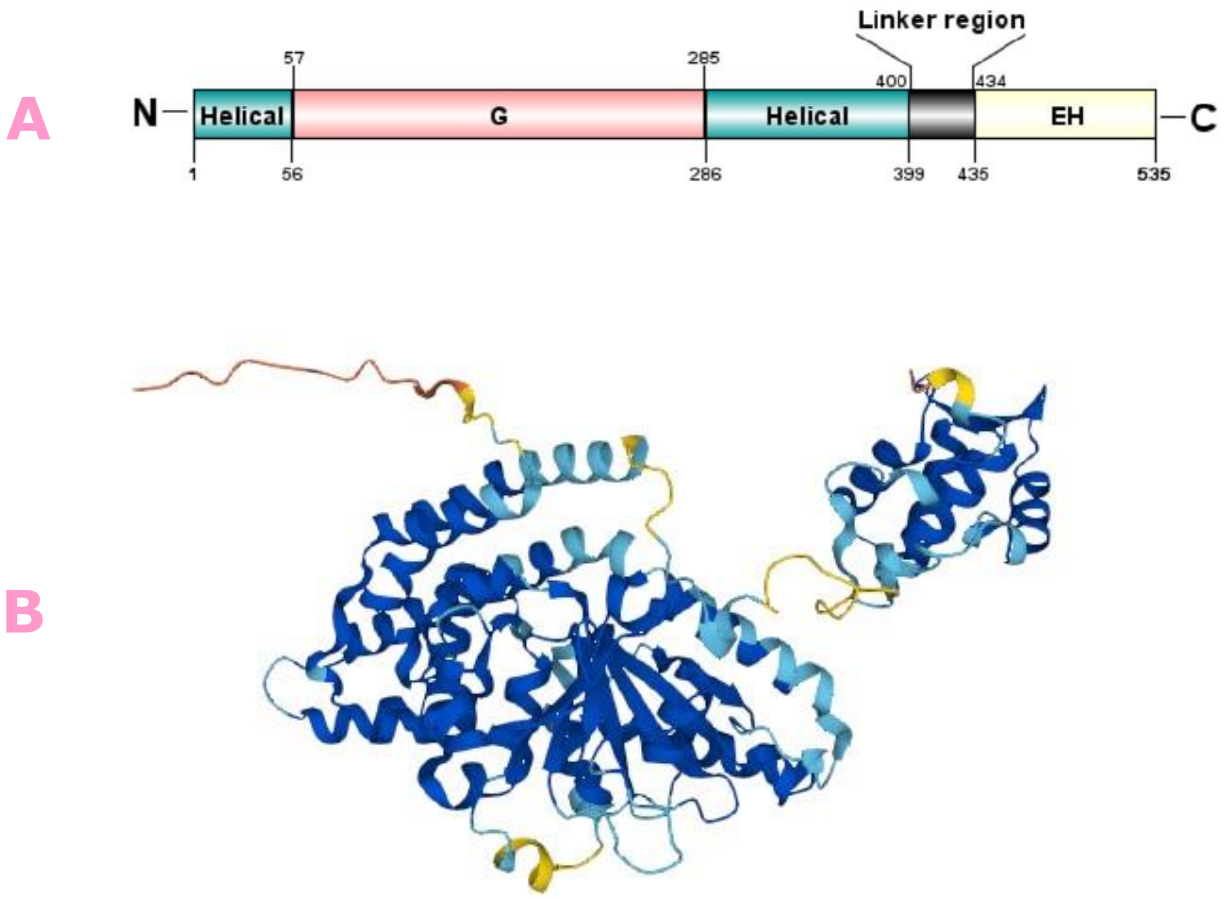


Figure 2. EHD3 protein structure (A-schematic (2D structure), B-predicted 3D structure). EHD3 protein consists of the two-part helical region that facilitates EHD oligomerization and lipid binding, a G domain that binds ATP, linker region and the EH domain connected to the C-terminal domain. Figure A taken from (32), B from Alphafold (AFQ9NZN3-F1)

EHD protein family is implied to have a role in the bending and fission of tubular recycling endosomes (TRE). TRE are an elaborate network of

dynamic lipid membranes that coordinate the process of endocytic recycling in mammalian cells. Endocytic recycling is not only important for the maintenance of cellular homeostasis, but also for the regulation of many processes inside cells, from nutrient uptake, ion channels and surface receptors to cell migration and cell adhesion (24)(25).

The location of the EHD3 gene is on chromosome 2p22-23. The EHD3 protein is expressed in the heart, brain, kidney, liver and placenta (22). The EHD3 protein shares the highest level of sequence identity with EHD1, whose role is regulating the transport and recycling of receptors. These two proteins interact with each other inside cells. The EHD3 protein is essential for TRE biogenesis, while EHD1 induces the vesiculation of TRE. EHD3 is also involved in the regulation of endosome to Golgi transport. Intracellular sorting receptors and extracellular toxins are transported through this pathway. In the absence of EHD3, Golgi morphology is altered, and carrier vesicles that are derived from early endosomes would not reach their target- the recycling endosome (26). It is believed that EHD1 and EHD3 are necessary for ciliogenesis, in a process where the formation of ciliary vesicles happens. Also, the function of these two proteins affects mother centriole reorganization (27). In the last few years, research has proven that the EHD3 protein is connected to microtubules. With nocodazole treatment (a microtubule disruptive agent), the vast majority of EHD3 in cells was in punctate forms (33), which I will try to replicate in my thesis. This could potentially be due to EHD3 commuting between the cytoplasm and the nucleus (29). Also, I will be looking at whether actin is involved with EHD3, which is not yet known. In my assays I will include nocodazole, as well as actin depolymerizing drug (cytochalasin B) to see if the effects are cumulative.

It has been shown that EHD3 affects dopaminergic transmission by internalizing D1 receptors. Disruption of the dopaminergic pathway has been linked with many pathological conditions, together with schizophrenia (28). EHD3 was found to be differentially expressed in neurons of schizophrenia patients, and it also tended to aggregate (30). This discovery was interesting because aggregation of EHD3 may be a possible cause of development of the SMIs.

Recent research in our lab confirmed that EHD3 forms aggregates in human neuroblastoma cells. These aggregates are seen to be mostly localized in the cytoplasm of the cell, around the nucleus (31). EHD3 was also co-expressed with other proteins that have the possibility of aggregation in SMI: dysbindin-1 and TRIOBP-1. It was proven that EHD3 does not co-aggregate with these proteins (32). Our lab wanted to determine a region that is crucial for the aggregation of EHD3. It was thought that the region critical for aggregation of EHD3 is linker region (33), but research showed that the EH domain is the one that is responsible for aggregation (32).

Further investigation showed that more than one region influences EHD3 aggregation. The EH domain is necessary for the initialization of EHD3 aggregation, but other structural regions like the helical and/or the G domain interacts and recruits other molecules that make up the aggregates (34). Also, aggregation of EHD3 fragments 1-399 and 1-434 was investigated, and it was proven that they do not aggregate alone, but when co-expressed with full length EHD3, 1-399 did aggregate (32). These fragments were designed to exclude the EH-domain and/or linker region and these numbers refer to amino acid numbers. The expression pattern of some of these constructs resembles the cytoskeleton, so now, a question that remains open is whether EHD3 interacts with actin inside cells. In my thesis, I will try to find out if EHD3 interacts with actin using an actin extension assay, as well as immunofluorescent microscopy. Also, I will investigate aggregation of 3, still unexplored plasmids from the EHD family- EHD1, EHD2 and EHD4.

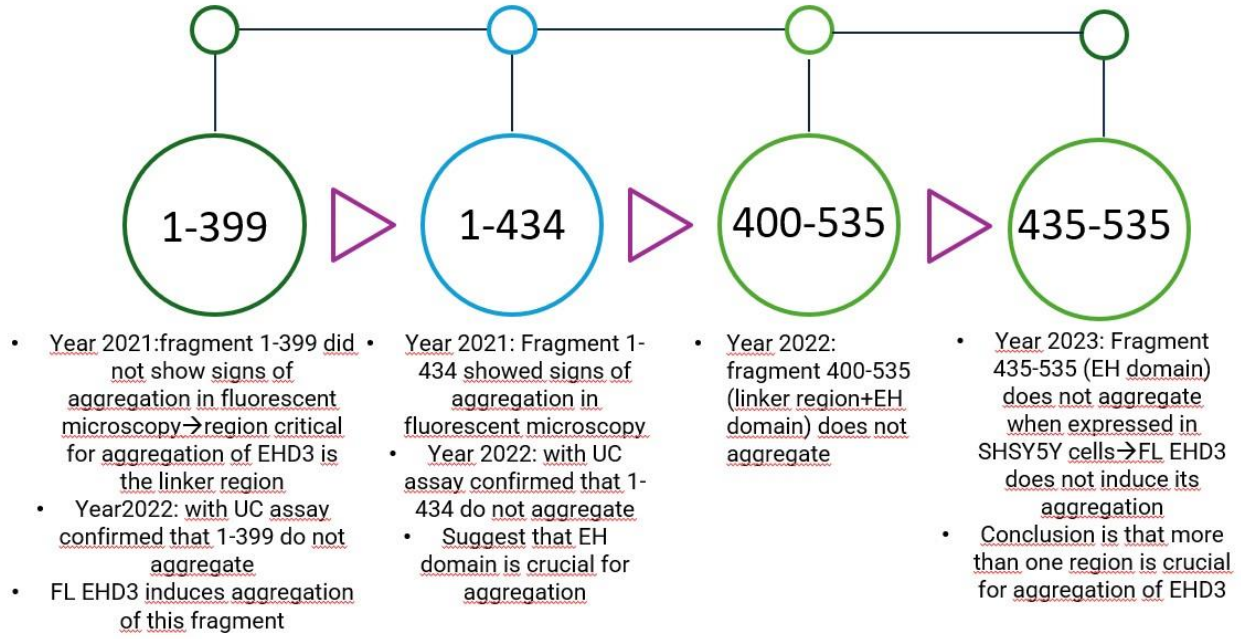


Figure 3. Scheme of previous research of FL EHD3 and different fragments in our lab. Results from years 2021-2023 (32)(33)(34).

2. Aims of the thesis

EHD3 is a member of the EHD protein family, observed to form aggregates in the brains of schizophrenia patients. It has been proven that more than one region is crucial for aggregation of EHD3. The EH domain is necessary for the initialization of EHD3 aggregation but other structural regions such as the helical and/or the G domain interact and recruit other molecules to form the aggregates. Also, it is hypothesized that the EHD3 protein is not only associated with microtubules but that it could also be associated with actin and interacts with it inside cells. In my thesis, I will test this hypothesis. Gaining further insight into these aspects of EHD3 behaviour would help us understand the mechanism underlying EHD3 aggregation and potentially lead to new findings in the field of schizophrenia.
Furthermore, it is also unknown whether other proteins from the EHD family tend to aggregate and if they have a role the in development of schizophrenia. Their mechanism and behaviour within cells are yet to be discovered.

In this thesis we aimed to:

1. Test the effect of depolymerising chemicals nocodazole and cytochalasin B on the tubulin and actin cytoskeleton to see if the EHD3 protein and its truncated version are associated with actin

2. Further test the association of full length EHD3 and fragments 1-399 and 1-434 with actin by using an actin extension assay

3. Compare aggregation of EHD3 to other EHD proteins, by generating EHD1, 2 and 4 protein and testing their solubility with an ultracentrifugation assay and a blinded immunocytochemistry assay

3. Methods and materials

3.1. Plasmids and vectors

The following plasmid vectors were used in my thesis:

Table 1. List of plasmids

Vector	Encoded protein	Source
pdcDNA-Flag	EHD3 full length	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-FlagMyc	empty	Prof. Dr. F. Van Roy & Dr. B. Janssens, Ghent University
pENTR223	EHD1	DNASU Plasmid Repository
pENTR223	EHD2	DNASU Plasmid Repository
pENTR223	EHD4	DNASU Plasmid Repository
pdcDNA-Flag	EHD1 full length	Generated in this thesis
pdcDNA-Flag	EHD2 full length	Generated in this thesis
pdcDNA-Flag	EHD4 full length	Generated in this thesis

3.2. Size markers

The following size markers were used in my thesis:

- My-Budget Prestained Protein Ladder 10-180 kDa -Bio Budget Technologies GmbH. Contain proteins with size ranges between 10kDa-180kDa. It was used for Western Blots.

- My-Budget 1kb DNA Ladder - Bio Budget Technologies GmbH. Contains 13 blunt-ended fragments in the range from 250 bp (base pairs) to 10 kbp (kilobase pairs). It was used for gel electrophoresis.

3.3. Antibodies and fluorescent markers

The following antibodies and fluorescent markers were used in my thesis:

Table 2. List of antibodies

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

3.4. Bacterial transformation

In an Eppendorf tube 1 µL of plasmid and 50 µL NEB5-α bacterial cells were pipetted and then incubated on ice for 30 minutes. After incubation, bacterial solution was heat shocked at 42˚C for 30 seconds. After heat shock, the Eppendorf tubes were immediately placed back on ice for a further 5-minute incubation. Transformed bacteria were spread on LB agar (1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 1.5 g Agar, dH2O added up to 100 mL) plates that contained appropriate antibiotics and then incubated at 37˚C overnight.

3.5. Bacterial liquid culture and plasmid DNA purification

After overnight incubation, a single colony from the agar plate was transferred into a Falcon tube using a pipette tip. The tube contained 5 mL of LB media and 5 µL (100 µg/mL) of the appropriate antibiotics. After transfer, the tube was left in the shaking incubator at 37 °C and 250 rpm overnight.

The next day, the cultures were centrifuged for 15 minutes at 4°C and 13000 rpm. After centrifugation, the supernatant was discarded, and the pellet was used for plasmid DNA purification. Purification was done using a commercially available kit- QIAprep Spin Miniprep Kit. Firstly, the bacterial pellet was resuspended in 250 µL of P1 buffer and then transferred to an Eppendorf tube. Then, 250 µL of P2 buffer was added to lyse the bacterial cells which were visible when the suspension turned blue. Immediately after, 350 µl of buffer N3 was added to stop the reaction and the tubes were inverted until the suspension turned white. The tubes were then centrifuged for 10 minutes at 13000 rpm. After centrifugation, the supernatant was transferred to a QIAprep 2.0 spin column and centrifuged for 60 seconds at 13000 rpm. The spin column was then washed with 750 µl of Buffer PE and again centrifuged for 60 seconds at 13000 rpm. The flow through was discarded, and the column was centrifuged one more time to completely remove the wash buffer. The column was placed in Eppendorf tube and then washed using 50 µl of previously warmed TE buffer (0.5 mL 1 M Tris pH 7.4, 200 µL 0.25 M EDTA, dH2O added up to 50 mL). After one minute incubation, the tubes were centrifuged for 60 seconds at 13000 rpm.

3.6. Measuring DNA concentrations

The concentration of purified plasmid DNA was measured using a BioDrop µLite spectrophotometer with absorbance wavelength set to µLite 0.5 mm. Distilled water was used as a blank probe. 1 µL of a sample was used for measuring concentration in ng/mL.

3.7. LR clonase reaction

To generate a final (new) expression vector, open reading frames were transferred from vectors by Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific) into a destination vector. 100 ng of an entry vector, 150 ng of a destination vector, 1 µL of LR clonase and the amount of TE/EB buffer needed to reach total volume of 10µL were added into an Eppendorf tube. This mixture was then incubated for 1 hour at 25 ° C. After 1 hour incubation, 1 µL of Proteinase K (Thermo Fisher Scientific) was added into a tube and incubated for 5 minutes at 37°C. To select destination vectors that had taken up the gene of interest, plasmids were then transformed as described above. Samples were then prepared for sequencing so they could later be used for transforming bacteria.

3.8. Cell culture

In this thesis, two different mammalian cell lines were used: HEK293 and SH-SY5Y. HEK293 is a human embryotic kidney cell line that is characterised by fast proliferation and high transfection rate. These cells require DMEM media (500 mL DMEM, 50 mL FCS, 5 mL non-essential amino acids, 5 mL penicillin/streptomycin-Thermo Fisher Scientific) for growth. They were used for Western blotting, actin extension assay and the aggregome purification method. The other cell line, SH-SY5Y is a human neuroblastoma cell line. It requires DMEM-F12 media (500 mL DMEM, 50 mL FCS, 5 mL 100X non-essential amino acids, 5 mL 1x penicillin/streptomycin-Thermo Fisher Scientific) for growth. When compared to HEK293 cells, this cell line is more difficult to sustain, and it grows more slowly. SH-SY5Y cells were used only for immunocytochemistry. Both cell lines are adherent, so they were grown in T25 cell culture flasks. They were used when they reached a confluency of around 80%. Working with both HEK293 and SH-SY5Y cell lines requires a sterile environment to avoid contamination. This was insured by sterilising the surface of the inside of the hood using 70% ethanol, incidin, 70% isopropanol and DNA erase solution. Also, everything that went into the hood was previously sprayed with 70% ethanol.

3.8.1. Cell splitting

When cells reached the wanted confluency (around 80%) they were ready for splitting. Before splitting, the hood and the UV light inside the hood were turned on. After a few minutes, when the UV turned off, we could start our work. Also, trypsin (Thermo Fisher Scientific) and media were preheated before use at 37 °C. Firstly, the media was removed from the T25 cell culture flask, and 1 mL of trypsin was added to break the bonds between the cells and the flask surface. The flask was then incubated in the hood for approximately 5 minutes. After incubation, the flask was hit firmly few times to make sure all the cells detached from the flask surface. To neutralize the trypsin reaction, 4mL of DMEM or DMEM-F12 media was added to the flask. A volume of the cells (see below) was transferred to a new flask containing 5 mL fresh media. A newly prepared flask was then stored in the incubator at 37 °C until it was ready for using or splitting again. When splitting cells for transfections, cells were transferred into plates that contained a different number of wells (6, 12 or 24). Depending on the experiment, splitting cells into wells required different amounts. For Western Blot and Actin extension assay, 500-700 µL of cells were split into a 6 well plate diluted to 3mL of media. For Immunocytochemistry, 70-250 µL of cells were split into 12/24 well plates with coverslips and diluted to 1mL of media. Plates were then stored overnight in the incubator at 37°C and 5% CO2.

3.8.2. Transfecting cells

For transfection of cells, DMEM (for HEK293) and DMEM-F12 (for SH-SY5Y) media without antibiotics or serum was used (-/- media). Metafectene PRO (Biontex) was used when working both with HEK293 cells and SH-SY5Y cells. Firstly, in an Eppendorf tube two solutions were prepared. In a tube, 100 µL of -/- media and 2 µL of Metafectene PRO were mixed (per well). DNA solution consisted of 0.5 µg of plasmid mixed with 100 µL of -/- media. After 5-minute incubation at room temperature, 100 µL of prepared Metafectene solution was mixed with each DNA solution. This solution was then left for a further 30-minute incubation. In the meantime, media from the plates was removed and 500 µL of -/- media was added per well. To make sure there were no antibiotics or serum left in the wells, this media was also removed and then 300µL of fresh -/- media added. After a 30-minute incubation, DNA and Metafectene solution mix was added into the correct well. The plate was then incubated at 37°C for 6 hours, after which the media was removed and replaced with 1 mL of fresh media containing antibiotics and serum (+/+ media). After changing media, the plate was then left in the incubator overnight. This was the protocol when using a 12/24 well plate. For a 6 well plate (used for ultracentrifuge), the protocol is the same except that the volumes are multiplied by 3.

3.8.3. Cell lysis

Firstly, media was removed from wells that incubated overnight and then washed twice using 500 µL of PBS (80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, dH_2O up to 1 L, pH 7.4). After removing PBS, 200 µL of cell lysis buffer solution (50 mL 10X PBS, 500 µL Triton X-100, 1 mL 1M $MgCl_2$, dH_2O up to 50 mL, protein inhibitor cocktail (1:50) and DNasel (1:1000)) was added and left to incubate for 5 minutes. The lysed cell suspensions were then transferred to Eppendorf tubes and left on the rotor for 30 minutes. These samples were stored at -20°C or prepared for Western blotting.

3.9. Western blot

3.9.1. Preparing acrylamide gels

Based on the sizes of the samples, different concentrations of running gels were used. Running gels were prepared as described in Table 3. Gels were then poured between glass plates (1 or 1.5mm thick) leaving 1 cm empty at the top, which was filled with distilled water. The gel was left to solidify while preparing a stacking gel (2.6 mL dH2O, 1mL 30% acrylamide mix, 625 µL 1M Tris pH 6,8, 50 µL 10% SDS, 50 µL 10% APS, 5 µL TEMED). Water was poured off, the stacking gel was poured on top of the running gel, a well comb was added, and the gel left to set.

Table 3. Chemical volumes used for preparation of acrylamide gels

	8% gel	10% gel	15% gel
Sample size (kDa)	>60	30-60	5-15
dH_2O (mL)	5.5	4.8	3.9
30% acrylamide (mL)	3.2	3.9	4.8
1.5M Tris pH 8.8 (mL)	3	3	3
10% SDS (µL)	120	120	120
10% APS (µL)	120	120	120
TEMED (µL)	12	12	12

3.9.2. SDS Page

Cell lysates (100 µL per sample) were mixed with 1x sample volume of loading buffer (6.25 mL 1M Tris, 10 mL glycerol, 20 mL 10% SDS, 3.75 mL dH2O, 5 mg bromophenol blue) and 0.2x sample volume of 1M DTT. The samples were then heated to denature them at 95 °C for 5 minutes after which they were placed on ice. Previously prepared acrylamide gels were placed into an SDS-PAGE tank filled with running buffer (30 g Tris, 144 g glycine, 10 g SDS, dH_2O up to 1 L). Then, combs were removed and 2 µL of the maker and 20 µL of samples were loaded into the wells. The gels were run for approximately 45 minutes at 180 V.

3.9.3. Transferring to membrane

The gels were taken out of the SDS-PAGE tank, unnecessary parts were cut off and the gels were then transferred to plastic trays containing 1x transfer buffer (5.8 g Tris, 2.9 g glycine, 4 mL 10% SDS, 200 mL methanol, dH2O up to 1 L). The buffer was poured out and replaced with fresh transfer buffer and left incubating on the shaker for 10 minutes. PVDF membrane (8x6cm) was cut and activated with methanol. The membrane was then placed in a plastic tray containing 1x transfer buffer and left to incubate on the shaker for 5 minutes. Two blotting papers were cut in half, wetted and placed on the transfer cassette. Then, a membrane was placed on top of it and on top of the membrane a gel was placed. The other half of the blotting paper was put on top of everything. Using a roller, air bubbles were removed, and the cassette was locked and placed in the Transblot system (Bio-Rad). The gels were transferred for 30 minutes at 25 V. After the transfer, the membranes were put in plastic trays containing dH2O which was then removed and replaced with a fresh amount of dH2O. The dH2O was poured out and Ponceau S staining solution (1g Ponceau S, 4 mL acetic acid, dH2O up to 200 mL) was added. The ponceau was poured off after 30 seconds and the membranes were then washed with dH2O until the red color faded. The membranes were washed one more time with PBS-T (50 mL 10x PBS, 450 mL dH2O, 250 mL Tween 20).

3.9.4. Antibody staining

The membranes were blocked by placing them in 50 mL PBS-T containing 5% milk powder for 1 hour. After 1 hour on the shaker, the membranes were washed twice using PBS-T. The membranes were then incubated overnight in 6 mL primary antibody solution (6 mL of PBS-T, 0.3 g of milk powder, 60 µL of 2% sodium azide and 3 µL of either anti-FLAG, anti-EGFP or anti-Actin primary antibody). The next day, primary antibody was poured off, and the membranes were washed 3 times with PBS-T with 10-minute

intervals between washes. Then, the membranes were incubated in a secondary antibody solution (6 mL PBS-T and 0.6 µL Goat-anti-Mouse (GAM) secondary antibody) for 1 hour. After incubation, secondary antibody was poured off and membranes were again washed 3 times using PBS-T. The membranes were covered in ECL visualising agent (ThermoScientific) before visualisation by the Bio-Rad ChemiDoc Imaging system.

3.10. Actin extension assay

Before starting the actin extension assay, HEK293 cells were placed in a 6-well plate (600 µL of cells per well and +/+ media up to 3 mL). The next day, transfection was done using appropriate constructs. The day after, actin extension assay was started. Firstly, cells were washed twice using 1x PBS. Then, to lyse the cells 150 µL of Lysis & F-actin Buffer (50mM PIPES, 50mM NaCl, 5mM $MgCl_2$, 5mM EGTA, 5% glycerol, 0.1% NP-40, 0.1% Triton X-100, 1% Tween 20 and 1x protease inhibitor) were added to the pellet. This solution was pipetted up and down to lyse the cells and then incubated for 10 minutes at 37 ℃. The eppendorf tube was put in a desk centrifuge at 350xg for 5 minutes. After short centrifugation, a 20 µL fraction was put in a new tube labelled **`Lysate´** and 100 µL fraction was transferred to an ultracentrifuge tube for further analysis. These samples were then centrifuged at 100 000xg for 60 min at 30 ℃. The supernatant (the initial G-actin fraction) was carefully removed and transferred to a new ultracentrifuge tube while a 20 µL fraction of it was stored as **`S1´**. The rest of supernatant was re-centrifuged as above. The pellet was resuspended in 80 µL G-actin Buffer (5mM Tris, 0.2mM $CaCl_2$, 0.2mM ADP, 0.5mM DTT and 1x protease inhibitor) and 20 µL of it was stored as **`P1´** while the rest was put in the fridge for 22 hours. After a second centrifuge, the supernatant was removed and stored as **`S1S´** and the pellet was then resuspended in 60 µL Lysis & F-actin Buffer as **`S1P´**. Once the P1 had been in the fridge for approximately 22 hours, it was centrifuged at 100 000xg for 60 min at 4 ℃. The supernatant was removed and stored as **`P1P´** and the pellet was resuspended in 40 µL G-actin Buffer and stored as **`P1S´**. The process is schematically shown in Figure 5.

3.11. Aggregome Purification Method

First, media was removed from a 6-well plate containing HEK293 cells and washed twice with PBS. The cells were lysed with 100 µL of Aggregome Lysis Buffer (50 mM HEPES pH 7.5, 250 mM sucrose, 5mM MgCl2, 100 mM KAc, 2 mM PMSF, 1x PI). The aggregome lysis buffer was left for 5 minutes, the cells were scraped and then transferred to ultracentrifuge (UC) tubes. 25 µL of Triton X-100 was added per tube and then 30 µL of lysate was

transferred to an Eppendorf tube before ultracentrifugation and stored at -20 °C. The samples in UC tubes were then spun at 20 000 g for 20 minutes at 4 °C. After centrifugation, the supernatant was removed and aggregome lysis buffer and Triton X-100 were added to the pellet. The samples were spun as above. The supernatant was again removed, and the pellet was resuspended with 100 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 spun at 130 000 g for 45 minutes at 4°C. The supernatant was removed, and the same volume of A1 was added to the pellet. The samples were again centrifuged at 130 000g for 45 minutes and 4°C. Supernatant was removed and the pellet was resuspended with 100 µL of buffer B1 (50 mM HEPES pH 7.5, 1M NaCl, 20 mM MgCl2, 30 Mm Ca2+, 100 U/mL DNAse, 1x PI). The samples 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, 4°C for 45 minutes. After centrifugation, supernatant was removed, and the pellet was resuspended with 100 µL of buffer B1 (but without DNAse). The samples were again centrifuged at 130 000g, 4°C and for 45 minutes. The supernatant was removed, and the pellet was resuspended in 100 µL of buffer C1 (50 mM HEPES pH 7.5, 0.5% Sarcosyl). The pellet was dissolved with the help of an insulin syringe and needle 0.4 mm. The samples were then incubated on ice for about 1 hour on the shaking tray. After incubation, the samples were centrifuged at 112 000g, 4 °C for 45 minutes. The supernatant was removed, and the previous step was repeated. The samples were again spun at 112 000g, 4 °C and for 45 minutes. Lastly, the supernatant was removed, and the pellet was resuspended in 20 µL of loading buffer and 2 µL of DTT. Homogenates were prepared with 30 µL of loading buffer and 3 µL of DTT. The prepared samples were then heat shocked at 95°C for 5 minutes and stored at -20°C or Western blotted.

3.12. Immunocytochemistry

3.12.1. Cell treatment

Firstly, 4 wells of SH-SY5Y cells were set up on coverslips for each plasmid (full length, 1-399 and/or 1-434) that we wanted to look at. Cells were then transfected as normal and left in the incubator to grow overnight. The next day, the following solutions were prepared in the hood:

X: 1 mL -/- media, 4 µL DMSO
N: 1 mL -/- media, 2 µL DMSO, 2 µL nocodazole solution (2.5 mM stock - 5 µL final)(supplier-MCE)
C: 1 mL -/- media, 2 µL DMSO, 2 µL cytochalasin B solution (5 mg/mL stock - 10 µg/mL final)(supplier-MCE)
NC: 1 mL -/- media, 2 µL nocodazole solution, 2 µL cytochalasin B solution
(all amounts are per well)

The media was removed from the cells and was replaced with one of these solutions per well instead. The cells were incubated in the incubator (37℃) for a further 30 minutes, after which they were fixed and stained as described below.

3.12.2. Fixation and permeabilization

SH-SY5Y cells in 12-well plates transfected the day before were washed once with 1x PBS and fixed using approximately 500 µL of fixation buffer (8g paraformaldehyde, 20 mL 10x PBS, dH2O up to 200 mL, pH 7.4) per well for 15 minutes. After 15 minutes incubation, the fixation buffer was removed and 250 µL of permeabilization buffer (10 mL 10x PBS, 500 µL Triton X-100, dH2O up to 50 mL) was added. After 10 minutes, the buffer was removed, and the wells were carefully washed 3 times using 1x PBS.

3.12.3. Immunocytochemistry

Coverslips were blocked with 10% goat serum/PBS (200 µL per well for a 12 well plate) for 45 minutes. After incubation, blocking media was removed and 100 µL of primary antibody solution (10% goat serum/PBS, 1:1000 Flag M2) was added and then incubated on the shaker for 3 hours. After 3 hours, the cells were washed three times with PBS over 15 minutes. 100 µL of the secondary antibody solution (Goat Anti Mouse RED 555 (1.500), DAPI (1:500), 10% goat serum/PBS, fluorescently labelled phalloidin) was added and incubated for 1 hour in the dark. After 1 hour, the cells were washed using PBS 3 times and once using miliQH2O. Lastly, coverslips were attached to microscopy slides using commercial mounting medium and stored in the fridge. The samples were visualised using an Olympus IX83 fluorescent microscope with CellSens software.

4. Results

4.1. The expression of EHD3 1-399 and 1-434 is dependent on both the actin and tubulin cytoskeletons

For this part of my thesis, I used 3 plasmids: full-length human EHD3, EHD3 fragments that include amino acids 1-399 and 1-434 (Figure 4). It is already known that FL EHD3 forms clear aggregates inside cells (31). EHD3 1-399 and 1-434 were previously designed to exclude the EH-domain and/or the linker region. Based on the previous research it was proven (by ultracentrifugation and fluorescent microscopy) that fragment 1-399 does not aggregate (32). Fragment 1-434, observed by fluorescent microscopy showed aggregate-like structures in SH-SY5Y cells, however ultracentrifugation showed that this fragment is not present in the aggregome of HEK293 cells (32). One hypothesis is that fragment 1-434 does not aggregate but is nonetheless insoluble because it is bound to the cytoskeleton: therefore, I depolymerised the cytoskeleton to see what effect this has.

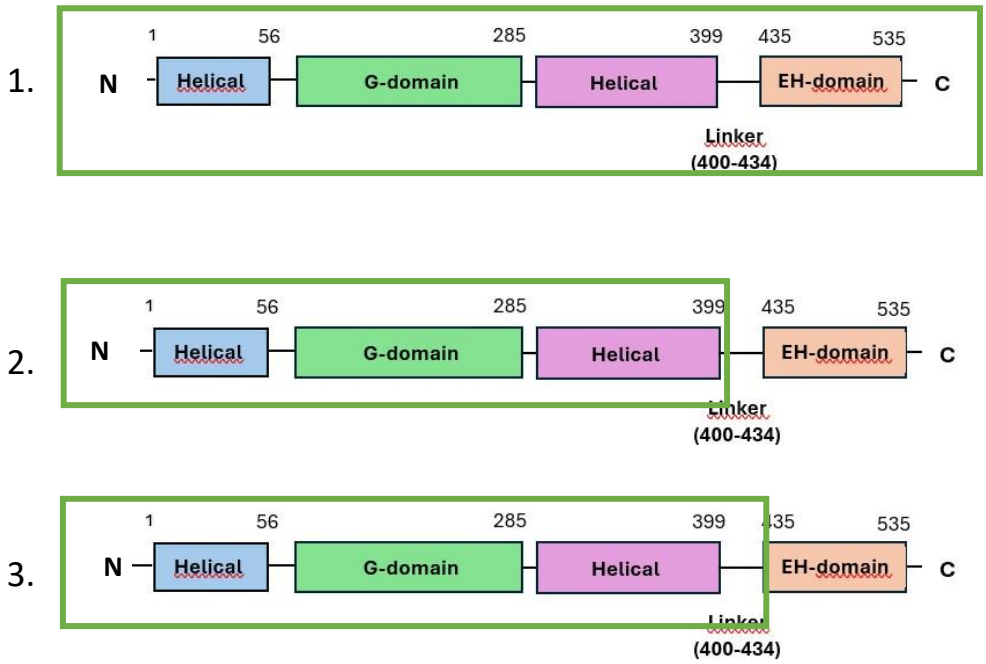
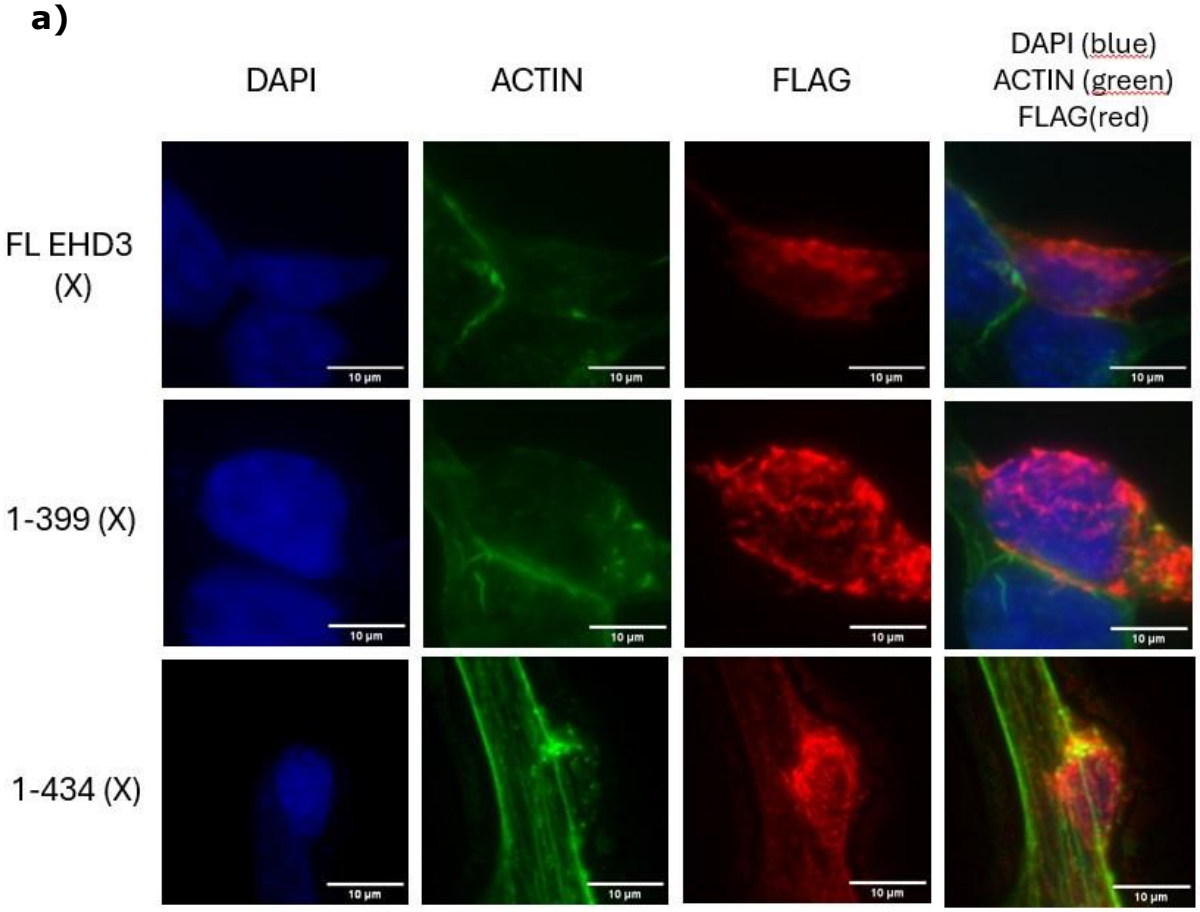
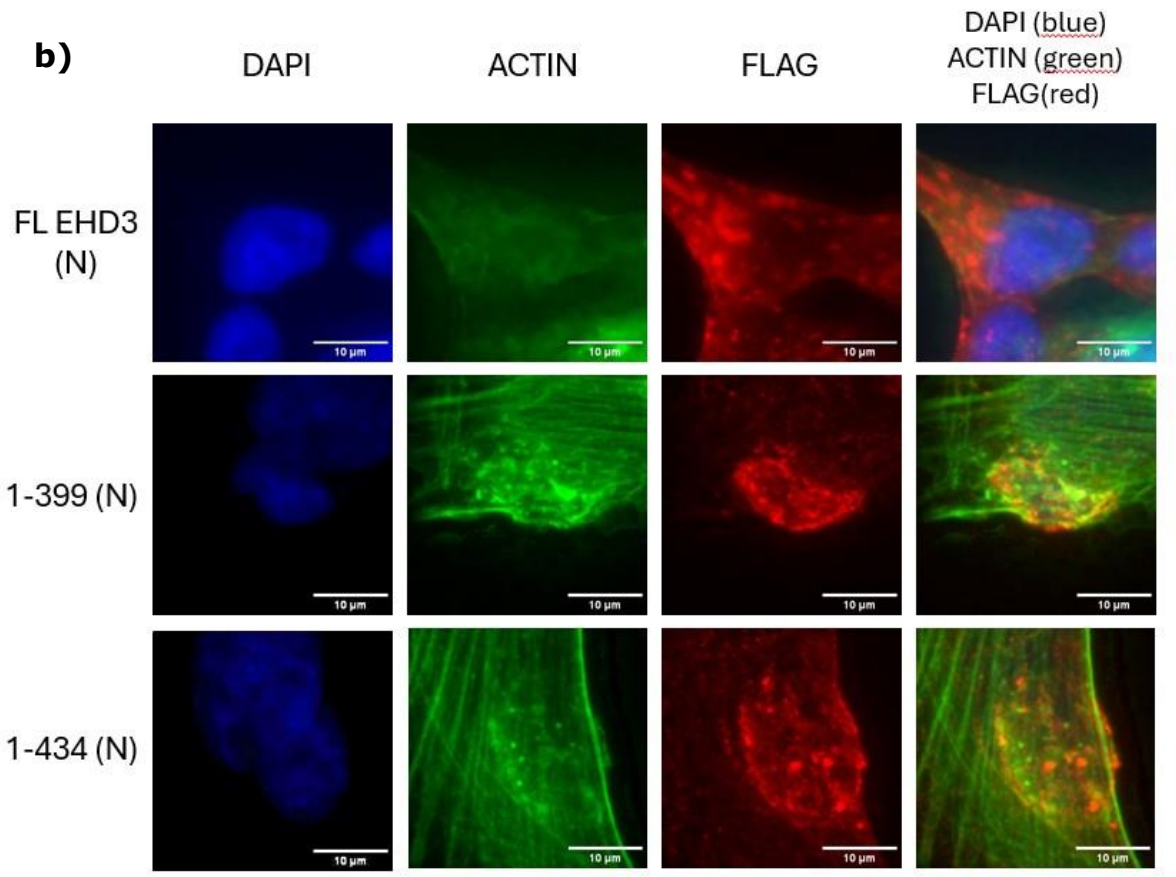


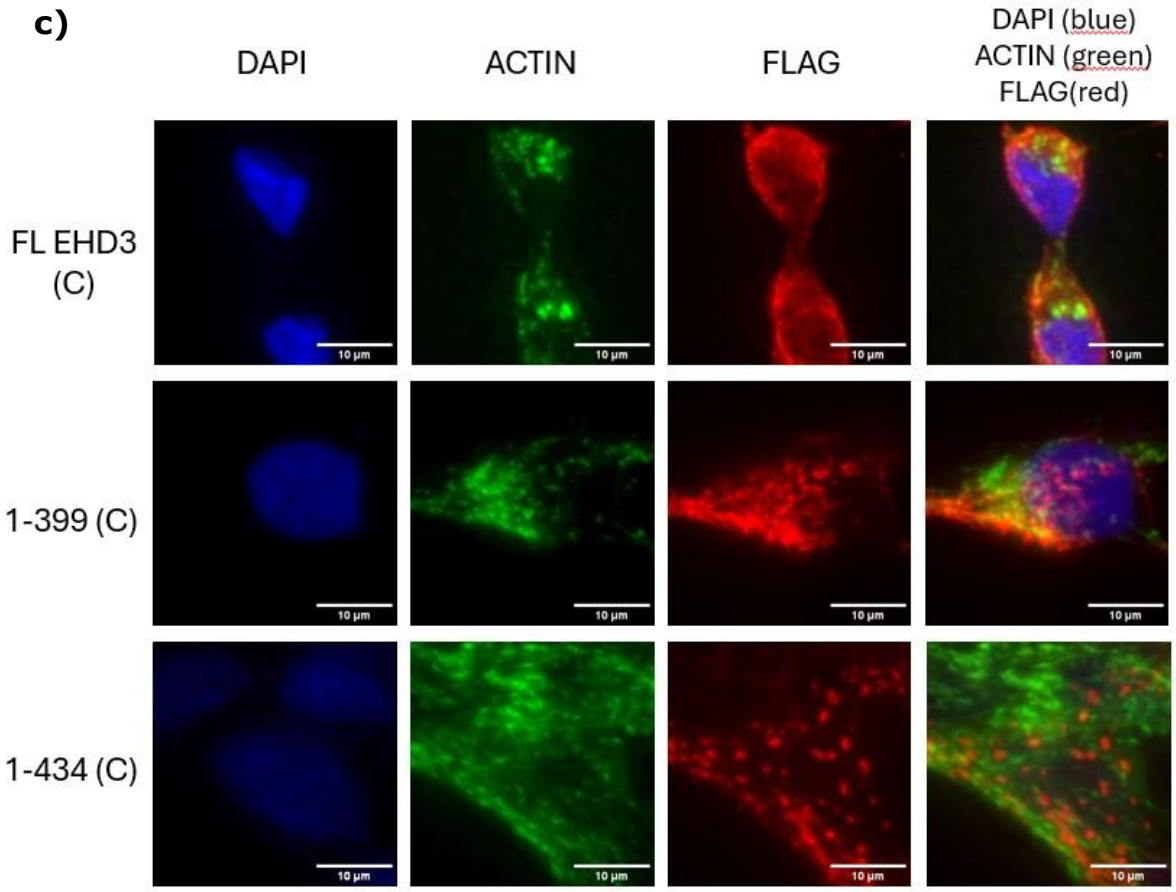
Figure 4. **Schematic representation of 3 plasmids I have been using for this experiment**. 1.Full length human EHD3 (FL), 2. EHD3 1-399, 3. EHD3 1-434. Numbers represent amino acids numbers.

To test the association of FL EHD3 and its constructs (1-399 and 1-434) with actin and tubulin cytoskeleton, I did fluorescent microscopy with SH-SY5Y cells. Before fixation and permeabilization, cells were treated with the following chemicals: nocodazole and cytochalasin B. Nocodazole is a chemical that depolymerises tubulin, while cytochalasin B depolymerises actin. For each plasmid (FL, 1-399 and 1-434) 4 samples were made. `X´ is a negative control and will therefore show us how the plasmids look normally, `N´ will show how cells look when the microtubule cytoskeleton is disrupted with nocodazole, `C´ will show how cells look when the actin cytoskeleton is disrupted with cytochalasin B and `NC´ how they look when disrupted with both chemicals. Results are shown in figure 5.

a)




b)
DAPI
ACTIN
FLAG
DAPI (blue)
ACTIN (green)
FLAG(red)
FL EHD3 (N)
1-399 (N)
1-434 (N)
10 µm


c)
DAPI
ACTIN
FLAG
DAPI (blue)
ACTIN (green)
FLAG(red)
FL EHD3 (C)
1-399 (C)
1-434 (C)
10 µm

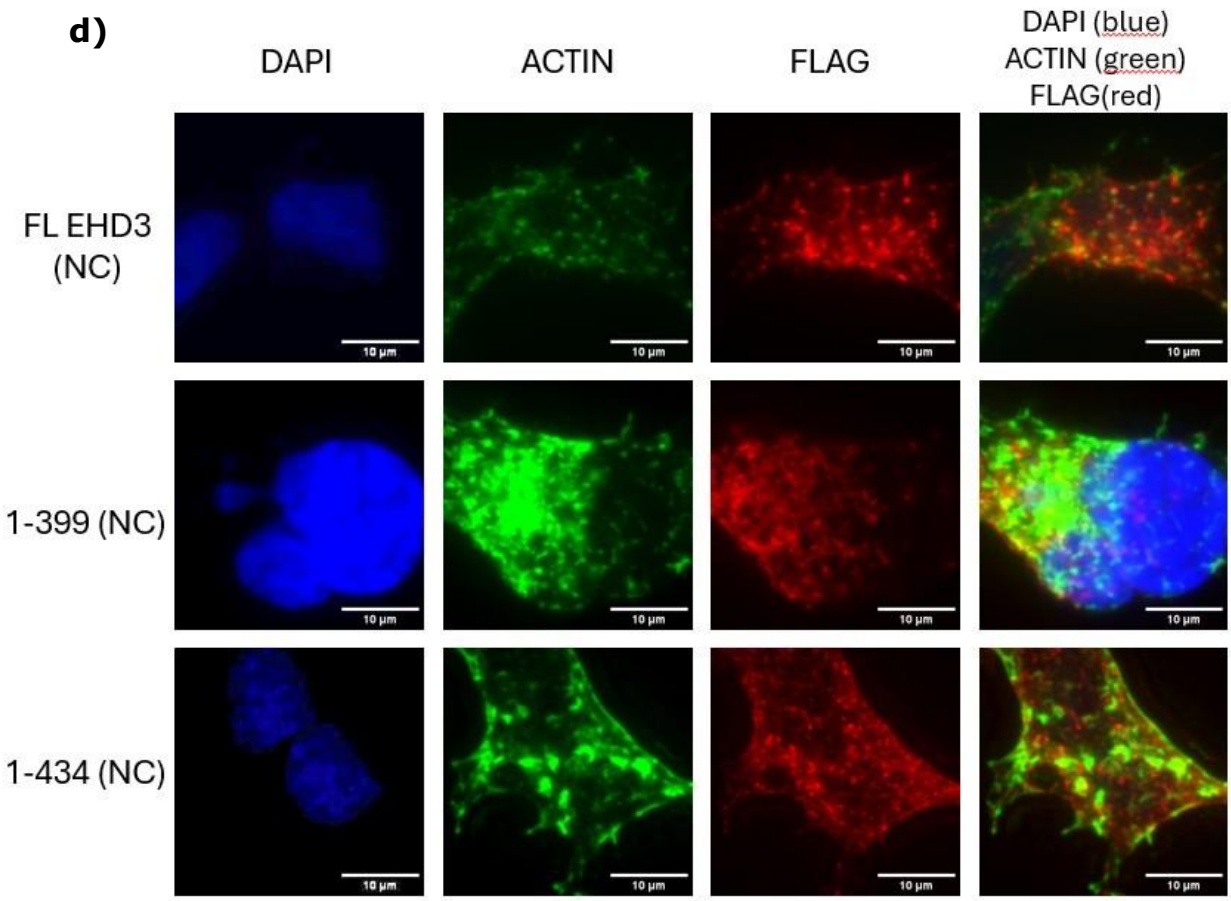


Figure 5. a) Negative control of FL EHD3, 1-399 and 1-434. Each protein is mostly localized in cytoplasm, around the nucleus. Apparent aggregation of FL EHD3 and 1-434 is seen, which agrees with previous studies **b) Nocodazole does not affect FL EHD3 but does affect the truncated versions: 1-399 and 1-434. c) Cytochalasin B depolymerises actin in all 3 versions of EHD3. d) Disruption of both actin and tubulin cytoskeleton in FL EHD3, 1-399 and 1-434**. Fluorescent microscopy of neuroblastoma (SH-SY5Y) cells transfected with full length EHD3 and 1-399 and 1-434 versions. Images were obtained using CellSens software on an immunofluorescent microscope with 60x magnification. Proteins were stained using anti-FLAG (red signal) primary antibody and GAM 555 secondary antibody, while nucleuses were stained using DAPI (blue signal). The scale bar corresponds to 10 µm.

It was previously confirmed that nocodazole does not affect distribution of FL EHD3 and its truncated versions in SH-SY5Y cells (31). Also, it has been proven that the expression and aggregation pattern of FL EHD3 did not change after nocodazole treatment, while expression and aggregation of EHD3 versions, 1-399 and 1-434 was exaggerated after treatment (31). In my assay, I confirmed these results (Figure 5.b). The actin cytoskeleton of FL EHD3 remained almost the same after the treatment, there were no obvious changes. On the other hand, the actin cytoskeleton of EHD3 fragments 1-399 and 1-434 changed. The green signal was exaggerated, and the actin cytoskeleton was partly in punctuated form and partly string-shaped form. Treating SH-SY5Y cells showed that FL EHD3 both with

fragments 1-399 and 1-434 was affected by cytochalasin B (Figure 5.c). The actin cytoskeleton can be seen in punctuated form, partly degraded which confirms the hypothesis that EHD3 is associated with actin inside cells. Also, red signal is exaggerated in treated cells. Aggregates and aggregate-like structures are more visible and round. This could also be a sign of association of EHD3 protein and its truncated version with cytoskeleton. I will further investigate this hypothesis with an actin extension assay.

4.2. Actin (de)polymerisation assay

Following on from my previous immunocytochemistry assay, treating cells with depolymerising agent cytochalasin B showed possible association of full length EHD3 and EHD3 constructs 1-399 and 1-434 with actin inside cells (29)(33). To further confirm that EHD3 and its constructs interact with actin, I set up a (de)polymerisation assay. Using 3 plasmids: FL EHD3, 1-399 and 1-434, a 6-well plate with HEK293 cells was set up, transfected and prepared for ultracentrifugation. Whole lysate samples were ultracentrifuged and split on S1 and P1 fractions. These fractions were additionally centrifuged and split on S1S, S1P and P1S, P1P fractions. S1S and S1P (derived from S1) show how much additional polymerization occurred in vitro, while P1S and P1P (derived from P1) show which proteins become soluble with the de-polymerizing of actin in vitro and whether de-polymerization of actin is reduced. This process is shown in Figure 6. After preparing these samples, Western blotting was done to identify our proteins of interest and to quantify actin concentrations in each sample.

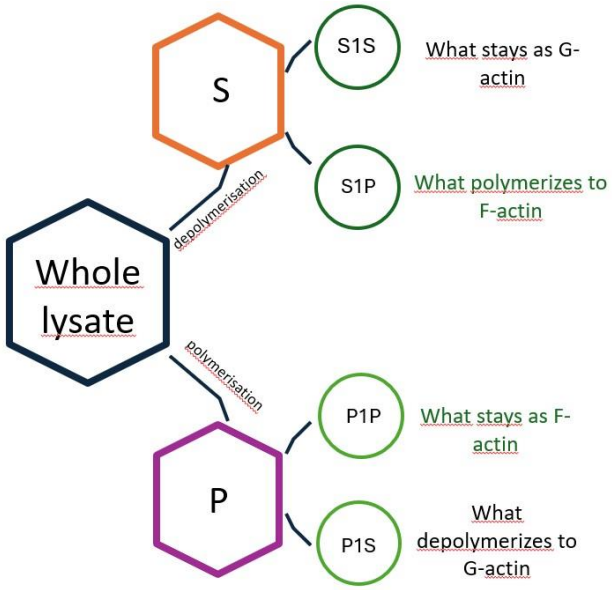


Figure 6. Process of ultracentrifugation

4.2.1. Actin depolymerisation is reduced in the presence of full length EHD3

Three independent experiments were conducted and analysed. Figure 7 shows an example of one experiment and data from the three experiments is quantified in figure 8.

When I analysed 3 consecutive experiments, I noticed that in general there is more actin in P1S and S1S than in P1P and S1P. This suggests that our assay favours depolymerisation of actin slightly over polymerisation.

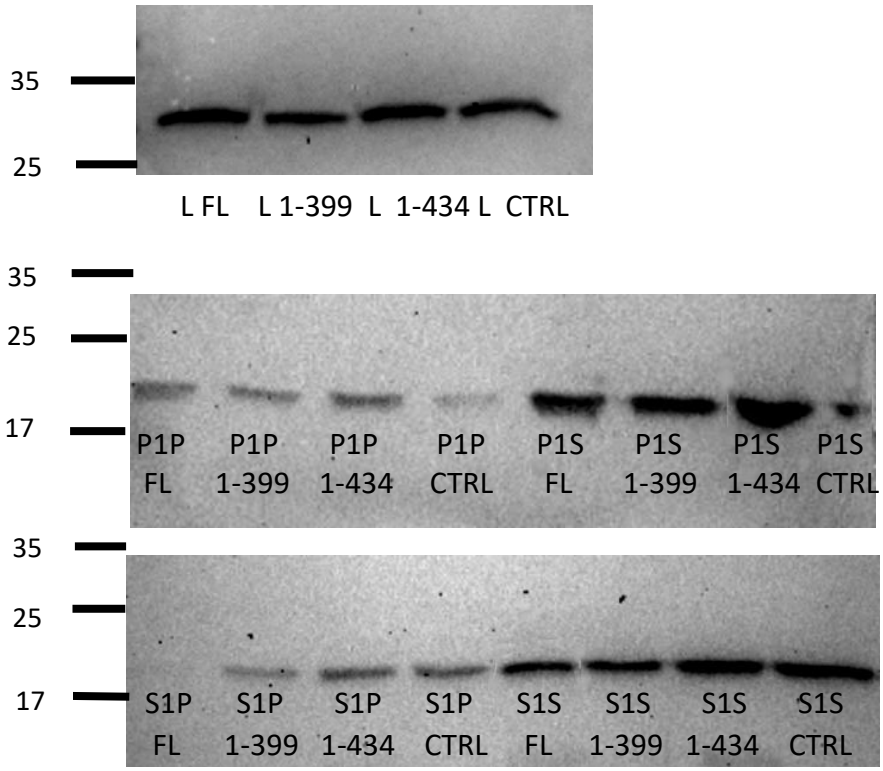


Figure 7. Western blots of ultracentrifuge samples stained for actin. The first membrane is a membrane of lysate samples. On the second upper membrane are samples derived from P1 (P1P and P1S) and on the second lower membrane are samples derived from S1 (S1P and S1S). Proteins were detected with primary anti-Actin antibody and secondary GAM antibody. Control represents mock transfected HEK293 cells. Protein size was compared to Prestained Protein Ladder 10-180 kDa.

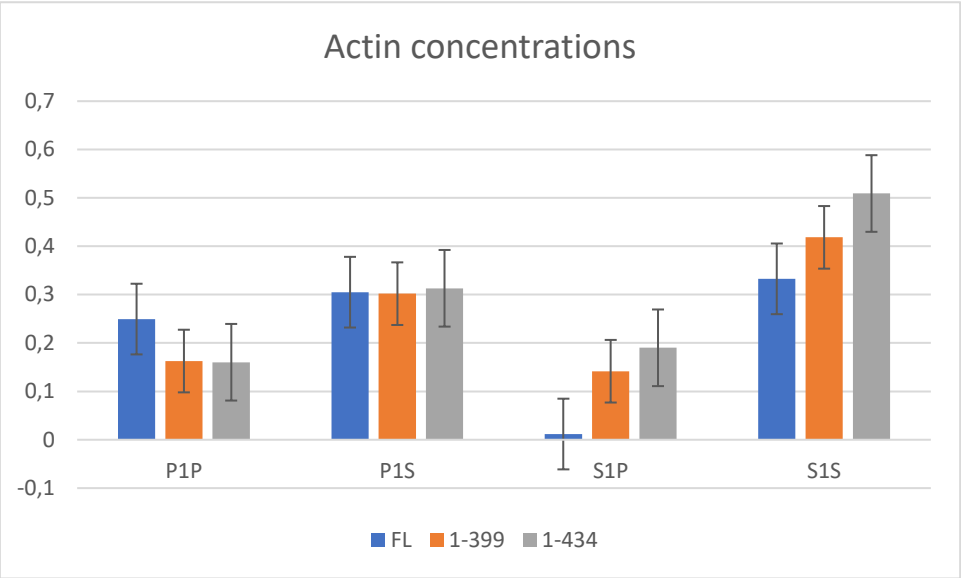


Figure 8. 2D bar chart with average actin concentrations, showing highest in P1S and S1S. Based on this chart our hypothesis was confirmed: the actin extension assay favours depolymerisation of actin slightly over polymerisation. This chart represents average numbers of 3 independent experiments. The error bars represent standard error of the mean. Numbers are normalised to a common value -divided by the amount of actin in full length lysate.

Furthermore, full length EHD3 when compared to the control, shows more actin in P1P than in S1P. This suggests a weak effect of EHD3 on preventing depolymerisation of actin. After quantification, it has been shown that this only happens with full length EHD3. Full length EHD3 reduces depolymerisation of actin, and seemingly the EH domain (435-535) is required for this.

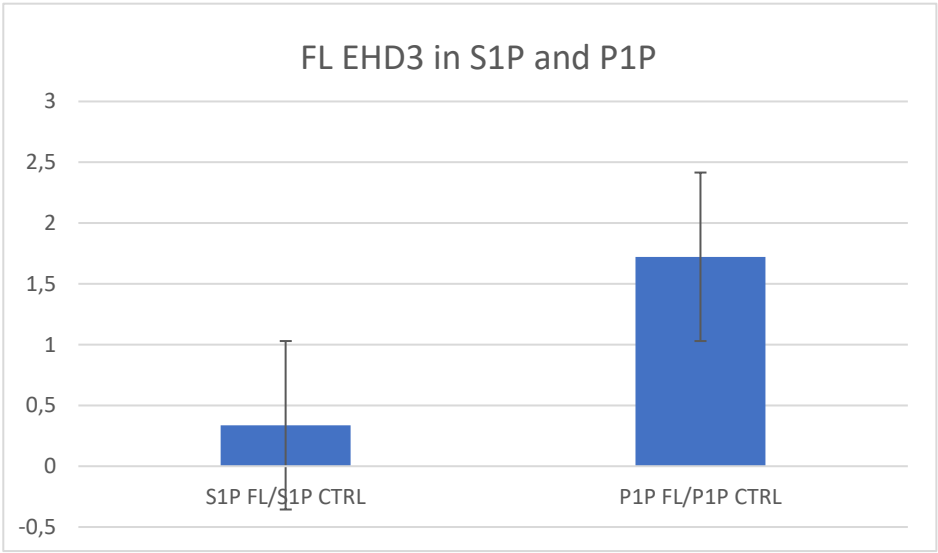


Figure 9. P1P concentrations of full length EHD3 are statistically higher than S1P concentrations. Average concentrations of S1P FL divided by S1P control on the left side and average concentrations of P1P FL divided by P1P control

on the right. This assay was repeated 3 times and to test significance of results, t-test was done (p value=0.037, Student's t-test). Based on this result, it is proven that depolymerisation of actin is reduced in the presence of full length EHD3.

4.2.2. Full length EHD3 co-segregates with actin, while versions lacking the EH domain do not

To further look at EHD3, the previously membranes were further stained with Flag antibody, revealing that FL EHD3 is mostly seen in P1P and S1S, but little in the other two fractions. This suggests that full length EHD3 co-segregates well with actin. It initially co-segregates with polymerised F-actin but follows it to the supernatant when F-actin depolymerises to G-actin. This infers that EHD3 interacts with F-actin. The fact that it does not move back to S1P with repolymerised F-actin suggests that this is not a simple direct interaction. One possibility is that EHD3 binds to something that is associated with actin. Based on what we know about EHD3 functions inside cells, it is most likely that EHD3 endosomes associate with actin.

Fragments 1-399 (583) and 1-434 (584) are mostly seen in S1S, sometimes seen in other fractions, but not consistently. Comparing this to the full length EHD3, this strongly suggests that the EH domain (435-535) is required for the association of EHD3 with actin.

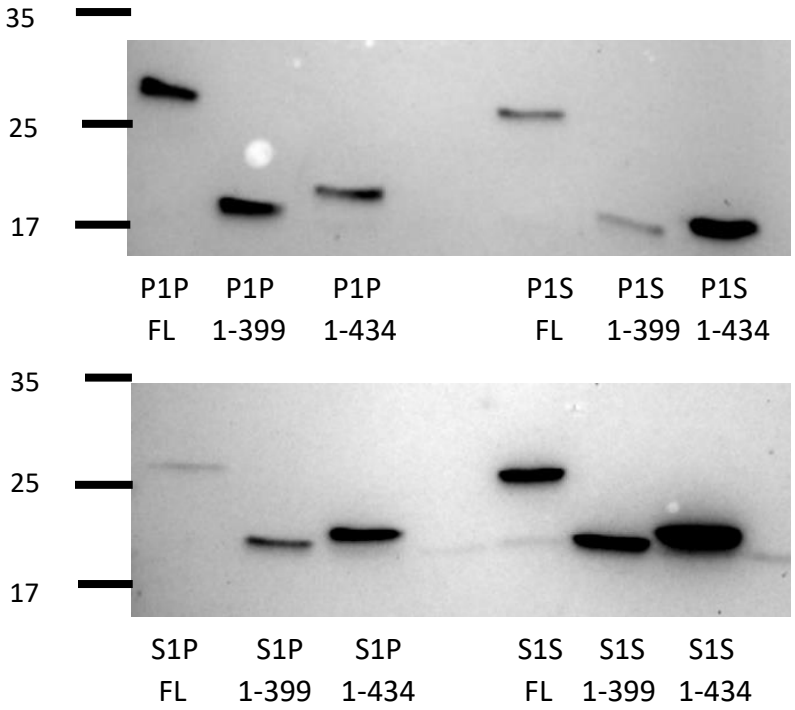


Figure 10. Western blots of ultracentrifuge samples. On the upper membrane are samples derived from P1 (P1P and P1S) and on the lower membrane are samples derived from S1 (S1P and S1S). Proteins were detected

with primary anti-Flag antibody and secondary GAM antibody. Protein size was compared to Prestained Protein Ladder 10-180 kDa.

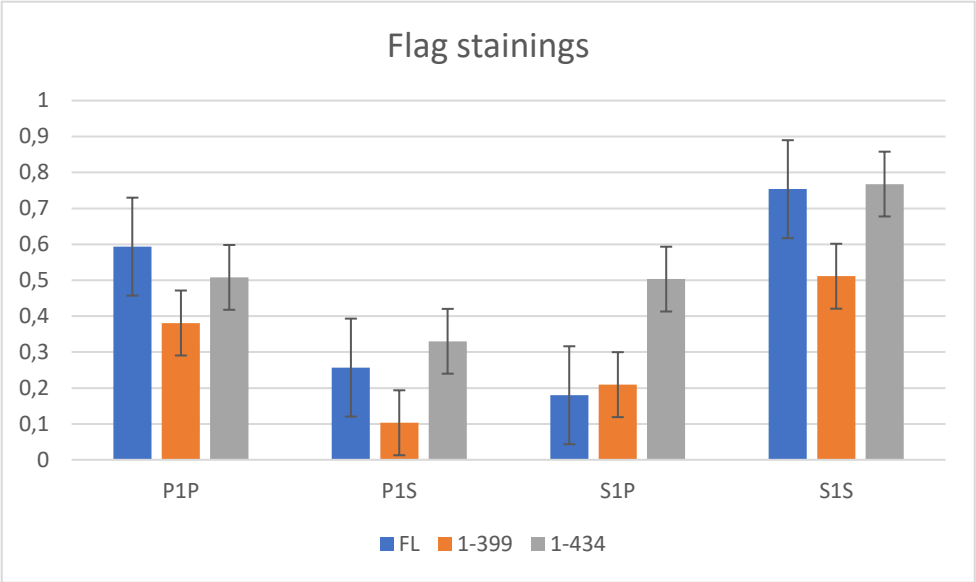


Figure 11. Full length EHD3 prevails in P1P and S1S fragment while 1-399 and 1-434 prevail in S1S. The error bars represent standard error of the mean. Numbers are normalised to a common value -divided by the amount of actin in full length lysate.

4.3. Testing protein insolubility of EHD1, EHD2 and EHD4 in comparison to EHD3 by ultracentrifugation assay

It has been shown that EHD3 clearly forms aggregates inside cells, but it is not yet known whether other EHD proteins do the same. To investigate aggregation of the EHD protein family (EHD1,2,3 and 4) an ultracentrifugation assay was used. By LR clonase reaction, I generated EHD1, 2 and 4 plasmids that I used for this assay. These were expressed in HEK293 cells, and then an aggregome purification method ultracentrifuge was based on the principle that aggregated proteins show greater insolubility than correctly folded proteins (20). For each plasmid, two fractions were separated: a lysate that represents all proteins from the cells and an aggregome that represents only insoluble proteins. The aggregome fraction was purified by ultracentrifugation, after which both fractions were Western blotted.

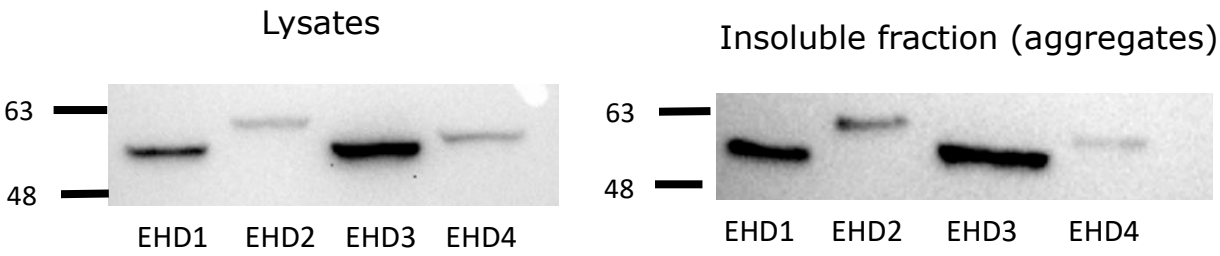


Figure 12. Western blots of second ultracentrifugation assay. Assay was done 3 times to confirm the results.

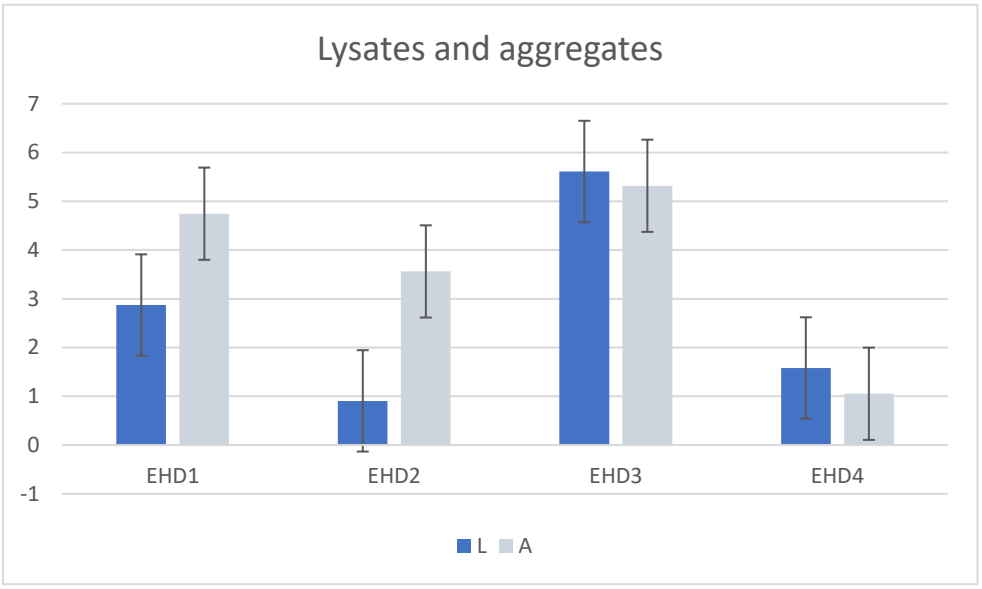


Figure 13. 2D bar chart with average concentrations where EHD3 samples (both lysates and aggregates) prevail. The error bars represent standard error of the mean. Assay was done 3 times. Numbers are normalised to a common value -divided by the amount of actin in full length EHD3.

When we look at the aggregate membrane at first, we could say that EHD1 and EHD3 are insoluble and therefore could be aggregating, while EHD2 and EHD4 show weaker signs of possible insolubility. For the lysate membrane, we can say that EHD1 and EHD3 show higher protein concentrations in lysate fractions than EHD2 and EHD4 when expressed in this system. This assay was done 3 times to confirm the results. This is not what we expected so therefore this is an experiment for future research. Additionally, I will try to investigate aggregation of these plasmids by fluorescent microscopy in SH-SY5Y cells.

4.4. Testing aggregation of EHD1, 2, 3 and 4 plasmids-blinded assay

After ultracentrifugation assay, to further investigate aggregation of the EHD protein family (EHD1, 2, 3 and 4), I transfected SH-SY5Y cells with these plasmids. To achieve objectivity, this was set up as blinded assay. For each plasmid, 6 wells were set up (a 24-well plate was used). For each well, 10 cells were found, and images saved. Fluorescent microscopy pictures were then analysed, cells that aggregate were counted as well as aggregates in each cell that showed signs of aggregation. After unblinding, statistical analysis was made.

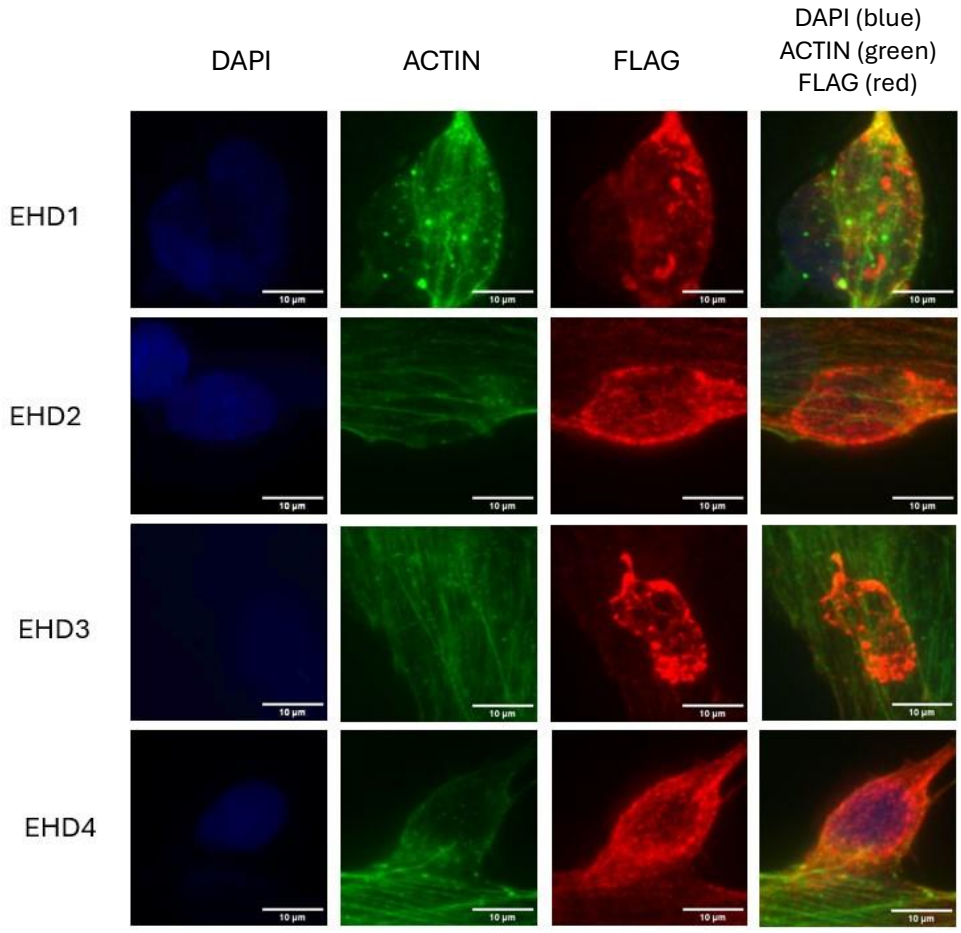


Figure 14. EHD1 and EHD3 protein show signs of aggregation, while EHD2 and EHD4 do not. Images were obtained using CellSens software on an immunofluorescent microscope with 60x magnification. Proteins were stained using anti-FLAG (red signal) primary antibody and GAM 555 secondary antibody, while nucleuses were stained using DAPI (blue signal). The scale bar corresponds to 10 μm.

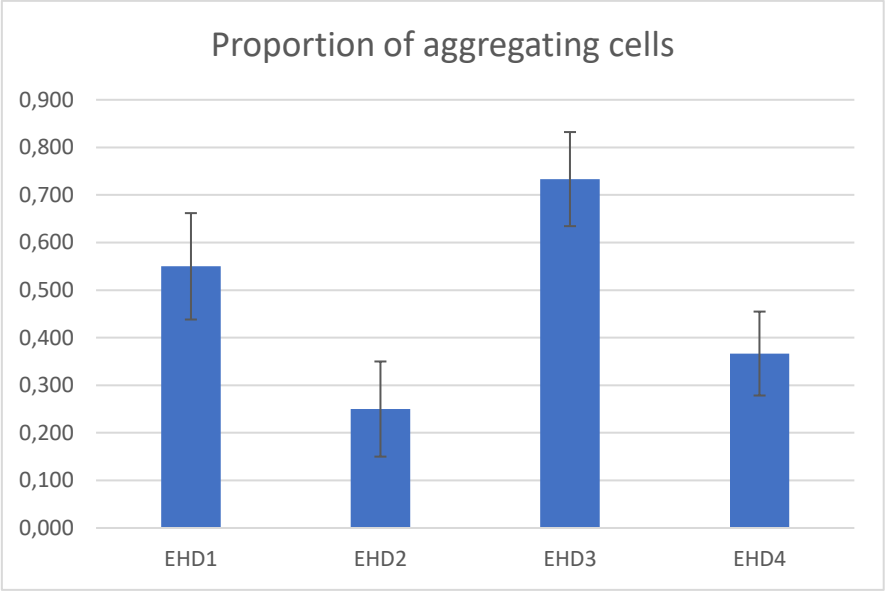


Figure 15 a). Proportion of cells that form aggregate-like structures in blinded assay (out of 10). Average of aggregating cells was calculated and is shown for each protein. The error bars represent standard error of the mean. As expected, EHD3 shows the highest number of aggregates.

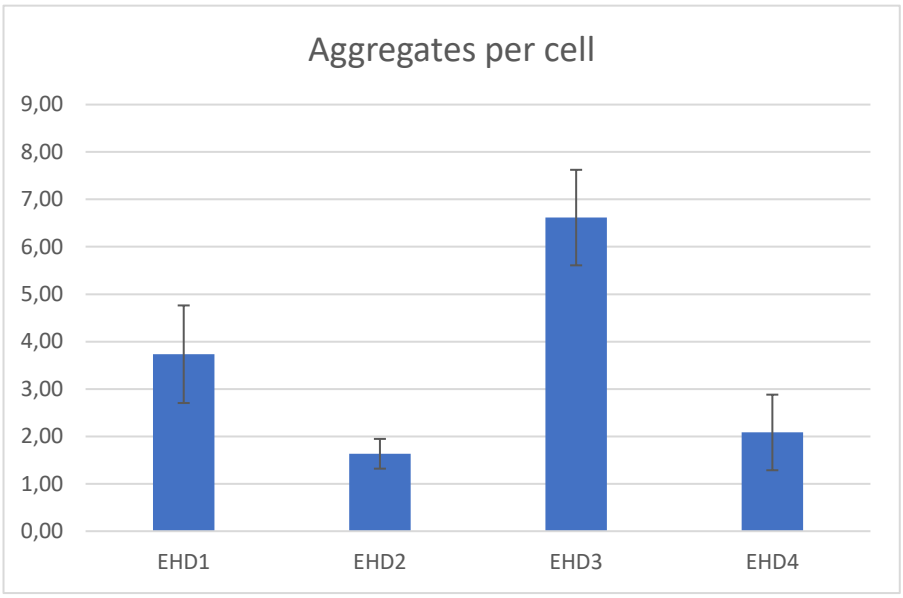


Figure 15 b). EHD3 shows the highest number of aggregate-like structures per cell (out of 10), then EHD1, while EHD2 and EHD4 show a smaller number of aggregate-like structures per cell. The error bars represent standard error of the mean

Based on this blinded assay, the aggregation pattern of EHD3 inside cells was once again confirmed. EHD1 also showed possible signs of aggregation and should therefore be further investigated. The lowest proportion of cells that form aggregate-like structures are seen in cells transfected with EHD2 and EHD4 plasmids.

5. Discussion

EHD3 is a protein that belongs to the EHD protein family, together with EHD1, 2 and 4. The EHD protein family is a group of four endocytic regulatory proteins that are involved in recycling and endocytic trafficking (Table 4). Recently, the EHD3 protein has also become the subject of research in the field of schizophrenia. It was found in insoluble aggregates that were formed in the brain of schizophrenia patients, while in healthy controls it was not. Recent SMI studies have focused on the protein aggregation and its role in the emergence of mental illness. When combined with other risk factors, EHD3 may potentially contribute to the development of schizophrenia. To better comprehend the function of EHD3 in mental illness, we need to learn more about its behavior inside cells and the mechanism underlying its aggregation. In the last few years, research in our lab confirmed that EHD3 forms aggregates (31)(32)(33). Aggregates were proven both in HEK293 cells with an ultracentrifuge assay and in SH-SY5Y cells with fluorescent microscopy. To determine which region is crucial for EHD3 aggregation, a few fragments missing EH and/or linker region were created. I also used two of these fragments in my thesis. I used fragment 1-399 which did not form aggregates and fragment 1-434 which formed aggregate-like clusters viewed with fluorescent microscopy (33). A previous ultracentrifugation assay showed solubility of fragment 1-434 so therefore, these aggregate-like structures do not represent aggregates (32). Based on this research, it was proven that the EH domain is necessary for the initialization of EHD3 aggregation, but more than one structural region is involved in this process. What was still unknown is how other proteins from EHD family behave inside cells and whether they also form aggregates.

Table 4. Localization and functions of proteins from EHD protein family (22)(26)

EHD protein	Localization	Function
EHD1	• Expressed in bone narrow, heart, testis	• role in regulating recycling of receptors and their transport to the plasma membrane • it regulates the internalisation of the low- density lipoprotein (LDL) receptor • controls membrane reorganization/tubulation upon ATP hydrolysis

EHD2	• Highly expressed in heart, moderately in placenta and lungs	• links internalization with the actin microfilament system • involved in myoblast fusion • it interacts with EH domain binding protein 1 (EHBP1)
EHD3	• expressed in brain, heart, placenta, liver and kidney	• regulator of endosome-to-Golgi transport→trafficking protein • affects dopaminergic transmission by internalizing D1 receptors
EHD4	• Highly expressed in pancreas and heart	• primarily involved in the regulation of early endosome transport

5.1. Full length EHD3 co-segregates with actin, while versions lacking the EH domain do not

Based on previous immunocytochemistry assays, it has been suggested that full length EHD3 and EHD3 constructs 1-399 and 1-434 might interact with actin inside cells, so I wanted to test this hypothesis and then to determine whether and how EHD3 affects the cytoskeleton. To test this, I did an actin extension assay with HEK293 cells transfected with full length EHD3 and EHD3 constructs with amino acid fragments 1-399 and 1-434. Results suggested that this assay favours depolymerisation of actin slightly over polymerisation. Also, when depolymerisation of actin, which then suggested that depolymerisation is reduced in its presence.

Furthermore, after quantification of WB membranes with actin extension assay samples, results showed that FL EHD3 is mostly seen in two of the fractions- containing F-actin and G-actin. Specifically, FL EHD3 initially co-segregates with polymerised F-actin but then follows it to the supernatant when F-actin depolymerises to G-actin. When F-actin repolymerises back, EHD3 does not follow it, which suggests that this is not a simple process and not a simple direct interaction. There is a possibility that EHD3 binds to something inside cells that is associated with actin. Based on what we know

about EHD3 functions and its primary role as a trafficking protein, one possibility is that EHD3 endosomes associate with actin. It has been previously described that EHD3 is localized to endocytic vesicles and membrane tubules, which are part of the endocytic recycling compartment (29). It is known that the actin filament and microtubule cytoskeletal networks, which are distinct but interact with each other, control vesicular trafficking. Vesicles move over the surface of microtubules or actin filaments when they attach to molecular motors. This information is valuable and together with our assay, allow us to conclude that trafficking protein EHD3 interacts with actin inside cells.

Two fragments lacking the EH domain, fragments 1-399 and 1-434 are mostly seen in a fraction where they stay as G-actin. Sometimes they are seen in other fractions, but not consistently. When I compare this to the full length EHD3, this strongly suggests that the EH domain (435-535) prevents the association of EHD3 with actin. When there is a lack of the EH domain, an EHD3 fragment remains localized in the nucleus and does not move between cytoplasmic compartments (29).

5.2. The expression of FL EHD3 is dependent on the actin cytoskeleton, while 1-399 and 1-434 EHD3 are dependent on both the actin and tubulin cytoskeletons

Previous research showed that EHD3 might be associated with microtubules. To confirm that hypothesis, cells were treated with the microtubule depolymerizing agent nocodazole. After treatment, FL EHD3 did not show any difference in aggregation or expression pattern. On the other hand, EHD3 fragment 1-399 showed an exaggerated, tubular expression phenotype in SH-SY5Y cells. Fragment 1-434 showed an exaggerated aggregation pattern. Also, nocodazole did not affect distribution of FL EHD3 and its truncated versions in SH-SY5Y cells. These results were not expected, so therefore to confirm the association with tubulin and actin, further assays were proposed.

In my research, I confirmed these results. Following treatment, the full length EHD3 cytoskeleton essentially did not change. However, the cytoskeleton of fragments 1-399 and 1-434 changed. The actin cytoskeleton was partially string-shaped and partially punctuated, while the green signal was accentuated. The overexpressed strand signal of EHD3 fragments 1-399 and 1-434 may suggest that protein is associated with actin, rather than microtubules. To further test this, I also treated cells with the actin

depolymerizing agent: cytochalasin B. Treating SH-SY5Y cells with this showed that FL EHD3, both with fragments 1-399 and 1-434 was affected by cytochalasin B. The actin cytoskeleton disintegrated and was mostly seen in punctuated form. Also, treating cells with a combination of nocodazole/cytochalasin B showed that cells were partly degraded and in punctuated form. Red signal in treated cells was also exaggerated and aggregate-like structures were more visible. These results confirmed the hypothesis that FL EHD3 is associated with actin inside cells. Following on my previous actin extension assay, which showed that FL EHD3 co-segregates with actin, now association with actin was confirmed. Regarding fragments 1-399 and 1-434, this assay suggests that they are associated both with the actin and tubulin cytoskeleton. In contrast, the actin extension assay suggests that these fragments are not associated with actin because of a lack of the EH domain. More research is needed for gaining information about association of fragments with actin inside cells.

5.3. As well as EHD3, EHD1 protein shows signs of aggregation inside cells

After determining that EHD3 clearly aggregates inside cells and it is potentially involved in the development of schizophrenia, the next step is to determine whether other proteins from the EHD family do the same. To test this, I did an ultracentrifugation assay with HEK293 cells and fluorescent microscopy with SH-SY5Y cells transfected with plasmid EHD1,2,3 and 4. With the aggregome purification method ultracentrifuge, I obtained two fraction samples, lysate and insoluble fraction (aggregate). After Western blotting aggregate samples, on the membrane two lines prevailed: EHD1 and EHD3. In contrast, EHD2 and EHD4 were barely visible on both the lysate and aggregate membrane. This assay once again confirmed the insolubility, and therefore likely aggregation of the EHD3 protein, while it also showed the potential insolubility of EHD1. As other causing for insolubility may exist, we cannot conclude based on this assay alone, whether EHD1, 2 or 4 aggregate, so I did one more assay.

I did a blinded immunocytochemistry assay to test the aggregation patterns of these proteins. Based on its results, I again confirmed the previous results where the EHD3 protein showed obvious signs of aggregation pattern, confirmed both in HEK293 cells with ultracentrifugation assay and in SH-SY5Y cells with fluorescent microscopy. EHD3 appeared to aggregate in 73% of cells. Ultracentrifugation results indicated that EHD1 could

potentially aggregate inside cells, while EHD2 and EHD4 showed low signs of potential insolubility. With this assay, I confirmed that EHD1 shows aggregate-like structures inside cells. It appeared to aggregate in 55% of cells. These results are somewhat expected because EHD1 and EHD3 share the highest level of sequence identity and interact with each other inside cells (22). On the other hand, EHD2 and EHD4 did not appear aggregate as much inside cells. EHD2 showed an aggregation pattern in only 25% of cells and EHD4 in 37%. What I noticed is that EHD2 and EHD4 were densely localized only around the nucleus.

We can conclude by combining data from both my assays is that EHD3 is only one that is both insoluble and clearly forms visible aggregate-like structures. It appears likely that EHD1 may also form aggregate-like structures inside cells as well as EHD3, but more research is needed. One possibility is that these aggregate-like structure are only vesicles seen in fluorescent microscopy. Also, based on these results, while EHD2 and EHD4 show some insolubility, it is less likely that they form aggregates inside cells.

5.4. Future research

In my thesis, I confirmed the aggregation pattern of FL EHD3 in HEK293 and SH-SY5Y (human neuroblastoma) cells. Then, I investigated the association of EHD3 and its truncated versions with tubulin and actin. Based on ultracentrifuge and fluorescent microscopy assays, it was proven that FL EHD3 co-segregates and is associated with actin inside cells. Truncated versions, EHD3 1-399 and 1-434 showed association both with actin and tubulin cytoskeleton in fluorescent microscopy, in SH-SY5Y cells but not in the actin extension assay with HEK293 cells. What remains unknown is what exactly EHD3 binds to inside cells and to further test how the EH domain affects association with actin. One possibility is that FL EHD3 binds to endosomes associated with actin, but the mechanism has yet to be discovered. The association of actin polymerization with endocytosis has previously been established (35). To further test this, an assay of in vitro polymerization of F-actin on early endosomes could be done. The protocol for this assay has been previously described (36). To test binding of EHD3 to structures inside cell, a few assays could be set up. First, fluorescent microscopy could be used. The endosome could be colored with one color and actin with another to see if these two signals co-localize. Also, to test binding, immunoprecipitation could be used. An assay with actin and some protein bound on the endosome surface could give us information in this field. For further research, I would also suggest repeating the fluorescent microscopy with nocodazole/cytochalasin B treatment but with co-expressing FL EHD3 with its fragments to see how this changes association

of FL EHD3 with tubulin. Also, more assays could be set up to investigate association with the actin cytoskeleton, how it affects aggregation of EHD3 and how lack of the EH domain affects this mechanism. These and future findings in this field are very important because with them we would have a better picture of EHD3's role in a physiological setting.

In the case of other proteins from EHD family, a lot more research is needed. I have just scratched the surface when it comes to researching these proteins. With the ultracentrifuge assay, aggregation of EHD3 was confirmed, while EHD1 showed possible signs of aggregation and insolubility. Because of the lack of consistency of data between lysate and aggregate membranes, I would suggest, for future research, to do more replicates with this protocol. It would also be useful to possibly repeat the assay in the presence of nocodazole or cytochalasin B in case proteins bound to the cytoskeleton affects the results by causing them to be insoluble. With the blinded assay and fluorescent microscopy with neuroblastoma cells transfected with EHD1,2,3 and 4, aggregation of EHD3 and EHD1 was confirmed. Based on these results, it seems likely EHD2 and EHD4 do not aggregate but more research is needed. In the future, fluorescent microscopy could be done with co-expressing proteins from the EHD family. Firstly, I would do co-expression of EHD1 and EHD3 because of their structural similarity and aggregation pattern. Then, to test if they induce aggregation, co-express them with EHD2 and EHD4. Because of its aggregation pattern, EHD3 protein has already been a subject of research in the field of schizophrenia. Now, the potential of EHD1 protein to aggregate inside cells has become something worth investigating. Further research of EHD1 and EHD3 proteins could help in the understanding of the molecular mechanisms behind the development of schizophrenia and the protein aggregation associated with SMIs. More fluorescent microscopy assays could be set up to test this. EHD1 and EHD3 could be co-expressed in SH-SY5Y cells with proteins that have already been known to aggregate in mental illnesses, such as TRIOBP, DISC1 and dysbindin. Also, co-expression with GAD1 and GAD2 could be investigated to see how this changes the aggregation pattern of these proteins. All these assays could give us insight in the potential causes of the development of schizophrenia.

6. Conclusion

One theory about the molecular basis of SMIs, including schizophrenia, is that it involves protein aggregation. This happens when a variety of biological processes are disrupted, leading to an accumulation of misfolded proteins in the brain. Other theories include environmental and genetic risk factors, which could also, possibly affect protein aggregation. EH-domain containing 3 (EHD3) protein is a member of the EHD protein family and is recognized as a modulator of endocytic recycling. Among others, it plays a significant role in the internalization of D1 dopamine receptors, the expression of which is reduced in schizophrenia. EHD3 was found to aggregate in the brains of schizophrenia patients and was therefore interesting for further investigation. Previous research initially showed that the EH domain is the one responsible for aggregation, however it was later proven that the EH domain does not aggregate independently (32). The EH is necessary for the initialization of EHD3 aggregation, but other structural regions interact and recruit other molecules that make up the aggregates (34). In my thesis, we wanted to test the association of EHD3 with actin and how it affects the cytoskeleton. It was proven that full length EHD3 co-segregates with actin and is associated with it, and with tubulin inside cells. This is somewhat expected because of the role of EHD3. Initially, it is a trafficking protein and is involved in the mechanism of endocytic recycling. On the other hand, EHD3 fragments 1-399 and 1-434 did not show association with actin, which led to the conclusion that EH domain prevents this association. Also, in this thesis aggregation of other proteins from the EHD protein family: EHD1, 2 and 4 was investigated. EHD3 showed aggregate-like structures in most of the cells, while EHD1 showed this pattern in 55% of cells. In the blinded assay, EHD2 and EHD4 showed low signs of potential insolubility. These results led to the conclusion that EHD3 is the only EHD protein confirmed to aggregate, although data for EHD1 show its potential for aggregation. This suggests that EHD1 protein is worth more attention, as well as association of EHD3 with actin and structures inside cells. Understanding EHD3 mechanisms and its physiological functions could help to better comprehend the potential causes and pathology behind the development of schizophrenia.

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Andrea Montan

Date of birth: 16/11/2000 | **Nationality:** Croatian | **Phone number:** (+385) 955205873 (Mobile) | **Email address:** andrea.montan16@gmail.com | **Address:** Banjol 360, 51280, Rab, Croatia (Home)

WORK EXPERIENCE

2021 – 2021 Rab, Croatia
INTERN MEDICAL AND BIOCHEMICAL LABORATORY OF THE HEALTH CENTER OF PGŽ

01/2024 – 06/2024 Rijeka, Croatia
ASSOCIATE IN RESEARCH PROJECT -"DEPRESSION, SUICIDE AND PROTEINOPATHY: ELUCIDATING THE RELATIONSHIPS BETWEEN AGGREGATION AND PATHOLOGICAL DEVELOPMENT (DESPERADO)"
UNIVERSITY OF RIJEKA, FACULTY OF BIOTECHNOLOGY AND DRUG DEVELOPMENT

EDUCATION AND TRAINING

2022 – 2024 Rijeka
MASTERS DEGREE IN DRUG RESEARCH AND DEVELOPMENT Faculty of biotechnology and drug development

2019 – 2022 Rijeka
BACHELOR´S DEGREE IN BIOTECHNOLOGY AND DRUG RESEARCH Faculty of biotechnology and drug development

2015 – 2019 Rab
HIGH SCHOOL EDUCATION General Gymnasium Markantuna de Dominisa

2007 – 2015 Rab
PRIMARY SCHOOL Primary school Ivana Rabljanina Rab

LANGUAGE SKILLS

Mother tongue(s): **CROATIAN**

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken production	Spoken interaction	
ENGLISH	B2	C1		B2	B2
GERMAN	B1			A2	

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

DIGITAL SKILLS

MS Office (Word Excel PowerPoint) | Google Drive | Internet | Outlook | Molecular Imaging | PyMol software for molecular visualization | Social Media | Laboratory and Research skills

PUBLICATIONS

2022
mRNA vaccines against COVID-19

Bachelor thesis

NETWORKS AND MEMBERSHIPS

2021 – 2022 Faculty of biotechnology and drug development
Project Students mentors

CONFERENCES AND SEMINARS

2024 Faculty of biotechnology and drug development, Rijeka
Conference Future and Perspective

HONOURS AND AWARDS

2024
The City of Rab Scholarship for Academic Excellence

2014
First place at the National Youth First Aid Competition – Croatian Red Cross

HOBBIES AND INTERESTS

Sport activities