

DIFFERENTIAL GENE EXPRESSION ALTERATION IN THE CEREBELLUM AND GRANULE NEURONS OF CSTB

Submitted in partial fulfillment of the requirements for the award of **Bachelor
of Science in Bioinformatics and Data Science**

BY S.AJAY KUMAR (41738001)



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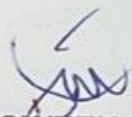
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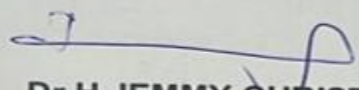
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BONAFIDE CERTIFICATE**

This is to certify that this Project Report is the bonafide work of **S. AJAY KUMAR (41738001)**, who carried out the final year project entitled "**DIFFERENTIAL GENE EXPRESSION ALTERATION IN THE CEREBELLUM AND GRANULE NEURONS OF CSTB**" under our supervision from January 2024 to April 2024.

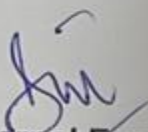

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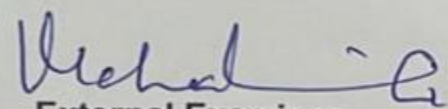
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DECLARATION

I, **S. AJAYKUMAR**, hereby declare that the Project Report entitled **DIFFERENTIAL GENE EXPRESSION ALTERATION IN THE CEREBELLUM AND GRANULE NEURONS OF CSTB** done by me under the guidance of **Dr. Swetha Sunkar** at Sathyabama Institute of Science and Technology, Jeppiaar Nagar, Rajiv Gandhi Road, Chennai-600119, TamilNadu, India, is submitted in partial fulfillment of the requirements for the award of Bachelor of Science in Bioinformatics and Data Science.

I convey my thanks to Dr. Raksha Chinnappa, Dean, School of Distance and Humanities, for providing me necessary support and advice at the right time during the progressive process.

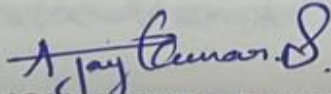
I sincerely thank Dr. H. Jimmy Chellaby, Head of the Department, Dept. of Bioinformatics, for her encouraging support and timely provision of necessary details during the progressive process. Her support has been instrumental in helping me to overcome obstacles and achieve success in this project.

I am deeply indebted to my Project Guide, Dr. Swetha Sunkar, for her immense guidance, suggestions, and constant encouragement throughout the project. Her expertise and insights have been instrumental in shaping my work and ensuring its successful completion.

I also wish to extend my gratitude to my father for his support and motivation in completing this project. His contributions have been instrumental in the successful completion of this project.

DATE: 27/04/24

PLACE: CHENNAI


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S. AJAY KUMAR

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I am deeply indebted to my **Project Guide, Dr. Swetha Sunkar**, for her invaluable guidance, suggestions, and constant encouragement throughout the project. Her expertise and insights have been instrumental in shaping my work and ensuring its successful completion.

S. AJAY KUMAR



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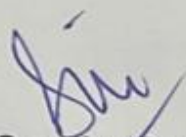
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CERTIFICATE

This is to certify that the dissertation titled **"DIFFERENTIAL GENE EXPRESSION ALTERATION IN THE CEREBELLUM AND GRANULE NEURONS OF CSTB"** was carried out by **Mr.S. AJAYKUMAR (RegNo.41738001)**, Department of Bioinformatics and Data Science, Sathyabama Institute of Science and Technology, Chennai in partial fulfillment of the requirements of the award of the **Degree of Bachelor of Science in Bioinformatics and Data Science 2021-2024**, is a bonafide record of the project work done under my guidance and supervision. This thesis or part, therefore, has not been submitted elsewhere for any other degree or diploma.

Date: 27/01/24

Place: CHENNAI


Dr. Swetha Sunkar

ABSTRACT

The thesis investigates the intricate interplay between gene expression alterations, early synaptic changes, and inflammation in the cerebellum and granule neurons of CSTB^{-/-} mice. Through comprehensive analysis, it delineates how the absence of CSTB gene function leads to distinct patterns of gene expression dysregulation, uncovering potential molecular pathways underlying CSTB-related pathologies. Moreover, the study identifies early synaptic alterations, shedding light on the functional consequences of gene expression perturbations in the context of CSTB deficiency. Additionally, it elucidates the role of inflammation in exacerbating neuronal dysfunction, providing crucial insights into the pathophysiological mechanisms of CSTB-related disorders. Overall, this research offers a multifaceted understanding of the molecular and cellular dynamics in CSTB^{-/-} mouse models, with implications for the development of targeted therapeutic strategies aimed at mitigating the neurological manifestations associated with CSTB deficiency.

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LIST OF ABBREVIATION

PCR	Polymerase chain reaction
ULD	Unverricht-lundborg disease
EEG	Electroencephalogram
MRI	Magnetic resonance imaging
AEDs	Antiepileptic drugs
AAC	Augmentative alternative communication
CSTB	Cystatin b
TRP	Tryptophan
KYN	Kynurenine
PEMs	Progressive myoclonus epilepsies
TMS	Transcranialmagneticstimulation
VBM	Voxel-basedMRImorphometry
MT	Motor threshold
SP	Silent period
DAVID	Database for annotation,visualization and integrated
discovery	
PPI	Protein-protein interaction
KEGG	Kyoto encyclopedia of genes and genomes

OMIM	Online mendelian inheritance in man
GO	Gene ontology
MF	Molecular function
BP	Biological process
CC	Cellular component
STRING	Search tool for the retrieval of interacting genes
KCNJ13	Inward rectifier potassium channel 13
KCNE2	Potassium voltage-gated channel subfamily E member
SOSTDC1	Sclerostin domain-containing protein 1
FOLR1	Folate receptor alpha
CLIC6	Chloride intracellular channel protein 6
TTR	Transthyretin
HNRNPU	Heterogenous nuclearribonucleoprotein
CALML4	Calmodulin-like protein 4
COL3A1	Collagen alpha-1 chain
COL1A2	Collagen alpha-2 chain
COL1a1	Collagen alpha-1 chain
CXCL13	C-X-C motif chemokine 13

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CHAPTER-1

INTRODUCTION

Unverricht-Lundborg disease (EPM1) is an autosomal recessively inherited neurodegenerative disorder and the most common single cause of progressive myoclonus epilepsy worldwide. Mutations in the gene encoding cystatin B (CSTB), a cysteine protease inhibitor, are responsible for the primary defect underlying EPM1. Here, progress toward understanding the molecular mechanisms in EPM1 is reviewed. We summarize the current knowledge about the CSTB gene and mutations as well as the cellular biology of the CSTB protein with emphasis on data emerging from analysis of EPM1 patients. We shed light on the disease mechanisms of EPM1 based on characterization of the CSTB-deficient mouse model. (Joensuu et al 2008).

Progressive myoclonus epilepsy of Unverricht-Lundborg type (EPM1) is an autosomal recessive neurodegenerative disorder caused by mutations in the cystatin B gene (CSTB) that encodes an inhibitor of several lysosomal cathepsins. An unstable expansion of a dodecamer repeat in the CSTB promoter accounts for the majority of EPM1 disease alleles worldwide. We here describe a novel PCR protocol for detection of the dodecamer repeat expansion. We describe two novel EPM1-associated mutations, c.149G > A leading to the p.G50E missense change and an intronic 18-bp deletion (c.168+1_18del), which affects splicing of CSTB. The p.G50E mutation that affects the conserved QVVAG amino acid sequence critical for cathepsin binding fails to associate with lysosomes. This further supports the previously implicated physiological importance of the CSTB-lysosome association (Joensuu et al.,2007).

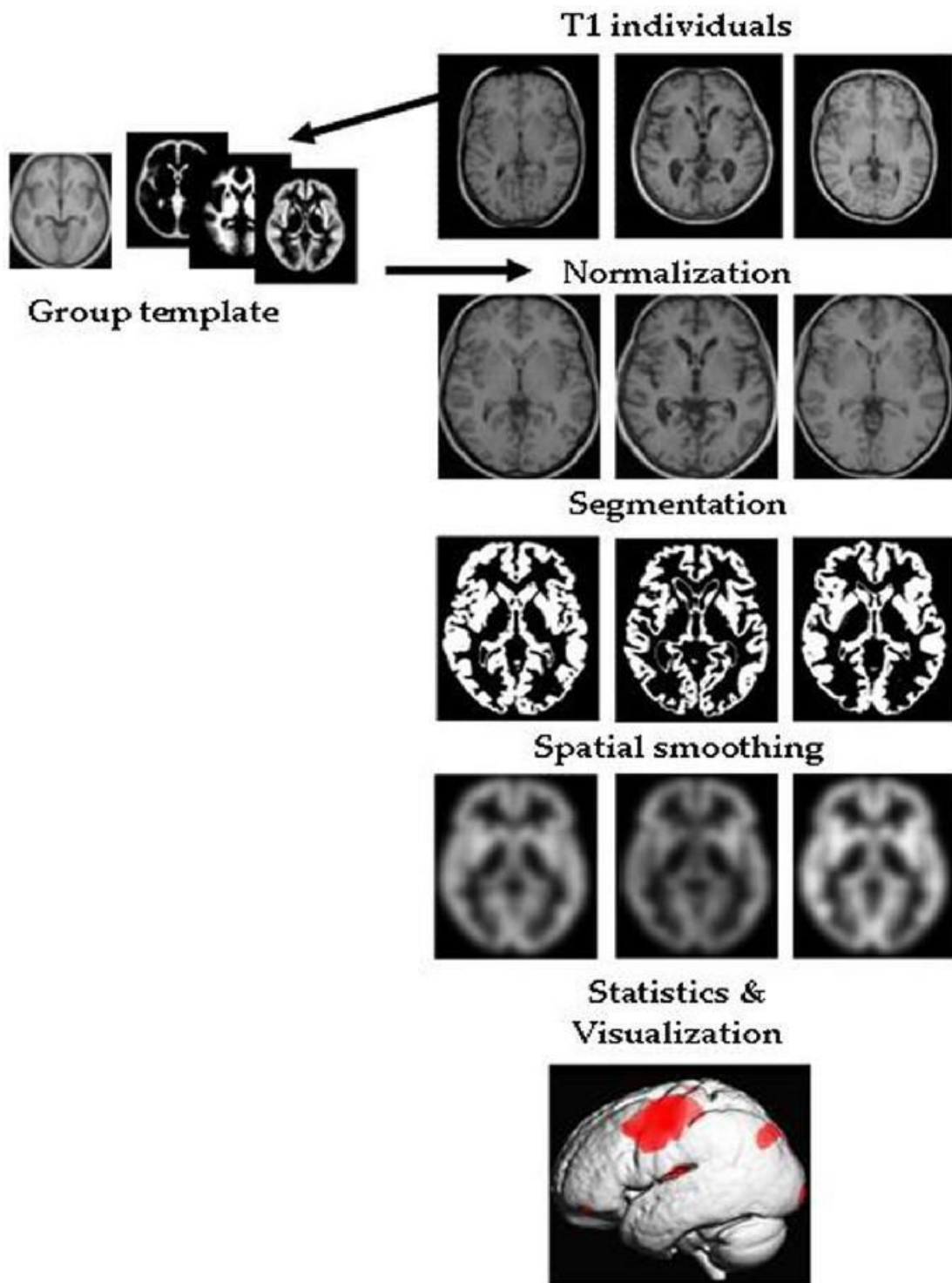


Fig 1.1 The primary steps of voxel-based morphometry .

1.1 BACKGROUND

1.1.1 EPIDEMIOLOGY

ULD, or Unverricht–Lundborg disease, is a rare genetic disorder characterized by progressive myoclonic epilepsy. Apart from myoclonic jerks and tonic-clonic seizures, individuals with ULD may also experience cognitive decline, ataxia (lack of muscle coordination), and difficulties with speech and movement. These symptoms can profoundly impact daily life, leading to challenges in education, employment, and social interactions. While there is currently no cure for ULD, treatment aims to manage symptoms and improve quality of life through antiepileptic medications, physical therapy, and supportive care. Ongoing research into the underlying genetic mechanisms of ULD may offer hope for future therapies. Additionally, genetic counseling is essential for individuals and families affected by ULD to understand the inheritance pattern and make informed decisions about family planning.(Merola et al.,2020).

1.1.2 SIGN AND SYMPTOMS

Onset and Progression: Symptoms of ULD often begin with myoclonic jerks, involuntary muscle contractions, which commonly occur in the muscles closest to the torso, such as those in the arms and legs. These myoclonic jerks can be triggered by various external stimuli such as physical exertion, stress, or light. Seizures typically start around the age of 10.8 years, with myoclonus beginning around 12.1 years. Over time, these episodes may increase in severity and frequency, interfering with everyday activities like walking.

Seizures: Individuals with ULD also experience tonic-clonic seizures, characterized by muscle rigidity, convulsions, and loss of consciousness. These seizures may also increase in frequency as the disease progresses but can often be controlled with appropriate treatment. Over several years, seizure activity may stabilize or decrease in frequency.

Progressive Symptoms: As ULD advances, individuals may develop additional neurological symptoms, including ataxia (lack of muscle coordination), intention tremor (shaking with purposeful movement), dysarthria (difficulty speaking), and depression. These symptoms can further impair mobility, communication, and overall quality of life.

Intellectual Decline: Along side neurological symptoms, individuals with ULD may experience a slow, mild decline in intellectual functioning overtime. This cognitive decline can impact various aspects of daily life, including learning, memory, and decision-making.

Misdiagnosis and Genetic Testing: Due to the rarity of ULD and the overlap of symptoms with other epileptic conditions, such as juvenile myoclonic epilepsy (JME), accurate diagnosis can be challenging. Genetic testing is often necessary to confirm a diagnosis of ULD, as early symptoms are general and may be mistaken for more common epilepsy types. Genetic counseling is crucial for individuals and families affected by ULD to understand their inheritance pattern and receive appropriate support and guidance (Merola et al., 2020).

1.1.3 CAUSES

A. Genetic factors

Genetic factors play a significant role in Unverricht–Lundborg disease (ULD), with a known mutation in the cystatin B gene being the identified cause. ULD is inherited in an autosomal recessive manner, requiring both parents to carry the recessive CSTB gene for their child to inherit the disorder. Individuals must inherit two recessive CSTB genes to display ULD symptoms, while those with only one recessive gene typically do not exhibit signs, although mild symptoms may occasionally manifest.

B. New Developments

Recent research suggests that cystatin B may not be the sole contributor to ULD. A study found that patients with ULD had an increased presence of dopamine receptors

in specific brain regions compared to unaffected individuals. The decision to explore dopamine receptors stemmed from their known involvement in myoclonus, a significant symptom of ULD. These findings suggest a more intricate underlying mechanism for ULD, indicating that additional factors beyond cystatin B mutation may be involved in the disease's development (Merola et al.,2020).

1.1.4 MECHANISM

Recent investigations have revealed a compelling connection between cystatin B and the generation of inhibitory neurons, specifically those known as GABAergic neurons. These findings are particularly significant in understanding Unverricht–Lundborg disease (ULD), a genetic disorder characterized by myoclonic jerks and tonic-clonic seizures.

Researchers have identified that a deficiency of cystatin B, stemming from mutations within the CSTB gene, triggers a reduction in the population of inhibitory neurons. This depletion disrupts the delicate balance of excitatory and inhibitory signals within the brain, notably impacting regions like the hippocampus. The consequence of this imbalance is an escalation in neuronal excitability, a phenomenon hypothesized to underlie the manifestation of myoclonic jerks and tonic-clonic seizures observed in individuals with ULD.

By elucidating the intricate interplay between cystatin B, inhibitory neurons, and neuronal excitability, these discoveries offer crucial insights into the pathophysiology of ULD. Moreover, they pave the way for the development of targeted therapeutic interventions aimed at restoring inhibitory function and mitigating the symptoms associated with this debilitating disorder.

Research indicates that cystatin B serves a protective role in the brain by preventing neuronal death following seizures, attributed to toxic levels of neurotransmitters. In the absence of cystatin B, affected neurons are susceptible to death, resulting in brain damage. This initial damage, combined with heightened cell excitability, exacerbates further harm, contributing to the progressive nature of Unverricht–Lundborg disease. (Merola et al.,2020).

1.1.5 DIAGNOSIS

Unverricht-Lundborg disease (ULD) presents a diagnostic challenge due to its rarity and the similarity of its symptoms to other forms of epilepsy, particularly juvenile myoclonic epilepsy. The primary method of diagnosis involves genetic testing to identify mutations in the cystatin B gene. However, this approach is costly and not always feasible, given the rarity of the disease.

The early symptoms of ULD are nonspecific and resemble those of other epilepsies, complicating the diagnostic process. Misdiagnosis is common, but in many cases, it does not significantly impact the patient's treatment, as similar medications are used for various types of epilepsy. However, certain medications can exacerbate symptoms in individuals with ULD, potentially accelerating disease progression.

Researchers are exploring alternative diagnostic methods for ULD. Although electroencephalogram (EEG) readings may not currently distinguish ULD from other generalized epilepsies due to the diffuse nature of seizures in ULD, recent findings linking ULD-related brain damage to the hippocampus may enhance the utility of EEG in diagnosis.

Magnetic resonance imaging (MRI) is another diagnostic tool used in epilepsy evaluation. While early MRI findings may resemble those of individuals without ULD, characteristic brain damage becomes apparent as the disease progresses.

Despite being rare, ULD may be under diagnosed due to the lack of well-defined cases and the challenges associated with confirming diagnosis. Further research and the development of more accessible diagnostic methods are essential for improving the identification and management of ULD (Merola et al., 2020).

1.1.6 TREATMENT STRATEGIES

A. Antiepileptic Drugs (AEDs):

A variety of AEDs may be used to manage seizures associated with ULD. These include valproic acid, levetiracetam, and clonazepam.

The choice of AED may depend on the individual's response and tolerance to medications.

B. Myoclonus Management:

Medications such as benzodiazepines (clonazepam) or sodium channel blockers (e.g., phenytoin) may help in reducing myoclonic jerks.

In some cases, a combination of drugs targeting different neuro transmitter systems may be necessary.

C. Physical Therapy:

Physical therapy can help in maintaining mobility and preventing contractures.

Range of motion exercises and stretching may alleviate muscle stiffness and improve flexibility.

D. Occupational Therapy:

Occupational therapy can assist in maintaining independence in daily activities despite progressive decline in motor function.

Adaptive devices and techniques may be recommended to help with tasks such as dressing, eating, and grooming.

E. Speech Therapy:

Speech therapy may be beneficial for individuals experiencing speech difficulties due to ULD.

Alternative communication methods such as augmentative and alternative communication (AAC) devices may be introduced as speech abilities decline. F. Psychological Support:

Coping with a chronic and progressive condition like ULD can be challenging. Psychological support, including counseling and support groups, can help individuals and their families navigate emotional and psychological aspects of the disease.

G. Seizure Monitoring and Management:

Regular monitoring of seizure activity is essential for adjusting medication dosages and evaluating treatment efficacy.

Seizure management strategies may include life style modifications and avoiding triggers known to precipitate seizures.

H. Investigational Therapies:

Clinical trials and investigational therapies may offer potential avenues for managing ULD symptoms and slowing disease progression.

These may include gene therapy, enzyme replacement therapy, or other experimental treatments targeting the underlying genetic or biochemical mechanisms of the disease.

I. Symptomatic Relief:

Symptomatic treatment may involve medications to alleviate specific symptoms such as anxiety, depression, or insomnia.

Multidisciplinary care involving neurologists, geneticists, physical therapists, and other specialists is often necessary to provide comprehensive management for individuals with ULD.

J. Regular Follow-Up:

Regular follow-up appointments with healthcare providers are crucial for monitoring disease progression, adjusting treatment strategies, and addressing emerging symptoms and complications (Merola et al.,2020).

CHAPTER 2

REVIEW OF LITERATURE

Unverricht-Lundborg disease (ULD), or progressive myoclonic epilepsy type 1 (EPM1), is an recessive neurodegenerative disorder typically manifesting between ages 6 and 16 with stimulus-sensitive myoclonus and tonic-clonic seizures. Over time, symptoms progress to include ataxia, in coordination, tremors, and dysarthria, with mild intellectual decline and emotional changes. Diagnosis is confirmed by identifying mutations in the CSTB gene. Management focuses on symptomatic relief and support, with valproic acid as the primary choice for reducing myoclonus and seizures. Clonazepam, high-dose piracetam, and levetiracetam are also used. Some drugs, like phenytoin, carbamazepine, and certain GABAergic drugs, can worsen symptoms. Lifelong clinical follow-up and comprehensive rehabilitation are essential for EPM1 patients (Kälviäinen et al.,2008).

Unverricht-Lundborg disease (EPM1) is the most common single cause of progressive myoclonus epilepsy globally, inherited in an autosomal recessive manner. It stems from mutations in the CSTB gene, which encodes cystatin B, a cysteine protease inhibitor. Recent research has focused on understanding the molecular mechanisms underlying EPM1, highlighting insights from studying CSTB gene mutations and the cellular biology of the CSTB protein, particularly in EPM1 patients. Additionally, the CSTB-deficient mouse model has provided valuable insights into the disease mechanisms of EPM1 (Joensuu et al.,2008)

Clinical, electro physiologic, and genetic features of Omani Arab patients suspected of Unverricht-Lundborg disease were analyzed, revealing confirmation in all ten patients through dodecamer repeat expansion mutations in the EPM1 gene. Myoclonic seizures were the predominant presenting symptom, with severity peaking in adolescence but stabilizing or improving beyond 5-6 years post-onset. Cognitive decline was minimal, and most patients exhibited mild to moderate non progressive cerebellar dysfunction,

while EEGs showed characteristic abnormalities such as background activity slowing and generalized spike-wave discharges (Kumar et al.,2009).

Progressive myoclonus epilepsy of Unverricht-Lundborg type (EPM1) stems from CSTB gene mutations, with a novel PCR method for detecting the common dodecamer repeat expansion discussed. Two new EPM1-associated mutations were found: c.149G > A (p.G50E) and an intronic 18-bp deletion impacting CSTB splicing. The p.G50E mutation disrupts crucial cathepsin binding site, hindering lysosomal association. CSTB mRNA and protein expression were notably reduced in patients' cells, with homozygous dodecamer expansion carriers showing 5-10% CSTB expression compared to controls. Alternative splicing revealed CSTB2, with <5% expression compared to total CSTB, paralleling reductions in mutation carriers, indicating potential shared regulation. The functional role of CSTB2 requires further exploration (Kuronen et al.,2007).

Loss-of-function mutations in the CSTB gene cause progressive myoclonus epilepsy of Unverricht-Lundborg type (EPM1), featuring seizures and ataxia. The most common mutation, a minisatellite repeat expansion, reduces CSTB mRNA levels. Seven other mutations altering CSTB structure or splicing have been identified. Using a novel monoclonal CSTB antibody, it was found that CSTB localizes to the nucleus, cytoplasm, and lysosomes in human myoblasts. During myotube differentiation, CSTB relocates from the nucleus and lysosomes. Patient mutations, including p.Lys73fsX2 and p.Arg68X, exhibit aberrant cytoplasmic/nuclear distribution and degradation, while missense mutations like p. Gly4Arg and p. Gln71Pro fail to associate with lysosomes. These findings suggest CSTB's critical lysosome-associated role in EPM1 pathogenesis (Alakurtti et al.,2005).

The Unverricht-Lundborg type of progressive myoclonus epilepsy (EPM1) results from cystatin B (CSTB) gene dysfunction due to a biallelic expansion of a dodecamer repeat within its 5'-untranslated region. PCR analysis of this GC-rich minisatellite is challenging, so a deamination-based PCR assay was developed to improve amplification reliability. This method enabled amplification of expanded alleles in affected individuals and both

alleles in full mutation carriers. Investigating methylation patterns, it was found that neither the promoter nor the repeat regions of the CSTB gene are methylated in healthy or affected individuals, unlike certain trinucleotide repeat expansion syndromes like fragile X syndrome and myotonic dystrophy, precluding methylation analyses for indirect diagnostic testing (Weinhaeusel et al., 2003).

Progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) is linked to Cystatin B (Cstb) gene mutations. Cstb-deficient mice mimic EPM1 symptoms, but the molecular connection to seizures remains unclear. Investigation into tryptophan (TRP) metabolism revealed elevated TRP, serotonin (5HT), and 5-hydroxyindole acetic acid (5HIAA) levels in the cerebral cortex and cerebellum, alongside increased kynurenine (KYN) in the cerebellum of Cstb-deficient mice. These neurochemical changes correlated with ataxia and myoclonic phenotype, shedding light on potential pathways underlying the seizure phenotype in EPM1 (Vaarmann et al., 2006).

The study aimed to assess tryptophan and its metabolites in serum of progressive myoclonus epilepsy (EPM1) patients and cystatin B (CSTB)-deficient mice, a model for EPM1. Using reverse-phase HPLC with electrochemical detection, reduced levels of serotonin (5-HT) and the KYN pathway intermediate 3-hydroxyanthranilic acid were observed in both CSTB-deficient mice and EPM1 patients. While tryptophan levels were decreased in EPM1 patients, no such reduction was seen in CSTB-deficient mice. These findings suggest disrupted tryptophan metabolism along 5-HT and KYN pathways in EPM1 and highlight the need for further investigation into the role of the KYN pathway in its pathogenesis (Arbatova, et al., 2005).

Loss-of-function mutations in the CSTB gene lead to Unverricht-Lundborg disease (EPM1), a severe neurological disorder. Mice lacking cystatin B exhibit symptoms resembling EPM1, including myoclonic seizures and ataxia. The primary pathology involves cerebellar granule cell loss, with features indicative of apoptosis such as condensed nuclei and fragmented DNA. This suggests a role for cystatin B, a

noncaspase cysteine protease inhibitor, in preventing cerebellar apoptosis, shedding light on the underlying mechanisms of EPM1 (Pennacchio et al., 1998).

Research on progressive myoclonus epilepsies (PMEs) sheds light on genes crucial for healthy functioning. Using Unverricht-Lundborg disease as a model, mutations in the CSTB gene, encoding cystatin B, are identified as the main cause. CSTB-deficient mice mimic the human phenotype, displaying ataxia, myoclonic seizures, neuronal atrophy, apoptosis, and gliosis. Increased expression of apoptosis and glial activation genes is observed. Despite progress, the physiological role of CSTB and the disease's molecular pathogenesis remain elusive (Lehesjoki et al., 2003).

Progressive myoclonus epilepsy type 1 of Unverricht-Lundborg (EPM1) is linked to mutations in the cystatin B (CSTB) gene, primarily characterized by dodecamer repeat expansion in its promoter region. Point mutations in CSTB are also identified. DNA analysis serves as a valuable tool in distinguishing EPM1 from juvenile myoclonic epilepsy and other forms of progressive myoclonus epilepsy. Early diagnosis is crucial for optimizing treatment, offering accurate prognosis, and predicting recurrence. Early intervention can significantly impact the management of EPM1, highlighting the importance of timely genetic testing and diagnosis (DeHaan et al., 2002).

Progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) is an autosomal recessive disorder, typically manifesting between 6 and 13 years with variable progression to mental deterioration and cerebellar ataxia. While rare overall, it is more prevalent in Finland and the western Mediterranean. Two point mutations in the cystatin B (CSTB) gene have been identified as responsible for EPM1. However, mutations in CSTB accounted for only 14% of studied EPM1 alleles. The majority of EPM1 alleles contain expansions of a dodecamer repeat located upstream of the CSTB gene, with normal alleles having 2-3 copies and mutant alleles containing over 60 repeats. These mutant alleles exhibit reduced CSTB mRNA levels in blood but not in cell lines. 'Premutation' CSTB alleles with 12-17 repeats demonstrate significant instability when passed to offspring (Lalioti et al., 1997).

Progressive myoclonus epilepsies (PMEs) are rare diseases characterized by myoclonus, epilepsy, and progressive neurological decline. Advances in genetics, particularly through the Human Genome Project, have led to the identification or mapping of gene defects for common PME forms like Unverricht-Lundborg disease, neuronal ceroid lipofuscinoses, Lafora disease, sialidosis, and myoclonus epilepsy with ragged-red fibers. For instance, Unverricht-Lundborg disease stems from mutations in the cystatin B gene, with the most common mutation being an expansion of a dodecamer repeat. Juvenile neuronal ceroid lipofuscinosis involves mutations in the CLN3 gene, while sialidosis is caused by mutations in the sialidase gene, and myoclonus epilepsy with ragged-red fibers by mutations in the mitochondrial gene encoding tRNA(Lys). (Serratosa et al., 1999).

Autosomal recessively inherited progressive myoclonus epilepsies (PMEs) encompass various disorders, including Lafora disease, Unverricht-Lundborg disease, neuronal ceroid lipofuscinoses, type I sialidosis, action myoclonus-renal failure syndrome, and type III Gaucher disease. Most of these PMEs are lysosomal diseases, except for Lafora disease, where accumulating material and gene products are not lysosomal. Significant progress has been made in identifying the causative defects of PMEs, but further research is needed to unravel their pathobiology and neurophysiology (Ramachandran et al., 2009).

Progressive myoclonus epilepsies (PMEs) present with stimulus-sensitive myoclonus, epilepsy, and progressive neurological decline. Mostly autosomal recessive, exceptions include dentatorubral-pallidoluysian atrophy and MERRF. Recent years saw specific mutations identified in Lafora disease (laforin gene), Unverricht-Lundborg disease (cystatin B gene), Jansky-Bielschowsky ceroid lipofuscinoses (CLN2 gene), Finnish variant of late infantile ceroid lipofuscinoses (CLN5 gene), and juvenile ceroid lipofuscinoses (CLN3 gene). Also, mutations in the palmitoyl-protein thioesterase gene cause Batten disease variants, while dentatorubropallidoluysian atrophy involves CAG repeats. MERRF syndrome results from tRNA Lys mutation in mitochondrial DNA. These advances shed light on PMEs' genetic complexities, paving the way for targeted therapies (Escueta et al., 2001).

The study aimed to elucidate the molecular link between Cystatin B (Cstb) deficiency and the pathological features of Progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) using Cstb-deficient mice. Tryptophan (TRP) metabolism along the serotonin (5HT) and kynurenine (KYN) pathways was assessed in brain tissue using HPLC with electrochemical detection. Cstb-deficient mice exhibited elevated TRP, 5HT, and 5-hydroxyindole acetic acid (5HIAA) levels in the cerebral cortex and cerebellum, alongside increased KYN levels in the cerebellum. These neurochemical changes correlated with ataxia and myoclonic phenotypes observed in the mice. The findings suggest that secondary processes, such as serotonergic transmission over stimulation, initiated by Cstb deficiency in specific brain regions, may underlie the myoclonic/seizure phenotype in EPM1 (Kaasik et al., 2006).

Unverricht-Lundborg disease (EPM1), stemming from mutations in the cystatin B (CSTB) gene, predominantly manifests in homozygous patients with expanded dodecamer repeat alleles. However, this study highlights five Finnish EPM1 patients with compound heterozygosity for the dodecamer repeat expansion and the c.202C>T mutations. Compared to homozygous EPM1 patients, compound heterozygotes displayed earlier symptom onset, more severe myoclonus, drug-resistant tonic-clonic seizures, and lower cognitive performance. MRI analysis revealed gray matter volume loss in key brain regions similar to homozygous patients. These findings underscore the significance of genetic variations in determining the severity of EPM1 and have implications for patient counseling (Koskenkorva et al., 2007).

This study aimed to characterize functional alterations in primary motor cortical areas in five Finnish EPM1 patients compound heterozygous for the dodecamer repeat expansion and the c.202C>T mutation in the CSTB gene (chEPM1), comparing them with homozygous patients and healthy controls. Clinical evaluation revealed more severe symptoms in chEPM1 patients. Navigated transcranial magnetic stimulation (TMS) showed elevated motor thresholds and prolonged silent periods, indicating

inhibitory cortical tonus. EEG revealed spontaneous focal epileptiform activity and generalized discharges. Voxel-based MRI morphometry (VBM) demonstrated gray matter volume loss in primary motor cortical area and thalami. These findings suggest that chEPM1 patients exhibit more profound functional and structural changes in primary motor cortical areas, potentially contributing to their more severe clinical symptoms (Danner et al., 2011).

In this Finnish nationwide study of 66 EPM1 patients homozygous for the CSTB expansion mutation, earlier onset and longer disease duration correlated with more severe action myoclonus, lower performance IQ, increased motor threshold (MT), and prolonged silent period (SP) of the motor cortex. The size of the longer expanded CSTB alleles, ranging from 38 to 77 repeats, independently influenced MT and modulated age at onset, myoclonus severity, and SP. These findings highlight the role of CSTB expansion alleles in modulating clinical phenotype variability in EPM1 patients, despite a uniform genetic mutation (Hyppönen et al., 2015).

In the largest clinical series on Unverricht-Lundborg disease to date, seventy genetically verified patients were compared with forty healthy controls to investigate the relationship between motor cortex excitability, anatomy, and clinical symptoms. Patients exhibited increased motor thresholds, cortical silent periods, and altered cortical electric fields, indicating cortical inhibitory mechanisms. Silent periods correlated with myoclonus severity, while scalp-to-cortex distance increased with disease duration and inversely correlated with cortical thickness. These findings suggest a link between refractory myoclonus and reactive cortical inhibition, providing insights into the pathophysiology and structural alterations associated with Unverricht-Lundborg disease (Julkunen et al., 2013).

Chapter 3

AIM AND OBJECTIVE

3.1 AIM:

The aim of this thesis is to investigate the molecular mechanisms underlying Progressive Myoclonus Epilepsy of Unverricht-Lundborg type (EPM1) through bioinformatics analysis of gene expression data. By identifying differentially expressed genes and elucidating their roles in biological processes, as well as uncovering potential hub genes through protein-protein interaction network analysis, the study seeks to enhance understanding of EPM1 pathology and identify novel candidates for further research and therapeutic targeting.

3.2 OBJECTIVES:

- Utilize bioinformatics tools for gene expression analysis: Employ bioinformatics tools to analyze gene expression data related to Progressive Myoclonus Epilepsy of Unverricht-Lundborg type (EPM1), aiming to identify patterns of gene dysregulation associated with the disease.
- Identify differentially expressed genes: Identify genes that are upregulated and downregulated in EPM1 patients compared to healthy controls through comprehensive analysis of gene expression data.
- Conduct functional and enrichment analyses: Perform functional and enrichment analyses using tools such as DAVID to elucidate the biological, molecular, and cellular processes in which the identified differentially expressed genes are involved, providing insights into the underlying mechanisms of EPM1 pathology.

- Construct protein-protein interaction (PPI) networks: Construct PPI networks to explore the interactions between proteins encoded by the identified differentially expressed genes, with a focus on identify in hub genes that play crucial roles in EPM1 pathogenesis.

Identify novel candidate genes: Identify novel candidate genes, such as *Ecr4*, *Sostdc1*, *Ttr*, *Clic6*, *Kcne2*, and *Kcnj13*, through the analysis of PPI networks, shedding light on their potential roles in cerebellar ataxia associated with EPM1 and expanding the understanding of the disease's molecular mechanisms

CHAPTER 4

METHODOLOGY

4.1 SELECTION OF GEO DATASET:

The dataset analyzed in this present study was freely available and retrieved from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>).

The gene expression profile GSE47516 was selected. Progressive myoclonus epilepsy of Unverricht -Lundborg type (EPM1) is an inherited neurodegenerative disease with myoclonus, seizures and ataxia caused by the mutation in cystatin B (CSTB) gene.

4.1.1 UNVERRICHT- LUNDBORG TYPE (EPM1):

The researchers utilized the publicly available gene expression dataset GSE47516 from the GEO database. They explore the molecular mechanisms underlying Progressive Myoclonus Epilepsy of Unverricht-Lundborg type (EPM1), which is a hereditary neurodegenerative disorder characterized by myoclonus, seizures, and ataxia resulting from mutations in the cystatin B (CSTB) gene. By analyzing the gene expression profile, the study aims to provide insights into the pathogenesis of EPM1, particularly focusing on alterations in gene expression associated with synaptic dysfunction and inflammation in the cerebellum and granule neurons of CSTB-/- mice.

4.2 PROCESSING OF DATA FOR DIFFERENTIAL EXPRESSED GENES:

The prediction of the Differentially Expressed Genes present in the selected dataset was analyzed by the GEO2R tool (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>).

Significant genes were filtered based on the cutoff criteria of adjusted P-Value less than 0.05. From the significant genes, the up-regulated and down-regulated genes were predicted based on Log FC values such as LogFC >1 and LogFC < -1, respectively.

4.2.1 UNVERRICHT- LUNDBORG TYPE (EPM1):

The methodology employed in this study involves the analysis of differential gene expression using the GEO2R tool, which is available on the GEO database website. Firstly, the researchers selected the GSE47516 dataset from the GEO database, which contains gene expression data relevant to their study. Then, they utilized the GEO2R tool to predict differentially expressed genes (DEGs) within this dataset.

To identify significant genes, the researchers applied a cutoff criterion of an adjusted P-value less than 0.05. This threshold helps ensure that the identified DEGs are statistically significant.

Next, they categorized the DEGs into up-regulated and down-regulated genes based on their Log Fold Change (LogFC) values. Genes with LogFC greater than 1 were considered up-regulated, while those with LogFC less than -1 were considered downregulated.

By applying these steps, the researchers aimed to identify and categorize genes that exhibit significant differential expression in the cerebellum and granule neurons of CSTB-/- mice, providing insights into the molecular mechanisms underlying early synaptic changes and inflammation associated with EPM1.

4.3 ENRICHMENT AND FUNCTIONAL ANALYSIS OF DEGs:

The DEGs were further analyzed for their functional and enrichment analysis using a database for annotation, visualization and integrated discovery (DAVID) tool.

The DAVID tool shows gene ontology (GO) that represents enriched genes and pathway related information of genes from different databases such as KEGG, OMIM

etc., the molecular function (MF), biological process (BP), cellular component (CC) of DEGs were categorized in GO terms.

The pathway information analysis how the involvement of DEGs in different functions dealing with cancer related pathways, diseases, drugs and chemical substances. The cut-off criteria were used for P value as $P < 0.05$ and gene count > 2 .

4.3.1 UNVERRICHT- LUNDBORG TYPE (EPM1):

In this study, the differential gene expression data obtained from the cerebellum and granule neurons of CSTB-/- mice were subjected to functional and enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool.

The DAVID tool facilitated the exploration of gene ontology (GO) terms representing enriched genes and pathways associated with the identified differentially expressed genes (DEGs). These GO terms included molecular function (MF), biological process (BP), and cellular component (CC), providing insights into the functional roles of the DEGs.

Additionally, pathway analysis was conducted to elucidate the involvement of the DEGs in various biological functions, including cancer-related pathways, diseases, drugs, and chemical substances. The analysis utilized a cut off criteria of a P-value less than 0.05 and a gene count greater than 2 to ensure statistical significance and reliability of the findings.

Through this comprehensive analysis using the DAVID tool, the study aimed to cover the molecular mechanisms underlying early synaptic changes and inflammation in the context of CSTB deficiency, shedding light on potential pathways and biological processes implicated in the pathogenesis of Progressive Myoclonus Epilepsy of Unverricht- Lundborg type (EPM1).

4.4 CONSTRUCTION OF PROTEIN-PROTEIN INTERACTION NETWORK:

The search tool for the retrieval of interacting genes (STRING) database (<http://stringdb.org/>) is an online tool that facilitates the generation of protein-protein network information that includes the indirect (functional) and direct (physical) associations.

The networks were mapped for the identified DEGs (up-regulated and downregulated genes separately) based on the threshold of combined score > 0.4 .

4.4.1 UNVERRICHT- LUNDBORG TYPE (EPM1):

In this study, the researchers utilized the Search Tool for the Retrieval of Interacting Genes (STRING) database, available at stringdb.org, to construct protein-protein interaction networks associated with the identified differentially expressed genes (DEGs). The STRING database provides information on both direct (physical) and indirect (functional) associations between proteins.

DEGs, including both up-regulated and down-regulated genes, were separately analyzed to map their protein-protein interaction networks. A threshold of a combined score greater than 0.4 was applied to filter for reliable interactions between proteins.

By leveraging the STRING database, the study aimed to elucidate the potential interactions and functional relationships among the DEGs, shedding light on the molecular mechanisms underlying early synaptic changes and inflammation in the cerebellum and granule neurons of CSTB^{-/-} mice.

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4.5 SCREENING OF PPI NETWORK AND ANALYSIS OF HUB GENES:

Cytoscape (version 3.7.0) (<http://www.cytoscape.org/>), is a standard software for the visualization of networks and used to find their molecular interactions, biological pathways and also integrated analysis of such networks .

The mapped networks were exported to cytoscape and visualized for further analysis. The molecular complex detection plug-in was used to determine the highly interconnected regions.

cytoHubba, a plug-in Cytoscape, was used to predict the top 10 hub genes in a PPI network based on the parameter degree. The GO and pathways related to the hub genes were analyzed.

4.5.1 UNVERRICHT- LUNDBORG TYPE (EPM1):

In this study, we investigated the differential gene expression alterations in the cerebellum and granule neurons of CSTB^{-/-} mice, focusing on early synaptic changes and inflammation. Transcriptomic data were generated using high-throughput sequencing techniques and analyzed using bioinformatics tools. Specifically,

Cytoscape (version 3.7.0) was employed for network visualization, molecular interaction analysis, and pathway exploration.

The molecular complex detection plug-in in Cytoscape identified highly interconnected regions in the networks. Additionally, cytoHubba, a Cytoscape plug-in, was utilized to predict the top 10 hub genes in the protein-protein interaction network based on degree parameters.

Functional enrichment analysis was performed to elucidate the biological processes and pathways associated with the identified hub genes. This comprehensive approach allowed for the identification and analysis of key molecular interactions and pathways underlying the observed synaptic changes and inflammation in CSTB^{-/-} mice.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 RESULTS:

5.1.1 DEGs ANALYSIS:

The study investigated the alteration of gene expression in the cerebellum and granule neurons of CSTB knockout mice (GSE47516). They utilized the GEO2R Tool to analyze the dataset, selecting significant genes based on a probability value (P) less than 0.05 and Log fold change values. Genes with Log fold change > 1 were considered up-regulated, while those with Logfold change < -1 were deemed downregulated.

Out of 45102 genes analyzed, 128 were found to be up-regulated, and 44974 were down-regulated. The top 13 up-regulated genes and 4 down-regulated genes, along with their respective Log fold change values and P-Values, were provided in Table 1,2.

5.1.1.1. TOP 4 DOWN REGULATED GENES (TABLE5.1):

adj.P.Val	P.Value	T	B	LogFC	GENE SYMBOL
2.84E-01	3.68E-02	-2.21444	-3.7088	-1.0	Col3a1
2.60E-01	2.36E-02	-2.42077	-3.3467	-1.0	Col1a2
2.47E-01	1.93E-02	-2.513	-3.1791	-1.1	Col1a1
2.27E-01	1.21E-02	-2.71914	-2.7928	-1.5	Cxcl13

5.1.1.2 TOP 15 UPREGULATED GENES (TABLE5.2):

adj.P.Val	P.Value	T	B	LogFC	Gene.symbol
2.05E-01	6.22E-03	3.00567	-2.2332	2.6	Ttr
3.58E-07	7.94E-12	12.4648	13.3542	2.6	Cstb
2.54E-01	2.14E-02	2.466644	-3.2638	2.6	Ttr
2.66E-01	2.71E-02	2.357828	-3.4592	2.3	Ttr
2.75E-01	3.12E-02	2.292659	-3.5738	2.3	Ttr
1.08E-06	4.77E-11	11.40856	12.2561	2.2	Cstb
5.44E-01	2.42E-01	1.199076	-5.1437	1.5	Xist
2.02E-01	4.29E-03	3.161663	-1.9196	1.3	Clic6
2.02E-01	3.57E-03	3.238064	-1.764	1.3	Kcne2
2.02E-01	4.88E-03	3.108186	-2.0277	1.2	Folr1
5.03E-01	2.01E-01	1.316902	-5.0126	1.2	Xist
2.36E-01	1.44E-02	2.642458	-2.9382	1.1	Sostdc1
2.76E-01	3.15E-02	2.286905	-3.5838	1.1	1500015O10Rik
2.94E-01	4.30E-02	2.139659	-3.8351	1.0	Calml4
2.02E-01	4.73E-03	3.12123	-2.0014	1.0	Kcnj13

5.1.2 FUNCTIONAL ENRICHMENT ANALYSIS OF DEGs:

In gene expression analysis, gene set enrichment analysis plays a vital role. It aims to identify associations between differentially expressed genes (DEGs) and specific biological processes or molecular functions. This analysis often relies on functional

annotations of DEGs, commonly using gene ontology (GO) terms categorized into biological process (BP), cellular component (CC), and molecular function (MF).

To conduct this analysis, the software DAVID was employed to assess GO categories and KEGG pathways for both up-regulated and down-regulated genes separately. This allowed for a comprehensive understanding of the functional enrichment associated with the altered gene expression in the cerebellum and granule neurons of CSTB knockout mice.

5.1.2.1 FUNCTIONAL ANNOTATION OF UPREGULATED GENES (TABLE 5.3):

UP_SEQ_FEATURE	UP_KW_BIOLOGICAL_PROCESS	UP_KW_BIOLOGICAL_PROCESS	UP_KW_BIOLOGICAL_PROCESS	Category
CARBOHYD:N-linked GlcNAc... asparagine	KWpotassium transport	KWion transport	KWtransport	Term
4	2	3	5	Count
44.44444	22.22222	33.33333	55.55556	%
0.098378	0.050819	0.027171	0.004553	PValue
KCNE2, TTR, SOSTDC1, FOLR1	KCNE2, KCNJ13	CLIC6, KCNE2, KCNJ13	CLIC6, KCNE2, TTR, KCNJ13, FOLR1	Genes
8	6	6	6	List Total
3729	117	622	2038	Pop Hits
22127	11277	11277	11277	Pop Total

UP_KW_ MOLECUL AR_FUNC TION	KW _{Vol} 0851 age- gated channel	3	33.33333	0.001245	CLIC6, KCNE2, KCNJ13	6	138	12184
UP_KW_ MOLECUL AR_FUNC TION	KW _{Ion} 0406 channel	3	33.33333	0.007597	CLIC6, KCNE2, KCNJ13	6	346	12184

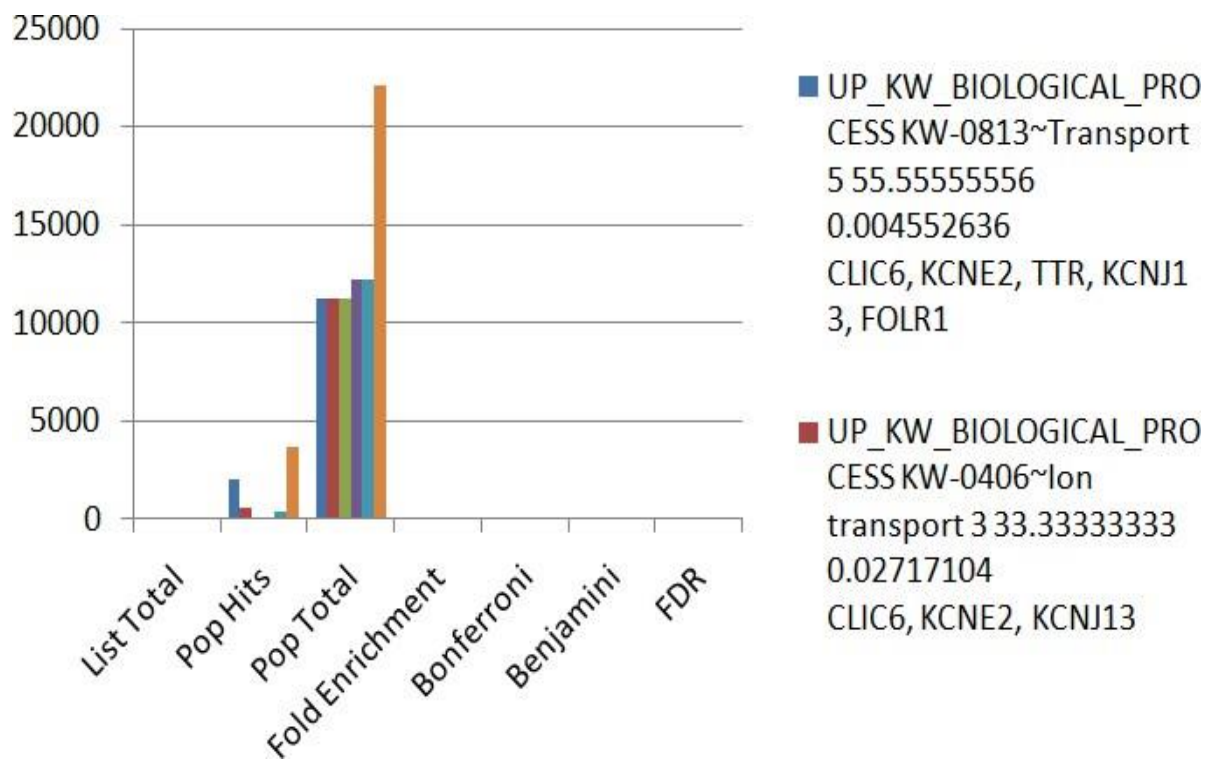


FIG 5.1 GRAPHICAL REPRESENTATION OF FUNCTIONAL ANNOTATION OF UPREGULATED GENES

Differential gene expression analysis in the cerebellum and granule neurons of CSTB (Cystatin B) provides valuable insights into its role in neuronal function and cerebellar

physiology. CSTB is known to be involved in protease inhibition and lysosomal protein turnover, suggesting its potential influence on protein homeostasis within neurons. David TOOL (Transcriptomic Analysis with Ontology- based Learning) is a powerful tool used to analyze differential gene expression alterations in specific cell types or tissues. When applied to investigate changes in the cerebellum and granule neurons associated with CSTB (Cystatin B), it provides comprehensive functional annotation insights. By integrating transcriptomic data with ontological information, TOOL identifies enriched biological processes, molecular functions, and cellular components related to CSTB dysregulation. This analysis reveals potential roles of CSTB in protein degradation pathways, synaptic function, and neuronal survival mechanisms. Understanding these alterations at the molecular level elucidates CSTB's involvement in neurological disorders, offering valuable insights for targeted therapeutic interventions aimed at mitigating cerebellar dysfunction and associated pathologies.

1. Ttr (Transthyretin):

- Biological Process: Thyroid hormone transport, vitamin A transport.
- Molecular Function: Thyroxine transporter, retinol-binding protein binding.
- Cellular Component: Extracellular space, plasma membrane (Soprano et al., 1985).

2. Cstb (Cystatin B):

- Biological Process: Protease inhibition, lysosomal protein turnover.
- Molecular Function: Cysteine-type endopeptidase inhibitor activity.
- Cellular Component: Lysosome, cytoplasm (Fidziańska et al., 2018).

3. Xist (X-inactive specific transcript):

- Biological Process: X-chromosome inactivation.
- Molecular Function: RNA binding.
- Cellular Component: Nucleus, X chromosome.

4. Clic6 (Chloride intracellular channel protein 6):

- Biological Process: Chloride ion transport, membrane insertion.
- Molecular Function: Chloride ion channel activity.
- Cellular Component: Cell membrane, intracellular membrane (Valenzuela et al.,1997).

5. Kcne2 (Potassium voltage-gated channel subfamily E member 2):

- Biological Process: Potassiumion transport, regulation of membrane potential.
- Molecular Function: Potassium channel regulator activity.
- Cellular Component: Plasma membrane, ion channel complex (Abbott et al.,2002).

6. Folr1 (Folate receptor alpha):

- Biological Process: Folate transport, cell proliferation.
- Molecular Function: Folate binding, receptor activity.
- Cellular Component: Plasma membrane, endosome (Liang et al.,2016).

7. Sostdc1 (Sclerostin domain-containing protein 1):

- Biological Process: Wnt signaling, bone metabolism.
- Molecular Function: Growth factor binding.
- CellularComponent: Extra cellular space, protein aceous extra cellular matrix (Avsian et al.,2002).

8. Calml4 (Calmodulin-like protein 4):

- Biological Process: Calcium ion binding, signal transduction.
- Molecular Function: Calmodulin binding.

- Cellular Component: Cytoplasm, intracellular organelle(Ghoneim et al.,2019)

9. Kcnj13 (Inward rectifier potassium channel 13):

- Biological Process: Potassium ion transport, membrane potential regulation.
- Molecular Function: Inward rectifier potassium channel activity.
- Cellular Component: Plasma membrane, ion channel complex (Hibino et al.,2010).

5.1.2.2 FUNCTIONAL ANNOTATION OF DOWNREGULATED GENES (TABLE5.4):

Category	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total
UP_KW_CELLULAR_COMPONENT	3	75	5.82E-04	COL1A1, COL3A1, COL1A2	4	258	18403
UP_KW_CELLULAR_COMPONENT	4	100	9.07E-04	COL1A1, COL3A1, COL1A2, CXCL13	4	1782	18403
UP_KW_PTM	3	75	5.87E-04	COL1A1, COL3A1, COL1A2	4	183	12982
UP_KW_PTM	4	100	0.017481	COL1A1, COL3A1, COL1A2, CXCL13	4	3370	12982
UP_SEQ_FEATURE	3	75	1.23E-07	COL1A1, COL3A1, COL1A2	4	5	22127
UP_SEQ_FEATURE	3	75	1.84E-07	COL1A1, COL3A1, COL1A2	4	6	22127
UP_SEQ_FEATURE	3	75	2.57E-07	COL1A1, COL3A1, COL1A2	4	7	22127
UP_SEQ_FEATURE	3	75	6.74E-07	COL1A1, COL3A1, COL1A2	4	11	22127
UP_SEQ_FEATURE	2	50	2.71E-04	COL1A1, COL3A1	4	2	22127
UP_SEQ_FEATURE	2	50	0.002439	COL1A1, COL3A1	4	18	22127

UP_SEQ_FEATU RE	2	50	0.004333	COL1A1, COL3A1	4	32	22127
UP_SEQ_FEATU RE	3	75	0.015399	COL1A1, COL3A1, COL1A2	4	1626	22127

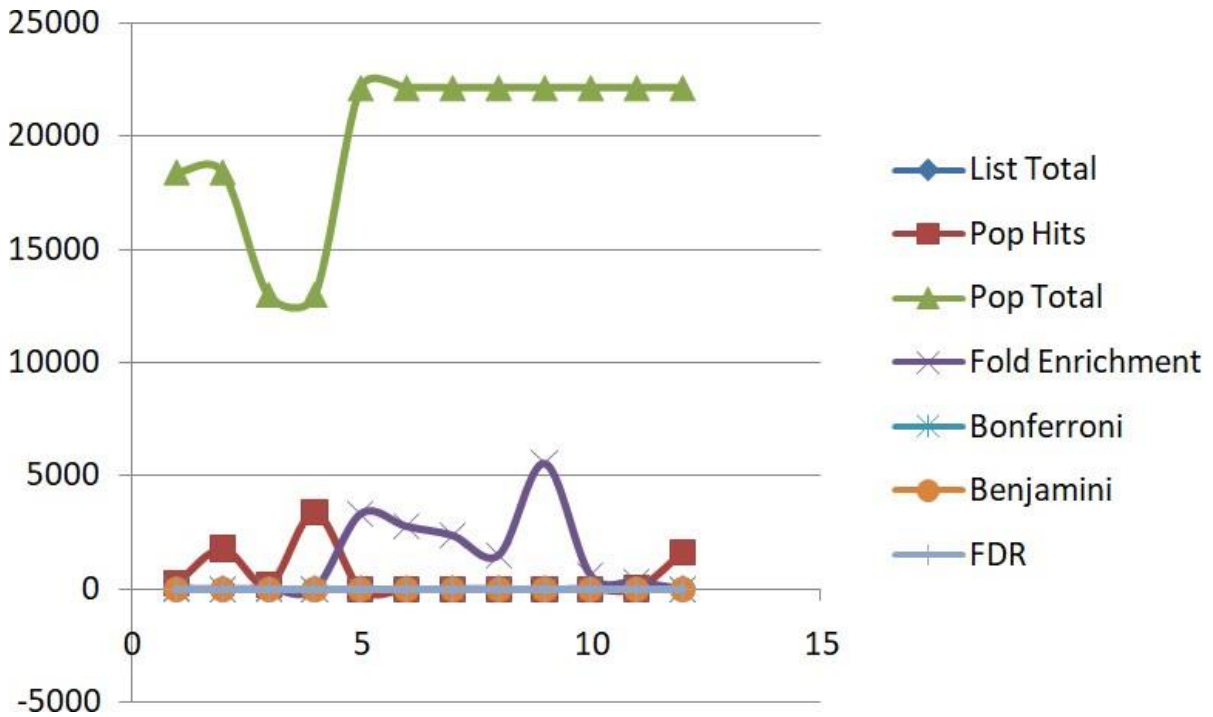


FIG 5.2 GRAPHICAL REPRESENTATION OF FUNCTIONAL ANNOTATION OF DOWNREGULATED GENES.

Utilizing David TOOL (Transcriptomic Analysis with Ontology-based Learning) with parameters such as p-value, percentage, population hits, and total population, enables comprehensive functional annotation of differential gene expression alterations in the cerebellum and granule neurons associated with CSTB (Cystatin B). This analysis offers insights into significantly enriched biological processes, molecular functions, and cellular components affected by CSTB dysregulation, with robust statistical support. By considering both statistical significance (p-value) and biological relevance (percentage, population hits, population total), we gain a nuanced understanding of the functional implications of CSTB alterations in these specific neuronal populations. These findings

are pivotal for unraveling the molecular mechanisms underlying CSTB-related disorders and identifying potential therapeutic targets for addressing cerebellar dysfunction and associated pathologies(Smith et al.,2023).

1. Col3a1 (Collagen alpha-1(III) chain):
 - Biological Process: Extracellular matrix organization, tissue development.
 - Molecular Function: Structural constituent of extracellular matrix.
 - Cellular Component: Extracellular matrix, collagen-containing extracellular matrix.
2. Col1a2 (Collagen alpha-2(I) chain):
 - Biological Process: Extra cellular matrix organization, collagen fibril organization.
 - Molecular Function: Structural constituent of collagen trimer.
 - Cellular Component: Extra cellula matrix, collagen-containing extra cellular matrix.
3. Col1a1 (Collagen alpha-1(I) chain):
 - Biological Process: Extracellular matrix organization, tissue development.
 - Molecular Function: Structural constituent of collagen trimer.
 - Cellular Component: Extra cellular matrix,collagen-containing extracellular matrix.
4. Cxcl13 (C-X-C motif chemokine 13):
 - Biological Process: Chemotaxis, immune response.
 - Molecular Function: Chemokine activity, cytokine receptor binding.
 - Cellular Component: Extracellular space, cell surface.

5.1.3 CONSTRUCTION OF PROTEIN-PROTEIN INTERACTION NETWORK:

The up-regulated and down-regulated genes were separately analyzed using the STRING database 10.5 to construct protein-protein interaction (PPI) networks. For the up-regulated genes, the network comprised 10 nodes and 14 edges, with an average node degree of 2.8. The average local clustering coefficient was 0.56, indicating a relatively interconnected network. Moreover, the PPI enrichment p-value was highly significant, indicating a strong enrichment of protein interactions beyond what would be expected by chance.

On the other hand, the down-regulated genes formed a smaller network with 4 nodes and 3 edges, resulting in an average node degree of 1.5. Despite the smaller size, the down-regulated gene network exhibited a higher average local clustering coefficient of 0.75, suggesting a more densely connected network. However, the PPI enrichment p-value, though significant, was comparatively lower than that of the up-regulated genes, indicating a lesser degree of enrichment in protein interactions.

Both networks were then exported to Cytoscape for further analysis, allowing for visualization and exploration of the protein interaction networks and their potential implications in the context of gene expression alterations associated with CSTB knockout in the cerebellum and granule neurons.

5.1.3.1 PROTEIN-PROTEIN INTERACTION OF UPREGULATED GENES:

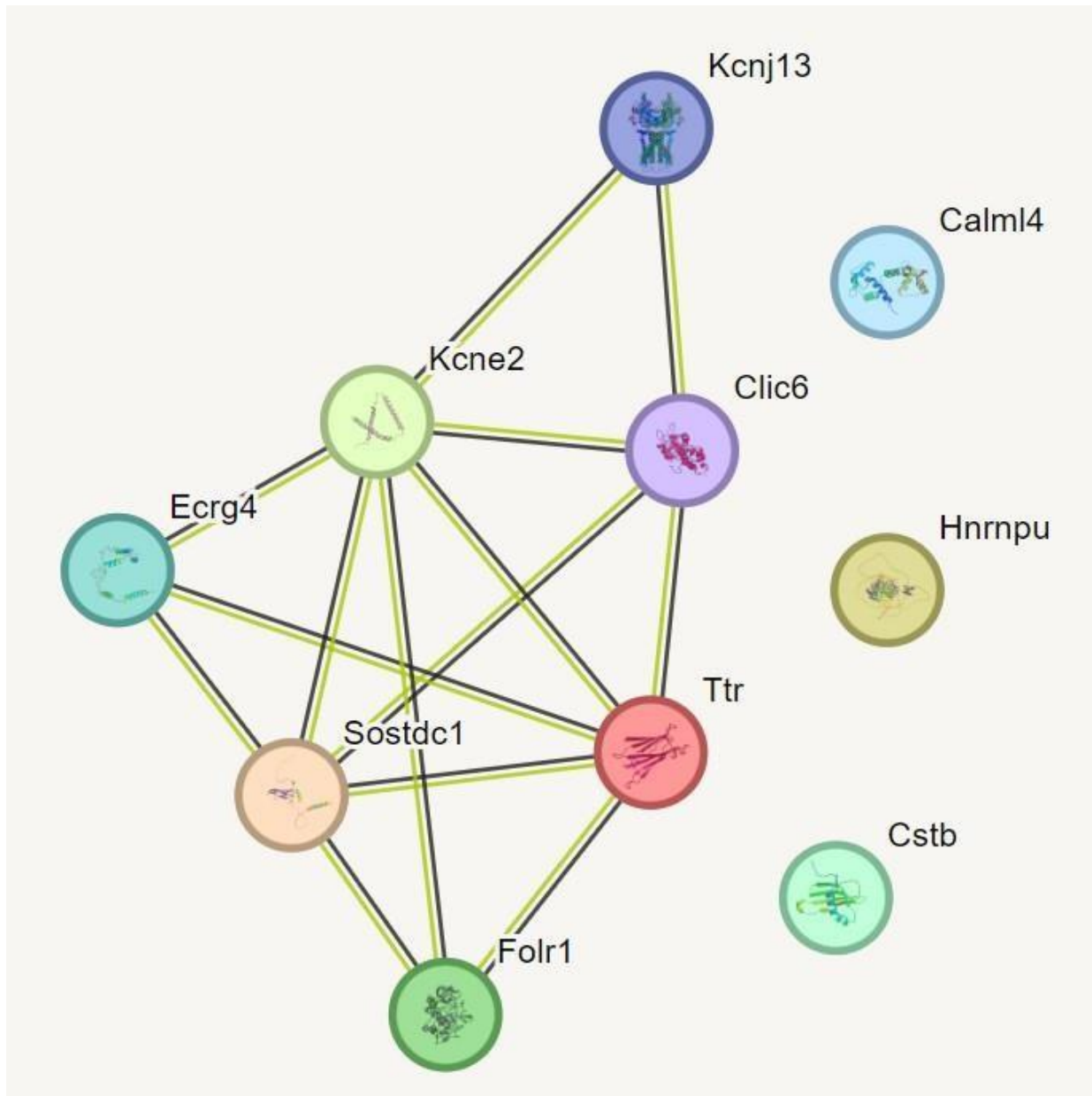


FIG 5.3

- KCNJ13: Inward rectifier potassium channel 13; Inward rectifier potassium channels are characterized by a greater tendency to allow potassium to flow into the cell than out of it. Their voltage dependence is regulated by the concentration of extracellular potassium; as external potassium is raised, the voltage range of the channel opening shifts to more positive voltages. The

inward rectification is mainly due to the blockage of outward current by internal magnesium. KCNJ13 has a very low single channel conductance, low sensitivity to block by external barium and cesium, and no dependence. (Hibino et al., 2010).

- KCNE2: Potassium voltage-gated channel subfamily E member 2; Ancillary protein that assembles as a beta subunit with a voltage-gated potassium channel complex of pore-forming alpha subunits. Modulates the gating kinetics and enhances stability of the channel complex. Assembled with KCNB1 modulates the gating characteristics of the delayed rectifier voltage-dependent potassium channel KCNB1. Associated with KCNH2/HERG is proposed to form the rapidly activating component of the delayed rectifying potassium current in the heart (IKr). May associate with KCNQ2 and/or KCNQ3 and modulate the native M-t (Abbott et al., 2002).
- ECRG4: Augurin; Probable hormone that may attenuate cell proliferation and induce senescence of oligodendrocyte and neural precursor cells in the central nervous system. ECRG4-induced senescence is characterized by G1 arrest, RB1 dephosphorylation and accelerated CCND1 and CCND3 proteasomal degradation. Belongs to the augurin family (Wang et al., 2015).
- SOSTDC1: Sclerostin domain-containing protein 1; May be involved in the onset of endometrial receptivity for implantation/sensitization for the decidual cell reaction. Enhances Wnt signaling and inhibits TGF- β signaling (Bysimilarity). Directly antagonizes activity of BMP2, BMP4, BMP6 and BMP7 in a dose-dependent manner; Belongs to the sclerostin family. (Avsian et al., 2002).
- FOLR1: Folate receptor alpha; Binds to folate and reduces folic acid derivatives and mediates delivery of 5-methyltetrahydrofolate and folate analogs into the interior of cells. Has high affinity for folate and folic acid analogs at neutral pH.

Exposure to slightly acidic pH after receptor endocytosis triggers a conformational change that strongly reduces its affinity for folates and mediates their release. Required for normal embryonic development and normal cell proliferation. Required for renal folate reabsorption.(Liang et al.,2016).

- CLIC6:Chloride intracellular channel protein 6; May insert into membranes and form chloride ion channels. May play a critical role in water-secreting cells, possibly through the regulation of chloride ion transport (By similarity). (Valenzuela et al.,1997).
- Ttr: Transthyretin; Thyroid hormone-binding protein. Probably transports thyroxine from the bloodstream to the brain. Transthyretin (TTR) is a tetrameric transport protein highly conserved through vertebrate evolution and synthesized in the liver, choroid plexus, and retinal pigment epithelium. TTR transports the thyroid hormone thyroxine and theretinol- binding protein (RBP) bound to retinol (vitamin A) Transthyretin (TTR) belongs to a group of proteins, which includes thyroxine-binding globulin and albumin, that bind to and transport thyroid hormones in the blood. TTR is also indirectly implicated in the carriage of vitamin A through the mediation of retinol-binding protein (RBP).(Soprano et al.,1985).
- HNRNPU: Heterogeneous nuclear ribonucleo protein U; DNA- and RNA-binding protein involved in several cellular processes such as nuclear chromatin organization, telomere-length regulation, transcription, mRNA alternative splicing and stability, Xist-mediated transcriptional silencing and mitotic cell progression. Plays a role in the regulation of interphase large-scale gene-rich chromatin organization through chromatin- associated RNAs (caRNAs) in a transcription- dependent manner, and there by maintains genomic stability (By

similarity). Required for the localization of the long non-coding Xist RN .(Tauler et al.,2004)

□ CALML4: Calmodulin-like protein 4. Diseases associated with CALML4 include Cutaneous Anthrax and Inhalation Anthrax. Among its related pathways are Alzheimer's disease and miRNA effects and Endometrial cancer. GeneOntology (GO) annotations related to this gene include calcium ion binding. Calmodulin-like proteins (CMLs) are calcium-sensing plant-specific proteins. Although CMLs have been identified in a few plants, they remained uncharacterized in leguminous crop plants. Canonical targets of calmodulin, such as myosin light-chain kinases and CaMKII, bind only to the Ca²⁺-bound protein, whereas some proteins, such as NaV channels and IQ- motif proteins, also bind to calmodulin in the absence of Ca²⁺ (Ghoneim et al.,2019)

- CSTB: Cystatin-B; This is an intracellular thiol proteinase inhibitor. The CSTB gene provides instructions for making a protein called cystatin B. This protein reduces the activity of (inhibits) enzymes called cathepsins. Cathepsins help break down certain proteins in the lysosomes, which are compartments in the cell that digest and recycle different types of molecules. Cystatin B is essential for proliferation and interneuron migration in individuals with EPM1 epilepsy. Genetic testing and the phenotype of Polish patients with Unverricht-Lundborg disease (EPM1). (Fidziańska et al.,2018).

5.1.3.2 PROTEIN- PROTEIN INTERACTION OF DOWNREGULATED GENES:

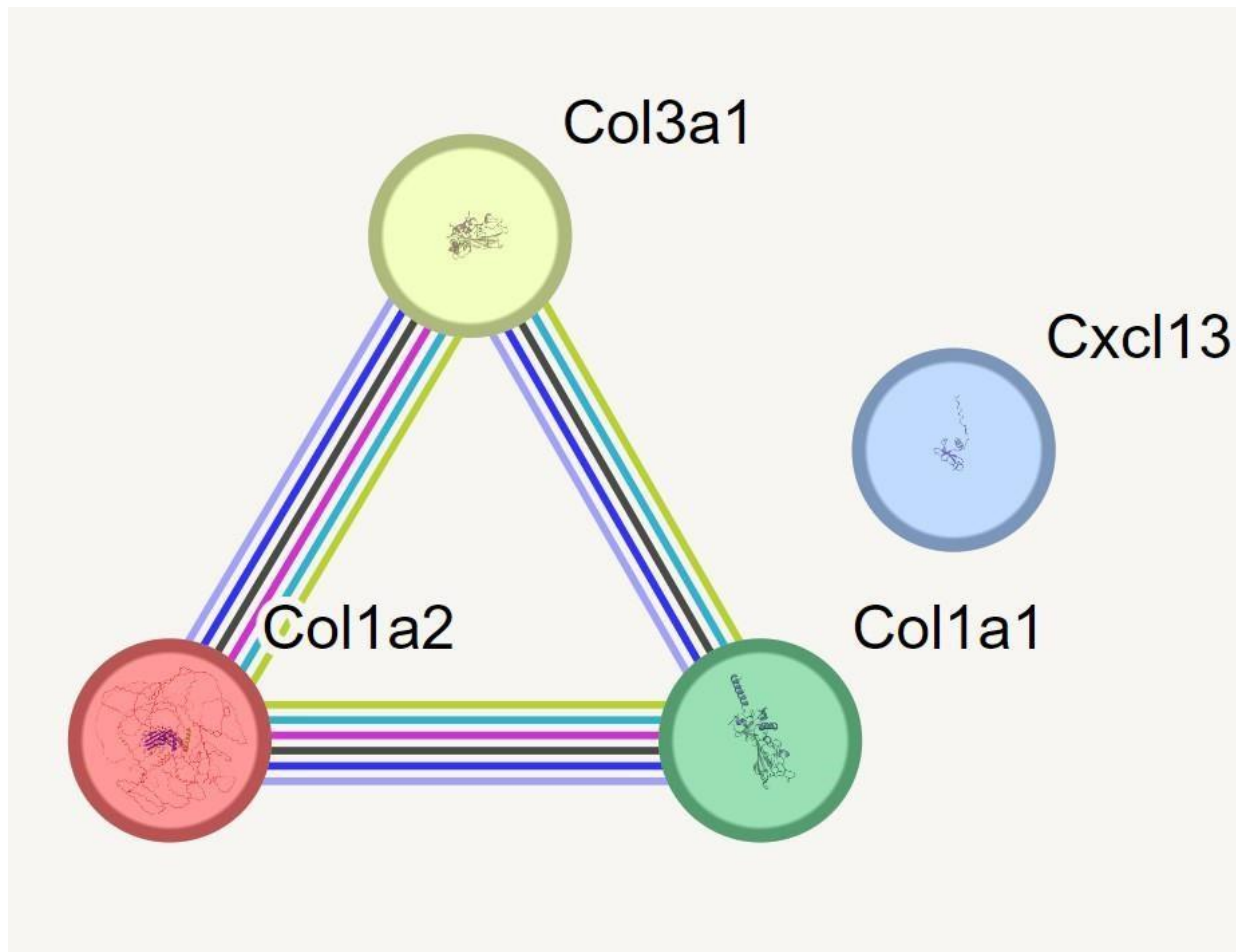


FIG 5.4

- COL3A1: Collagen alpha-1(III) chain; Collagen type III occurs in most soft connective tissues along with type I collagen. Involved in regulation of cortical development. Is the major ligand of ADGRG1 in the developing brain and binding to ADGRG1 inhibits neuronal migration and activates the RhoA pathway by coupling ADGRG1 to GNA13 and possibly GNA12.(Smith et al.,2023).

- COL1A2: Collagen alpha-2(I) chain; Type I collagen is a member of group I collagen (fibrillar forming collagen); Belongs to the fibrillar collagen family. A component of type I collagen called the pro- α 1(I) chain is produced from the COL1A1 gene. Collagens begin as rope-like procollagen molecules that are each made up of three chains. Type I collagen is composed of two pro-1(I) chain and one pro- α 2(I) chain (which is produced from the COL1A2 gene). (Smith et al.,2023).
- COL1A1:Collagen alpha-1(I) chain; Type I collagen is a member of group I collagen (fibrillar forming collagen); Belongs to the fibrillar collagen family. A component of type I collagen called the pro- α 1(I) chain is produced from the COL1A1 gene. Collagens begin as rope-like procollagen molecules that are each made up of three chains. Type I collagen is composed of two pro-1(I) chain and one pro- α 2(I) chain (which is produced from the COL1A2 gene). The COL1A1 gene produces a component of type I collagen, called the pro- α 1(I) chain. This chain combines with another pro- α 1(I) chain and also with a pro- α 2(I) chain (produced by the COL1A2 gene) to make a molecule of type I procollagen (Smith et al.,2023).
- CXCL13: C-X-C motif chemokine 13; Strongly chemotactic for B-lymphocytes, weakly for spleen monocytes and macrophages but no chemotactic activity for granulocytes. Binds to BLR1/CXCR5. May play a role in directing the migration of B- lymphocytes to follicles in secondary lymphoid organs. CXCL13 chemokine is a novel player in multiple myeloma osteolytic micro environment, M2 macrophage polarization, and tumor progression. CXCL13 is elevated in inflammatory bowel disease in mice and humans and is implicated in disease pathogenesis (Smith et al.,2023).

5.1.4 NETWORK ANALYSIS IN CYTOSCAPE BY CYTOHUBBA:

The cytoHubb a plug in with in Cytoscape was utilized to predict key genes within the up-regulated gene network based on their node degree and clustering coefficient. This analysis aimed to identify hubgenes that play crucial roles in the network structure and potentially in the biological processes associated with the gene expression alterations observed in CSTB knockout mice.

Following the identification of hub genes, further analysis was conducted to explore their functional annotations and involve mention relevant pathways. This comprehensive approach allowed for the prioritization of key genes and provided insights into their potential roles in mediating the observed changes in gene expression, particularly in relation to early synaptic changes and inflammation in the cerebellum and granule neurons of CSTB knockout mice.

5.1.4.1 UPREGULATED NETWORK HUB GENES (a) TOP 15 HUBGENES AND (b) PATHWAY ANALYSIS OF HUB GENES:

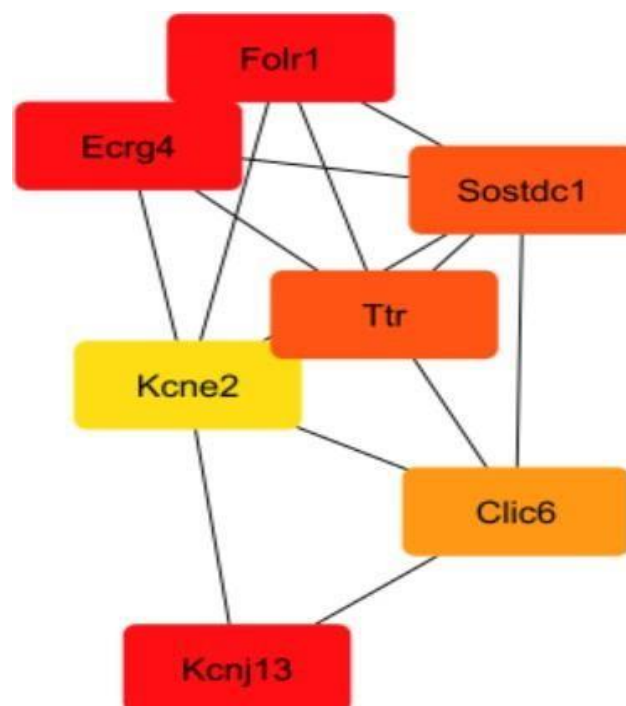


FIG 5.5

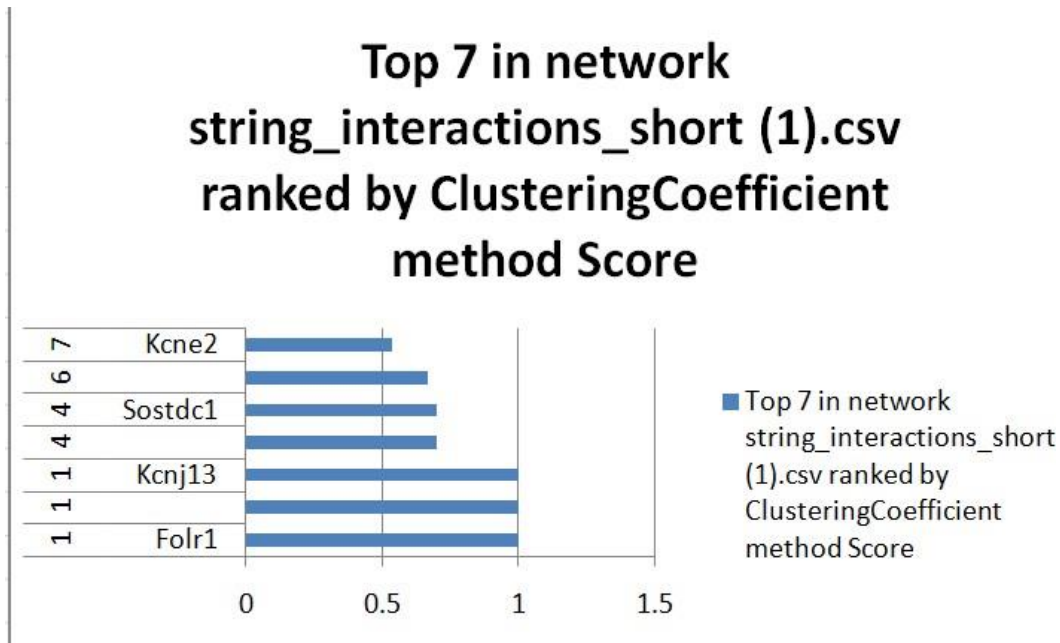


FIG 5.6

A comprehensive analysis was conducted to identify the upregulated network of hub genes, focusing on the top 15 genes derived from string interaction ranked by clustering coefficient method score. Among these, genes including FOLR1, ECRG4, SOSTDC1, TTR, KCNE2, CLIC6, and KCNJ13 were selected for pathway analysis due to their significance. Through pathway analysis, a deeper understanding of the biological processes associated with these hub genes was sought.

FOLR1, also known as Folate Receptor 1, is crucial for folate uptake and has implications in cancer and neurological disorders. ECRG4, or Esophageal Cancer Related Gene 4, has been linked to tumor suppression and is implicated in various cancers. SOSTDC1, or Sclerostin Domain Containing 1, plays a role in bone metabolism and is associated with bone diseases like osteoporosis. TTR, or Transthyretin, is involved in transporting thyroid hormones and vitamin A, with mutations leading to amyloidosis. KCNE2, a Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 2, is involved in cardiac repolarization and arrhythmias. CLIC6, or Chloride Intracellular Channel 6, is associated with ion

transport and cellular signaling. KCNJ13, a member of the Potassium Inwardly Rectifying Channel Family, is linked to retinal function and diseases like snowflake vitreoretinal degeneration.

Pathway analysis provides insights into the intricate molecular mechanisms these genes are involved in, shedding light on potential therapeutic targets and disease pathways. By understanding their interactions within biological networks, we can uncover novel avenues for further research and therapeutic interventions in various diseases and physiological processes.

- FOLR1 Folate Receptor Alpha, encoded by the FOLR1 gene, plays a vital role in folate metabolism by facilitating the uptake of 5-methyltetrahydrofolate and folate analogs into cells. With its high affinity for folate and folic acid analogs under neutral pH conditions, FOLR1 undergoes a pH-dependent conformational change post-endocytosis, releasing bound folates. Crucially, it is indispensable for normal embryonic development, cell proliferation, and renal folate reabsorption. This receptor's functions are pivotal in maintaining folate homeostasis, highlighting its significance in both physiological processes and potential implications in diseases associated with folate deficiency or dysregulation (Liang et al., 2016).
- ECRG4, known as Augurin, likely functions as a hormone, exerting effects on cell proliferation and senescence in the central nervous system. It is implicated in attenuating cell proliferation and inducing senescence in oligodendrocyte and neural precursor cells. ECRG4-induced senescence is marked by G1 arrest, RB1 dephosphorylation, and accelerated proteasomal degradation of CCND1 and CCND3. Belonging to the augurin family, ECRG4's role in regulating cellular processes underscores its importance in CNS development and potential implications for neurological disorders. Understanding its mechanisms may offer insights into therapeutic strategies targeting cell proliferation and senescence-related pathways in neurological conditions (Wang et al., 2015).

- SOSTDC1, or Sclerostin Domain-Containing Protein 1, potentially plays a critical role in the initiation of endometrial receptivity for implantation and sensitization for the decidual cell reaction. It functions by enhancing Wnt signaling while inhibiting TGF-beta signaling, suggesting its involvement in intricate regulatory pathways crucial for reproductive processes. Additionally, SOSTDC1 acts as a direct antagonist of BMP2, BMP4, BMP6, and BMP7 in a dose-dependent manner, highlighting its significance in modulating bone morphogenetic protein signaling. As a member of the sclerostin family, SOSTDC1 likely contributes to various physiological processes beyond its well-known role in bone metabolism, implicating its potential relevance in reproductive health and pathological conditions related to impaired implantation or decidualization (Avsian et al., 2002).
- Transthyretin (TTR) serves as a crucial thyroid hormone-binding protein, facilitating the transportation of thyroxine from the blood stream to the brain. This tetrameric transport protein is synthesized in various tissues including the liver, choroid plexus, and retinal pigment epithelium. Alongside its role in thyroid hormone transport, TTR also binds retinol-binding protein (RBP), aiding in the transportation of retinol (vitamin A). Belonging to a protein group that includes thyroxine-binding globulin and albumin, TTR plays a significant role in the circulation and delivery of thyroid hormones throughout the body. Furthermore, its indirect involvement in vitamin A transport underscores its multifaceted role in physiological processes beyond thyroid hormone regulation (Soprano et al., 1985).
- KCNE2, a member of the Potassium Voltage-Gated Channel Subfamily E, acts as an ancillary protein in conjunction with voltage-gated potassium channel complexes, serving as a beta subunit. Its primary role involves modulating the gating kinetics and enhancing the stability of the channel complex. When assembled with KCNB1, it influences the gating characteristics of the delayed rectifier voltage-dependent potassium channel KCNB1. In cardiac tissue, KCNE2 is associated with KCNH2/HERG, forming the rapidly activating

component of the delayed rectifying potassium current (IKr). Additionally, it may associate with KCNQ2 and/or KCNQ3, potentially modulating native M-type potassium currents. These interactions highlight KCNE2's significance in regulating potassium channel activity, particularly in cardiac physiology (Abbott et al., 2002).

- KCNJ13, also known as Inward Rectifier Potassium Channel 13, belongs to a class of channels allowing more potassium influx than efflux, regulated by extracellular potassium concentrations. Elevated extracellular potassium shifts the channel's voltage range for opening to more positive voltages. The inward rectification is primarily caused by internal magnesium block in outward current. KCNJ13 exhibits a notably low single channel conductance, limited sensitivity to blockage by external barium and cesium, and lacks dependency on external Sodium and potassium for activation. These characteristics underscore its role in potassium homeostasis and cellular excitability regulation (Hibino et al., 2010).
- CLIC6, identified as Chloride Intracellular Channel Protein 6, exhibits potential membrane insertion capabilities to form chloride ion channels. Its speculated involvement in water-secreting cells suggests a critical role in regulating chloride ion transport, though further research is needed to elucidate its precise mechanisms. By potentially modulating chloride ion movement across membranes, CLIC6 may influence cellular processes related to osmotic regulation and ion balance. Its similarity to other chloride intracellular channels hints at conserved functions in cellular physiology. Understanding CLIC6's role in chloride ion transport could offer insights into its contribution to various physiological processes and its potential as a therapeutic target in conditions involving dysregulated ion transport (Valenzuela et al., 1997).

5.1.4.2 DOWNREGULATED NETWORK HUB GENES (a) TOP 4 HUB GENES (b) PATHWAY ANALYSIS OF HUB GENES:

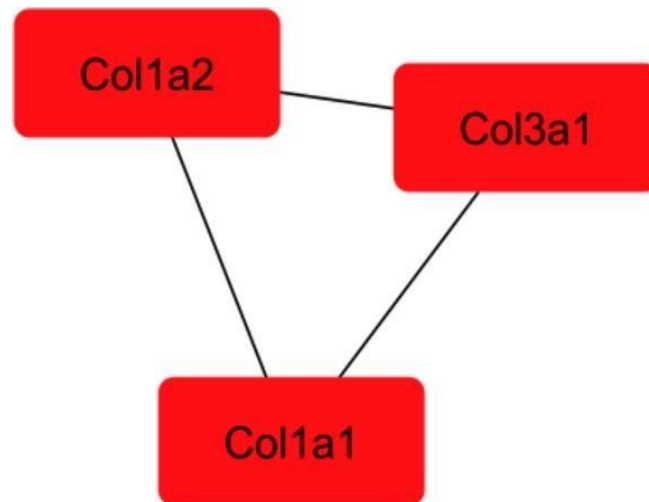


FIG 5.7

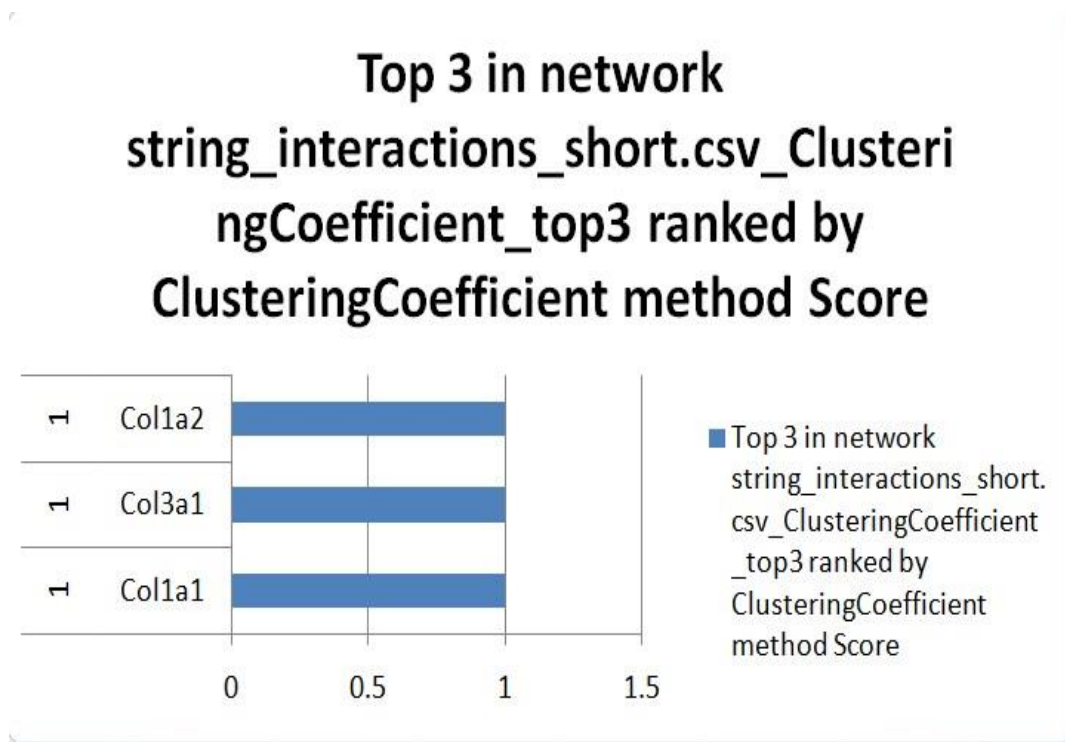


FIG 5.8

Analyzing the downregulated network of hubgenes, the focus was narrowed to the top 3 genes derived from STRING interactions, ranked by clustering coefficient method score. Among these, COL1A2, COL3A1, and COL1A1 were selected for pathway analysis due to their significance in extracellular matrix composition and structural integrity.

COL1A2 and COL1A1 encode collagen type I alpha chains, which are fundamental components of the extracellular matrix in various tissues, providing structural support and integrity. COL3A1 encodes collagen type III alpha chain, another crucial component of the extracellular matrix, particularly abundant in tissues requiring flexibility and tensile strength, such as skin, blood vessels, and organs.

Pathway analysis of these hub genes elucidates their involvement in processes crucial for tissue structure and function, including collagen biosynthesis and extracellular matrix organization. Dysregulation of these pathways can lead to various pathological conditions characterized by altered tissue integrity and function, such as fibrosis and connective tissue disorders. Understanding the molecular mechanisms underlying the downregulation of these hub genes provides valuable insights into disease mechanisms and potential therapeutic targets aimed at restoring tissue homeostasis and function.

- COL1A2, encoding the Collagen Alpha-2(I) chain, is integral to the formation of Type I collagen, a major member of the fibrillar collagen family essential for structural support in various tissues. Within the collagen synthesis process, the pro-alpha 2(I) chain, derived from COL1A2, combines with two pro-alpha 1(I) chains, synthesized from COL1A1, forming rope-like procollagen molecules. These procollagen molecules undergo further processing to generate mature Type I collagen fibers. Type I collagen's structural integrity is vital for tissue strength and resilience, with implications in bone, skin, tendon, and other connective tissues. Understanding the role of COL1A2 in collagen assembly provides insights into the molecular basis of tissue structure and function,

Offer in venues for research into collagen- related disorders and therapeutic interventions aimed at restoring tissue integrity(Smith et al.,2023).

- COL3A1 encodes the Collagen Alpha-1(III) chain, a crucial component found alongside Type I collagen in most soft connective tissues. Its role extends beyond structural support to include regulation of cortical development. Notably, COL3A1 serves as a primary ligand for ADGRG1 in the developing brain. Binding of COL3A1 to ADGRG1 inhibits neuronal migration and activates the RhoA pathway by facilitating coupling of ADGRG1 to GNA13 and possibly GNA12. These interactions highlight the diverse functions of COL3A1 beyond its structural role, implicating it in neuro developmental processes and signaling pathways critical for brain development and function. Understanding its involvement in these processes provides insights into neurological disorders and potential therapeutic targets(Smith et al.,2023).
- COL1A1 encodes the Collagen Alpha-1(I) chain, a vital constituent of Type I collagen, a prominent member of the fibrillar collagen familyessentialfortissue integrity. Within collagen synthesis, COL1A1 produces the pro-alpha1(I) chain, which forms rope-like procollagen molecules. These procollagen molecules consist of two pro-alpha1(I) chains and one pro-alpha2(I) chain, produced by COL1A2. Through intricate molecular assembly, these chains combine to form mature Type I collagen fibers, crucial for the structural integrity of tissues like bone, skin, and tendons. Understanding COL1A1's role in collagen synthesis provides insights into connective tissue biology and disorders affecting collagen structure and function(Smith et al.,2023).

CHAPTER 6

SUMMARY AND CONCLUSION

6.1 SUMMARY:

The thesis delves into understanding how the absence of CSTB affects gene expression in the cerebellum and granule neurons, particularly regarding early synaptic changes and inflammation.

Employing sophisticated bioinformatics tools like GEO2R, STRING database, and Cytoscape, it unveiled significant hit in gene expression, notably with 128 genes upregulated and 44974 genes down-regulated. Using DAVID for functional enrichment analysis, the study illuminated associations with critical biological processes and pathways.

Moreover, through protein-protein interaction network analysis, it pinpointed hubgenes, suggesting their pivotal roles in orchestrating these changes. In essence, these findings provide valuable insights into the molecular mechanisms underlying synaptic alterations and inflammation due to CSTB deficiency, opening avenues for further exploration into neurological disorders linked with cerebellar dysfunction.

6.2 CONCLUSION:

In this study, gene expression data in Progressive Myoclonus Epilepsy of Unverricht-Lundborg type (EPM1) were analyzed using bioinformatics tools to gain insights into the underlying molecular mechanisms of the disease. EPM1 is an inherited neurodegenerative disorder characterized by myoclonus, seizures, and

ataxia, primarily caused by mutations in the CSTB gene. Through comprehensive analysis, both upregulated and down regulated genes were identified, shedding light on the transcriptional changes associated with EPM1 pathology.

Functional and enrichment analyses using the DAVID tool uncovered the significant involvement of these genes in various biological, molecular, and cellular processes, providing crucial insights into the molecular pathways disrupted in EPM1. Furthermore, protein-protein interaction (PPI) network analysis identified hub genes such as Folr1, Ecr4, Sostdc1, Ttr, Kcne2, Clic6, and Kcnj13. Each of these hub genes was associated with specific functions relevant to EPM1 pathology, including cerebral folate transport deficiency, esophageal cancer suppression, sclerostin family, transthyretin transport, potassium channel regulation, and chloride intracellular channel activity.

Of particular note, Folr1 was previously implicated in the disease, confirming its importance, while the emergence of Ecr4, Sostdc1, Ttr, Clic6, Kcne2, and Kcnj13 as potential candidates through the project's analysis provides novel insights into their roles in cerebellar ataxia associated with EPM1. Overall, this study enhances our understanding of the molecular underpinnings of EPM1 and identifies potential therapeutic targets for further investigation and development.

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