Investigation of novel genetic causes of epilepsy

Chloe Lawrence

Thesis submitted to the University of Ottawa in partial fulfillment of the requirements for the Master's degree in Biochemistry with a Specialization in Human and Molecular Genetics

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

© Chloe Lawrence, Ottawa, Canada, 2025

Abstract

Epilepsy is a common neurological disorder with many underlying causes. The single gene contributions to the epilepsies have historically been considered rare and a challenge to diagnose. And yet, a molecular diagnosis in patients with epilepsy can be important for their treatment and management plans as well as for family planning. With the advent of next generation sequencing in the early-mid 2010s, there was an increase in the identification of disease-causing genes and variants in relation to epilepsy. Despite these advances, 50-80% of individuals with epilepsy do not have a genetic diagnosis after initial NGS testing. A straightforward and practical approach to improve the diagnostic rate is by ***re***analysis of the exome data after a period of time has elapsed. Studies in rare disease have shown that reanalysis can increase the number of diagnoses by 5-15%. The second chapter of this thesis demonstrates the benefit of using exome reanalysis for diagnostic purposes and for novel gene identification. The reanalysis of a cohort of 20 patients resulted in a 10% diagnostic rate (2/20) and candidate variants identified in an additional 35% (7/20) of cases. The third chapter of this project is a study of a candidate gene, *UBE2R2,* in a patient with genetic epilepsy and global developmental delay. To date, this gene has not been associated with disease or any form of epilepsy. Trio exome sequencing and research reanalysis identified a variant (c.21dup p.(Ser8GlnfsTer56)) in *UBE2R2* that was rare (absent from gnomAD) and *de novo* and therefore *UBE2R2* was considered a candidate gene for epilepsy and developmental delay. GeneMatcher was used to "match" with others interested in this gene, but no matches were, at this time, forthcoming. Functional studies were then performed that included assessment of cell cycle progression, cell growth, apoptosis, mitophagy, and interrogation of p53 and the β-catenin pathway. Results show that the expression of UBE2R2 was significantly decreased, mRNA expression of only one other E2 ubiquitin conjugating enzyme (*UBE2R1)* was increased, and expression of mRNA as well as protein expression of one of three Wnt target genes (*AFF3*) was significantly decreased in patient cells. The other functional work investigating proteins and pathways suspected to be impacted by the *UBE2R2* variant (cell cycle progression, cell growth, apoptosis, mitophagy, p53 and β-catenin) did not demonstrate any statistically significant differences. Thus, while the functional assays performed were not able to provide significant evidence in favor of a disease-gene association, it also does not rule out *UBE2R2* as the explanation for the phenotype of the patient. Taken together, these findings illustrate the benefits of whole exome sequencing and subsequent reanalysis for patients with

genetic epilepsy that remain without a diagnosis and demonstrates the tools and methods available by which compelling candidates in putative novel disease genes can be further interrogated.

Co-Authorship Statement

I participated in the design and execution of all experiments presented in this thesis with the following exceptions:

In Chapter Two, the demographic information collection was performed in part by Andrea Goodman at Care4Rare.

In Chapter Three, the final step of flow cytometry (running the samples through the flow cytometer) resulting in Figure 3-4A was completed by Dr. Vera Tang.

Acknowledgements

This thesis, and the work completed over the course of my degree, would not have been possible without the support of the many people in my life.

To start, I would like to express my sincere thanks and deepest gratitude to my supervisors, Dr. David Dyment and Dr. Kristin Kernohan. Your knowledge, support, patience, insight and overall guidance throughout this process has been invaluable to me. You have both taught me how to become a scientist, to think outside the box, challenged me to find solutions to any difficult situation and have allowed me to learn both independently and with your help. Thank you for being such skilled, inspiring mentors these past few years.

I would also like to thank the members of my thesis advisory committee, Dr. Melanie Lacaria and Dr. Steffany Bennett, for their continued support, constructive feedback, and encouragement throughout this project. Your expertise was an incredible advantage during my graduate studies.

Furthermore, I would like to thank the lab members and collaborators over the years. Xueqi Wang, who taught me many of the lab skills I have used throughout my research and whose help in analysis of my results was invaluable. Wendy Mears, your help with cell culture was a tremendous asset for my lab work. Dr. Vera Tang, your support in troubleshooting and finalizing experiments for flow cytometry was indispensable. Giulia Del Gobbo, Aren Marshall, Alexanne Cuillerier, Minh Hieu Tran and Leanne de Kock thank you for your continued help as I learned new techniques, asked for help with furthering my understanding and supporting me throughout my degree.

To my friends, you’ve been some of my biggest champions, always understanding when I would be unavailable or hiding away to work for long periods of time. Your continued support, friendship, advice, and adjusting the things we do so I can have breaks to spend time with you is so greatly appreciated. I value your friendships and appreciate all of you so much.

Finally, I would like to thank my family. My amazing parents, you have shaped the person I am, and this would not have been possible without you. The endless support and encouragement, lending an ear even though you don’t understand what I’m talking about, and the unconditional love you’ve given me will never be forgotten. To the rest of my immediate and extended family, I’m so grateful to have this amazing support system and my gratitude is endless for your encouragement.

Abbreviations

Abbreviation	Meaning
μg	Microgram
μL	Microliter
μM	Micromolar
°C	Degrees Celsius
7-AAD	7-aminoactinomycin D
ACMG	American College of Medical Genetics and Genomics
AFF3	ALF transcription elongation factor 3
β-catenin	Catenin beta-1
β-TrcP	Beta-transducin repeat containing protein
BrdU	Bromodeoxyuridine
cDNA	Complementary DNA
C4R	Care4Rare
CADD	Combined annotation dependent depletion
CDC34	Cell division cycle 34 ubiquitin conjugating enzyme
CHD5	Chromodomain-helicase-DNA-binding protein 5
CHEO	Children's Hospital of Eastern Ontario
CHRNA4	Cholinergic receptor nicotinic alpha 4 subunit
CUL4B	Cullin 4B ubiquitin ligase
DNA	Deoxyribonucleic acid
DZ	Dizygotic
Exac	Exome aggregation consortium database
FBS	Fetal bovine serum
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDD	Global developmental delay
GEFS+	Genetic epilepsy with febrile seizures plus
GLUT1	Glucose transporter protein type 1
gnomAD	Genome Aggregation Database
GUS	Gene of uncertain significance

ILAE	International League Against Epilepsy
JAKMIP1	Janu kinase and microtubule interacting protein 1
kDa	Kilodalton
KINSSHIP	Horseshoe kidney, Nievergelt.Savarirayan type of mesomelic dysplasia, seizures, hypertrichosis, intellectual disability, and pulmonary involvement
KLH20	Kelch like family member 20
LC3B	Microtubule-associated protein 1 light chain 3B
LEF1	Lymphoid enhancer binding factor 1
LP	Likely pathogenic
MADD	MAP kinase activating death domain
mAMP	Milliamp
mL	Milliliter
mRNA	Messenger ribonucleic acid
MYC	MYC proto-oncogene, bHLH transcription factor
MZ	Monozygotic
NEDD4-2	Neural precursor cell expressed developmentally down-regulated 4-like
NGS	Next-generation sequencing
OPA1	Optic atrophy 1
p53	Tumor protein p53
p62	Sequestosome 1
P	Pathogenic
PBS	Phosphate buffered saline
PINK1	PTEN-induced putative kinase 1
pLI	Probability of loss of function intolerance
PVDF	Polyvinylidene fluoride
RD	Rare disease
RGD	Rare genetic disease
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid

RPMI	Roswell Park memorial institute medium
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SATB2	Special AT-rich sequence-binding protein 2
SBNO1	Strawberry notch homolog 1
SCF	Skp1-cullin-F-box protein
SCN1A	Sodium voltage-gated channel alpha subunit 1
SMYD3	SET and MYND domain-containing protein 3
SSC	Side scatter
STXBP1	Syntaxin-binding protein 1
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TRIO	Trio rho guanine nucleotide exchange factor
UBE2G2	Ubiquitin conjugating enzyme E2 G2
UBE2G1	Ubiquitin conjugating enzyme E2 G1
UBE2R1 (CDC34)	Cell division cycle 34, ubiquitin conjugating enzyme
UBE2R2	Ubiquitin conjugating enzyme E2 R2
USP9X	Ubiquitin specific peptidase 9 X-linked
VUS	Variant of uncertain significance
Wnt	Wingless-related integration site
WES	Whole exome sequencing
ZBTB7A	Zinc finer and BTB domain-containing protein 7A

Table of Contents

Abstract....ii

Co-Authorship....iv

Acknowledgements....v

List of Abbreviations....vii

List of Figures....xii

List of Tables....xiv

Chapter 1: Thesis overview and background information....1

1.1 Thesis Overview....1

1.1.1 Chapter one: introduction....1

1.1.2 Chapter two: exome reanalysis for patients with epilepsy and intellectual disability is an effective diagnostic tool....1

1.1.3 Chapter three: functional studies for a novel *UBE2R2* variant detected in a patient with seizures and intellectual disability....2

1.1.4 Chapter four: discussion and future directions....2

1.2 Introduction to epilepsy and the importance of genetic diagnoses....2

1.2.1 Genetics of epilepsy....3

1.2.2 A brief history in diagnosing genetic epilepsy....5

1.2.3 Whole exome sequencing for epilepsy patients....7

1.*2*.4 Whole exome sequencing reanalysis for epilepsy patients....7

1.2.5 Care4Rare consortium and Care4Rare-SOLVE....8

1.2.6 Steps to confirm diagnosis in putative novel disease genes....9

Chapter 2: Exome reanalysis for patients with epilepsy and intellectual disability is an effective diagnostic tool....11

2.1 Introduction....11

2.2 Materials and methods....13

2.2.1 Study design....13

2.2.2 Data collection....13

2.2.3 Data transfer and reprocessing 13
2.2.4 Exome sequencing and data reanalysis 13
2.3 Results 14
2.3.1 Patient demographic, previous clinical testing and patient phenotype 14
2.3.2 Reanalysis results 16
2.3.3 Testing strategy and time since reanalysis 24
2.4 Discussion 26
Chapter 3: Functional studies for a novel *UBE2R2* variant detected in a patient with rare genetic epilepsy and intellectual disability 30
3.1 Introduction 30
3.1.2 UBE2R2 gene and protein 32
3.1.3 Ubiquitin-proteasome degradation pathway 32
3.1.4 Pathways affected by UBE2R2 33
3.1.5 Literature review to support the downstream effects of the *UBE2R2* variant as the cause for epilepsy 34
3.2 Materials and methods. 35
3.2.1 Lymphoblast cell culture 35
3.2.2 Cell harvesting 36
3.2.3 Quantitative reverse polymerase chain reaction (RT-qPCR) 37
3.2.4 Western blot 37
3.2.5 Cell growth curve 38
3.2.6 Flow cytometry 39
3.2.7 Cell treatment 39
3.3 Results 40
3.3.1 mRNA and protein expression of E2 ubiquitin conjugating enzymes 40
3.3.2 Cell growth and cell cycle analysis 44
3.3.3 Interactions with proteins of interest and downstream pathways 47
3.3.4 Degradation pathways: apoptosis and mitophagy 51
3.4 Discussion 55

3.5 Supplemental figures 57

3.6 Supplementary methods 63

3.6.1 Western blot 63

Chapter 4: Discussion and future directions 67

4.1 Discussion 67

4.2 Future directions and remaining questions 68

4.3 Conclusions 70

References 72

List of Figures

Figure 1-1 Care4Rare solve workflow………………………………………………………….....9

Figure 2-1 Patient demographic and clinical presentation……………………………….…………15

Figure 2-2 Candidate identification and solve status for the cohort of exome sequencing reanalysis cases (n=20) ………………………………………………………………………….24

Figure 2-3 Testing strategy and time since initial testing……………………………………..26

Figure 3-1 RT-qPCR for analysis of mRNA expression of *UBE2R2* and three other E2 ubiquitin conjugating enzymes (*UBE2R2, UBE2R1, UBE2G1*, and *UBE2G2*) demonstrates only *UBE2R1* mRNA expression has been affected in patient cells……………………………………………41

Figure 3-2. Western blot to analyze expression of E2 ubiquitin conjugating enzymes is unaffected by the identified *UBE2R2* variant.……………….……………………………...……42

Figure 3-3 Growth curve of patient and control cells demonstrates no significant difference in cell growth between patient and control lymphoblast cells……………………………………...45

Figure 3-4 Progression through cell cycle phases is unchanged between patient and control samples……………………………………………………………………………………...46

Figure 3-5 Western blot to analyze protein expression of β-catenin shows no difference in expression.……………………………………………………………………………………47

Figure 3-6 mRNA and protein expression of Wnt target genes demonstrates significant differences in only *AFF3* expression……………………………………………….……….49

Figure 3-7 Western blot to analyze protein expression of p53 shows no difference in expression between control and patient samples………………………………………………….……51

Figure 3-8 Apoptosis and mitophagy measured through western blotting of key proteins does not show a significant impact on the degradation pathways…………………………………………54

Supplemental Figure 3-1 Western blot replicates for E2 ubiquitin conjugating enzymes............57

Supplemental Figure 3-2 Western blot replicates for β -catenin...59

Supplemental Figure 3-3 Western blot replicates of Wnt target genes..59

Supplemental Figure 3-4 Western blot replicates of p53..61

Supplemental Figure 3-5 Replicates of western blots detecting key apoptosis proteins..............61
Supplemental Figure 3-6 Replicates of western blots detecting LC3B...62

List of Tables

Table 2-1 Exome reanalysis with cohorts with overlapping phenotypes to the reanalysis cohort presented (rare disease, neurodevelopmental disorders, epilepsy, intellectual disability)...……..25

Table 2-2 Re-interpretation of variants identified in initial testing……………………….……..30

Table 2-3 Candidate information……………………………………...…………………....34

Supplemental Table 3-1: Optimized antibody dilution used for western blot experiments.…….76

Chapter 1 Thesis overview and background

1.1 Thesis overview

The overarching objective of this thesis was to identify potential candidate variants and genes for individuals who present with epilepsy and development delay by using exome reanalysis as well as to further interrogate one such candidate gene with functional assays to demonstrate any evidence of causality.

1.1.1 Chapter one: introduction

Chapter one will provide background information for key concepts that will permit a thorough comprehension of the genetics of epilepsy. This will include historical aspects of genetics in addition to the current status of genetic testing for epilepsy. This chapter will also present a brief overview of the aims in each chapter. Much of this work was included, in part, in a book chapter co-written by myself and supervisors (Lawrence et al., 2024).

1.1.2 Chapter two: exome reanalysis for patients with epilepsy and intellectual disability and intellectual disability is an effective diagnostic tool

Chapter two describes whole exome sequencing reanalysis in an epilepsy cohort. For this chapter, I reanalyzed 20 exomes for patients that presented with epilepsy as well as developmental delay and/or intellectual disability and lacked a genetic diagnosis. Broadly, I followed the reanalysis protocol described by Hartley et al., 2022, where filtering protocols were applied and variants in known genes were re-analyzed first, followed by analysis into variants of genes of uncertain significance (i.e., genes not yet associated with disease). Both genetic diagnoses and the identification of candidate variants were highlighted, leading me to the conclusion that this is a technique that should be implemented routinely for patients that remain without a genetic diagnosis. This work was subsequently included in a cohort of 87 individuals that was submitted for publication. The manuscript is currently in revisions (Cuillerier et al., 2025).

1.1.3 Chapter three: functional studies for a novel *UBE2R2* variant detected in a patient with rare genetic epilepsy and intellectual disability

Chapter three is focused on a novel variant in *UBE2R2* that was detected through exome reanalysis in a patient presenting with seizures and developmental delay just prior to the start of my thesis. This chapter describes the functional experiments pursued to accumulate evidence to demonstrate that a variant in a putative novel disease gene is pathogenic. It has been established that UBE2R2 interacts with numerous proteins, many of which are involved in key pathways. Many of these pathways and cellular functions implicated by these interactions have all been found to contribute to epilepsy when dysregulated. As such, it was hypothesized that the detected variant may be causing significant molecular defects to cause the phenotype of the patient. I performed a series of functional assays that examined these pathways and potentially impacted molecules described in order to determine if this was the case.

1.1.4 Chapter four: discussion and future directions

Chapter four will summarize the results from chapters two and three in a succinct manner, in addition to providing a discussion as to the meaning of these results in a broader context. Furthermore, this chapter will discuss the future directions and next steps to be taken with regards to both the exome reanalysis cases and the individual with the variant identified in *UBE2R2*.

1.2 Introduction: epilepsy and the importance of genetic diagnoses

The International League Against Epilepsy (ILAE) has defined epilepsy as (i) the occurrence of at least two unprovoked seizures 24 hours apart, (ii) an unprovoked seizure leading to increased risk of experiencing another, or (iii) the diagnosis of an epilepsy syndrome (Fisher et al., 2014). Epilepsy is a relatively common condition with an accepted prevalence of 5-8 per 1000 individuals and an overall lifetime risk of 3.0% (Myers et al., 2019; Rastin et al., 2023; Tellez-Zenteno et al., 2004). The underlying cause of epilepsies are heterogeneous, with autoimmunity, trauma, tumors, infection, and stroke responsible for the seizures in many individuals (Thomas & Berkovic, 2014). However, it has been estimated that 70%, if not more, of all epilepsies have a

genetic contribution to their underlying etiology (Hildebrand et al., 2013; Li et al., 2019; Rastin et al., 2023; Thomas & Berkovic, 2014). ILAE states that these genetic epilepsies include epilepsies with monogenic and/or complex inheritance (Fisher et al., 2014; Rastin et al., 2023; Scheffer et al., 2017). Rare monogenic forms of epilepsies, or epilepsies caused by a genetic variant in a single gene, account for a portion of the genetic epilepsies with the majority hypothesized to be the result of complex or multifactorial causes. There is evidence suggesting that genetic factors may even contribute to epilepsy in those with acquired forms of epilepsy (for example, epilepsy resulting from a stroke or autoimmune disease; Rastin et al., 2023; Thomas & Berkovic, 2014).

A genetic diagnosis is important for management, treatment, and planning appropriate supports and surveillance regimes for an affected individual (Panayiotopoulos, 2005; Thomas & Berkovic, 2014). In theory, once a genetic diagnosis has been made, additional diagnostic investigations should no longer be required and this will reduce the discomfort experienced by the patient, as well as the costs associated with the various testing and diagnostic methods (Enatsu & Mikuni, 2016; Sánchez Fernández et al., 2019). A diagnosis can also inform disease management. For example, Glucose Transporter Type 1 Deficiency Syndrome (GLUT1 deficiency; MIM 606777) is a deficiency syndrome whose classical characterization includes infantile onset seizures, atypical absence epilepsy and movement disorders (Wang et al., 1993). This epilepsy is typically drug-resistant, but the ketogenic diet in these patients is effective in seizure management and moreover improves developmental outcomes (Kass et al., 2016; Klepper et al., 2020; Ramm-Pettersen et al., 2014). The ketogenic diet can provide the brain with an alternate energy source and thereby reduces the impact of the GLUT1 deficiency (Sandu et al., 2019). This particular example highlights that an understanding of the underlying genetics, biology, treatment and disease-management can be of benefit to patients with epilepsy.

1.2.1 Genetics of epilepsy

The study of families with epilepsy, particularly twin studies, has provided convincing evidence supporting a genetic contribution (Berkovic et al., 1998, 1998; Kjeldsen et al., 2001, 2003, 2005; Poduri & Lowenstein, 2011; Steinlein, 2004; Vadlamudi et al., 2014). Higher concordance rates in monozygotic twins, as well as increased risk of epilepsy in first degree relatives of probands with generalized epilepsy, provides evidence that there is a heritable contribution to epilepsy

(Poduri & Lowenstein, 2011; Steinlein, 2004; Vadlamudi et al., 2014). In general, when determining if there is a genetic contribution to a disease, if the concordance in identical (monozygotic; MZ) twins is greater than that of fraternal (dizygotic; DZ) twins, a genetic contribution to the disease is implied (Helbig et al., 2008). Consistently, across studies, the concordance of epilepsy and seizures in monozygotic (MZ) twins is greater than that of dizygotic (DZ) twins with specific findings including: febrile seizures (MZ = 0.58; DZ = 0.14), partial (focal) epilepsies (MZ = 0.36; DZ = 0.05), idiopathic (genetic) epilepsies (MZ = 0.76; DZ = 0.33), symptomatic epilepsies (MZ = 0.83; DZ = 0), and genetic epilepsies with febrile seizures plus (MZ = 0.85; DZ = 0.25; Berkovic et al., 1998; Vadlamudi et al., 2014).

In terms of the underlying molecular causes of epilepsy, at this time there are several hundred genes that have been identified as being associated with epilepsy (Chi & Kiskinis, 2024; Wang et al., 2017). This includes genetic variants for diseases with epilepsy as the primary symptom as well as those diseases where epilepsy is one of many symptoms, such as developmental malformation syndromes or metabolic disorders that impact the nervous system (Wang et al., 2017). The genes themselves are involved with many cellular processes, although it has been estimated that 25% of the identified epilepsy-related genes encode ion channels (Lerche et al., 2012; Oyrer et al., 2018). A well-known and common ion-channel-associated gene is *SCN1A*, and the resulting disorder associated with this gene highlights the complexities associated with even monogenic epilepsy (Miller & Sotero de Menezes, 1993; Wang et al., 2017). The gene was first identified as an epilepsy gene in the early 2000s (Claes et al., 2003). The gene encodes the alpha subunit of a voltage gated sodium channel - the dysfunction associated with *SCN1A* variants cause hyperexcitability in the brain, which results in seizures and Dravet syndrome (Brunklaus et al., 2022; Ma et al., 2022; Valassina et al., 2022). Dravet syndrome is a well-known epilepsy disorder wherein the patient experiences epilepsy and subsequent intellectual disability, behavioural issues and abnormal gait (Jansson et al., 2020; Miller & Sotero de Menezes, 1993; MIM 607208). Even within this well-established syndrome, with a clearly identified genetic cause, there is a wide range of phenotypes within families and across variants, as well as a multitude of variants (missense, nonsense, frameshift, splice and deletion/duplication) within the gene itself that can result in Dravet syndrome (Ma et al., 2022; Miller & Sotero de Menezes, 1993). Broadly, loss of function variants in this gene can result in Dravet syndrome as well as a milder epilepsy known as GEFS+, and even migraine (Escayg et

al., 2000, 2001; Escayg & Goldin, 2010; Fan et al., 2016). Gain-of-function missense variants can result in a different syndrome known as Developmental Epileptic Encephalopathy type 6B (Brunklaus et al., 2022; MIM 619317).

Targeted multi-gene epilepsy panel sequencing is, currently, a common strategy to identify the pathogenic variant in *SCN1A* and other channelopathies (Lee et al., 2020). Once diagnosed, there can be a number of treatment options available to the patient with Dravet syndrome. For example, valproic acid, clobazam and stiripentol are recommended first-tier options (Strzelczyk & Schubert-Bast, 2022). Furthermore, there are precision medicine advancements and new approaches that can provide new seizure treatments for Dravet syndrome patients; for example, the use of cannabinoids and fenfluramine are relatively newer options that are now available to those with refractory seizures (Isom & Knupp, 2021; Knowles et al., 2022; Samanta, 2025). Importantly, a diagnosis can aid in avoiding medications that could worsen seizures (such as, sodium channel blockers in Dravet syndrome) and provide informed genetic counselling for the families (Connolly, 2016; Millichap et al., 2009). While Dravet syndrome is typically the result of a heterozygous *de novo* variation, there can be individuals with low-level parental mosaicism or gonadal mosaicism that can increase the sibling recurrence risk. Prenatal counseling and invasive prenatal diagnostic testing would be available to families in Ontario after a diagnosis is made. Pathogenic variation in *SCN1A* provide a specific example of how a genetic diagnosis can impact the care provided to a patient and their family members as well as demonstrates the variability and complexity of the genetic contribution to epilepsy, even with a single gene cause that has been known for over two decades.

1.2.2 A brief history in diagnosing genetic epilepsy

Disease gene discovery for epilepsy started with the identification of *CHRNA4* as the causative gene in autosomal dominant nocturnal frontal lobe epilepsy (Helbig & Lowenstein, 2013; Steinlein et al., 1995). The earliest identified genes, including *CHRNA4*, were autosomal dominant genes in familial epilepsy wherein there were many affected individuals, in the same family, with similar clinical presentations (Helbig & Lowenstein, 2013; Yozawitz & Moshé, 2022). These early epilepsies were not typically associated with global developmental delays or intellectual disability. The technologies for identifying genetic cause(s) for disease involved linkage analysis followed by Sanger sequencing and this often required large, multi-generational

families with many affected individuals. Given these requirements, there were very few epilepsy-causing genes identified (Helbig & Lowenstein, 2013; Myers et al., 2019). As a result, the severe, early-onset monogenic forms of epilepsy were historically difficult to diagnose given the limitations and costs associated with Sanger sequencing (Ottman et al., 2010). Advances such as the Human Genome Project (completed in 2003) improved the ability of researchers to identify disease genes (Collins & Fink, 1995; Hood & Rowen, 2013; Moraes & Góes, 2016). Next generation sequencing (NGS) was subsequently developed and was able to overcome many of the limitations of prior sequencing technologies which allowed for larger amounts of sequencing to occur at a reduced cost (Satam et al., 2023). Next generation sequencing techniques were initially applied to cohort studies of intellectual disability and autism, and this highlighted the frequency of *de novo* variants which were also identified when applied to the study of epilepsy (Helbig & Lowenstein, 2013; Lohmann & Klein, 2014). The pace of gene discovery increased significantly with the introduction of such NGS technologies (Helbig & Lowenstein, 2013). Some of the earliest examples of NGS techniques being implemented in a clinical setting, versus the research laboratory, involved the use of epilepsy gene panels that analyze a targeted set of genes known to cause epilepsy (Chambers et al., 2016; Kodera et al., 2013; Lemke et al., 2012). Multiple gene panels such as those are used for diagnostic testing methods today (Chambers et al., 2016; Kodera et al., 2013; Lemke et al., 2012). Initial studies were often biased in the ascertainment of the epilepsy cohorts but, today, the diagnostic rate of multi-gene panels for patients with epilepsy is approximately 19%, and a separate study completed from CHEO with a cohort of patients with pediatric genetic epilepsy reported a diagnostic yield of 17% with the use of multi-gene panels (Leduc-Pessah et al., 2022; Sheidley et al., 2022). These results demonstrate the benefits of using NGS to achieve a genetic diagnosis in cases of genetic epilepsy. Many of these multigene panels are analyzed from sequence data derived from an exome "backbone" – essentially, wherein the laboratory applies a customized informatics workflow to an individual's exome sequencing in order to analyze a particular set of genes (Rehm et al., 2023). Multi-gene panels may be selected as an initial testing method as it can be completed quicker than an exome sequencing analysis and in cases where there are genes strongly associated with the patient's phenotype, the targeted gene panel allows for possible variants in known disease genes to be analyzed at a faster rate (Wilson et al., 2020; Xue et al., 2015). Other benefits can include cost, as it is less expensive to utilize a multi-gene panel than whole exome sequencing and analysis,

and with consistent use of the same labs for multigene panels, they can become “local experts” for a subset of genes such as epilepsy-associated genes (Feliubadaló et al., 2017).

1.2.3 Whole exome sequencing for epilepsy patients

As described, whole exome sequencing (WES) is a next generation sequencing technique that has emerged as a useful tool for individuals with epilepsy and has also been identified as useful for such individuals with other neurologic disorders and rare genetic diseases (Frésard & Montgomery, 2018; Hartley et al., 2020; Kim et al., 2020).

WES alone provides a high diagnostic yield (up to 60% in select groups) in addition to being a cost-effective way to ascertain a genetic diagnosis (Akbar et al., 2022; Demos et al., 2019; Sánchez Fernández et al., 2019). It has been established that WES is a useful tool in achieving a diagnosis for adult and paediatric epilepsy patients (Benson et al., 2020). However, in addition to this, such paediatric cohorts have achieved higher diagnostic yields than cohorts of adult patients, with a study directly comparing two patient groups finding a 27% diagnostic rate in the adult cohort and a 47% diagnostic rate in the paediatric cohort (Benson et al., 2020). In addition, studies have found that patients with other neurodevelopmental disorders (such as intellectual disability or autism) in addition to epilepsy have a higher diagnostic rate in whole exome sequencing studies than patients presenting with only epilepsy (Habela et al., 2024; Palmer et al., 2018; Sheidley et al., 2022).

Another factor that improves the diagnostic rate is the availability of sequencing data from parents. In cases where only the affected patient’s exome sequencing is made available for analysis it is termed a “singleton”, but when both parents have an exome sequencing available alongside their child (the patient), it is termed a “trio”. Trio sequencing permits the identification of *de novo* variants, the ability of viewing inheritance from unaffected parents which can immediately determine if a variant has been inherited, and there is a faster turn-around time (Kingsmore et al., 2019; Tan et al., 2019). Overall, when focusing on epilepsy, it is widely accepted that whole exome sequencing is capable of achieving a 24% diagnostic yield in such individuals (Sheidley et al., 2022). It is also thought that the use of trio exome sequencing can allow for a higher diagnostic yield (Slavotinek et al., 2023; Tan et al., 2019).

1.2.4 Whole exome sequencing ***re*analysis** for epilepsy patients

Whole exome sequencing ***re***analysis is an emerging tool to bridge the diagnostic gap in patients with epilepsy that remain without a genetic diagnosis after initial investigation (Hartley et al., 2020, 2023). It is recommended that the referring clinician seek out reanalysis for patients without genetic diagnoses after they have gone through the initial clinical testing pathway. This is promising, as numerous factors from new information on genetic variants to new information or changing patient presentation can lead to different interpretations of the data (Hartley et al., 2023; Krey et al., 2022). WES reanalysis is a systematic, updated review of WES data – where a researcher or clinician will review the WES data and interpret previously identified variants differently, or identify new potentially pathogenic variants (Leung et al., 2022). Reanalysis has been shown to provide a genetic diagnosis in 4% and identify novel gene candidates in 21% of individuals in a Canadian study of patients with rare disease (Hartley et al., 2023). Furthermore, a study focusing solely on epilepsy achieved a diagnostic yield of 5.8% of their cohort, with the average from studies of cohorts of patients with epilepsy and/or intellectual disability reaching approximately 9.5% (Al-Nabhani et al., 2018; Epilepsy Genetics Initiative, 2019; Li et al., 2019; Salinas et al., 2021; van Slobbe et al., 2024). Overall, exome **re**analysis is a viable option for those with rare genetic diseases and lacking a genetic diagnosis after initial testing methods.

1.2.5 Care4Rare consortium and Care4Rare-SOLVE

Although rare diseases individually affect few, as a whole, rare diseases affect many with estimates of 300 million people worldwide (or 1 in 12 Canadians) presenting with a rare disease (Health, 2024; McMillan & Campbell, 2017). As a result, there are many groups and organizations that focus on researching rare diseases. One such group is Care4Rare (C4R). C4R is a research program based in the CHEO (Children's Hospital of Eastern Ontario) Research Institute, but with collaborating sites across Canada. One of the aims of C4R is to provide a diagnosis following negative exome sequencing (Boycott et al., 2022). Once a patient has been enrolled in this program, WES reanalysis is completed (Figure 1-1; Boycott et al., 2022). If a candidate variant is identified, there are two pathways that can be followed: (i) if there is sufficient evidence to support a diagnosis the line of inquiry can be stopped or (ii) further analysis is required to confirm or refute pathogenicity of a candidate. This thesis work will follow this path (ii): wherein a candidate genetic variant has been identified in a putative novel

disease gene, and functional analysis is completed with the aim of acquiring evidence of pathogenicity (Ciesielski et al., 2024; Osmond et al., 2022). Functional analysis is targeted to the highlighted proteins, pathways, and cellular functions that are suspected to be affected by the gene and/or variant. This second path of functional analysis can occur in cases where the candidate genetic variant is in a gene not yet associated with a disease (putative novel disease gene candidate) as well as a variant of unknown significance (VUS) in a known disease gene, wherein the effect of that particular variant is not yet established. Overall, many pieces of compelling evidence, from matches in GeneMatcher to functional assays showing significant effects, are required to confirm a newly identified candidate variant is pathogenic.

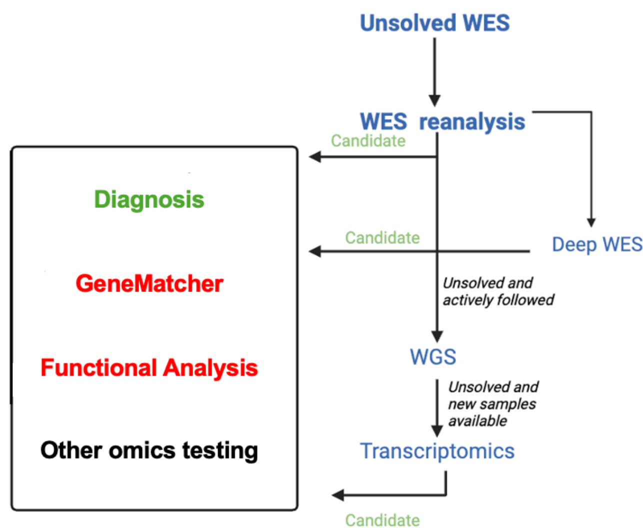


Figure 1-1 Care4Rare Solve Workflow. General flow demonstrating the C4R-SOLVE workflow. The first step is WES reanalysis where a candidate identification can lead to diagnosis or other testing (functional analysis, omics testing such as transcriptomics and metabolomics) coupled with GeneMatcher. If a candidate is not identified, enrolment continues and can undergo whole genome sequencing or deep WES (where a small number of cells are deep sequenced; de Kock et al., 2024). This figure was created using BioRender.com.

1.2.6 Steps to confirm diagnosis in putative novel disease genes

In the case where the candidate variant would represent a putative novel disease gene, multiple similarly affected individuals with similar variants in the same gene can provide necessary evidence required to prove a new disease-gene association. One tool used to provide this

evidence is matchmaking using GeneMatcher, which is a large database where genetic variants in putative novel disease genes along with patient phenotypes can be uploaded (Sobreira et al., 2015). If there is another entry (or entries) of variants in the same gene, the two uploaders will be connected. Subsequent review of the information provided from programs such as GeneMatcher can allow for a "match" to occur. With this, it is possible to determine if there are multiple un-related individuals with similar phenotypic features and rare variants within the same gene of interest (Osmond et al., 2022; Sobreira et al., 2015).

Chapter 2 Exome reanalysis for patients with epilepsy and intellectual disability is an effective diagnostic tool

2.1 Introduction

Several-hundred epilepsy genes have been identified within the last decade as a result of applying NGS techniques, and these genes can now be tested on a clinical basis (de Ligt et al., 2012; Dunn et al., 2018; Krygier et al., 2024). One commonly used NGS strategy is whole exome sequencing – where the majority of the protein coding complement of the genome is sequenced and genetic variants can be analyzed (Laurie et al., 2025, Minardi et al., 2020; Ostrander et al., 2018). Despite the advancements in gene discovery and genetic testing that have been implemented as part of routine care, most of these patients, seen today, do not obtain a genetic diagnosis (Ruggiero et al., 2023; Sheidley et al., 2022). Exome ***re***analysis is an emerging strategy that can improve diagnostic rates in patients with unexplained epilepsy whose standard of care and genetic tests were non-diagnostic. Improvements in the diagnostic rate have been made in epilepsy studies that implement exome reanalysis (Table 2-1; Epilepsy Genetics Initiative, 2019; Johannesen et al., 2023; Koh et al., 2023; Li et al., 2019; Palmer et al., 2021; Salinas et al., 2021). This method does not require any additional testing or sample collection which, in addition to being convenient for researchers/clinicians as well as patients and family members, contributes to its cost-effectiveness (Ewans et al., 2018). The utility of exome reanalysis has been demonstrated with a focus on rare disease with prior studies achieving a diagnostic yield of 13% (Hartley et al., 2023). Furthermore, in exome reanalysis studies with cohorts of epilepsy patients a diagnostic yield of 5.8% was achieved (Epilepsy Genetics Initiative, 2019). Reanalysis targeting specific phenotypes, as is being completed in this study by targeting patients with epilepsy/seizures in addition to intellectual disability and/or developmental delay, has not yet been broadly explored. With these factors to consider, the aim of this chapter was to assess any benefits that exome reanalysis is to individuals presenting with epilepsy/seizures as well as intellectual disability and/or developmental delay. The research reanalysis was performed in a cohort of 20 exome sequencing datasets from patients with such phenotypes.

Table 2-1 Exome reanalysis with cohorts with overlapping phenotypes to the reanalysis cohort presented (rare disease, neurodevelopmental disorders, epilepsy, intellectual disability).

Publication Title	Author(s)	Year of Publication	Diagnostic Rate	Notes (patient information?)
The Epilepsy Genetics Initiative: Systematic reanalysis of diagnostic exomes increases yield	Epilepsy Genetics Initiative	2019	8/139 (5.8%)	Individuals with epilepsy who underwent whole exome sequencing were enrolled into this study
Reanalysis of whole exome sequencing data in patients with epilepsy and intellectual disability/mental retardation	Li, J., et al	2019	8/76 (10.5%)	Patients with epilepsy and intellectual disability/mental retardation
Reanalysis of whole-exome sequencing (WES) data of children with neurodevelopmental disorders in a standard of patient care context	Slobbe, M.V., et al	2024	20/159 (12.6%)	Children with neurodevelopmental disorders with prior whole exome sequencing analysis
Reanalysis of exome sequencing data of intellectual disability samples: Yields and benefits	Al-Nabhani, M., et al	2018	6/50 (12%)	Patients with intellectual disability were enrolled
Clinical next generation sequencing in developmental and epileptic encephalopathies: Diagnostic relevance of data re-analysis and variants re-interpretation	Salinas, V., et al	2021	1/27 (3.7%)	Pediatric patients with developmental epileptic encephalopathy were selected for whole exome sequencing. After initial analyses, those with undetermined genetic diagnoses and negative genetic diagnoses were reanalyzed.

Total			43/451 (9.5%)	

2.2 Materials and methods

2.2.1 Study design

Through the local Genetics Clinic at the Children's Hospital of Eastern Ontario (CHEO), a cohort of 20 families – including the proband and any family members – were recruited and proper consent was obtained. Individuals were recruited as part of the C4R-SOLVE project (CTO-1577) and were eligible for this if they had non-diagnostic clinical testing that used exome sequencing. In order to be included in this study, the proband needed to present with seizures or epilepsy with global developmental delay and/or intellectual disability.

2.2.2 Data collection

Medical records from the Care4Rare database (Genomics4RD, www.genomics4rd.org) were used to collect the phenotypic and demographic information manually (Driver et al., 2022). The remaining information used for analysis was obtained from the electronic medical records and the initial clinical laboratory reports. From the medical records, a clinical geneticist obtained information regarding the patient's seizure type, refractory status and whether they presented with any syndromic features and/or brain malformations. The initial test name, sequencing strategy and information regarding any variants of interest were taken from the laboratory reports.

2.2.3 Data transfer and reprocessing

The data transfer, analysis and processing follow the methods previously described (Hartley et al., 2023). All clinical laboratories involved worked to create a custom data transfer process. All individuals in Care4Rare (patients and any family members) provided consent to access their data with established release forms. Secure transfer portals or Citrix ShareFile accounts were used to transfer the sequencing files available. These sequencing files were then processed using the most recent bioinformatics pipeline which has been previously described (Kernohan et al., 2018).

2.2.4 Exome sequencing data reanalysis

I completed the exome reanalysis for the 20 cases. The steps taken are part of a protocol used by Care4Rare. The first step of the reanalysis per the previously described methods, begins with what is termed the "clinical reanalysis". In this first step, the focus of the analysis is the examination of rare variants in genes *already* associated with human diseases as catalogued in the OMIM and Orphanet databases. Such known disease gene variants were considered plausible only if the patient presentation aligned with the known disease-gene association. The variants were then interpreted and given a classification according to the criteria set by the American College of Medical Genetics and Genomics (ACMG; Richards et al 2015). If the classification per these criteria was "pathogenic" or "likely pathogenic", a genetic diagnosis could be made with the individual and their case receiving a classification of "diagnosed". The next step in the process is the "translational reanalysis". In this portion of the analysis, variants in *genes of uncertain significance* (GUS) were analyzed – simply, variants in genes not yet associated with disease. These variants were prioritized according to the following criteria, in order: segregation in the family (for example, if an unaffected parent has this variant, it may be excluded), rarity (internal and control databases such as gnomAD are used to determine how frequently, if at all, the variant is detected), the biological plausibility of the variant contributing the patient's disease (analysis of predicted function, any implicated pathways and analysis of protein type), and, finally, determining if the gene was intolerant to the type of variant detected (for example, if an amino acid substitution variant would significantly affect the protein structure and/or function) as assessed by *in silico* tools.

2.3 Results

2.3.1 Patient demographic, previous clinical testing and patient phenotype

The exomes of 20 patients enrolled in the Care4Rare research program were reanalyzed. All patients were cases of epilepsy or seizures and also had intellectual disability and/or developmental delay that did not have a genetic diagnosis. The clinical testing was accomplished for all patients on an "exome backbone".

The sex of participants between male and female individuals was 13:7 (Figure 2-1A). Individuals experienced seizure onset at 0-3 years of age (10/20: 50%), 4-6 years of age (5/20: 25%), 7-9

years of age (5%: 1/20) and over 10 years of age (20%: 4/20) (Figure 2-1B). Of the 20 participants, 4 presented with focal seizures, 9 presented with generalized seizures, 5 presented with mixed seizures and the specific seizure type for 1 patient was not available (Figure 2-1C). In 15 patients the seizures were not refractory to treatment, in 4 patients the seizures were refractory to treatment and 1 patient's seizures were not treated (Figure 2-1C). Only one participant reported a family history of epilepsy where a sibling was also affected. Syndromic features (congenital anomalies or dysmorphic appearance) were reported in 7 patients (35%), and brain malformations were reported in 7 patients (35%). There were 3 patients within those classifications that presented with both brain malformation and syndromic features (15%). With regards to clinical testing, 7 of the 20 participants had a multigene panel (performed on an exome backbone) and the other 13 had a clinical exome prior to enrollment into Care4Rare. The initial clinical testing identified candidate variants (but no genetic diagnosis) in close to half (9/20; 45%) of patients (Figure 2-1D).

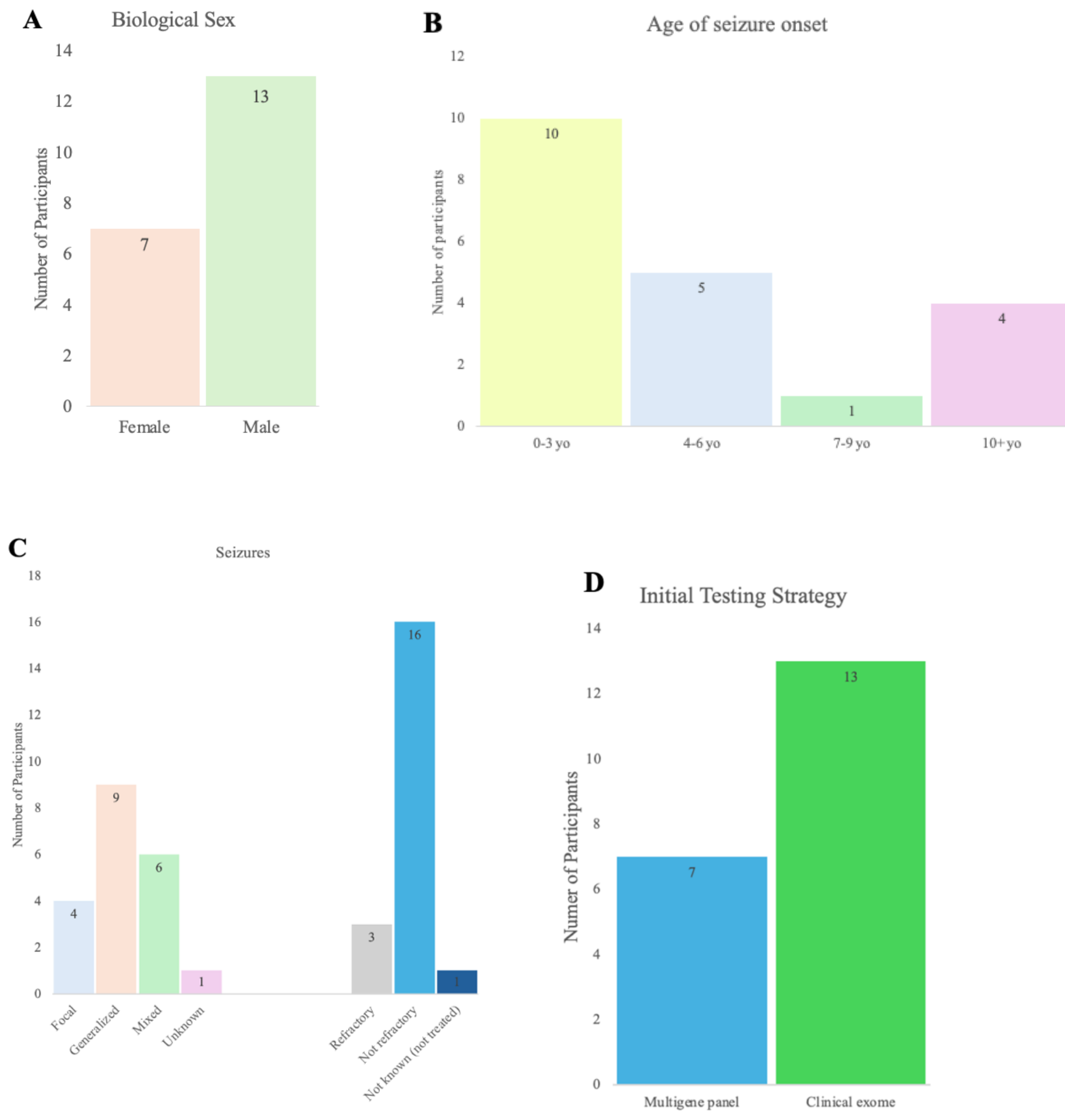


Figure 2-1 Patient demographic and clinical presentation. (**A**) Representative graph of the proportion of participants whose biological sex is female vs. male (7:13). (**B**) Age of seizure onset for each participant was separated by year: 0-3 years of age (n=10), 4-6 years of age (n=5), 7-9 years of age (n=1) or 10+ years of age (n=4). (**C**) Participant seizure type was separated into four categories; focal seizures (those on only one side of the brain, n=4), generalized seizures (those on both sides of the brain, n=9), or mixed seizures (more than one type of seizure, n=6) as well as one case where patient's type of seizure was unknown (n=1). The cohort was also separated if their seizures were refractory (seizures that persist despite the use of antiepileptic medications, n=3), not refractory (seizures that can be controlled effectively with the use of antiepileptic medication, n=16), and one case where the patient has not been treated with anti-

epileptic medication and cannot be classified as refractory or not refractory (n=1). **(D)** Initial testing strategy of each participant wherein a proportion initially had a multigene panel (n=7) and the others initially had a clinical exome (n=13) completed.

2.3.2 Reanalysis results

As a first step, the variants previously reported by the clinical laboratory were re-analyzed. Of the 17 variants that were classified in this cohort, none changed their ACMG classification status. Of the initial variants, 12/17 were classified as a variant of uncertain significance (VUS), 2/17 were pathogenic or likely pathogenic variants in disease genes associated with bi-allelic inheritance and 3/17 were listed on the clinical reports though classified as "benign" (Table 2-2). In one case, a variant in *OPA1* was initially considered a variant of uncertain significance. With re-interpretation, the variant status was again a variant of uncertain significance. However, the clinician had segregated this variant and showed that it was present in an adult individual (the mother) without a similar phenotype and absent in the sibling who shared the same phenotype as the proband. As such, this variant could be re-interpreted as "benign", with the additional information provided by the clinician.

Table 2-2 Re-interpretation of variants identified in initial testing. The initial ACMG classification for variants identified, when applicable, through the initial testing for patients is compared to the current ACMG classification. Additional notes are present for cases where the variant has a "likely pathogenic" or "pathogenic" classification as well as those where the current classification could be updated as a result of additional testing.

Study ID	Initial gene and variant	Initial ACMG classification	Current ACMG classification	Additional notes
1358	*TBC1D24* NM_001199107.2 :c.1633A>G:p.Ile545Val	VUS	VUS	
1472	*ASPM* NM_018136.4:c.8009T>C :p.Ile2670Thr	VUS	VUS	
2146	*ZBTB7A* NM_001317990.2:c.267G>A :p.Met89Ile	VUS	VUS	

2359	*ARSA* NM_000487.6:c.1283C>T :p.Pro428Leu *NGLY1* NM_001145293.2:c.1201A>T :p.(Arg401Ter)	Pathogenic Likely pathogenic	Pathogenic Likely pathogenic	These gene/variants were ruled out as the cause of the individuals presentation as they are known-disease genes with an autosomal recessive inheritance, and there was only one variant detected in the patient for each gene.
2608	*TRIO* NM_007118.4:c.3641C>G :p.(Ala1214Gly)	VUS	VUS	
1254	*NARS2* NM_024678.5:c.847A>G :(p.Thr283Ala) *NEXMIF* NM_001008537.2:c.136C>T :(p.Pro46Ser) *NPC1* NM_000271.4:c.398T>G :p.(Val133Gly) *OPA1* NM_130837.2:c.1262G>A :p.(Arg421Gln) *VPS13B* NM_152564.4:c.584C>G :p.(Thr195Ser) NM_152564.4:c.1864A>G :p.(Thr622Ala)	Likely benign Likely benign VUS VUS VUS VUS	Likely benign Likely benign VUS VUS* VUS VUS	*Segregation analysis completed for this individual confirmed that the *OPA1* variant was not detected in an affected sibling (sister) but present in an unaffected parent. With this additional information it would now be classified as “benign” for this individual.
1440	*VPS13B* NM_017890.4:c.2600A>G :p.Lys867Arg NM_017890.4:c.8645C>T :p.Pro2882Leu *CNOT2* NM_001199302.1:c.1621T>A: p.541Lysext65	VUS Benign VUS	VUS Benign VUS	
2159	*SLC6A1*	VUS	VUS	

	NM_001348250.2:c.989C>T :p.Thr330Ile			
2560	*AGAP2* NM_001122772.2:c.113C>T :p.Ala38Val *DLG2* NM001206769.1:c.269G>A :p.Gly90Glu	VUS VUS	VUS VUS	
0258G	*DCBLD2* NM_080927.3:c.2102T>C :p.Phe701Ser NM_080927.3:c.1445C>A :p.Ala482Asp NM_080927.3:c.1433C>T :p.Pro478Leu	VUS VUS VUS	VUS VUS VUS	

Next, the exome sequencing for all 20 patients and available relatives were reanalyzed following the protocol previously described by Hartley et al., 2023 and variants were then classified using the ACMG criteria (Richards et al., 2015). A classification of "diagnosed" was assigned to individuals if the candidate variant satisfied one of the following conditions: (1) the candidate, in a known disease gene, was classified as "pathogenic" or "likely pathogenic" by the ACMG criteria with the appropriate inheritance pattern and phenotypic overlap in the patient (as confirmed by the referring clinician), or (2) in the case of a novel disease gene candidate, there were two additional families with the same or overlapping condition.

For a candidate to be classified as "further evidence required" (a compelling candidate) one of the following conditions would need to be satisfied: (1) a known disease gene with adequate phenotypic overlap that was classified as a VUS with the ACMG criteria, (2) the candidate was classified as pathogenic/likely pathogenic in a gene associated with an autosomal recessive inheritance but only one variant was present, or (3) the candidate was in a convincing novel disease gene, but fewer than three families with overlapping phenotypes or the proposed presentation had been identified.

In this cohort, 9 participants had a candidate variant identified (Table 2-3), the other 11 remain without a genetic diagnosis or "undiagnosed" (Figure 2-2 A). Of the 9 cases where new

candidates were identified, 2 were subsequently classified as "diagnosed", and 7 required further evidence to demonstrate causality (Figure 2-2B).

In the first of those diagnosed cases, the variant was in a known disease gene (*STXBP1*) and received a classification of "likely pathogenic" with confirmation of phenotypic overlap by the clinician. This patient initially had a clinical exome where no candidate genes were reported in a trio-exome testing strategy (both unaffected parents and the affected child's exome sequences were all used). We contacted the clinical laboratory (Genome Sequencing Ontario; GSO), and they were also able to "see" the variant, though at the time of their original interpretation it was considered a false positive based on the available clinical description. As a result, it was not formally reported. With reanalysis, it was determined that leaving this variant out of the report was an error and the variant has been identified as the genetic diagnosis for this patient.

The second genetic diagnosis was also a variant in a known disease gene (*USP9X*) from an individual who initially had a multigene panel on an exome backbone. This variant was identified in the reanalysis, classified as pathogenic and confirmed by the clinician. The original multigene panel test did *not* include *USP9X* as this was not a known epilepsy gene at the time of initial testing (and was therefore not formally included in the panel content).

With regards to the 8 other compelling variants seen in the 7 individuals, 2 of the candidates were novel disease genes (*JAKMIP1* and *SBNO1*) where both participants initially had a clinical exome. The remaining candidates in 5 cases are variants in known disease genes (Table 2-2). One patient (who initially had a research exome) had two candidates identified, further evidence into both variants is required to resolve the interpretation status from uncertain to benign or pathogenic. These variants were considered compelling candidates for a variety of reasons including, but not limited to, their rarity (not being found in the gnomAD database), *de novo* status (as with case 2146, *ZBTB7A*), and in the case of some known disease genes, being associated with disease or disorder that has an overlapping clinical presentation to the clinical presentation of the patient.

Of the other participants that remain undiagnosed, one initially had a panel whereas the rest initially had a clinical exome. To note, of the cases without any candidates identified, 3 initially had a multigene panel and 8 initially had a clinical exome.

Overall, the exome sequencing reanalysis in cases of epilepsy with intellectual disability or developmental delay that did not have a genetic diagnosis, yielded a diagnosis in 2 of the 20 cases or 10%.

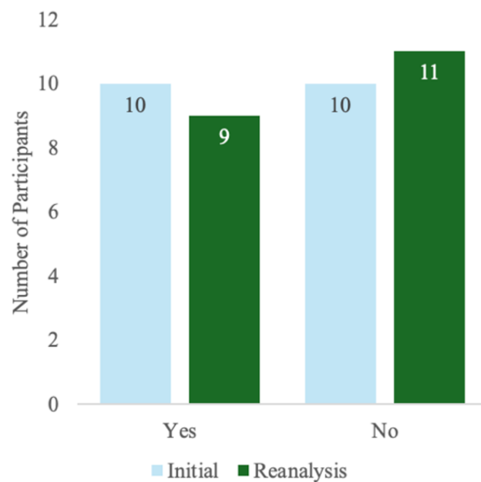
With regards to the phenotype for the individuals that received a genetic diagnosis, one patient had additional/syndromic features (cerebellar atrophy), and the other did not. In individuals who initially received a gene panel (35% of participants), 1 participant achieved a genetic diagnosis (14.3%), 3 had compelling candidates identified (35%), and 3 had no compelling candidates identified and remain undiagnosed (35%). For those who initially received a clinical exome, 1 participant was diagnosed (7.7%), 4 had compelling candidates identified (30.8%) and 8 had no compelling candidates identified and remain undiagnosed (61.5%). Regarding the particular case classifications, the cases with a diagnosis and compelling candidates were fairly evenly split between participants with a panel and those with a clinical exome at initial testing (1:1, 3:4). The results here suggest that utilizing exome sequencing reanalysis in cases with multigene panel testing and clinical exomes is a useful tool for improving the diagnostic rate.

Table 2-3 Candidate information: This is an overview of information that summarized candidates identified. The study ID is the number assigned to each individual and their case upon enrolment. The frequency in databases includes gnomAD and the internal Care4Rare database to determine how frequently the variant has been detected. Following this are the in silico scores, which are a series of metrics that are used to predict the effects of a variant on a gene or protein. CADD scores predict the deleteriousness of a single nucleotide variation, some types of variants will not receive a CADD score (“none”). Higher scores indicate that the variant is more likely to be deleterious. Another scoring system used is the Exac pLI score, shortened to pLI, which predicts whether or not the protein encoded by the gene is loss of function intolerant. As the score approaches 1, the more intolerant the variant is predicted to be. Notes on literature lists any associated diseases or disorders, as well as whether the resulting protein is detected in the brain. ACMG classification as well as the code that encompasses this follow, with the final column listing any possible next steps or conclusions. There are 2 candidates wherein the patients received a diagnosis, and 8 candidates that require further investigation from 7 cases. Case 2735 had 2 candidates identified through reanalysis.

Study ID	Gene and variant(s)	Frequency in databases	In silico scores	Notes on literature	ACMG classification with codes	Next steps
528	*USP9X* NM_001039590.3:c.435+1G>A	Seen 0x in gnomAD and 1x in C4R	CADD: 35. pLI: 1	Associated with intellectual development disorder X-linked 99 (MIM 300919) and intellectual development disorder, X-linked 99, syndromic, female-restricted (OMIM 300968). USP9X is expressed in the brain.	**P** PS2 PM2 PVS1 Segregated in parents and found to be de novo Original panel (2018) did not carry *USP9X*	This case has been diagnosed.
2543	*STXBP1* NM_001032221.6:c.316_318del:p.Phe106	Seen 0x in gnomAD and 0x in C4R	CADD: none pLI: 1	Associated with developmental and epileptic encephalopathy 4 (OMIM 612164). STXBP1 is expressed in the brain.	**LP** PS2 PM2 PM4	This case has been diagnosed.
1358	*MADD* NM_001135943.2:c.3238G>A:p.(Ala1080Thr) NM_001135943.2:c.2615C>G:p.(Ser872Cys)	Seen 0x in gnomAD and C4R	CADD: 29.4 pLI: 0.000000000000012008	Neurodevelopmental disorder with dysmorphic facies, impaired speech and hypotonia (OMIM 619005) and DEEAH syndrome (OMIM 619004). MADD is expressed in the brain.	VUS PM2	Segregated in 3 unaffected siblings – no siblings with same genotype. Found to be *in trans.*
1472	*JAKMIP1*	Seen 0x in gnomAD and 1x in C4R	CADD: 22.5 pLI: 0. 99987	Putative novel disease gene (OMIM).	A variant in a GUS	Submitted to GeneMatcher

	NM_001099433.2:c.217A>G:p.(Lys73Glu) *De novo*			JAKMIP1 is expressed in the brain.		
2146	*ZBTB7A* NM_001317990.2:c.267G>A:p.(Met89Ile) De novo Mosaic 25/148 (17%)	Seen 0x in gnomAD and C4R	CADD: 23.5 pLI: 0. 96083	Macrocephaly, neurodevelopmental delay, lymphoid hyperplasia, and persistent fetal hemoglobin (OMIM 619769). ZBTB7A is expressed in the brain.	VUS PS2 PM2	MD attempted to contact the family with no response. At this time considered “lost to follow-up”
2359	*SBNO1* NM_001167856.3:c.3480T>G:p.(Asp1160Glu) NM_001167856.3:c.2300A>G:p.((Glu767Gly) Biallelic, *in trans*	Seen 0x in C4R and 7x in gnomAD Seen 0x in C4R and gnomAD	CADD: 15.17 pLI: 1 CADD: 23.1 pLI: 1	Putative novel disease gene (OMIM). SBNO1 is expressed in the brain. 2025-07-30 2:24:00 PM	A variant in a GUS	Submitted to GeneMatcher
2403	*CHD5* NM_015557.3:c.1063A>G:p.(Ile355Val)	Seen 0x in gnomAD and C4R	CADD: 25.1 pLI: 1	Parenti-Mignot neurodevelopmental syndtrome (OMIM 619873). CHD5 is expressed in the brain.	VUS PP3 PM2	Ruled out by segregation study (paternally inherited).
2608	*TRIO* NM_007118.4:c.3641C>G:p.(Ala1214Gly)	Seen 0x in gnomAD or C4R	CADD: 29.3 pLI: 1	Intellectual developmental disorder, autosomal dominant 44 with microcephaly and intellectual developmental disorder,	VUS PP3 PM2	Clinical review by physician did not consider this variant a genotype:phe

				autosomal dominant 63, with macrocephaly (OMIM 609823). TRIO is expressed in the brain.		notype match – this has been removed from consideration.
2735	*SATB2* NM_001172509.2:c.1072G>A:p.(Val358Met)	Seen 0x in C4R and 2x in gnomAD	CADD: 26.1 pLI: 0.99999	Glass syndrome (OMIM 612313). SATB2 is expressed in the brain	VUS PP3	Advancement in gnomAD since reanalysis revealed the variant is seen 10x and no longer a compelling candidate.
2735	*TRIP12* NM_001284214.2:c.1225A>T:p.(Asn409Tyr)	Seen 0x in C4R and gnomAD	CADD: 23 pLI: 1	Global developmental delay/intellectual disability, epilepsy (OMIM 617752). TRIP12 is expressed in the brain.	VUS PP3 PM2	Parents have declined further testing/investigations



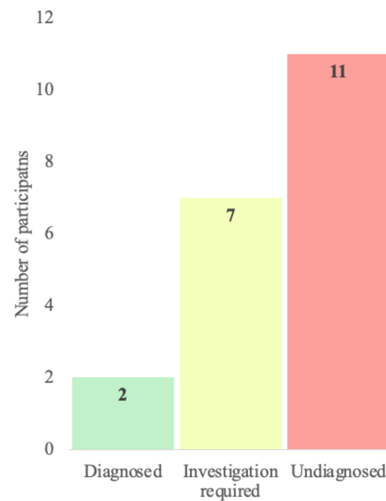


Figure 2-2 Candidate identification and diagnostic status for the cohort of exome sequencing cases (n=20). (**A**) Graph representing the proportion of participants that had candidates identified with initial testing (that were not classified as pathogenic or "diagnosed") and identified with reanalysis. (**B**) Representation of the final classification for each participant. They were classified as diagnosed (n=2), requiring further investigation (n=7), or undiagnosed (n=11) based on the criteria as described above.

2.3.3 Testing strategy and time since reanalysis

Factors that have been hypothesized as influencing a genetic diagnosis in exome sequencing as well as re-analysis are the (1) initial testing strategy performed and the (2) time to re-analysis (Ewans et al., 2018; Fung et al., 2020; Tan et al., 2019). First, the testing strategy (i.e., singleton, duo or trio) wherein the exomes of unaffected or affected family members available for analysis was only changed between one participant, and this case had no change in result (no candidate was identified with initial testing or with the exome reanalysis: Figure 2-3A). The number of cases with a change in testing strategy was too small to detect any conclusive changes.
When analyzing the time that passed between initial testing and reanalysis, most cases (10/20; 50%, Figure 2-3B) were reanalyzed within 2-3 years of initial testing. The two cases that were diagnosed were reanalyzed at 2 years and 5 years from initial clinical testing. In cases where compelling candidates were identified without being classified as having received a genetic diagnosis, 2 were reanalyzed within 6-7 years, and the remaining 5 cases were reanalyzed within 2-3 years. There were 3 cases analyzed ≤1 year after initial testing, and only one of these had a candidate identified.
One of the first steps that can be taken as part of the reanalysis process is to re-interpret variants that were identified in the initial testing the patients obtained. More information for variants may have been uncovered or established in the time since initial interpretation, which may impact its ACMG classification. This change may provide a diagnosis (should the variant be re-interpreted as likely pathogenic or pathogenic with the appropriate number of variants) or reject the variant as a candidate (should the variant be re-interpreted as likely benign or benign). In all cases for reanalyzed for this study that had candidate variants identified with initial testing, no classification changes were found with re-interpretation (Table 2-3) though we appreciate that the sample size was modest.

Given the small sample size, the effect of testing strategy on diagnostic yield was inconclusive. Similarly, it is not possible to determine if the time since initial testing has any significant impact on diagnostic yield or candidate identification for exome sequencing reanalysis. To note, the individual from case 2543 with the genetic diagnosis in *STXBP1* was reanalyzed after 2 years, whereas the individual from case 528 with the genetic diagnosis in *USP9X* was reanalyzed after 5 years.

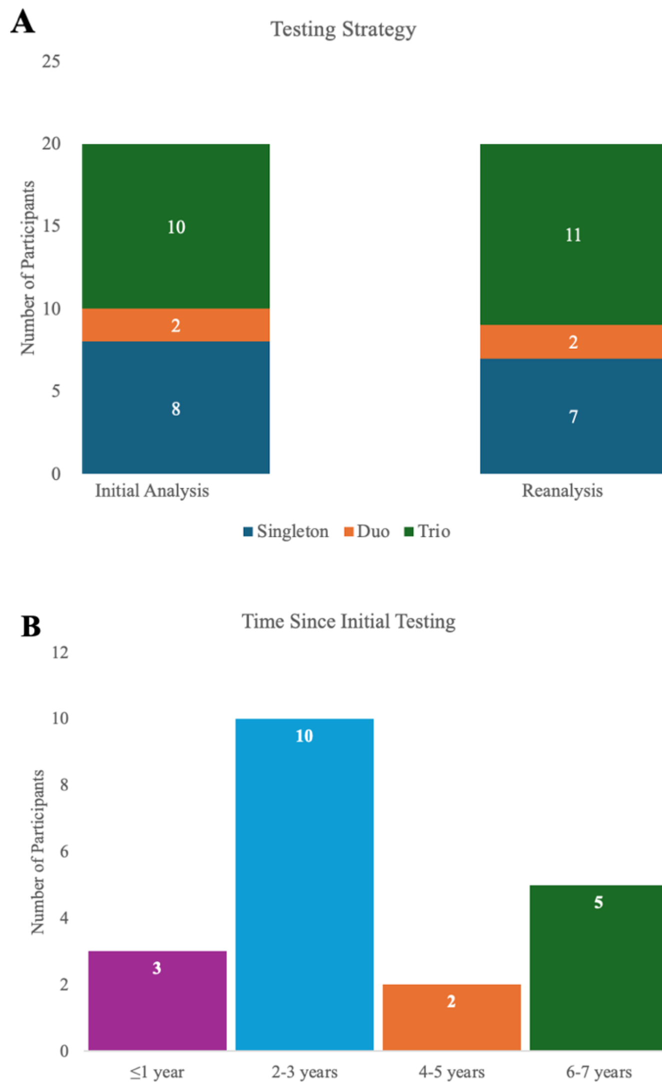


Figure 2-3 Testing strategy and time since initial testing. (**A**) Representative graph of the testing strategy in initial testing and for reanalysis. Singleton analysis is where only the affected participant has testing completed, a duo strategy is where one additional family member has been sequenced, and trio indicates both parents have also been sequenced. (**B**) Participants were separated based on the time since initial testing.

2.4 Discussion

Achieving a genetic diagnosis for an individual with a presumed genetic epilepsy can be difficult. There are numerous pathways for testing that can be taken or recommended by specialists and clinicians, which take into account the patient's clinical symptoms such as age, type of seizure and associated comorbidities (such as global developmental delay or intellectual disability). Individuals that do present with epilepsy and additional symptoms, such as intellectual disability, can qualify for whole exome or whole genome sequencing currently in Ontario (https://gsontario.ca/). The availability and access to comprehensive testing can impact the diagnostic odyssey for these patients, making an earlier genetic diagnosis possible. Treatment can be impacted by a genetic diagnosis and can also discontinue additional investigations that can be costly and even invasive (for example, brain imaging or muscle biopsies). Many patients that present with such suspected forms of epilepsy receive singleton, multigene panel testing, which can be performed on an exome backbone and can thus be used for exome sequencing reanalysis.

Initial clinical testing utilizing a multigene panel has a diagnostic rate of 19% and exome sequencing testing has a diagnostic rate of 24% (Sheidley et al., 2022). The remaining proportion of individuals, the vast majority, remain without a genetic diagnosis (up to 81%; Sheidley et al., 2022). Utilizing exome reanalysis is a cost effective, straightforward tool to increase the number of diagnoses that can be achieved. It is a method that can be used in a research setting to further understand the specific mechanisms of the disease in each patient especially when novel epilepsy genes (and pathways) are identified. In the research completed here, "pathogenic" and "likely pathogenic" variants that explain the patient's clinical symptoms were identified in 10% (2/20) of the participants through reanalysis. This is consistent with exome reanalysis studies of epilepsy exome sequencing data, as well as reanalysis studies for rare diseases and in children presenting with epilepsy (Table 2-1; Epilepsy Genetics Initiative, 2019; Hartley et al., 2023; van Slobbe et al., 2024). In both cases in this reanalysis project where a diagnosis was achieved, 2 or more years had passed since the initial testing. Although the small size of the cohort does not permit conclusions as to how time since initial testing effects the result of exome reanalysis, it does show a trend that over 2 years since reanalysis may allow for increased diagnosis or candidate identification. The diagnosis involving the *USPX* variant highlights the advancement

of genetic knowledge over time. In the time since the initial testing, improved bioinformatics pipelines, new variant interpretation tools, variant reclassification and overall understanding or knowledge on genes all contribute to the identification of a candidate variant or even identifying the causative variant (Ji et al., 2021; Olimpio et al., 2024.; van der Geest et al., 2024).

As mentioned, reanalysis can identify candidates that while not diagnostic or classified as pathogenic, are compelling enough to lead to further testing in a research setting. This permits additional "-omics" studies to be applied that may not be available or accessible in the clinical setting (Quezada et al., 2017). This can help to acquire the evidence needed to make a diagnosis or simply understand the mechanism of disease more thoroughly. Not only this, but such candidate genes can be enrolled in "match making" portals such as the GeneMatcher database – an international database where researchers and clinicians can be connected if their patients have variants in the same putative disease gene. Should they have overlapping clinical presentations a "match" can be made (Hamosh et al., 2022; Sobreira et al., 2015).

Progress in cases where compelling candidates were identified that required further investigation

Since the reanalysis was completed for this project, "next steps" have been implemented. In several instances, the subsequent testing allowed the candidate variant to be "ruled out". For example, one of the candidates from case 2735 (*SATB2*) was re-interpreted with advancements in the gnomAD database. The updated population frequency of the variant (gnomAD, version 4; variant seen 10x) was considered too high for a multisystem, infantile-onset syndrome (MIM 612313) and therefore not likely contributing to the clinical presentation. For case 2403, segregation studies found that the variant in *CHD5* was paternally inherited and therefore it has been "ruled out" as the father did not have any history of seizures or any developmental delays or intellectual disability. In case 2608 the candidate (*TRIO*) was ruled out by the most responsible physician who did not consider the candidate gene a good fit for the patient presentation. The patient had a brain malformation (lissencephaly) that has not been reported in those with pathogenic *TRIO* variant(s).

The variants in *TRP12* and *ZBTB7A* could not be studied further as the families declined additional testing or were lost to follow-up (respectively).

There were several variants that continue to be compelling candidates. For example, case 1472 had a *de novo* variant in *JAKMIP1*. A literature review showed another substitution in the same

gene in an individual with a similar phenotype (macrocephaly, obesity and intellectual disability; Loviglio et al., 2016). The next step was to identify a cohort with others interested in the gene *JAKMIP1*, and the variant has been submitted to GeneMatcher. A "match" was obtained, and the cohort is currently being compiled by researchers in Pittsburgh. This project is currently in the cohort ascertainment phase but there is reportedly one additional individual with macrocephaly and cognitive deficits. The family is aware and has given consent for this collaboration.

The biallelic variants in *MADD* (family 1358) continue to be compelling as they were found to be *in trans* in the proband and not *in trans*, in the 3 unaffected siblings. The plan for this family is to follow on a routine basis (every 1-2 years) to re-interpret the variants in *MADD*.

In summation, this research has supplied further evidence that exome reanalysis is a useful tool for patients presenting with presumed genetic epilepsy as well as intellectual disability and/or developmental delay. As scientific knowledge increases for genetic variants associated with diseases, and as the understanding as to the function of genes in general, increases in the coming years, it will become an increasingly valuable step for patients without a genetic diagnosis so as to implement this knowledge in their diagnostic odyssey that would not have been available at the time of their initial testing.

Chapter 3 Functional studies for a novel *UBE2R2* variant detected in a patient with rare genetic epilepsy and intellectual disability

3.1 Introduction

The research presented in this chapter is in relation to an individual with borderline intellectual disability and epilepsy. She presented to the outpatient Genetics Clinic at the Children's Hospital of Eastern Ontario at 10 years of age with developmental delay, intellectual disability, absence seizures, and a history of chronic encopresis and enuresis. Genetic testing in the clinic-setting included *SCN1A* sequencing, microarray, and epilepsy multigene panel testing which all yielded non-diagnostic results. This individual was then enrolled into the Care4Rare Canada Research program in 2018, at an early stage of NGS implementation - prior to the widespread availability of clinical exome sequencing in Ontario. The participant and her parents had exome sequencing performed on a research basis, and this was also non-diagnostic. After the non-diagnostic research exome, the sequence data for proband and parents was placed into the Care4Rare (C4R) reanalysis pipeline. Exome sequence reanalysis was then performed periodically every 12 months. Given the lack of consanguinity in the family and no affected family members, it was hypothesized that this individual had epilepsy and intellectual disability due to variants inherited as a *de novo* variant or an autosomal recessive-acting gene. After variants in known disease genes as well as multi-heterozygous variants in genes not currently associated with disease were ruled out in exome sequencing reanalysis, a novel *de novo* loss of function variant in *UBE2R2* was identified. This is a *de novo* duplication at position c.21 predicted to result in a frameshift mutation, NM_017811.3: c.21dup p.(Ser8GlnfsTer56). The depth of coverage was 110X in the mother, 113X in the father and 153X in the proband at this location. The *de novo* variant was detected in 77 reads in the proband and was absent in both the mother and father. This variant has not been observed in affected individuals in the literature, the Care4Rare database or in ClinVar. It has also not been observed in presumed healthy control databases (gnomAD 4.1.0

(most recent)). Specific roles of UBE2R2 are not well known aside from its function as an E2 ubiquitin-conjugating enzyme (i.e., adding ubiquitin to proteins). This reanalysis, and identification of the *UBE2R2* variant, was performed just prior to the start of my thesis project. Based on the reanalysis data, rarity, *de novo* status as well as biological plausibility due to its involvement in the ubiquitination pathway, the *UBE2R2* variant was considered the most promising candidate. At the time of reanalysis, there were no other loss of function variants reported in this gene of uncertain significance reported in gnomAD (V2.1). In an updated version (V4.1.0), four other frameshift variants (p.Asn50ThrfsTer29, p.Met81CysfsTer21, p.Met147CysfsTer35, and p.Glu160ArgfsTer4) and one stop gained variant (p.Glu220Ter) in *UBE2R2* have been reported, with the variant of interest for this patient remaining unreported. The variant was considered a viable candidate to move forward with further testing. Not only this, but the UBE2R2 protein contains a glycyl thioester intermediate at position 93, an active site that functions as an intermediate in the ubiquitin process (Song et al., 2009, UniProt Q712K3). Within the ubiquitination pathway, E2 ubiquitin conjugating enzymes form a thioester bond with the activated ubiquitin to hold it prior to transfer to the substrate (David et al., 2010; Pruneda et al., 2011). The predicted truncation that would result from the detected variant would end the protein at position 56 – indicating that this suspected protein would not contain this domain. It is therefore suspected that processes requiring the use of this active site will be significantly affected by the protein truncation, and that normal functions in general will be affected as a result of the truncated protein suspected to arise from the variant.

To demonstrate that a GUS is associated with a disease, there are several tools that can be used to generate sufficient evidence that this gene is associated with the phenotype or disease seen in the participant. Within the C4R research workflow, the identification of similarly affected individuals with similar variants in the same gene is one such tool. Therefore, to provide evidence for disease causality of *UBE2R2*, we first sought a gene match through submission to GeneMatcher and Matchmaker Exchange, which are two large databases where clinical geneticists, or other interested individuals, from different organizations upload genes of interest along with the associated patient features (Sobreira et al., 2015). If another clinical geneticist has uploaded similar patient features and the same gene highlighted, the two geneticists are notified and can communicate with one another – this is considered a “match”. With this process, it is possible to determine that there are multiple individuals (>2) with similar phenotypic features

and rare variants within the same gene of interest; thus providing further evidence in favour of this particular gene being disease-causing. At this time, the *UBE2R2* entries in Matchmaker Exchange (entered 2022) and GeneMatcher (entered in 2023) have had no matches; however, this is a continuous process, and the gene remains enrolled in both wherein the appropriate individual (myself or a genetic counselor in C4R) will be notified if there is a match in the future.

Another step in providing evidence that a gene is associated with a disease is through functional laboratory studies to determine the effect a variant may be having on the protein function and associated pathways. The following research examines some of the implicated proteins and pathways suspected to be affected by the UBE2R2 truncated protein to assess its pathogenicity.

3.1.2 UBE2R2 gene and protein

UBE2R2 is a gene that encodes for an E2 ubiquitin conjugating enzyme, which ubiquitinates proteins that can then be targeted for degradation through the 26S proteasome degradation pathway (NCBI, Gene ID: 54926). From the latest assembly, this gene is 105 232 bases long and located on chromosome 9 (MANE transcript, ENSP00000263228). The resulting protein is comprised of 238 amino acids with a molecular weight of approximately 27.2 kDa (MANE transcript ENSP00000263228, GeneCards, ID: GC09P061619) .The Human Protein Atlas (currently the largest database for protein distribution in tissues and cells) does not have data for protein expression for UBE2R2 at this time, but RNA expression data indicates that *UBE2R2* RNA is expressed in the brain along with most other tissues (Thul & Lindskog, 2017; *Tissue Expression of UBE2R2 - Summary - The Human Protein Atlas*, n.d.). Presently, *UBE2R2* is not associated with any disease(s) in OMIM (MIM 612506). However, research regarding long non-coding *UBE2R2* RNA and antisense *UBE2R2* RNA suggests that it may be a prognostic marker in some cancers (Wang et al., 2023; Xu et al., 2024).

3.1.3 Ubiquitin-proteasome degradation pathway

The ubiquitin-proteasome degradation pathway uses ubiquitin to target substrates for degradation through proteolysis (Ciechanover & Schwartz, 1998; Glickman & Ciechanover, 2002; Hegde, 2004). In this pathway, three enzymes polyubiquitinate the substrate, which the subunits of the

26S proteasome will recognize, as they have high affinity for ubiquitin, and will bind and degrade the substrate through unfolding the protein, removing the ubiquitin and translocating these to proteolytic sites (Collins & Goldberg, 2017; Grice & Nathan, 2016; Saeki, 2017). The function of interest for the purpose of this research is with regards to the three enzymes that will ubiquitinate the substrate. There are many E1, E2 and E3 enzymes, but broadly the E1 activating enzyme will activate ubiquitin before transferring it to the E2 ubiquitin conjugating enzyme (Guo et al., 2024; Stewart et al., 2016). The E3 ubiquitin protein ligases have a substrate recognition subunit that allows it to bind to the substrate; E2 enzymes and E3 ligases can interact with each other and with all other steps completed, the activated ubiquitin from the E2 enzyme is transferred to the substrate protein bound to the E3 ligase (Guo et al., 2024; Wang et al., 2016; Wijk et al., 2009). This degradation pathway is involved in numerous functions and pathways in the cell including, but not limited to: protein quality control, cell cycle progression, apoptosis and signal transduction (Aminake et al., 2012; Majumder & Baumeister, 2019; Nunes & Annunziata, 2018; Park et al., 2018).

3.1.4 Pathways affected by UBE2R2

UBE2R2 is implicated in numerous pathways by virtue of being an E2 ubiquitin conjugating enzyme and the numerous proteins and pathways wherein the proteasome degradation is involved. To begin, UBE2R2 (also known as UB3CB) has a subunit that, when phosphorylated, will interact with β-TrCP (Semplici et al., 2002). β-TrCP is the substrate recognition subunit of an SCF E3 ligase. This subunit recognizes and will bind to β-catenin which will target it for ubiquitination (Fuchs et al., 2004; Semplici et al., 2002). As β-catenin is a signal transducer (a substrate outside the cell that can activate signaling) for the Wnt signaling pathway, this is another pathway wherein the variant may affect its function (Liu et al., 2022; Pai et al., 2017). The Wnt/β-catenin pathway is involved a multitude of functions/pathways such as embryonic development, regulating cellular functions such as apoptosis and cell proliferation to name a few, and its dysregulation has been associated with multiple diseases (Liu et al., 2022; Pai et al., 2017).

Further indicative that the variant may impact normal apoptotic functions is the interaction of UBE2R2 with SMYD3, a protein involved in the control of apoptosis (Zhang et al., 2019).

SMYD3 is considered a novel E3 ligase that recognizes p53 as its substrate, it promotes p53 ubiquitination and thusly regulates its expression (Han et al., 2024; Zhang et al., 2019). Apoptosis is known to be impacted by the expression of p53 and, as UBE2R2 has been proven to interact with SMYD3, it is suspected to affect apoptosis through this mechanism as well (Han et al., 2024; Polyak et al., 1997; Zhang et al., 2019).
Yet another degradation pathway where this variant may affect its function is the mitophagy pathway. Parkin is an E3 ligase that functions as a regulatory molecule for mitophagy that utilizes E2 ubiquitin-conjugating enzymes to transfer ubiquitin to mitochondria during normal mitophagy (Fiesel et al., 2014; Iguchi et al., 2013). It has been established that the depletion of UBE2R1 affects the translocation of Parkin and p62 to mitochondria - affecting mitochondrial dynamics and function (Fiesel et al., 2014). As UBE2R1 is an E2 ubiquitin conjugating enzyme that functions in many of the same pathways as UBE2R2, it suggests that UBE2R2 may affect the mitophagy pathway through a similar fashion.
Cell cycle progression is yet another pathway where UBE2R2 contributes to its function. UBE2R2 helps to extend the ubiquitin chain on a key molecule involved in cell cycle progression from G1 to S phase (Du et al., 2021; Wijk & Timmers, 2010). Specifically, UBE2R2 helps elongate the ubiquitin chain of cell cyclin E, an essential molecule that activates its catalytic partner CDK2 to allow progression through the cell cycle (Mazumder et al., 2004). Cell cyclin E is regulated through this ubiquitination which is one way cell cycle is controlled (Clurman et al., 1996; Dang et al., 2021). This further demonstrates how ubiquitination itself is a regulatory process for many cellular functions, including the apoptotic proteasomal degradation pathways and cell cycle (Chen & Qiu, 2013; Kimura & Tanaka, 2010). A result of ubiquitination, and proteasome degradation, and impacting a multitude of pathways, is that E2 ubiquitin conjugating enzymes can be associated with many and have a significant influence over several downstream substrates (Wijk & Timmers, 2010).

3.1.5 Literature review to support the downstream effects of the *UBE2R2* variant as the cause for epilepsy

Broadly, the disruption of ubiquitination and the proteasome degradation pathway is known to be pathogenic and associated with epilepsy (Poliquin & Kang, 2022). Furthermore, it has been

found that disruption in the ubiquitination pathway through E3 ligases has been found to cause some rare comorbid epilepsies, with research focusing specifically on the disruption in ubiquitination as the cause of epilepsy in such cases (Kim et al., 2018; Zhu & Tsai, 2020). Examples of genes that encode for E3 ligases or components of CUL3-RING E3 ligases that cause or have been associated with epilepsy include *KLHL20*, *CUL4B*, and *NEDD4-2* (Sleyp et al., 2022; Tarpey et al., 2007; Zhu et al., 2017).

As UBE2R2 may influence the regulation of β-catenin expression, it is theorized that defects in its function will induce apoptosis (Kim et al., 2000). Mouse model research suggests that high levels of β-catenin increase susceptibility to seizures (Campos et al., 2004). It has also been observed that increased β-catenin leads to increased excitatory synapse density, altered synaptic maturation and function, and aberrant dendritic and axonal branching which makes β-catenin and its associated pathways an interesting target in relation to patients with epilepsy (Hormaechea-Agulla et al., 2018; Kimura & Tanaka, 2010; Seong et al., 2015). Wnt signaling is involved in brain development, and its regulation is required for normal development to occur (Chenn, 2008; Harrison-Uy & Pleasure, 2012). The Wnt/β-catenin signaling pathway regulates many of the changes seen in the brains of those with epilepsy and its differential regulation has been linked previously to epilepsy in epilepsy rat models (Hodges & Lugo, 2018; Rawat et al., 2023).

Since UBE2R2 interacts with the apoptosis pathway through the Wnt/β-catenin signaling pathway as well as through regulation of p53 and β-catenin, this is yet another avenue to explore with regards to its contribution to epilepsy. Seizures can activate apoptotic pathways of cells in the brain, which can impact the pathogenesis of epilepsy (Bazhanova & Kozlov, 2022; Henshall, 2007). It has also been suggested that dysregulated apoptosis may be involved in childhood epilepsy (Ahmed et al., 2024). Another degradation pathway UBE2R2 may be impacting is mitophagy, as Parkin (an E3 ligase) is known to interact with UBE2R2 and, as such, it is theorized that impacting UBE2R2 function will therefore affect the ubiquitination occurring through Parkin in normal mitophagy (Hayashida et al., 2023; Kazlauskaite et al., 2014). Dysfunctional mitophagy is linked to neurological disorders such as Parkinson's and Alzheimer's (Gao et al., 2024; Wang et al., 2022; Zhang et al., 2021). Increased rates of mitophagy are also considered to be a major trigger in epilepsies (Liang et al., 2000; Zhong et al., 2022).

These findings suggest that multiple pathways may be impacted by a variant in *UBE2R2* which may then ultimately result in genetic epilepsy.

3.2 Materials and methods

3.2.1 Lymphoblast cell culture

A lymphoblast cell line was established in late 2022 at the CHEO Research Institute. Control cell lines from the apparently healthy individual database at the Coriell Institute for Medical Research were used throughout all experiments for cell cultures, protein lysates and RNA extraction. Control cell line 1: AG15995 (Female, 14 years old), control cell line 2: AG15803 (Female, 12 years old) and control cell line 3: AG15792 (Female, 14 years old) were the cell lines selected. Aliquots were prepared for each of the samples to be placed in cryostorage which were then used for cell culturing. Three aliquots of patient cells and one aliquot from each control cell line were removed and placed immediately into a 37°C water bath until thawed. Each aliquot of the cells were then transferred to a falcon tube with 9 mL of RPMI cell culture media (containing 1% glutamine, 1% penicillin-streptomycin, 10% fetal bovine serum (FBS)) which was centrifuged at 300g for 5 minutes. The supernatant was removed, 5 mL media was used to re-suspend the cells and they were transferred to a flask with 25 mL media. These flasks were placed in a 37°C incubator with 5% CO_2. These flasks were monitored over the course of the research conducted using only the RPMI media containing 1% glutamine, 1% penicillin-streptomycin and 10% fetal bovine serum to ensure cells continued to grow appropriately.

3.2.2 Cell harvesting

Harvesting for RNA extraction

Ten mL of cell culture from the flasks described in section 3.2.1 were placed into falcon tubes and centrifuged at 300 g for 5 minutes. The supernatant was then removed, and the remaining pellet was frozen at -80°C until needed for RNA extraction. This procedure was repeated for three patient samples and each of the three control cell lines at a time.

Harvesting for protein lysate extraction

At least 10 mL of cell culture from the flasks described in section 3.2.1 was placed into falcon tubes and centrifuged at 300 g for 5 minutes. The supernatant was then removed, and the cells were resuspended in 3-5 mL of 1x PBS before centrifugation again at 300 g for 5 minutes. The supernatant was removed once again, and the pellet was resuspended in 1.8 mL of PBS before

immediately being transferred to a 2 mL Eppendorf tube. These tubes were then centrifuged for 15 minutes at 15000g, and the supernatant was removed. These pellets were frozen at -80°C until needed for protein lysate extraction.

3.2.3 Quantitative reverse polymerase chain reaction (RT-qPCR)

Total RNA was obtained from three patient samples and each control line using the RNeasy mini kit (QIAGEN, cat. 74104) and reverse transcribed into complementary DNA (cDNA) at a concentration of 7.5 ng using the iScript™ Advanced cDNA Synthesis Kit (BioRad, cat. 1725037). Control reactions without reverse transcriptase were prepared at the same time. cDNA was amplified using gene specific primers. Primers were tested with reverse transcriptase positive and negative cDNA in cDNA generated from the AG15995 cell line, followed by temperature gradient to determine the optimal annealing temperature for each primer. cDNA was amplified with the iQ™ SYBR® Green master mix (BioRad). Experiments were performed on a BioRad T100 Thermal Cycler. The following standard conditions were followed: (1) 95°C for 5 minutes, (2) 95°C for 10 seconds, (3) annealing temperature for 30 seconds, (4) 72°C for 30 seconds, (5) return to step 2 and repeat 34 cycles, (6) 95°C for 10 seconds, (7) final melting curve in increments of 0.5°C from 65°C to 95°C. The annealing temperature for step (3) was optimized per primer as follows: *UBE2R2, UBE2R1, UBE2G2, UBE2G2*: 55°C, *LEF1*: 61.4°C, *MYC*: 57°C, and *AFF3*: 59°C. RT-qPCR was completed in triplicate for each gene-specific primer, with each sample having biological duplicates for every RT-qPCR completed (patient sample 1, patient sample 2, patient sample 3, control 1, control 2, control 3) wherein three separate frozen pellets as described in 3.2.2 were used. Gene expression data were corrected against *GAPDH* gene-specific primer as an internal control for all analyses. All error bars represent the standard error of the mean.

3.2.4 Western blot

RIPA lysis buffer and p8430 protease inhibitor were mixed at a 1:100 ratio (inhibitor: buffer) where 300-500 uL was then added to pellets as described in section 3.2.2 for protein lysate extraction. Vortexing intermittently over the course of 1-2 hours occurs before centrifugation at 15000 g for 15 minutes. The supernatant was removed into a new Eppendorf tube and 200 uL of the RIPA/p8430 protease inhibitor mix was added to the remaining pellet. The vortexing and

subsequent centrifugation was repeated. All accumulated supernatant was the total protein lysate. Protein concentration was measured using the Pierce™ BCA Protein Assay Kit (cat. 23225). Standard curve was generated using the albumin standard provided in duplicate and each protein lysate was measured in triplicate. This total process was completed in triplicate for each sample type, resulting in biological triplicates for each sample (patient sample 1, patient sample 2, patient sample 3, control 1, control 2, control 3). Using the calculation *concentration = mass/volume*, 30 ng – 50 ng of protein lysate was placed into an Eppendorf tube and mixed with 1x NuPage™ LDS Sample Buffer. These mixes were then frozen overnight at -20°C. Western blot analysis was carried out using anti-UBE2R2 (ThermoFisher, cat. 14077-1-AP), anti-UBE2R1 (Novus Biologicals, cat. NBP1032153), anti-UBE2G1 (ThermoFisher, cat. PA5-30201), anti-UBE2G2 (ThermoFisher, cat. PA5-98226), anti-p53 (Cell Signaling Technology, cat. 9282T), anti-β-catenin (Abcam, cat. ab16051), anti-MYC (ThermoFisher, cat. 10828-1-AP), anti-AFF3 (ThermoFisher, cat. PA5-68628), or anti-LEF1 (ThermoFisher, cat. 14972-1-AP).

3.2.5 Cell growth curve

Cells from flasks described in section 3.2.1 were used. Cells were mixed thoroughly to ensure all clumps of cells were separated and distributed evenly. Using a pipette, 0.5 mL of cells were placed into an Eppendorf tube. This was further mixed, and 15 uL was placed into a separate Eppendorf tube. An equal amount (15 µL) of Trypan Blue Solution, 0.45 (ThermoFisher, cat. 15250061) was added and mixed by pipetting up and down 3 to 5 times. Ten µL of this mix was added to each side of a two-chamber haemocytometer provided with the Corning® Cell Counter (cat. CLS6749). The haemocytometer was then placed on the aforementioned cell counter, the view was adjusted to ensure the cells were in view. Four images were taken of each side of the haemocytometer, and the software counted the live cells, thus providing a final concentration in cells/mL. Using the formula $C_1V_1 = C_2V_2$, where C indicates the cell concentration in cells/mL and V indicates volume in mL, the amount of the cell mixture required for a final volume of 5 mL with a concentration of $2x10^5$ cells/mL was determined. Additional media was added as needed to achieve a final volume of 5 mL. Should the volume of the cells be higher than 5 mL, that calculated volume was placed in a falcon tube and centrifuged at 300g for 5 minutes. The supernatant was removed, and the pellet resuspended in 5 mL of RPMI media as described in section 3.2.1. The 5 mL of $2x10^5$ cells/mL for each cell line (patient flask 1, patient flask 2, patient flask 3, control 1, control 2, control 3) was then placed into a well from a Corning®

Falcon® Cell Culture Plate (6 wells, cat. CLS353224). After ensuring the cells are mixed within the wells, the cell culture plate was then placed into a 37°C incubator with 5% CO_2 overnight; this was Day 0. The following morning, for each well the counting process was repeated. The wells were mixed thoroughly, 150 μL was then removed to a new Eppendorf tube. This was mixed again and 15 μL was placed into another Eppendorf tube. This was mixed with 15 μL of Trypan blue. Ten μL of this mixture was added to each side of the haemocytometer and counted using the Corning Cell Counter. This was done for each well for each sample – these were used as the Day 1 results. This process was repeated up to Day 4. Analysis was completed by determining the number of proliferations each cell line went through each day by dividing the cell concentration by the starting concentration (i.e., $x/2x10^5$). This process was completed in triplicate with each individual growth curve representing one well from each cell line. All error bars represent the standard error of the mean.

3.2.6 Flow cytometry

After 5 mL of cells were treated with 2.5 μL BrdU, cells were treated with the Phase-Flow™ BrdU Kit (BioLegend, cat. 370704). Cells were fixed immediately after BrdU was added for staining at a later time overnight before the protocol provided was completed. After the final step, where cells were stained with the anti-BrdU antibody followed by a final wash with Buffer B, cells were resuspended in PBS containing 7-AAD (1μg/sample). They were then incubated for 10 minutes before flow cytometry was performed. Each cell line was analyzed in triplicate. All error bars represent the standard error of the mean.

3.2.6 Cell treatment

Mitophagy Induction

In new flasks, following the culture protocol described in section 3.2.1, cells from each cell line were cultured in the described RPMI media for 4-5 days until active proliferation was seen. In Corning® Falcon® Cell Culture Plate (6 wells, cat. CLS353224), 6mL of each cell line was placed into one well. These were cultured for 8 hours, mixed partway through with pipetting up and down, with spermidine at a concentration of 50 μM. The spermidine (ThermoFisher, cat. A19096.06) was diluted to 50 mM and 1 μL/mL was added to cell culture. After culturing, the cells were harvested for protein lysate extraction as described in sections 3.2.1 and 3.2.4. This

was completed in triplicate for each cell line. These samples were then analyzed using the western blot protocol, with untreated samples compared to the samples treated with spermidine.

Apoptosis Induction

In new flasks, following the culture protocol described in section 3.2.1, cells from each cell line were cultured in the described RPMI for 4-5 days until active proliferation was seen. In Corning® Falcon® Cell Culture Plate (6 wells, cat. CLS353224), 6 mL of each cell line was placed into one well. These were cultured for 5 hours. Actinomycin D was added at a concentration of 1µM at the start, and each well was further mixed partway through with pipetting up and down. Actinomycin was kindly provided by Martine St. Jean for this purpose. After culturing, the cells were harvested for protein lysate extraction as described in sections 3.2.1 and 3.2.4. This was completed in triplicate for each cell line.

3.3 Results

3.3.1 mRNA and protein expression of E2 ubiquitin conjugating enzymes

The first step in this functional analysis was assessing the impact of the *UBE2R2* variant on mRNA and protein expression. UBE2R2 is part of a family of E2 ubiquitin conjugating enzymes that encompasses other E2 enzymes such as UBE2R1, UBE2G1 and UBE2G2 (Stewart et al., 2016). A study completed in 2019 found that UBE2G1, another E2 ubiquitin conjugating enzyme, may functionally compensate for the loss of UBE2R1 and UBE2R2 in *UBE2R1/2* knockout cell lines (Hill et al., 2019). Therefore, in addition to testing mRNA and protein levels of the gene of interest, three additional E2 ubiquitin conjugating enzymes were included in these analyses with the goal of observing any significant changes in their expression that may indicate functional compensation for the loss of functioning UBE2R2.

To determine if the mRNA levels were affected, RT-qPCR was completed for all E2 ubiquitin conjugating enzymes of interest (Figure 3-1). Furthermore, western blot analysis for each E2 ubiquitin conjugating enzyme of interest was completed while optimized for each antibody to observe protein expression (Supplemental Table 3-1, Supplemental Figure 3-1,Figure 3-2). Only western blot analysis for UBE2R2 protein expression demonstrated a significant decrease in patient samples, and only *UBE2R1* mRNA expression demonstrated a significant increase in patient samples through RT-qPCR analysis.

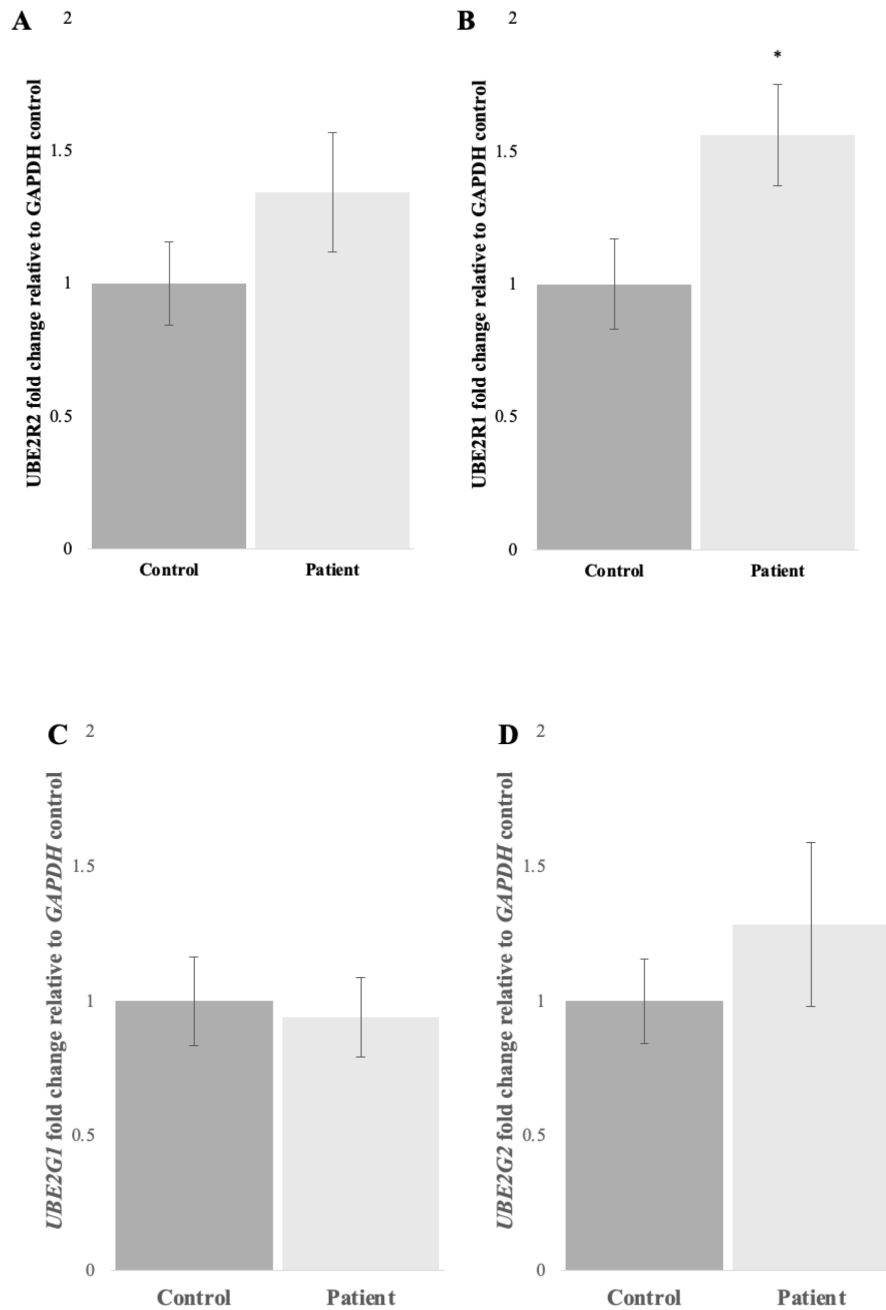
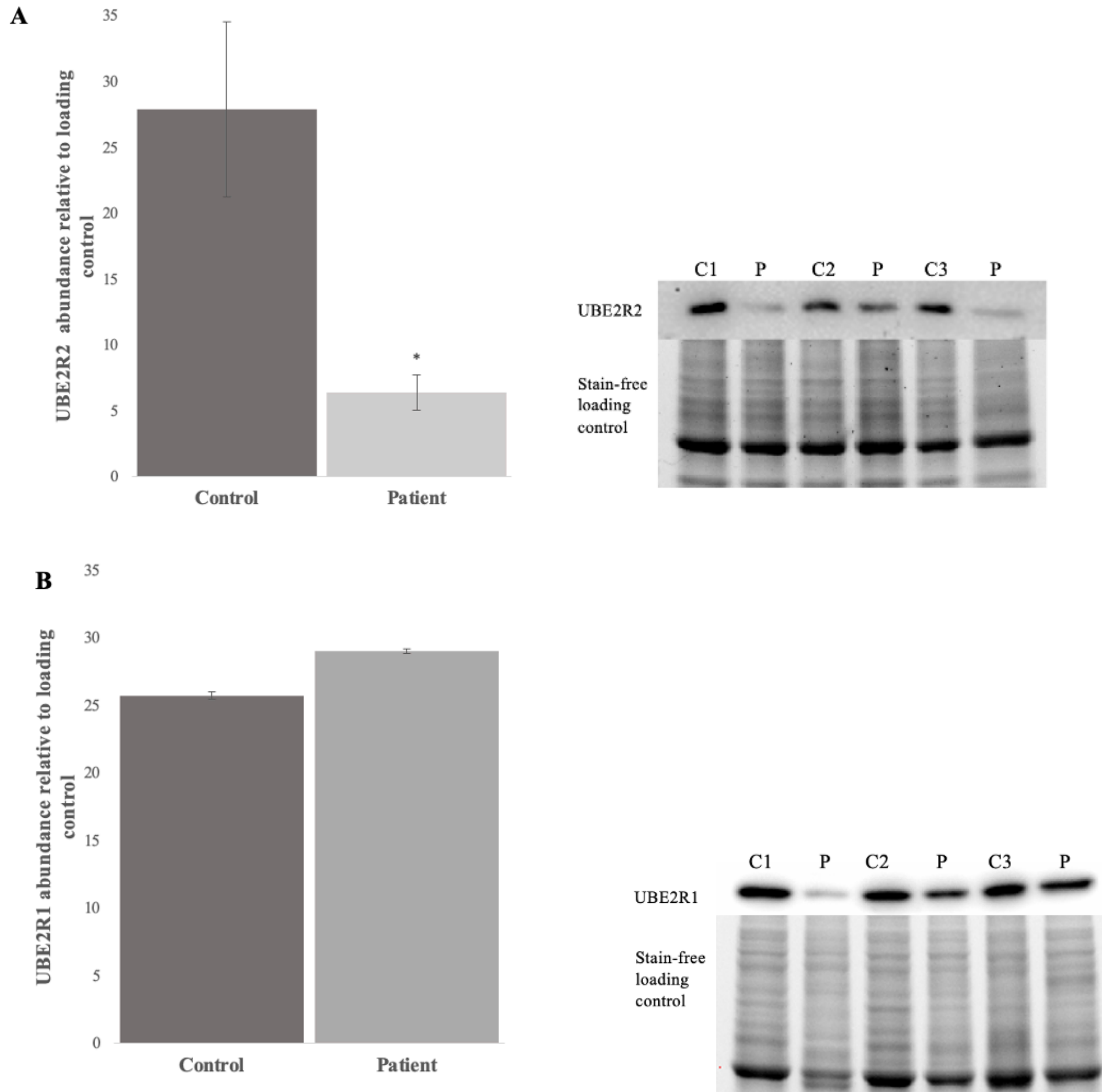


Figure 3-1 RT-qPCR for analysis of mRNA expression of *UBE2R2* and three other E2 ubiquitin conjugating enzymes (*UBE2R2, UBE2R1, UBE2G1*, and *UBE2G2*). Total RNA was extracted from lymphoblast cell lines for three patient samples and three different control samples and mRNA was then reverse transcribed into DNA. RT-qPCR was performed on these samples with gene specific primers designed for (**A**), *UBE2R2* (**B**), *UBE2R1* (**C**) *UBE2G1,* and

(**D**) *UBE2G2*. The Ct values were normalized to *GAPDH*, with the values from control samples normalized to 1. Representative bar graphs (n=3) were generated from the normalization values. A significant increase was detected in *UBE2R1* expression (p=0.04). The other E2 ubiquitin conjugating enzymes had no significant differences.



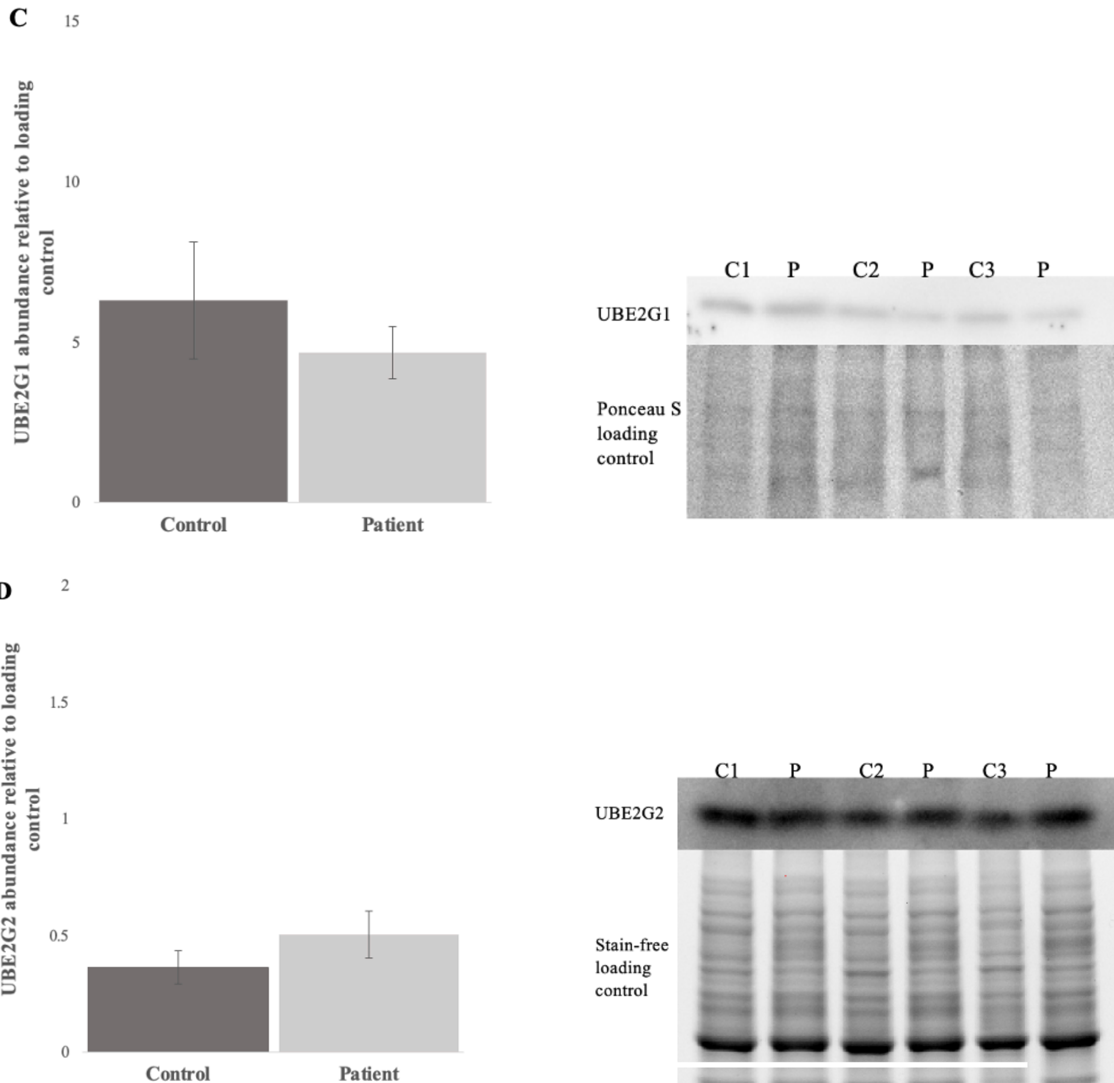


Figure 3-2. Western blots to analyze expression of other E2 ubiquitin conjugating enzymes demonstrate that protein expressions are not significantly affected by the identified *UBE2R2* variant. Protein lysate was extracted from each cell line with 30ng used and run following the western blot protocol. (**A)** Western blot membranes were treated with a UBE2R2 antibody. A representative western blot from all runs (n=3) shows the detected UBE2R2 protein bands. To the right of the graph representing the relative protein abundance, a representative western blot image is aligned with its loading control (stain free gel). The protein detected was normalized against this control for calculations. There was a significant difference in protein expression from the combined results of each western blot completed for this antibody (p= 0.006). (**B)** Western blot membranes were treated with a UBE2R1 antibody. A representative western blot from all runs (n=3) shows the detected UBE2R1 protein bands. To the right of

graph representing the relative protein abundance, a representative western blot image is aligned with its loading control (stain free gel). The protein detected was normalized against this control for calculations. There is not a significant difference in protein expression (p=0.69). **(C)** Western blot membranes were treated with a UBE2G1 antibody. A representative western blot from all blots (n=3) shows the detected UBE2G1 protein bands. To the right of the graph representing the relative protein abundance, a representative western blot image is aligned with its loading control (Ponceau S, also referred to as ponceau red). The protein detected was normalized against this control for calculations. There is no significant difference in protein expression (p=0.60). (**D**) Western blot membranes were treated with a UBE2G2 antibody. A representative western blot from all blots (n=3) shows the detected UBE2G2 protein bands. To the right of the graph representing the relative protein abundance, a representative western blot image is aligned with its loading control (stain free gel). The protein detected was normalized against this control for calculations. There is not a significant difference in protein expression (p=0.27).

3.3.2 Cell growth and cell cycle analysis

Prior studies established that UBE2R2 is implicated in the progression of the cell cycle from the G1 to S phase (Du et al., 2021; Wijk & Timmers, 2010). Specifically, UBE2R2 participates in elongating the ubiquitin chain of cyclin E, an essential molecule that activates its catalytic partner CDK2 in order to progress through the cell cycle (Mazumder et al., 2004). Lymphoblast cells are rapidly growing cultures derived from peripheral blood samples and undergo the typical cell cycle with a standard growth curve (American cancer society, 2003; Jankauskaitė et al., 2017; Ligasová et al., 2023). Due to cyclin E being a key component of cell cycle machinery, disrupting its regulation would have significant impacts on cell growth and the overall cell cycle process (Chu et al., 2021; Guertin & Sabatini, 2015). It was thus theorized that the *UBE2R2* variant, through disrupting the regulation of cyclin E and cell cycle machinery, would result in differences in the growth curve of patient cells as well as how patient cells progress through the cell cycle.

To analyze cell growth, a growth curve was conducted over the course of 5 days (starting with day 0) where patient and control cells started with the same number of cells per well and were counted for the remaining 4 days to determine if growth rates were affected by the variant. The resulting growth curves demonstrated no significant differences in patient and control cell growth (Figure 3-3). Following this, flow cytometry was used to analyze the number of cells in

each phase of the cell cycle. One method commonly used to analyze cell cycle with flow cytometry is by using BrdU, a thymidine analog that can be incorporated into DNA during the S-phase of the cell cycle as new DNA is synthesized (Glasper et al., 2010). This can be coupled with 7-AAD (7-aminoactinomycin D) which is another staining reagent that binds to double stranded DNA (*Flow Cytometry Protocol: 7-AAD Cell Viability: R&D Systems*, n.d.). As this binds to the total DNA in cells, it can be used to differentiate cells with different amounts of DNA; therefore, 7-AAD used in conjunction with BrdU can be used to distinguish cells in different phases of the cell cycle. This double-staining method was used in a flow cytometry experiment to analyze the proportion of cells in each phase of the cell cycle, ultimately demonstrating no differences between the patient and control cell samples (Figure 3-4).

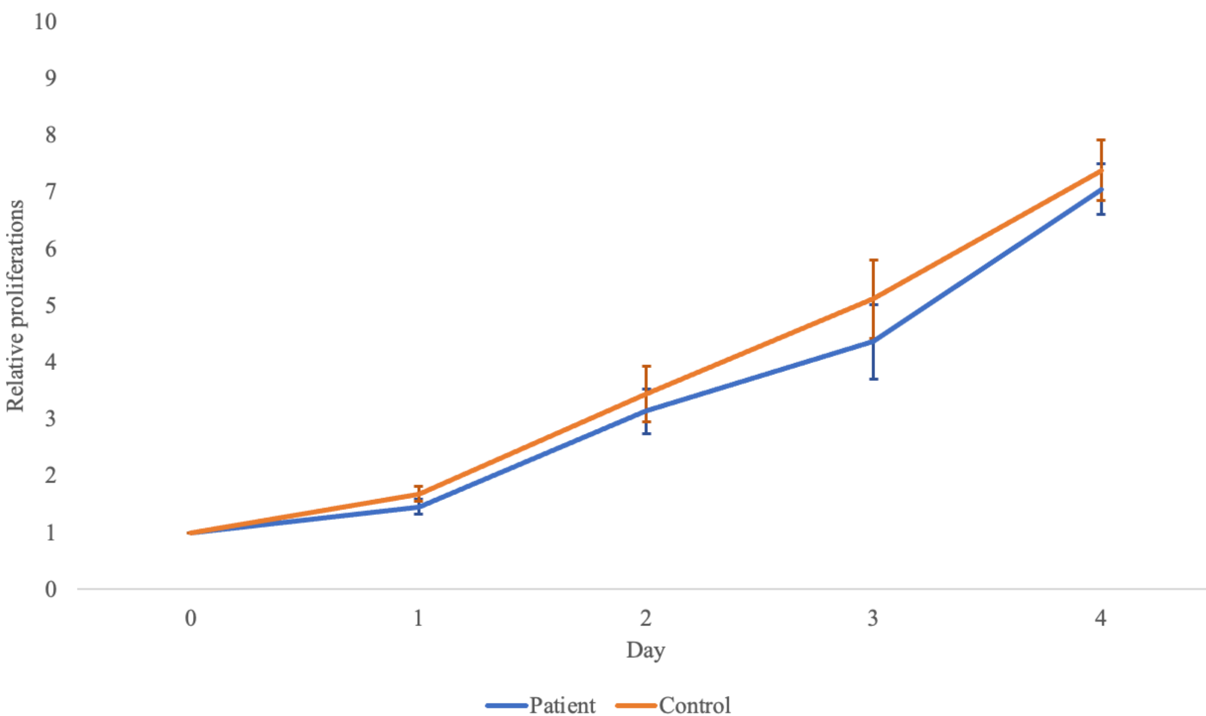


Figure 3-3 Growth curve of patient and control cells demonstrates no significant difference in cell growth between patient and control lymphoblast cells. On day 0, $2.0x10^5$ cells/mL for three separate cultures of patient cells and one culture for each control sample were placed in an incubator, cells were counted for days 1-4. The total concentration of cells was divided by the starting concentration to determine the "relative proliferations" of each culture. The data is presented with standard error of the mean bars for each sample on each day.

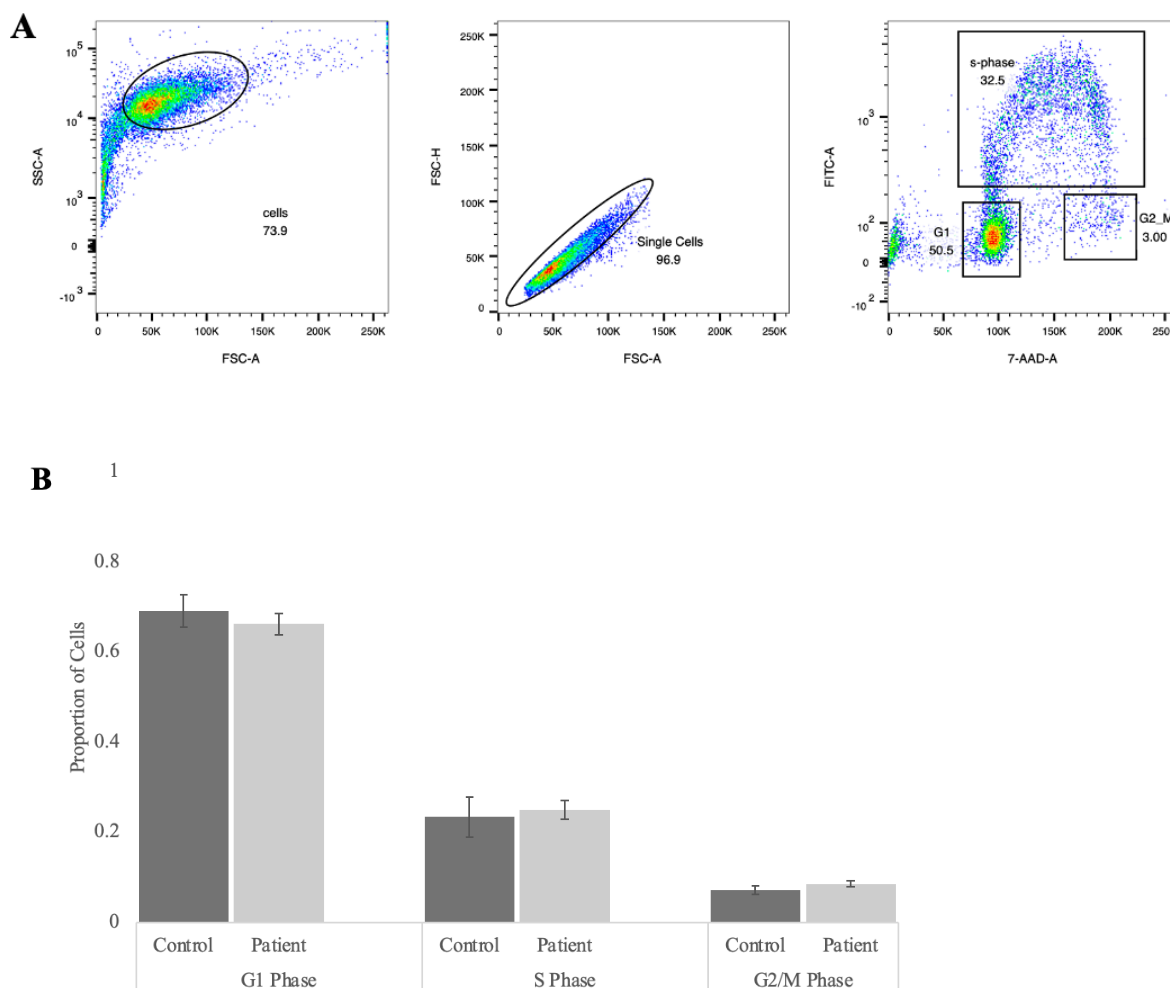


Figure 3-4 Progression through cell cycle phases is unchanged between patient and control samples. The gating strategy for all samples was (1) viable cells were detected by FSC (forward light scatter)-area by SSC (side scatter)-area followed by (2) gating for singlets using FSC-height by FSC-area. Negative controls were used following the manufacturer's instructions to gate all samples for each phase of the cell cycle. **(A)** Gating strategy for all samples. From right to left: Viable cells, single cells, phase of the cell cycle. The gating system was identical for every sample. **(B)** Representative graph of the proportion of cells detected in each phase of the cell cycle for control and patient samples. Staining and flow cytometry analysis was completed in triplicate for each cell line used. This flow cytometry experiment did not display any differences between control and patient samples.

3.3.3 Interaction with proteins of interest and downstream pathways

It has been established that UBE2R2 interacts with the substrate recognition subunit of an SCF (Skp, Culling, F-box containing complex) protein, β-TrCP, which is an E3 ligase. This subunit recognizes β-catenin for ubiquitination, which is subsequently degraded through the 26S proteasome degradation pathway. Since it is thought that the protein from the *UBE2R2* variant would be truncated, and the data collected demonstrates a significant decrease in the UBE2R2 protein, it was theorized that the degradation of β-catenin would be significantly decreased in patient cells. Western blotting for β-catenin was completed, with no significant differences found between patient and control cells (Figure 3-5, Supplemental Figure 3-2). As β-catenin is a signal transducer for the Wnt pathway, investigation into Wnt target genes was completed to observe if the *UBE2R2* variant would impact their expression. These were: *MYC, AFF3* and *LEF1* (Lefèvre et al., 2015; Ramakrishnan & Cadigan, 2017). RT-qPCR and western blot experiments were completed for all three. A significant decrease in *AFF3* (mRNA and protein) was observed in patient cells (Figure 3-6, Supplemental Figure 3-3).

Prior studies also confirmed the interaction between UBE2R2 and SMYD3, an E3 ligase that targets p53. As this interaction increases ubiquitination and subsequent degradation of p53 through the 26S proteasome degradation pathway, exploration of the potential effect on p53 protein levels in patient cells was explored through western blots. There was no statistically significant difference in p53 expression between patient and control samples (Figure 3-7, Supplemental Figure 3-4).

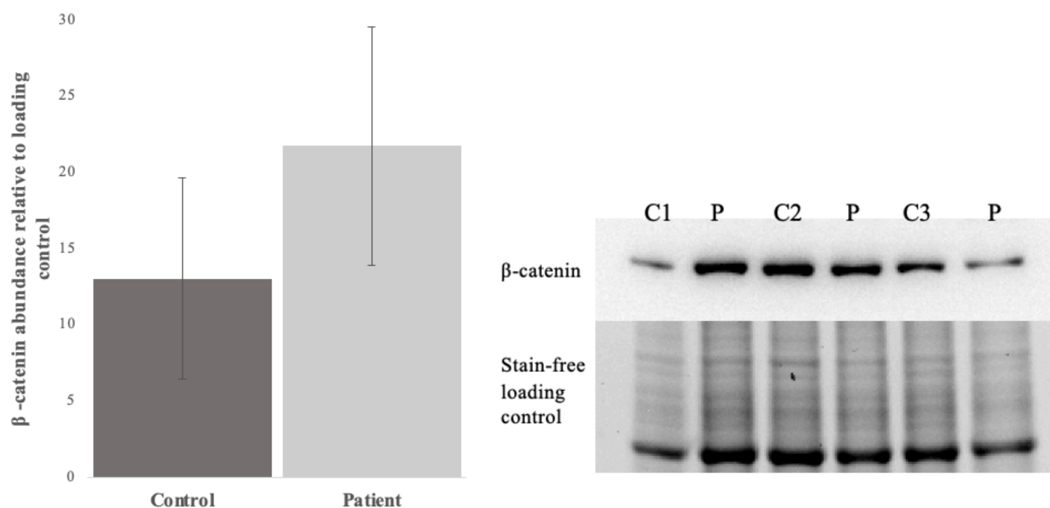
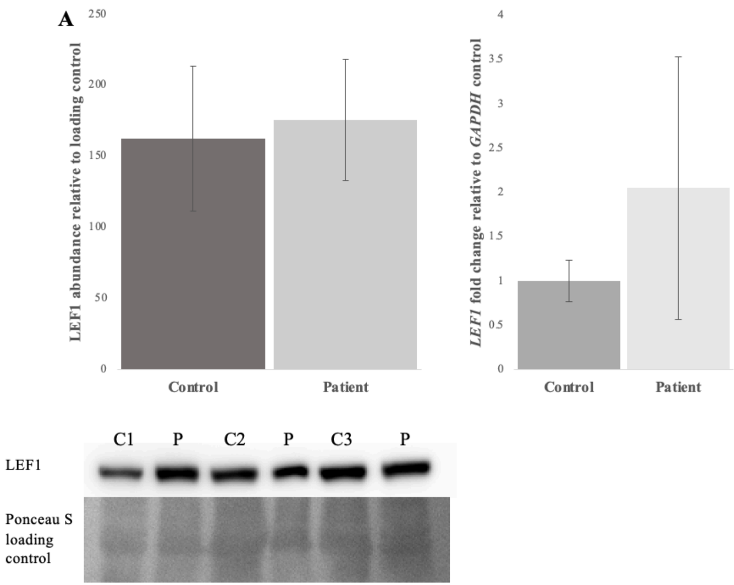
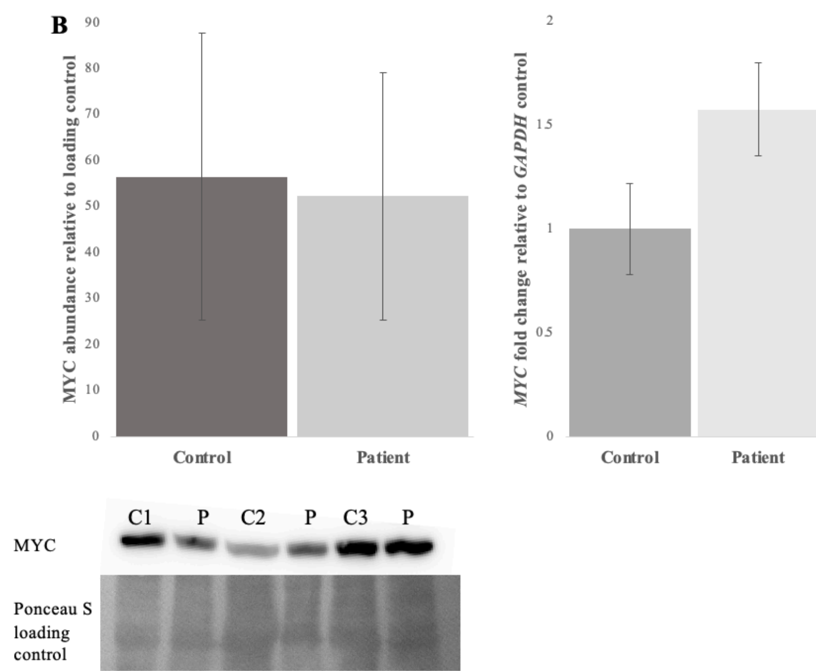


Figure 3-5 Western blot to analyze protein expression of β-catenin shows no difference in expression. Protein extracted from patient and control cells were treated with a β-catenin antibody. A representative western blot from all blots (n=4) shows the detected β-catenin protein bands in the right panel. The detected protein was normalized against the loading control (stain-free gel), represented in the panel below the β-catenin bands. As seen in the graph, to the left of the representative blot, no significant difference between control and patient samples were detected.


A
LEF1 abundance relative to loading control
250
200
150
100
50
0
Control
Patient
LEF1 fold change relative to GAPDH control
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Patient
C1
P
C2
P
C3
P
LEF1
Ponceau S
loading
control


B
MYC abundance relative to loading control
90
80
70
60
50
40
30
20
10
0
Control
Patient
MYC fold change relative to GAPDH control
2
1.5
1
0.5
0
Control
Patient
C1
P
C2
P
C3
P
MYC
Ponceau S
loading
control

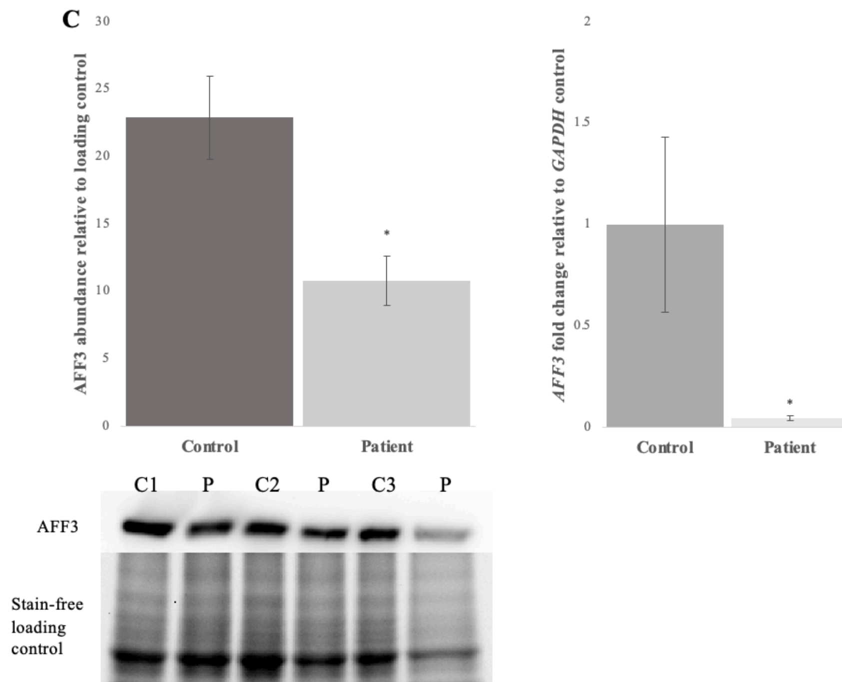


Figure 3-6 mRNA and protein expression of Wnt target genes demonstrates significant differences in only *AFF3* expression. (**A**) Protein extracted from control and patient cells were treated with a LEF1 antibody. A representative western blot from all blots (n=3) shows the detected LEF1 protein bands. The detected protein was normalized against the loading control (Ponceau S, also referred to as ponceau red). The leftmost graph and the panels below are representative of these results, showing no difference between control and patient samples. The rightmost panel represents the RT-qPCR completed with mRNA extracted from patient and control samples, with a gene-specific primer designed for *LEF1*. This also demonstrates no difference in mRNA expression. (**B**) Protein extracted from control and patient cells were treated with a MYC antibody. A representative western blot from all blots (n=3) shows the detected MYC protein bands. The subsequent protein detected was normalized against the loading control (Ponceau S, also referred to as ponceau red). The leftmost graph and panels below are representative of these results, showing no difference in protein expression. The graph to the right represents the RT-qPCR that was completed using mRNA extracted from patient and control cells, using a gene-specific primer designed for *MYC*. These results do not demonstrate a significant difference in expression (**C**) Protein extracted from control and patient cells were treated with an AFF3 antibody. A representative western blot from all blots (n=3) shows the detected AFF3 protein bands. The protein detected was normalized against the loading control

(stain-free gel). These results demonstrate a significant decrease in AFF3 protein in patient samples (p=0.0041). The rightmost graph represents RT-qPCR completed using a gene-specific primer designed for *AFF3* using mRNA extracted from control and patient cells. There was a significant decrease in mRNA expression (p=0.045).

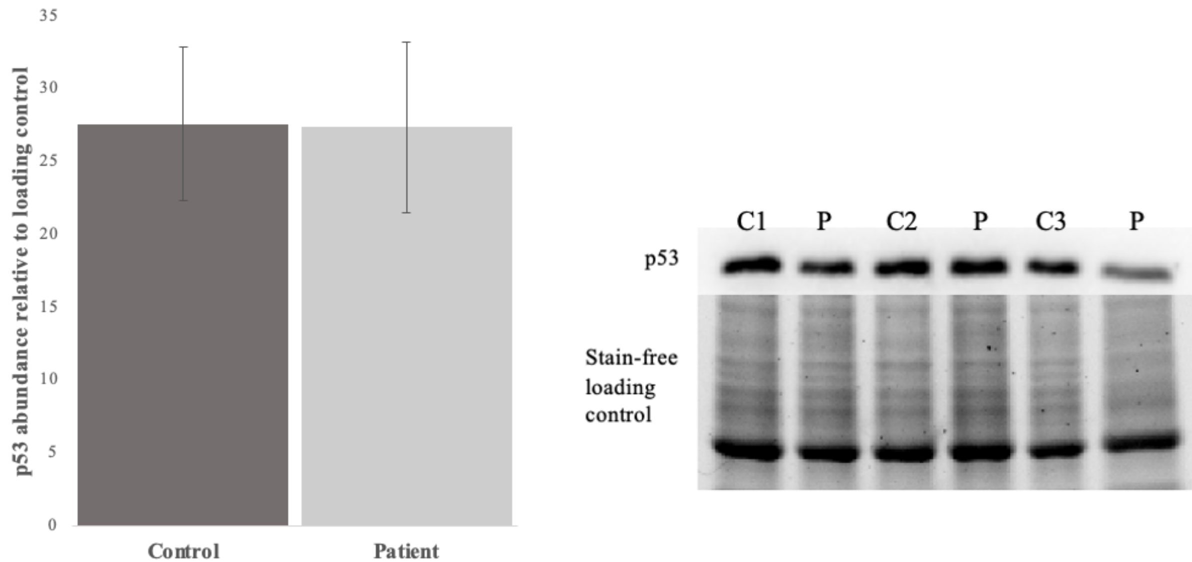


Figure 3-7 Western blot to analyze protein expression of p53 shows no difference in expression between control and patient samples. Protein extracted from patient and control cells were treated with a p53. A representative western blot from all blots (n=3) shows the detected p53 protein bands in the right panel. The detected protein was normalized against the loading control (stain-free gel), represented in the panel below the p53 bands. As seen in the representative graph, to the left of the representative blot, no significant difference between control and patient samples were detected.

3.3.4 Degradation pathways: apoptosis and mitophagy

With established research demonstrating that both p53 and β-catenin dysregulation can lead to increased apoptosis, it was suspected that the *UBE2R2* variant and predicted dysfunction in degradation of those molecules would result in significant differences in apoptosis levels. To test this, western blots using an apoptosis cocktail was completed. Actinomycin D was used to induce apoptosis in cells; procaspase 3 and cleaved PARP were detected after this treatment. Actinomycin D is a drug that is used to induce apoptosis in cell culture and accomplishes this through inhibiting synthesis of RNA and proteins, as well as blocking cell cycle (Lu et al., 2015). Procaspase 3 is the precursor for caspase 3, which is the active form of the protein that induces

apoptosis (Budd, 2001). PARP is a hallmark of apoptosis – the cleaved form detected here is one of two proteins generated when PARP is cleaved by a protease during active apoptosis (Gobeil et al., 2001). As it was suspected that the predicted UBE2R2 protein truncation would result in consequential differences in molecules that affect apoptosis, it was suspected that significant differences in these proteins would be observed. This was not the case as no change between patient and control samples was detected (Figure 3-8A, Supplemental Figure 3-5). While procaspase 3 seems to trend towards a significant increase (p=0.06), it is not possible to confirm that apoptosis has been affected through this mechanism as the cleaved PARP was not significantly affected.

Mitophagy, a specific type of autophagy that degrades mitochondria, can be regulated and caused by the Pink1/Parkin pathway (Khot et al., 2022; Yang et al., 2020). In this pathway, PINK1 activates Parkin which acts as an E3 ligase to ubiquitinate damaged or dysfunctional mitochondria to be targeted for degradation (Barodia et al., 2019; Torii et al., 2020). Prior studies have established that dysregulated mitophagy is detected in the brains of those with epilepsy, and that disruption of normal mitophagy function can cause epilepsy (Panda et al., 2022; Wang et al., 2024). As Parkin interacts with E2 ubiquitin conjugating enzymes to ubiquitinate mitochondria in normal mitophagy processes, it was suspected that the variant would affect ubiquitination and disrupt mitophagy in patient cells (Fiesel et al., 2014; Koszela et al., 2024). Furthermore, prior studies established that a decrease in the E2 ubiquitin conjugating enzyme UBE2R1 (a homolog of CDC34) affected the translocation of Parkin to mitochondria (Cocklin et al., 2011; Fiesel et al., 2014; Hill et al., 2019). As UBE2R2 is an E2 enzyme itself, and is another homolog of CDC34, it was suspected that a consequence of this variant would be dysfunctional mitophagy (Hill et al., 2019). Microtubule-associated protein 1 light chain 3 (LC3B) is a protein often used to detect autophagic activity in cells (El-Maraghy et al., 2023; Hwang et al., 2022). During autophagy, the cytosolic form of this protein (LC3BI) is converted to the membrane bound form (LC3BII) and the ratio of these two forms of the protein can be used to determine or detect differences in autophagic activity (El-Maraghy et al., 2023; Tanida et al., 2008). Thus, analysis of the expression levels of LC3BI and LC3BII can be used as a method to measure mitophagy changes in the cell (Chen et al., 2017). As such, western blots using an LC3B antibody that detects both LC3BI and LC3BII was used for patient and control. Spermidine was used to attempt to induce mitophagy in cell culture, chosen as prior studies have established that this

activated mitophagy through the PINK1/Parkin pathway (Naumova et al., 2023). However, the mitophagy induction was not successful. Cells before and after spermidine treatment were used for protein lysate extraction and analysis of LC3BI and LC3BII. In all cases, there were no differences in the LC3BII:LC3BI ratio between patient and control samples (Figure 3-8 B, Supplemental Figure 3-6).

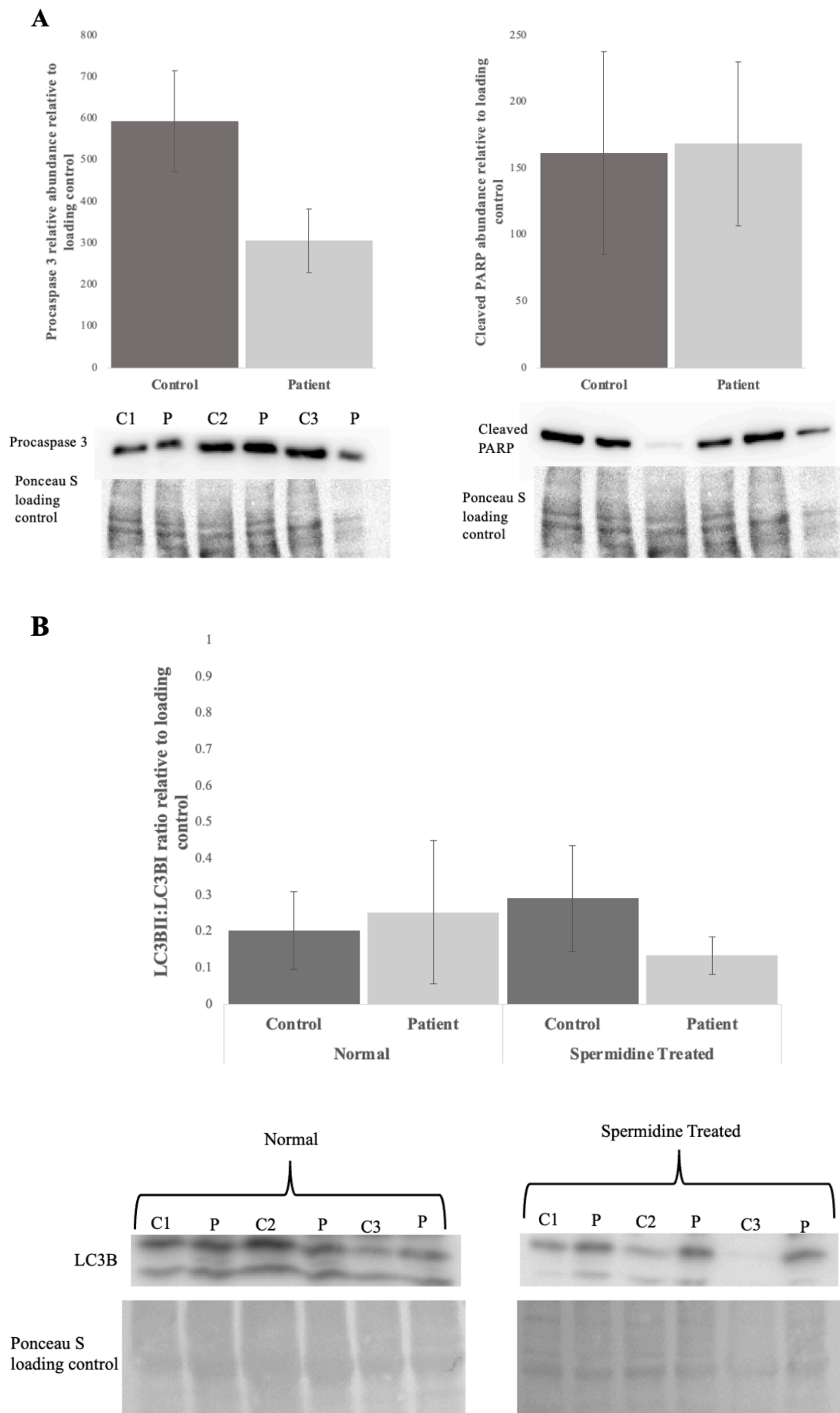


Figure 3-8 Apoptosis and mitophagy measured through western blotting of key proteins.

(**A)** Protein extracted from cells cultured with Actinomycin D was incubated with an Apoptosis

Cocktail antibody wherein procaspase 3 and cleaved PARP were detected. A representative western blot from all blots (n=3) shows the detected procaspase 3 and cleaved PARP protein bands. The detected protein was not significantly different between control and patient cells. The panel below the representative western blot represents the loading control (Ponceau S, known as ponceau red) to which the protein was normalized for calculations. (**B**) Protein extracted from cells treated with spermidine to induce mitophagy as well as normal protein lysate from the same cells were treated with an LC3B antibody. LC3BI and LC3BII were detected and the LC3B II:I ratio was calculated, demonstrating no significant difference between patient and control samples. A representative western blot from all blots (n=3) shows the detected LC3BI and LC3BII protein bands. The panel below the representative western blot represents the loading control (Ponceau S, also referred to as ponceau red). The detected protein was normalized to the loading control for calculations.

3.4 Discussion

In order to provide evidence that a putative novel disease gene is the cause for a disease or disorder, studying pathways or other molecules the gene interacts with can demonstrate if there are significant impacts of the variant. In this chapter, such targets of UBE2R2 function were explored including possible functional compensation, β-catenin, Wnt signaling pathway, cell cycle, p53 and degradation pathways.

The data presented does not demonstrate that functional compensation is occurring with other E2 ubiquitin conjugating enzymes UBE2R1, UBE2G1 or UBE2G2. We also determined that progression through the cell cycle and cell growth remain unaffected in patient cells. Analysis into β-catenin and p53 determine that the degradation of those proteins is unaffected.

With regards to the Wnt signaling pathway, only one of the three genes had significant differences in mRNA expression and protein abundance (*AFF3)*. Given that protein expression for two of these has not been affected in patient cells, coupled with no difference in β-catenin, the Wnt pathway is likely not significantly impacted in patient cells through β-catenin signal transduction. The majority of the research surrounding *AFF3* at this time is focused on cancer with little known about how the gene itself is regulated or its specific role or interaction with the Wnt signaling pathway (Lefèvre et al., 2015). This gene is part of a family that functions as transcriptional activators that promote RNA elongation and it has been found to be involved in

the development of the mesoderm and ectoderm (Bassani et al., 2024). This gene has previously been implicated in an autosomal dominant syndrome (KINSSHIP syndrome, MIM 619297), whose clinical features include developmental delay, impaired intellectual development, seizures, dysmorphic facial features and mesomelic dysplasia (Bassani et al., 2024). Homozygous *AFF3* knockout mice presented with brain malformations, neurological anomalies, kidney defects and skeletal anomalies (Voisin et al., 2021). As the association with epilepsy and developmental delay through KINSSHIP overlap with the clinical features of the patient, this is an intriguing pathway to explore. With this, it is also important to consider the entirety of the clinical features of KINSSHIP and our patient. The patient presented with developmental delay, intellectual disability, and absence seizures. The clinical presentation KINSSHIP syndrome overlaps with developmental delay, impaired intellectual development and seizures. The typical features of KINSSHIP that are not present in this patient include mesomelic dysplasia, dysmorphic facial features, horseshoe or hypoplastic kidney and failure to thrive. These results are inconclusive as to the effect of the *UBE2R2* variant on the Wnt signalling pathway. Despite this, it is possible to determine that the lack of effect on β-catenin degradation indicates that this mechanism of Wnt pathway activation (through β-catenin as the signal transducer) is not affected.

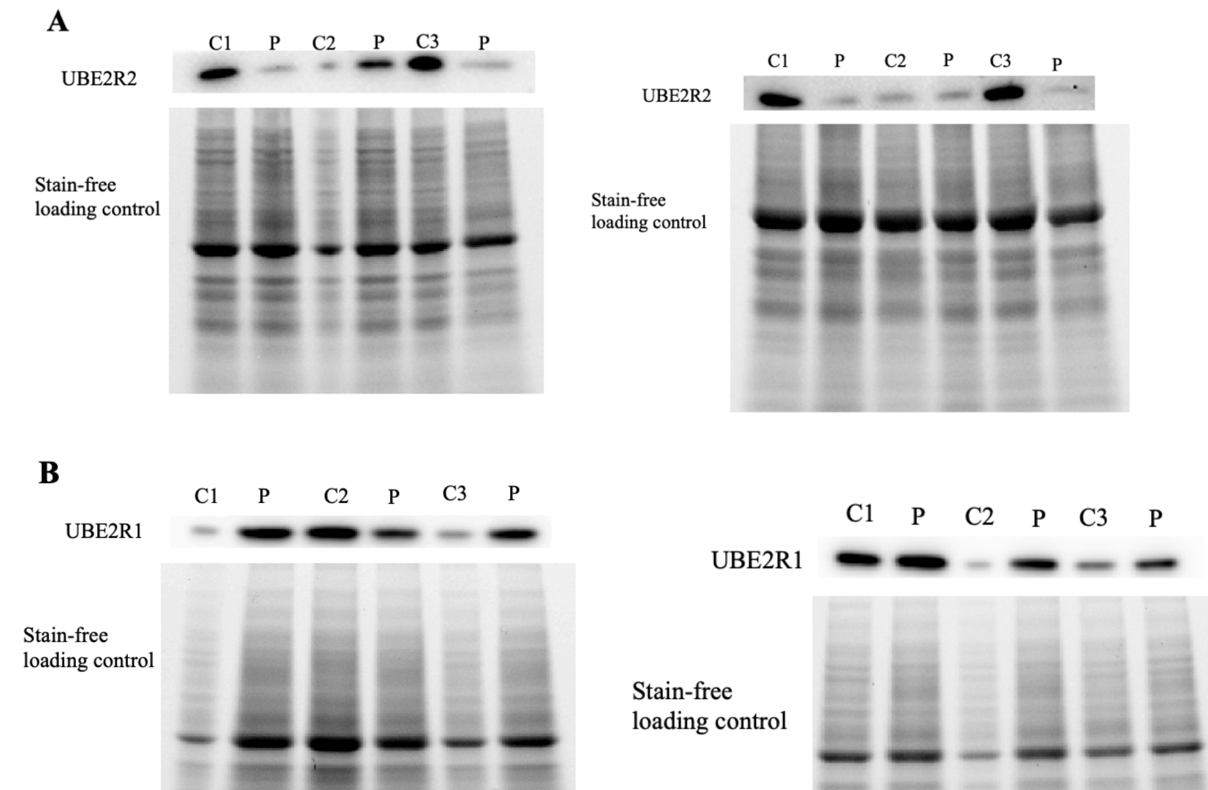
The results from the western blot analysis of actinomycin D cells treated with an antibody that detects key proteins involved in apoptosis (procaspase 3 and cleaved PARP) do not demonstrate any significant differences – which indicates that apoptosis has not been significantly impacted in these mechanisms. Although the procaspase 3 seems to trend towards a significant decrease ($p=0.062$), the other results do not present compelling evidence of significant apoptotic dysregulation in the patient cells with cleaved PARP as a hallmark feature.

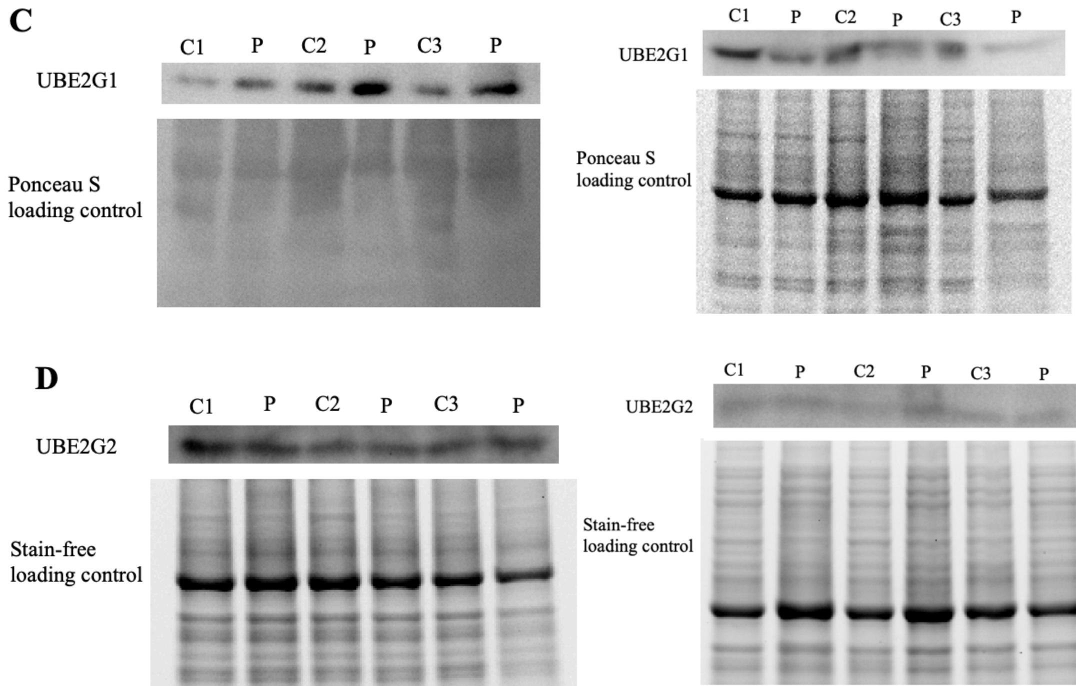
With regards to mitophagy, it was attempted to induce mitophagy with the use of spermidine in cell culture. The lack of significant change between LC3BI and LC3BII between the normal conditions and the spermidine conditions indicate that mitophagy induction through spermidine treatment. In-culture this was not successful. This may be a result of lymphoblasts being an immortalized cell line, and the required process to achieve this may have affected the efficacity of spermidine for these cells (Jankauskaitė et al., 2017). Given the ratio was unchanged in protein extracted from cells that were not treated, it was determined that mitophagy occurring

when the cells are not under stress ("normal" mitophagy) was unaffected by the p.(Ser6GlnfsTer56) variant.

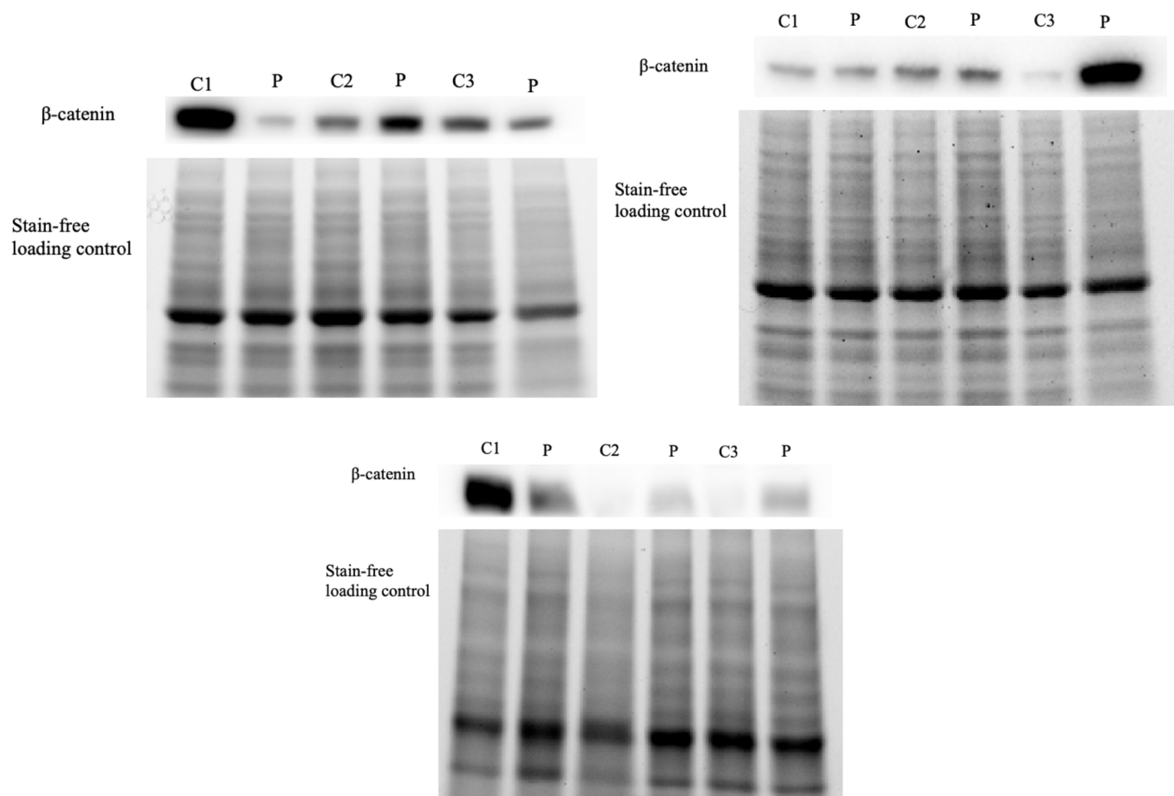
The results, taken in sum, do not provide sufficient evidence of disease-gene association for *UBE2R2*. While the variant impacts expression of UBE2R2, it does not seem to have an overt downstream functional effect. Although it is not possible to prove the disease-gene association, finding other patients through GeneMatcher (or other databases wherein unrelated patients with phenotypic and genotypic overlap are connected) would be required to suggest pathogenicity and continue laboratory research to further investigate the underlying mechanism in this case. Despite the lack of a genetic diagnosis, the research does present evidence for which pathways and interactions remain functional within the patient cells. This can be used for further research and analysis in the future to target different mechanisms for research by which evidence supporting diagnosis can be accumulated. It will be important to utilize all the information presented in this body of work to achieve a fuller understanding of the mechanism(s) of disease in this patient and in any future steps to achieve a diagnosis or differential treatment.

3.5 Supplemental figures

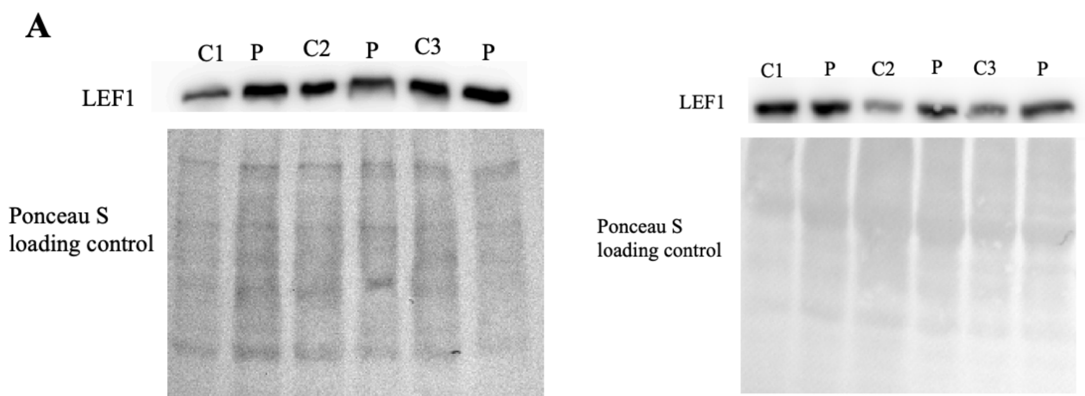




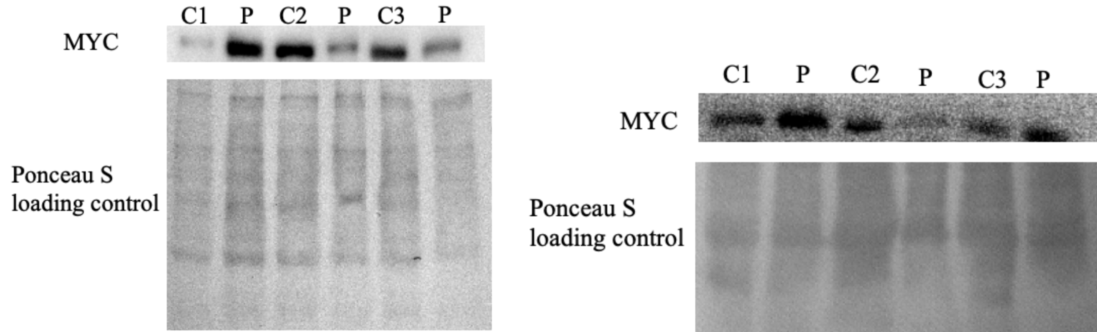
Supplemental Figure 3-1 Western blot replicates for E2 ubiquitin conjugating enzymes. Images of western blots replicates completed for all E2 ubiquitin conjugating enzymes using different protein lysates. (**A**) Western blot membranes were treated with a UBE2R2 antibody. Each western blot image is aligned with its loading control (stain free gel). The protein detected was normalized against this control for calculations. There was a significant difference in protein expression from the combined results of each western blot completed for this antibody (p= 0.006). (**B)** Western blot membranes were treated with a UBE2R1 antibody. Each western blot image is aligned with its loading control (stain free gel). The protein detected was normalized against this control for calculations. There is not a significant difference in protein expression (p=0.69). **(C)** Western blot membranes were treated with a UBE2G1 antibody. Each western blot image is aligned with its loading control (Ponceau S, also referred to as ponceau red). The protein detected was normalized against this control for calculations. There is no significant difference in protein expression (p=0.60). (**D**) Western blot membranes were treated with a UBE2G2 antibody. Each western blot image is aligned with its loading control (stain free gel). The protein detected was normalized against this control for calculations. There is not a significant difference in protein expression (p=0.27).



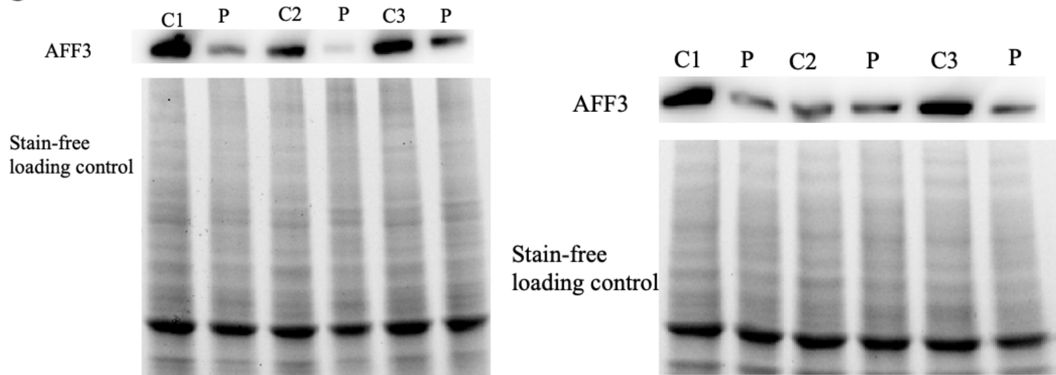
Supplemental Figure 3-2 Western blot replicates for β-catenin. Replicates of β-catenin western blot replicates using different protein lysates. Protein extracted from patient and control cells were treated with a β-catenin antibody. The detected protein was normalized against the loading control (stain-free gel), represented in the panel below the β-catenin bands. No significant difference between control and patient samples were detected.



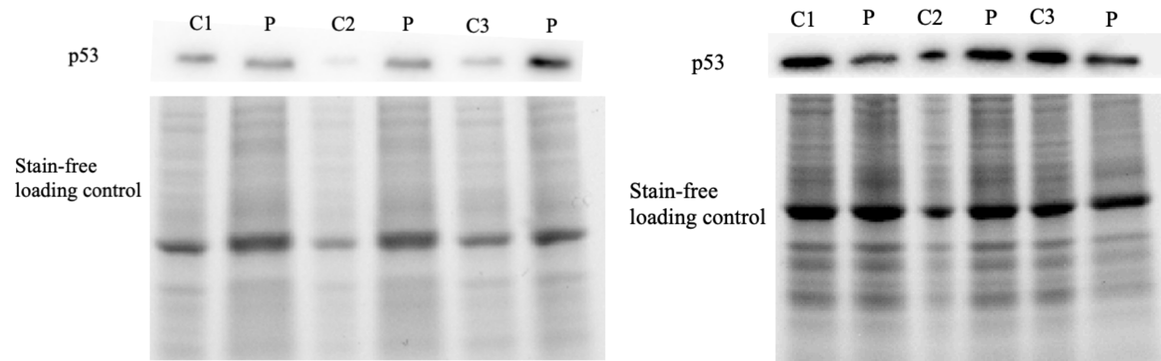
B



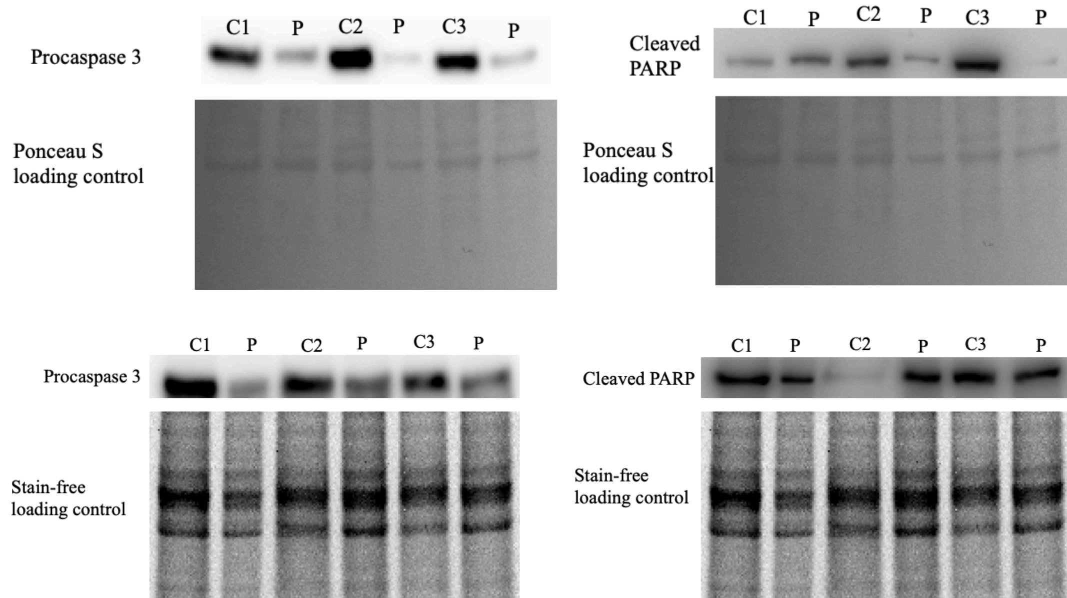
C



Supplemental Figure 3-3 Western blot replicates of Wnt target genes. Replicates of western blots for each Wnt target gene, using different protein lysates. (**A**) Protein extracted from control and patient cells were treated with a LEF1 antibody. The detected protein was normalized against the loading control (Ponceau S, also referred to as ponceau red). These results show no difference between control and patient samples. (**B**) Protein extracted from control and patient cells were treated with a MYC antibody. The subsequent protein detected was normalized against the loading control (Ponceau S, also referred to as ponceau red). The leftmost graph and panels below are representative of these results, showing no difference in protein expression. These results do not demonstrate a significant difference in expression (**C**) Protein extracted from control and patient cells were treated with an AFF3 antibody. The protein detected was normalized against the loading control (stain-free gel). These results demonstrate a significant decrease in AFF3 protein in patient samples (p=0.0041).

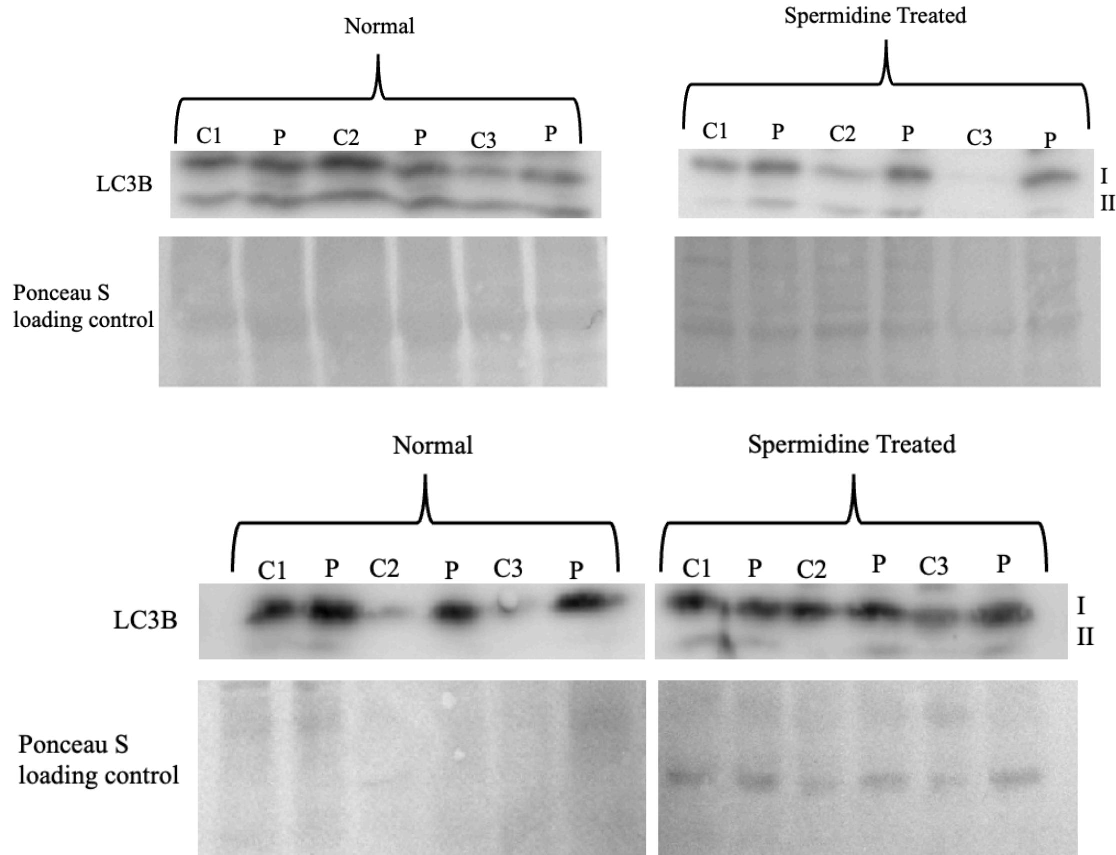


Supplemental Figure 3-4 Western blot replicates of p53. Replicates of p53 western blots, using different protein lysates. Protein extracted from patient and control cells were treated with a p53. The detected protein was normalized against the loading control (stain-free gel), represented in the panel below the p53 bands. As seen in the representative graph, to the left of the representative blot, no significant difference between control and patient samples were detected.



Supplemental Figure 3-5 Replicates of western blots detecting key apoptosis proteins. Replicates of western blots using an anti-apoptosis cocktail antibody, using different protein lysates. Protein extracted from cells cultured with Actinomycin D was incubated with an Apoptosis Cocktail antibody wherein procaspase 3 and cleaved PARP were detected. The detected protein was not significantly different between control and patient cells. The panel

below the representative western blot represents the loading control (Ponceau S or stain-free gel) to which the protein was normalized for calculations.



Supplemental Figure 3-6 Replicates of western blots detecting LC3B. Replicates of western blots detecting LC3B, using different protein lysates. Protein extracted from cells treated with spermidine to induce mitophagy as well as normal protein lysate from the same cells were treated with an LC3B antibody. LC3BI and LC3BII were detected and the LC3B II:I ratio was calculated, demonstrating no significant difference between patient and control samples. The panel below the representative western blot represents the loading control (Ponceau S, also referred to as ponceau red). The detected protein was normalized to the loading control for calculations.

Supplemental Table 3-1: Optimized antibody dilution used for western blot experiments.

Antibody	Dilution
UBE2R2	1:500
UBE2R1	1:500

UBE2G2	1:500
UBE2G1	1:500
LEF1	1:500
AFF3	1:500
MYC	1:1000
β-Catenin	1:1000
LC3B	1:1000
p53	1:1000
Apoptosis Cocktail	1:250

3.6 Supplementary Methods

3.6.1 Western Blot

Once all protein lysate and loading buffer mixes were made, they were frozen overnight and used for running gel(s) as follows.

Running gel(s)

10% gels made with the TGX Stain-Free ™ FastCast ™ Acrylamide Kit (BIORAD, cat. 1610183) or 12% gels made with the TGX ™ FastCast ™ Acrylamide Starter Kit, 12% (BIORAD, cat. 1610174) with 15 well combs were used based on protein size. One replicate from each UBE2G1, cleaved PARP and procaspase 3 were completed using 12%TGX Stain-Free ™ FastCast ™Acrylamide Kit (BIORAD, cat. 1610185). To note, a maximum volume of 30µL could be loaded into this well size. Prior to being loaded into the wells, the protein lysate and loading buffer mixes were heated at 70°C for 10 minutes. All gels were loaded as follows using the heated mixes along with BLUeye Prestained Protein Ladder (Sigma-Aldrich, cat. 94964):

5.5µL protein ladder	20 µL loading buffer	Control 1 (AG15995)	Patient Sample 1	Control 2 (AG15803)	Patient Sample 2	Control 3 (AG15792)	Patient Sample 3	20µL Loading Buffer	7.5µL protein ladder

The gels were run in an electroporator filled with running buffer at 10 mAmp until the dye front reached the bottom of the gel (or ~1cm from the bottom).

For 10% stain free gels once running was completed, the protein separation was visualized immediately using the BioRad Image Lab software on a ChemiDoc XRS+ System under the stain free gel application. This image was analyzed for total protein loading to normalize final western blot data. The transfer process occurred after this.

For 12% gels went immediately to transfer.

Transfer

For all western blots, polyvinylidene (PVDF) membranes with a 0.2µM pore size were used. Four filter sheets per gel were cut to fit the size of the gel as was one PVDF membrane. First, the PVDF membrane was activated by being submerged in methanol for 1-6 seconds, the methanol was removed and the membrane was then covered with 1x transfer buffer. The four filter papers and four sponges per gel were submerged in transfer buffer as well. A sandwich as created for transfer as follows:

2 Sponges
2 Filter papers
PVDF membrane
Gel
2 Filter papers
2 Sponges

A roller covered in transfer buffer was used between each addition to the sandwich to ensure no air bubbles were present. The PVDF membrane was on the positive side of the sandwich and the wet transfer system to ensure the protein was transferred from the gel to the membrane. The transfer system was then covered with 1x transfer buffer, with an ice pack filling in the remaining space, and placed in the fridge for an overnight transfer at 0.03 AMP.

For 12% gels the membrane was immediately transferred to a plastic container with a removable lid, covered in Ponceau S Staining Solution (ThermoFisher, cat. A40000279) and placed on a platform rocker for 15 minutes. The staining solution was then washed off with double distilled water before air drying completely. The membrane was then imaged using the same Image Lab software and machine using the Colorimetric application under “Blots”. This image was used to

normalize protein bands in the final analysis. The membrane was washed 3 times for 5 minutes each with TBST before moving on to blocking and antibody incubation.

For 10% gels the membrane was removed and immediately used in blocking and antibody incubation.

Blocking and antibody incubation

The membrane was removed from the transfer sandwich and immediately placed in a plastic container with a removable lid and covered with 5% skim milk made with TBST for 2 hours on a platform rocker. The milk was removed, and the membrane was then incubated with the primary antibody mix (made with 5% milk, containing 5% sodium azide and at a concentration dependent on each antibody). They were incubated with an anti-UBE2R2 (ThermoFisher, cat. 14077-1-AP), anti-UBE2R1 (Novus Biologicals, cat. NBP1032153), anti-UBE2G1 (ThermoFisher, cat. PA5-30201), anti-UBE2G2 (ThermoFisher, cat. PA5-98226), anti-p53 (Cell Signaling Technology, cat. 9282T), anti-β-catenin (Abcam, cat. ab16051), apoptosis western blot cocktail (Abcam, cat. ab136812), anti-LC3B (ThermoFisher, cat. PA1-46286), anti-MYC (ThermoFisher, cat. 10828-1-AP), anti-AFF3 (ThermoFisher, cat. PA5-68628), or anti-LEF1 (ThermoFisher, cat. 14972-1-AP) at 4°C on a rocker overnight.

The next morning, the membrane was washed quickly 3 times with TBST followed by 3 longer washes with TBST for 15 minutes each. The membrane was then incubated with the secondary antibody that would target the host species the primary antibody was derived from. This mixture was again made with 5% milk and the antibody at a 1:5000 ratio. Incubation on a rocker occurred for 1 hour before the washing steps (3 short washes, 3 long washes) with TBST were repeated. The membrane remained covered in TBST until imaging. Note: the apoptosis cocktail antibody used a customized secondary antibody at a 1:100 dilution.

Imaging

Clarity and clarity max ECL western blotting substrates (BIORAD) were used for imaging. Equal amounts of each substrate were mixed together, then the membrane was dried gently with a Kimwipe before being covered by the substrate mix. Colorimetric and chemiluminescent imaging using a ChemiDoc (BIORAD) was completed. Membranes were placed back into TBST and dried for further use as required.

Stripping and re-probing

Membranes were covered with Thermo Scientific^{TM} Restore^{TM} PLUS Western Blot Stripping Buffer and placed on a rocker for 15 minutes. It was then washed 3 times for 5 minutes with TBST. Blocking and antibody incubation was then performed as described above.

The total western blot process was completed in triplicate for each antibody.

All error bars represent the standard error of the mean.

Chapter 4 Discussion and future directions

4.1 Discussion

Genetic testing has become increasingly available and accessible as a result of the advancements in sequencing technologies (Krey et al., 2022; McKnight et al., 2022; Pellinen et al., 2024). It is now recommended that genetic testing (specifically exome and genome sequencing or multi-gene panels) be incorporated into the standard testing used in clinic for individuals with unexplained epilepsy (Smith et al., 2023). However, even with these comprehensive genetic testing becoming implemented into the standard clinical care of those with epilepsy, the vast majority of patients with genetic forms of epilepsy will remain without a molecular diagnosis (Sheidley et al., 2022).

In chapter two, I showed the benefits of the re-analysis of genetic data as a practical and easily accessible method that can be used to provide new diagnoses. By reanalyzing existing exome sequencing for a cohort of 20 patients, 10% of the participants were able to achieve a definite molecular diagnosis. An additional 35% had candidate genetic variants identified that have the potential to lead to a diagnosis. Initial testing techniques for this cohort were exome sequencing and multi-gene panels completed on an exome backbone, where one participant with each type of initial test received a diagnosis after reanalysis and candidate genetic variants were identified in individuals with both initial testing methods. These results, and others from the literature, suggest that reanalysis should be incorporated into routine clinical care. Results from reanalysis can lead to a diagnosis as well as provide information as to how research should proceed. Not only this, but as multigene panels and exome sequencing initial testing had similar results, this technique can be taken advantage of for patients who received either test initially if the muti-gene panel is completed on an exome backbone. Currently the Ontario-based multigene panel is not on an exome backbone (Dyment et al., 2020). These thesis results would provide evidence for the provincial laboratory stakeholders to consider modifying their targeted panel test to include an exome backbone and subsequent reanalysis as part of routine protocol.

Matchmaking efforts also have a role to play in the identifying novel epilepsy-associated genes. In the cohort of 20, we identified *JAKMIP1* and *SBNO1*. Both were added to the GeneMatcher portal and *JAKMIP1* matched to a research group in Pittsburgh. The developing cohort includes missense and loss-of-function variants. There is at least one other individual with a missense variant as well as macrocephaly, seizures and developmental delay in the new cohort and similar to the individual described in our study (personal communication with Dr. Collyer; UPMC Children's Hospital of Pittsburgh). This is also similar to an individual described, in brief, in a cohort study of individuals with delays (Loviglio et al., 2016). While detailed clinical descriptions, in multiple individuals are a necessity before *JAKMIP1* can be considered a bona-fide disease gene, this match is a promising initial step. It would also be reasonable to pursue functional studies for JAKMIP1 to demonstrate a deleterious effect of the substitution on protein function and/or downstream pathway.

Chapter three describes the process that can occur should a candidate gene be identified with exome reanalysis but requires further investigation to confirm or deny it as a disease gene. In this chapter, I performed a series of experiments in pathways or proteins that were theorized to be affected. The variant in *UBE2R2* was identified by exome reanalysis prior to the start of my thesis project. Using three control cell lines, and a lymphoblast cell line from the participant, I found that protein expression of other E2 ubiquitin conjugating enzymes were not affected (and therefore not functionally compensating for the loss of UBE2R2 function), cell cycle progression, cell growth, Wnt signaling pathway, p53 expression, β-catenin, apoptosis and mitophagy levels are unaffected. A difference was detected in *AFF3* mRNA and protein levels, *UBE2R1* mRNA expression and UBE2R2 protein expression which, while does not provide adequate evidence for pathogenicity of this variant, may offer a different direction for future testing or research to take place. As there is very little research into *UBE2R2*, this work also provides insight into the effects, or lack thereof, of a frameshift resulting in a truncation of this gene and protein.

4.2 Future directions and remaining questions

The body of work supports the benefits of exome reanalysis in cases where a clinical exome or multigene panel on an exome backbone have been completed in initial testing with no diagnosis.

The research also explores what can occur when a compelling candidate has been identified wherein further evidence is required to classify it as pathogenic.

Exome reanalysis cases that remain unsolved

As was described in chapter two, some of the cases with compelling candidates have progressed through next steps to demonstrate pathogenicity. The overarching paths forward when a compelling candidate has been identified includes (i) genotype: phenotype correlation, which is frequently completed by the most responsible physician relative to the patient(s), (ii) segregation studies to confirm inheritance, (iii) use of GeneMatcher to search for other patients with overlapping phenotypes and variants in the same gene , and (iv) the use of additional research studies, such as methylation studies or the type of experiments described in chapter three. In the future, should the variants in *JAKMIP1*, *MADD*, *TRIP12* and *ZBTB7A* be resolved to be the underlying cause of the seizures and global developmental delay, it would highlight the increased solve rate based on reanalysis from 2/20 (10%) to 6/20 (30%).

For the remaining 11 cases where no compelling candidates were identified, they will remain enrolled in the reanalysis program until such a time where consent is withdrawn, or the clinician removes them or Care4Rare has been completed. Reanalysis is also available on a clinical basis with funding from the Ministry of Health and Long-Term Care. The candidate may also progress through the C4R-SOLVE pathway with further research testing (for example, methylation analysis or long-read whole genome sequencing). As mentioned, the vast increases in genetic knowledge over the course of even one year can contribute to the identification of a candidate or provide the information required for a diagnosis.

***UBE2R2* as a disease gene?**

The body of work in chapter three does not provide sufficient evidence to declare the *UBE2R2* as a disease gene. However, it is also not possible to entirely rule-out this gene and variant as the cause of the clinical presentation seen in the patient. The pathways involved in ubiquitination and subsequently the ubiquitin-proteasome degradation pathways are comprehensive and varied and I may not have studied the appropriate pathway. The experiments in Chapter Three were based on existing knowledge, much of which is in relation to cancer. There may yet be other aspects of cellular function affected by the protein that has not been identified.

An actionable path forward at this time includes leaving this gene in the GeneMatcher and Matchmaker Exchange databases. As both systems are continuous, the relevant genetic counsellor and most responsible physician will be notified should another participant with a similar clinical presentation and genetic variant be uploaded to either database. Further functional assays into related pathways could also be explored to either rule out which pathways are affected by the variant or create a more detailed understanding of what cellular functions have been affected. In general, increased understanding can aid in guiding research in the future. In addition, the participants exome will continue to be re-analyzed on a routine basis (by Care4Rare) to determine if there is another candidate, in a gene other than *UBE2R2*, that may explain the seizures and delays.

Another potential path forward to prove a gene is associated with disease is the use of model systems. *UBE2R2* has an ortholog in mice (*Ube2r2*), indicating that a knockout mouse model could provide data that may be applicable to the human condition (*Ube2r2 Ubiquitin-Conjugating Enzyme E2R 2 [Mus Musculus (House Mouse)] - Gene - NCBI*, n.d.). Testing in this case could include tests such as monitoring mice at stages of development for seizures (Marshall et al., 2021; Teng et al., 2022). This could be in addition to monitoring for physical symptoms of seizures events, and cognitive function could be studied with a variety of behavioral tests (Ghafarimoghadam et al., 2022; Webster et al., 2014). There are currently knockout mouse models available that could be used to accomplish this detailed phenotyping (Cyagen, C57BL/6JCya-*Ube2r2*^{em1}/Cya, strain number: KOCMP-67615-Ube2r2-B6J-Va, Taconic Biosciences model TF3247, MGI: 1914865).

4.3 Conclusions

Overall, the work presented in this thesis has provided a diagnosis for two participants and candidates for further investigation in 7 more. The data from chapter two, following the path in a case where a candidate is identified requiring further investigation, does not provide satisfactory evidence towards pathogenicity of the variant. Levels of mRNA expression was significantly different in two genes (*AFF3* and *UBE2R1*) and protein expression was only significantly affected in UBE2R2 and AFF3 between control and patient cells. Whilst these results, as well as the current status in GeneMatcher (no matches), do not provide sufficient evidence for pathogenicity of this gene or variant, the results are still valuable in determining the possible

mechanism of disease in this patient. The information discerned from negative results can be useful in further determining the pathways that are affected by ruling out what is not affected from these functional assays.

This work emphasizes the utility of exome reanalysis for patients with unexplained epilepsy. It further highlights the process used to provide evidence should a novel disease gene candidate be identified through reanalysis as a likely cause of disease. Despite not demonstrating differences in the Wnt/β-catenin pathway, apoptosis, mitophagy, cell cycle, cell growth and a lack of functional compensation from other E2 ubiquitin conjugating enzymes, it is now possible to explore other avenues to attain a molecular diagnosis for this patient. The continued use of exome reanalysis, along with increased research into novel disease gene candidates, will be imperative for achieving a diagnosis for patients with epilepsy.

From these results, I would recommend implementing exome reanalysis in cases of epilepsy without a molecular diagnosis and performing multi-gene panels on an exome backbone so this avenue is possible. Moreover, functional analysis to investigate candidates can provide information on the patient's cellular function and is an important avenue to explore with putative novel disease genes.

References

Ahmed, A. E.-A., Hassan, M. H., Abdelfatah, A. A., & Bakri, A. H. (2024). Dysregulated Apoptosis and Autophagy in Childhood Epilepsy: Correlation to Clinical and Pharmacological Patterns. *Neuropediatrics*, *55*(5), 327–336. https://doi.org/10.1055/s-0044-1788032

Akbar, F., Saleh, R., Kirmani, S., Chand, P., Mukhtiar, K., Jan, F., Kumar, R., & Ibrahim, S. (2022). Utility of genetic testing in pediatric epilepsy: Experience from a low to middle-income country. *Epilepsy & Behavior Reports*, *20*, 100575. https://doi.org/10.1016/j.ebr.2022.100575

Al-Nabhani, M., Al-Rashdi, S., Al-Murshedi, F., Al-Kindi, A., Al-Thihli, K., Al-Saegh, A., Al-Futaisi, A., Al-Mamari, W., Zadjali, F., & Al-Maawali, A. (2018). Reanalysis of exome sequencing data of intellectual disability samples: Yields and benefits. *Clinical Genetics*, *94*(6), 495–501. https://doi.org/10.1111/cge.13438

American cancer society (Ed.). (2003). *Cancer medicine: An approved publication of the American cancer society* (6th ed). BC Decker.

Aminake, M. N., Arndt, H.-D., & Pradel, G. (2012). The proteasome of malaria parasites: A multi-stage drug target for chemotherapeutic intervention? *International Journal for Parasitology: Drugs and Drug Resistance*, *2*, 1–10. https://doi.org/10.1016/j.ijpddr.2011.12.001

Barodia, S. K., McMeekin, L. J., Creed, R. B., Quinones, E. K., Cowell, R. M., & Goldberg, M. S. (2019). PINK1 phosphorylates ubiquitin predominantly in astrocytes. *Npj Parkinson's Disease*, *5*(1), 1–9. https://doi.org/10.1038/s41531-019-0101-9

Bassani, S., Chrast, J., Ambrosini, G., Voisin, N., Schütz, F., Brusco, A., Sirchia, F., Turban, L., Schubert, S., Jamra, R. A., Schlump, J-U., DeMille, D., Bayrak-Toydemir, P., Nelson, G. R., Wong, K. N., Duncan, L., Mosera, M., Gilissen, C., Vissers, L. E. L. M., Pfundt, R., Kersseboom, R., Yttervik, H., Hansen, G. Å. M., Smeland, M. F., Butler, K. M., Lyons, M. J., Carvalho, C. M. B., Zhang, C., Lupski, J. R., Potocki, L., Flores-Gallegos, L., Morales-Toquero, R., Petit, F., Yalcin, B., Tuttle, A., Elloumi, H. Z., McCormick, L., Kukolich, M., Klaas, O., Horvath, J., Scala, M., Iacomino, M., Operto, F., Zara, F., Writzl, K., Maver, A., Haanpää, M. K., Pohjola, P., Arikka, H., Kievit, A. J. A., Calandrini, C., Iseli, C., Guex, N., & Reymond, A. (2024). Variant-specific pathophysiological mechanisms of AFF3 differently influence

transcriptome profiles. *Genome Medicine*, *16*(1), 72. https://doi.org/10.1186/s13073-024-01339-y

Bazhanova, E. D., & Kozlov, A. A. (2022). Mechanisms of Apoptosis in Drug-Resistant Epilepsy. *Neuroscience and Behavioral Physiology*, *52*(9), 1360–1367. https://doi.org/10.1007/s11055-023-01367-y

Benson, K. A., White, M., Allen, N. M., Byrne, S., Carton, R., Comerford, E., Costello, D., Doherty, C., Dunleavey, B., El-Naggar, H., Gangadharan, N., Heavin, S., Kearney, H., Lench, N. J., Lynch, J., McCormack, M., Regan, M. O., Podesta, K., Power, K., Rogers, A. S., Steward, C. A., Sweeney, B., Webb, D., Fitzsimons, M., Greally, M., Delanty, N., & Cavalleri, G. L. (2020). A comparison of genomic diagnostics in adults and children with epilepsy and comorbid intellectual disability. *European Journal of Human Genetics*, *28*(8), 1066–1077. https://doi.org/10.1038/s41431-020-0610-3

Berkovic, S. F., Howell, R. A., Hay, D. A., & Hopper, J. L. (1998). Epilepsies in twins: Genetics of the major epilepsy syndromes. *Annals of Neurology*, *43*(4), 435–445. https://doi.org/10.1002/ana.410430405

Boycott, K. M., Hartley, T., Kernohan, K. D., Dyment, D. A., Howley, H., Innes, A. M., Bernier, F. P., & Brudno, M. (2022). Care4Rare Canada: Outcomes from a decade of network science for rare disease gene discovery. *The American Journal of Human Genetics*, *109*(11), 1947–1959. https://doi.org/10.1016/j.ajhg.2022.10.002

Brunklaus, A., Brünger, T., Feng, T., Fons, C., Lehikoinen, A., Panagiotakaki, E., Vintan, M.-A., Symonds, J., Andrew, J., Arzimanoglou, A., Delima, S., Gallois, J., Hanrahan, D., Lesca, G., MacLeod, S., Marjanovic, D., McTague, A., Nuñez-Enamorado, N., Perez-Palma, E., Perry, M. S., Psyden, K., Russ-Hall, S. J., Scheffer, I. E., Sully, K., Syrbe, S., Vaher, U., Velayutham, M., Vogt, J., Weiss, S., Wirrell, E., Zuberi, S. M., Lal, D., Møller, R., Mantegazza, M., & Cestèle, S. (2022). The gain of function SCN1A disorder spectrum: Novel epilepsy phenotypes and therapeutic implications. *Brain*, *145*(11), 3816. https://doi.org/10.1093/brain/awac210

Budd, R. C. (2001). Activation-induced cell death. *Current Opinion in Immunology*, *13*(3), 356–362. https://doi.org/10.1016/S0952-7915(00)00227-2

Campos, V. E., Du, M., & Li, Y. (2004). Increased seizure susceptibility and cortical malformation in β-catenin mutant mice. *Biochemical and Biophysical Research Communications*, *320*(2), 606–614. https://doi.org/10.1016/j.bbrc.2004.05.204

Chambers, C., Jansen, L. A., & Dhamija, R. (2016). Review of Commercially Available Epilepsy Genetic Panels. *Journal of Genetic Counseling*, *25*(2), 213–217. https://doi.org/10.1007/s10897-015-9906-9

Chen, L., Ma, K., Han, J., Chen, Q., & Zhu, Y. (2017). Monitoring Mitophagy in Mammalian Cells. In *Methods in Enzymology* (Vol. 588, pp. 187–208). Elsevier. https://doi.org/10.1016/bs.mie.2016.10.038

Chen, Y.-S., & Qiu, X.-B. (2013). Ubiquitin at the crossroad of cell death and survival. *Chinese Journal of Cancer*, *32*(12), 640–647. https://doi.org/10.5732/cjc.012.10283

Chenn, A. (2008). Wnt/β-catenin signaling in cerebral cortical development. *Organogenesis*, *4*(2), 76. https://doi.org/10.4161/org.4.2.5852

Chi, W., & Kiskinis, E. (2024). Integrative analysis of epilepsy-associated genes reveals expression-phenotype correlations. *Scientific Reports*, *14*(1), 3587. https://doi.org/10.1038/s41598-024-53494-2

Chu, C., Geng, Y., Zhou, Y., & Sicinski, P. (2021). Cyclin E in normal physiology and disease states. *Trends in Cell Biology*, *31*(9), 732. https://doi.org/10.1016/j.tcb.2021.05.001

Ciesielski, T. H., Sirugo, G., Iyengar, S. K., & Williams, S. M. (2024). Characterizing the pathogenicity of genetic variants: The consequences of context. *Npj Genomic Medicine*, *9*(1), 1–11. https://doi.org/10.1038/s41525-023-00386-5

Claes, L., Ceulemans, B., Audenaert, D., Smets, K., Löfgren, A., Del-Favero, J., Ala-Mello, S., Basel-Vanagaite, L., Plecko, B., Raskin, S., Thiry, P., Wolf, N. I., Van Broeckhoven, C., & De Jonghe, P. (2003). De novo SCN1A mutations are a major cause of severe myoclonic epilepsy of infancy. *Human Mutation*, *21*(6), 615–621. https://doi.org/10.1002/humu.10217

Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M., & Roberts, J. M. (1996). Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin

phosphorylation. *Genes & Development*, *10*(16), 1979–1990. https://doi.org/10.1101/gad.10.16.1979

Cocklin, R., Heyen, J., Larry, T., Tyers, M., & Goebl, M. (2011). New Insight Into the Role of the Cdc34 Ubiquitin-Conjugating Enzyme in Cell Cycle Regulation via Ace2 and Sic1. *Genetics*, *187*(3), 701. https://doi.org/10.1534/genetics.110.125302

Collins, F. S., & Fink, L. (1995). The Human Genome Project. *Alcohol Health and Research World*, *19*(3), 190–195. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6875757/

Collins, G. A., & Goldberg, A. L. (2017). The Logic of the 26S Proteasome. *Cell*, *169*(5), 792. https://doi.org/10.1016/j.cell.2017.04.023

Connolly, M. B. (2016). Dravet Syndrome: Diagnosis and Long-Term Course. *The Canadian Journal of Neurological Sciences. Le Journal Canadien Des Sciences Neurologiques*, *43 Suppl 3*, S3-8. https://doi.org/10.1017/cjn.2016.243

Cooper, G. M. (2000). *The cell: A molecular approach* (2nd ed). ASM Press.

David, Y., Ziv, T., Admon, A., & Navon, A. (2010). The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. *The Journal of Biological Chemistry*, *285*(12), 8595–8604. https://doi.org/10.1074/jbc.M109.089003

de Kock, L., Cuillerier, A., Gillespie, M., Couse, M., Hartley, T., Mears, W., Bernier, F. P., Chudley, A. E., Frosk, P., Nikkel, S. M., Innes, A. M., Lauzon, J., Thomas, M., Guerin, A., Armour, C. M., Weksberg, R., Scott, J. N., Watkins, D., Harvey, S., Cytrynbaum, C., Care4Rare Canada Consortium, Kernohan, K. D., & Boycott, K. M. (2024). Molecular characterization of 13 patients with PIK3CA-related overgrowth spectrum using a targeted deep sequencing approach. *American Journal of Medical Genetics. Part A*, *194*(3), e63466. https://doi.org/10.1002/ajmg.a.63466

de Ligt, J., Willemsen, M. H., van Bon, B. W. M., Kleefstra, T., Yntema, H. G., Kroes, T., Vulto-van Silfhout, A. T., Koolen, D. A., de Vries, P., Gilissen, C., del Rosario, M., Hoischen, A., Scheffer, H., de Vries, B. B. A., Brunner, H. G., Veltman, J. A., & Vissers, L. E. L. M.

(2012). Diagnostic Exome Sequencing in Persons with Severe Intellectual Disability. *New England Journal of Medicine*, *367*(20), 1921–1929. https://doi.org/10.1056/NEJMoa1206524

Demos, M., Guella, I., DeGuzman, C., McKenzie, M. B., Buerki, S. E., Evans, D. M., Toyota, E. B., Boelman, C., Huh, L. L., Datta, A., Michoulas, A., Selby, K., Bjornson, B. H., Horvath, G., Lopez-Rangel, E., van Karnebeek, C. D. M., Salvarinova, R., Slade, E., Eydoux, P., Adam, S., Allen, M. I. V., Nelson, T. N., Bolbocean, C., Connolly, M. B., & Farrer, M. J. (2019). Diagnostic Yield and Treatment Impact of Targeted Exome Sequencing in Early-Onset Epilepsy. *Frontiers in Neurology*, *10*, 434. https://doi.org/10.3389/fneur.2019.00434

De Wachter, M., Schoonjans, A.-S., Weckhuysen, S., Van Schil, K., Löfgren, A., Meuwissen, M., Jansen, A., & Ceulemans, B. (2024). From diagnosis to treatment in genetic epilepsies: Implementation of precision medicine in real-world clinical practice. *European Journal of Paediatric Neurology*, *48*, 46–60. https://doi.org/10.1016/j.ejpn.2023.11.003

Driver, H. G., Hartley, T., Price, E. M., Turinsky, A. L., Buske, O. J., Osmond, M., Ramani, A. K., Kirby, E., Kernohan, K. D., Couse, M., Elrick, H., Lu, K., Mashouri, P., Mohan, A., So, D., Klamann, C., Le, H. G. B. H., Herscovich, A., Marshall, C. R., Statia, A., Care Rare Canada Consortium, Knoppers, B. M., Brudno, M., & Boycott, K. M. (2022). Genomics4RD: An integrated platform to share Canadian deep-phenotype and multiomic data for international rare disease gene discovery. *Human Mutation*, *43*(6), 800–811. https://doi.org/10.1002/humu.24354

Du, X., Song, H., Shen, N., Hua, R., & Yang, G. (2021). The Molecular Basis of Ubiquitin-Conjugating Enzymes (E2s) as a Potential Target for Cancer Therapy. *International Journal of Molecular Sciences*, *22*(7), 3440. https://doi.org/10.3390/ijms22073440

Dunn, P., Albury, C. L., Maksemous, N., Benton, M. C., Sutherland, H. G., Smith, R. A., Haupt, L. M., & Griffiths, L. R. (2018). Next Generation Sequencing Methods for Diagnosis of Epilepsy Syndromes. *Frontiers in Genetics*, *9*, 20. https://doi.org/10.3389/fgene.2018.00020

Dyment, D. A., Prasad, A. N., Boycott, K. M., Ediae, G. U., Hartley, T., Hassan, A., Muir, K. E., Potter, M., Boisse Lomax, L., Jarinova, O., Sadikovic, B., Stavropoulos, D. J., & Snead, O. C. (2020). Implementation of Epilepsy Multigene Panel Testing in Ontario, Canada. *Canadian*

Journal of Neurological Sciences / Journal Canadien Des Sciences Neurologiques, *47*(1), 61–68. https://doi.org/10.1017/cjn.2019.304

El-Maraghy, S. A., Reda, A., Essam, R. M., & Kortam, M. A. (2023). The citrus flavonoid "Nobiletin" impedes STZ-induced Alzheimer's disease in a mouse model through regulating autophagy mastered by SIRT1/FoxO3a mechanism. *Inflammopharmacology*, *31*(5), 2701. https://doi.org/10.1007/s10787-023-01292-z

Enatsu, R., & Mikuni, N. (2016). Invasive Evaluations for Epilepsy Surgery: A Review of the Literature. *Neurologia Medico-Chirurgica*, *56*(5), 221. https://doi.org/10.2176/nmc.ra.2015-0319

Epilepsy Genetics Initiative. (2019). The Epilepsy Genetics Initiative: Systematic reanalysis of diagnostic exomes increases yield. *Epilepsia*, *60*(5), 797–806. https://doi.org/10.1111/epi.14698

Escayg, A., & Goldin, A. L. (2010). Sodium channel SCN1A and epilepsy: Mutations and mechanisms. *Epilepsia*, *51*(9), 1650–1658. https://doi.org/10.1111/j.1528-1167.2010.02640.x

Escayg, A., Heils, A., MacDonald, B. T., Haug, K., Sander, T., & Meisler, M. H. (2001). A novel SCN1A mutation associated with generalized epilepsy with febrile seizures plus—And prevalence of variants in patients with epilepsy. *American Journal of Human Genetics*, *68*(4), 866–873. https://doi.org/10.1086/319524

Escayg, A., MacDonald, B. T., Meisler, M. H., Baulac, S., Huberfeld, G., An-Gourfinkel, I., Brice, A., LeGuern, E., Moulard, B., Chaigne, D., Buresi, C., & Malafosse, A. (2000). Mutations of SCN1A, encoding a neuronal sodium channel, in two families with GEFS+2. *Nature Genetics*, *24*(4), 343–345. https://doi.org/10.1038/74159

Ewans, L. J., Schofield, D., Shrestha, R., Zhu, Y., Gayevskiy, V., Ying, K., Walsh, C., Lee, E., Kirk, E. P., Colley, A., Ellaway, C., Turner, A., Mowat, D., Worgan, L., Freckmann, M.-L., Lipke, M., Sachdev, R., Miller, D., Field, M., Dinger, M. E., Buckley, M. F., Cowley, M. J., & Roscioli, T. (2018). Whole-exome sequencing reanalysis at 12 months boosts diagnosis and is cost-effective when applied early in Mendelian disorders. *Genetics in Medicine*, *20*(12), 1564–1574. https://doi.org/10.1038/gim.2018.39

Fan, C., Wolking, S., Lehmann-Horn, F., Hedrich, U. B., Freilinger, T., Lerche, H., Borck, G., Kubisch, C., & Jurkat-Rott, K. (2016). Early-onset familial hemiplegic migraine due to a novel SCN1A mutation. *Cephalalgia*, *36*(13), 1238–1247. https://doi.org/10.1177/0333102415608360

Feliubadaló, L., Tonda, R., Gausachs, M., Trotta, J.-R., Castellanos, E., López-Doriga, A., Teulé, À., Tornero, E., del Valle, J., Gel, B., Gut, M., Pineda, M., González, S., Menéndez, M., Navarro, M., Capellá, G., Gut, I., Serra, E., Brunet, J., Beltran, S., & Lázaro, C. (2017). Benchmarking of Whole Exome Sequencing and Ad Hoc Designed Panels for Genetic Testing of Hereditary Cancer. *Scientific Reports*, *7*(1), 37984. https://doi.org/10.1038/srep37984

Fiesel, F. C., Moussaud-Lamodière, E. L., Ando, M., & Springer, W. (2014a). A specific subset of E2 ubiquitin-conjugating enzymes regulate Parkin activation and mitophagy differently. *Journal of Cell Science*, *127*(16), 3488–3504. https://doi.org/10.1242/jcs.147520

Fiesel, F. C., Moussaud-Lamodière, E. L., Ando, M., & Springer, W. (2014b). Select E2 enzymes differentially regulate parkin activation and mitophagy. *Journal of Cell Science*, jcs.147520. https://doi.org/10.1242/jcs.147520

Fisher, R. S., Acevedo, C., Arzimanoglou, A., Bogacz, A., Cross, J. H., Elger, C. E., Engel, J., Forsgren, L., French, J. A., Glynn, M., Hesdorffer, D. C., Lee, B. I., Mathern, G. W., Moshé, S. L., Perucca, E., Scheffer, I. E., Tomson, T., Watanabe, M., & Wiebe, S. (2014). ILAE Official Report: A practical clinical definition of epilepsy. *Epilepsia*, *55*(4), 475–482. https://doi.org/10.1111/epi.12550

Flow Cytometry Protocol: 7-AAD Cell Viability: R&D Systems. (n.d.). Retrieved November 14, 2024, from https://www.rndsystems.com/resources/protocols/analysis-cell-viability-using-7-amino-actinomycin-d-7-aad

Frésard, L., & Montgomery, S. B. (2018). Diagnosing rare diseases after the exome. *Cold Spring Harbor Molecular Case Studies*, *4*(6), a003392. https://doi.org/10.1101/mcs.a003392

Fuchs, S. Y., Spiegelman, V. S., & Suresh Kumar, K. G. (2004). The many faces of β-TrCP E3 ubiquitin ligases: Reflections in the magic mirror of cancer. *Oncogene*, *23*(11), 2028–2036. https://doi.org/10.1038/sj.onc.1207389

Fung, J. L. F., Yu, M. H. C., Huang, S., Chung, C. C. Y., Chan, M. C. Y., Pajusalu, S., Mak, C. C. Y., Hui, V. C. C., Tsang, M. H. Y., Yeung, K. S., Lek, M., & Chung, B. H. Y. (2020). A three-year follow-up study evaluating clinical utility of exome sequencing and diagnostic potential of reanalysis. *Npj Genomic Medicine*, *5*(1), 37. https://doi.org/10.1038/s41525-020-00144-x

Gao, Y., Ma, L., Yuan, J., Huang, Y., Ban, Y., Zhang, P., Tan, D., Liang, M., Li, Z., Gong, C., Xu, T., Yang, X., & Chen, Y. (2024). GLS2 reduces the occurrence of epilepsy by affecting mitophagy function in mouse hippocampal neurons. *CNS Neuroscience & Therapeutics*, *30*(10), e70036. https://doi.org/10.1111/cns.70036

Ghafarimoghadam, M., Mashayekh, R., Gholami, M., Fereydani, P., Shelley-Tremblay, J., Kandezi, N., Sabouri, E., & Motaghinejad, M. (2022). A review of behavioral methods for the evaluation of cognitive performance in animal models: Current techniques and links to human cognition. *Physiology & Behavior*, *244*, 113652. https://doi.org/10.1016/j.physbeh.2021.113652

Glasper, E. R., Morton, J. C., & Gould, E. (2010). Environmental Influences on Adult Neurogenesis. In G. F. Koob, M. L. Moal, & R. F. Thompson (Eds.), *Encyclopedia of Behavioral Neuroscience* (pp. 485–492). Academic Press. https://doi.org/10.1016/B978-0-08-045396-5.00241-4

Gobeil, S., Boucher, C. C., Nadeau, D., & Poirier, G. G. (2001). Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): Implication of lysosomal proteases. *Cell Death & Differentiation*, *8*(6), 588–594. https://doi.org/10.1038/sj.cdd.4400851

Grice, G. L., & Nathan, J. A. (2016). The recognition of ubiquitinated proteins by the proteasome. *Cellular and Molecular Life Sciences: CMLS*, *73*(18), 3497. https://doi.org/10.1007/s00018-016-2255-5

Guertin, D. A., & Sabatini, D. M. (2015). 12—Cell Growth. In J. Mendelsohn, J. W. Gray, P. M. Howley, M. A. Israel, & C. B. Thompson (Eds.), *The Molecular Basis of Cancer (Fourth Edition)* (pp. 179-190.e1). W.B. Saunders. https://doi.org/10.1016/B978-1-4557-4066-6.00012-3

Guo, H. J., Rahimi, N., & Tadi, P. (2024). Biochemistry, Ubiquitination. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK556052/

Habela, C. W., Schatz, K., & Kelley, S. A. (2024). Genetic Testing in Epilepsy: Improving Outcomes and Informing Gaps in Research. *Epilepsy Currents*, 15357597241232881. https://doi.org/10.1177/15357597241232881

Hamosh, A., Scott, A. F., Amberger, J. S., Bocchini, C. A., & McKusick, V. A. (2004). Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders. *Nucleic Acids Research*, *33*(Database Issue), D514. https://doi.org/10.1093/nar/gki033

Hamosh, A., Wohler, E., Martin, R., Griffith, S., Rodrigues, E. da S., Antonescu, C., Doheny, K. F., Valle, D., & Sobreira, N. (2022). The impact of GeneMatcher on international data sharing and collaboration. *Human Mutation*, *43*(6), 668–673. https://doi.org/10.1002/humu.24350

Han, T.-S., Kim, D.-S., Son, M.-Y., & Cho, H.-S. (2024). SMYD family in cancer: Epigenetic regulation and molecular mechanisms of cancer proliferation, metastasis, and drug resistance. *Experimental & Molecular Medicine*, 1–12. https://doi.org/10.1038/s12276-024-01326-8

Harrison-Uy, S. J., & Pleasure, S. J. (2012). Wnt Signaling and Forebrain Development. *Cold Spring Harbor Perspectives in Biology*, *4*(7), a008094. https://doi.org/10.1101/cshperspect.a008094

Hartley, T., Lemire, G., Kernohan, K. D., Howley, H. E., Adams, D. R., & Boycott, K. M. (2020). New Diagnostic Approaches for Undiagnosed Rare Genetic Diseases. *Annual Review of Genomics and Human Genetics*, *21*(1), 351–372. https://doi.org/10.1146/annurev-genom-083118-015345

Hartley, T., Soubry, É., Acker, M., Osmond, M., Couse, M., Gillespie, M. K., Ito, Y., Marshall, A. E., Lemire, G., Huang, L., Chisholm, C., Eaton, A. J., Price, E. M., Dowling, J. J., Ramani, A. K., Mendoza-Londono, R., Costain, G., Axford, M. M., Szuto, A., McNiven, V., Damseh, N., Jobling, R., de Kock, L., Mojarad, B. A., Young, T., Shao, Z., Hayeems, R. Z., Graham, I. D., Tarnopolsky, M., Brady, L., Armour, C. M., Geraghty, M., Richer, J., Sawyer, S., Lines, M., Mercimek-Andrews, S., Carter, M. T., Graham, G., Kannu, P., Lazier, J., Li, C., Aul, R. B., Balci, T. B., Dlamini, N., Badalato, L., Guerin, A., Walia, J., Chitayat, D., Cohn, R., Faghfoury, H., Forster-Gibson, C., Gonorazky, H., Grunebaum, E., Inbar-Feigenberg, M., Karp, N., Morel, C., Rusnak, A., Sondheimer, N., Warman-Chardon, J., Bhola, P. T., Bourque, D. K., Chacon, I. J., Chad, L., Chakraborty, P., Chong, K., Doja, A., Goh, E. S-Y., Saleh, M., Care4Rare Canada,

Potter, B. K., Marshall, C. R., Dyment, D. A., Kernohan, K., & Boycott, K. M. (2023). Bridging clinical care and research in Ontario, Canada: Maximizing diagnoses from reanalysis of clinical exome sequencing data. *Clinical Genetics*, *103*(3), 288–300. https://doi.org/10.1111/cge.14262

Health, T. L. G. (2024). The landscape for rare diseases in 2024. *The Lancet Global Health*, *12*(3), e341. https://doi.org/10.1016/S2214-109X(24)00056-1

Helbig, I., & Lowenstein, D. H. (2013). Genetics of the epilepsies: Where are we and where are we going? *Current Opinion in Neurology*, *26*(2), 179–185. https://doi.org/10.1097/WCO.0b013e32835ee6ff

Helbig, I., Scheffer, I. E., Mulley, J. C., & Berkovic, S. F. (2008). Navigating the channels and beyond: Unravelling the genetics of the epilepsies. *The Lancet Neurology*, *7*(3), 231–245. https://doi.org/10.1016/S1474-4422(08)70039-5

Henshall, D. C. (2007). Apoptosis signalling pathways in seizure-induced neuronal death and epilepsy. *Biochemical Society Transactions*, *35*(Pt 2), 421–423. https://doi.org/10.1042/BST0350421

Hildebrand, M. S., Dahl, H.-H. M., Damiano, J. A., Smith, R. J. H., Scheffer, I. E., & Berkovic, S. F. (2013). Recent advances in the molecular genetics of epilepsy. *Journal of Medical Genetics*, *50*(5), 271–279. https://doi.org/10.1136/jmedgenet-2012-101448

Hill, S., Reichermeier, K., Scott, D. C., Samentar, L., Coulombe-Huntington, J., Izzi, L., Tang, X., Ibarra, R., Bertomeu, T., Moradian, A., Sweredoski, M. J., Caberoy, N., Schulman, B. A., Sicheri, F., Tyers, M., & Kleiger, G. (2019). Robust cullin-RING ligase function is established by a multiplicity of poly-ubiquitylation pathways. *eLife*, *8*, e51163. https://doi.org/10.7554/eLife.51163

Hodges, S. L., & Lugo, J. N. (2018). Wnt/β-catenin signaling as a potential target for novel epilepsy therapies. *Epilepsy Research*, *146*, 9–16. https://doi.org/10.1016/j.eplepsyres.2018.07.002

Hood, L., & Rowen, L. (2013). The human genome project: Big science transforms biology and medicine. *Genome Medicine*, *5*(9), 79. https://doi.org/10.1186/gm483

Hormaechea-Agulla, D., Kim, Y., Song, M. S., & Song, S. J. (2018). New Insights into the Role of E2s in the Pathogenesis of Diseases: Lessons Learned from UBE2O. *Molecules and Cells*, *41*(3), 168–178. https://doi.org/10.14348/molcells.2018.0008

Hwang, H. J., Ha, H., Lee, B. S., Kim, B. H., Song, H. K., & Kim, Y. K. (2022). LC3B is an RNA-binding protein to trigger rapid mRNA degradation during autophagy. *Nature Communications*, *13*(1), 1436. https://doi.org/10.1038/s41467-022-29139-1

Iguchi, M., Kujuro, Y., Okatsu, K., Koyano, F., Kosako, H., Kimura, M., Suzuki, N., Uchiyama, S., Tanaka, K., & Matsuda, N. (2013). Parkin-catalyzed Ubiquitin-Ester Transfer Is Triggered by PINK1-dependent Phosphorylation. *The Journal of Biological Chemistry*, *288*(30), 22019–22032. https://doi.org/10.1074/jbc.M113.467530

Isom, L. L., & Knupp, K. G. (2021). Dravet Syndrome: Novel Approaches for the Most Common Genetic Epilepsy. *Neurotherapeutics*, *18*(3), 1524. https://doi.org/10.1007/s13311-021-01095-6

Jankauskaitė, E., Bartnik, E., & Kodroń, A. (2017). Investigating Leber's hereditary optic neuropathy: Cell models and future perspectives. *Mitochondrion*, *32*, 19–26. https://doi.org/10.1016/j.mito.2016.11.006

Jansson, J. S., Hallböök, T., & Reilly, C. (2020). Intellectual functioning and behavior in Dravet syndrome: A systematic review. *Epilepsy & Behavior: E&B*, *108*, 107079. https://doi.org/10.1016/j.yebeh.2020.107079

Ji, J., Leung, M. L., Baker, S., Deignan, J. L., & Santani, A. (2021). Clinical Exome Reanalysis: Current Practice and Beyond. *Molecular Diagnosis & Therapy*, *25*(5), 529–536. https://doi.org/10.1007/s40291-021-00541-7

Johannesen, K. M., Tümer, Z., Weckhuysen, S., Barakat, T. S., & Bayat, A. (2023). Solving the unsolved genetic epilepsies: Current and future perspectives. *Epilepsia*, *64*(12), 3143–3154. https://doi.org/10.1111/epi.17780

Kass, H. R., Winesett, S. P., Bessone, S. K., Turner, Z., & Kossoff, E. H. (2016). Use of dietary therapies amongst patients with GLUT1 deficiency syndrome. *Seizure*, *35*, 83–87. https://doi.org/10.1016/j.seizure.2016.01.011

Kernohan, K. D., Hartley, T., Alirezaie, N., Consortium, C. C., Robinson, P. N., Dyment, D. A., & Boycott, K. M. (2018). Evaluation of exome filtering techniques for the analysis of clinically relevant genes. *Human Mutation*, *39*(2), 197–201. https://doi.org/10.1002/humu.23374

Khot, M., Sood, A., Tryphena, K. P., Khan, S., Srivastava, S., Singh, S. B., & Khatri, D. K. (2022). NLRP3 inflammasomes: A potential target to improve mitochondrial biogenesis in Parkinson's disease. *European Journal of Pharmacology*, *934*, 175300. https://doi.org/10.1016/j.ejphar.2022.175300

Kim, E. C., Zhang, J., Pang, W., Wang, S., Lee, K. Y., Cavaretta, J. P., Walters, J., Procko, E., Tsai, N.-P., & Chung, H. J. (2018). Reduced axonal surface expression and phosphoinositide sensitivity in K v 7 channels disrupts their function to inhibit neuronal excitability in Kcnq2 epileptic encephalopathy. *Neurobiology of Disease*, *118*, 76–93. https://doi.org/10.1016/j.nbd.2018.07.004

Kim, K., Pang, K. M., Evans, M., & Hay, E. D. (2000). Overexpression of β-Catenin Induces Apoptosis Independent of Its Transactivation Function with LEF-1 or the Involvement of Major G1 Cell Cycle Regulators. *Molecular Biology of the Cell*, *11*(10), 3509–3523. https://doi.org/10.1091/mbc.11.10.3509

Kim, M.-J., Yum, M.-S., Seo, G. H., Lee, Y., Jang, H. N., Ko, T.-S., & Lee, B. H. (2020). Clinical Application of Whole Exome Sequencing to Identify Rare but Remediable Neurologic Disorders. *Journal of Clinical Medicine*, *9*(11), 3724. https://doi.org/10.3390/jcm9113724

Kimura, Y., & Tanaka, K. (2010). Regulatory mechanisms involved in the control of ubiquitin homeostasis. *Journal of Biochemistry*, *147*(6), 793–798. https://doi.org/10.1093/jb/mvq044

Kingsmore, S. F., Cakici, J. A., Clark, M. M., Gaughran, M., Feddock, M., Batalov, S., Bainbridge, M. N., Carroll, J., Caylor, S. A., Clarke, C., Ding, Y., Ellsworth, K., Farnaes, L., Hildreth, A., Hobbs, C., James, K., Kint, C. I., Lenberg, J., Nahas, S., Prince, L., Reyes, I., Salz, L, Sanford, E., Schols, P., Sweeney, N., Tokita, M., Veeraraghavan, N., Watkins, K., Wigby, K., Wong, T., Chowdhury, S., Wright, M. S., Dimmock, D., Bezares, Z., Bloss, C., Braun, J. J. A., Diaz, C., Mashburn, D., Tamang, D., Orendain, D., Friedman, J., Gleeson, J., Barea, J., Chiang, G., Cohenmeyer, C., Coufal, N. G., Evans, M., Honold, J., Hovey, R. L., Kimball, A., Lane, B., Le, C., Le, J., Leibel, S., Moyer, L., Mulrooney, P., Oh, D., Ordonez, P., Oriol, A., Oritz-

Arechiga, M., Puckett, L., Speziale, M., Suttner, D., Van Der Kraan, L., Knight, G., Sauer, C., Song, R., White, S., Wise, A., & Yamada, C. (2019). A Randomized, Controlled Trial of the Analytic and Diagnostic Performance of Singleton and Trio, Rapid Genome and Exome Sequencing in Ill Infants. *The American Journal of Human Genetics*, *105*(4), 719–733. https://doi.org/10.1016/j.ajhg.2019.08.009

Kirk, E. (2021). Diagnostic Yield of Whole Genome Sequencing After Nondiagnostic Exome Sequencing or Gene Panel in Developmental and Epileptic Encephalopathies. *Neurology*, *96*(13), e1770–e1782. https://doi.org/10.1212/WNL.0000000000011655

Kjeldsen, M. J., Corey, L. A., Christensen, K., & Friis, M. L. (2003). Epileptic seizures and syndromes in twins: The importance of genetic factors. *Epilepsy Research*, *55*(1), 137–146. https://doi.org/10.1016/S0920-1211(03)00117-7

Kjeldsen, M. J., Corey, L. A., Solaas, M. H., Friis, M. L., Harris, J. R., Kyvik, K. O., Christensen, K., & Pellock, J. M. (2005). Genetic factors in seizures: A population-based study of 47,626 US, Norwegian and Danish twin pairs. *Twin Research and Human Genetics: The Official Journal of the International Society for Twin Studies*, *8*(2), 138–147. https://doi.org/10.1375/1832427053738836

Kjeldsen, M. J., Kyvik, K. O., Christensen, K., & Friis, M. L. (2001). Genetic and environmental factors in epilepsy: A population-based study of 11900 Danish twin pairs. *Epilepsy Research*, *44*(2–3), 167–178. https://doi.org/10.1016/s0920-1211(01)00196-6

Klepper, J., Akman, C., Armeno, M., Auvin, S., Cervenka, M., Cross, H. J., De Giorgis, V., Della Marina, A., Engelstad, K., Heussinger, N., Kossoff, E. H., Leen, W. G., Leiendecker, B., Monani, U. R., Oguni, H., Neal, E., Pascual, J. M., Pearson, T. S., Pons, R., Scheffer, I. E., Veggiotti, P., Willemsen, M., Zuberi, S. M., & De Vivo, D. C. (2020). Glut1 Deficiency Syndrome (Glut1DS): State of the art in 2020 and recommendations of the international Glut1DS study group. *Epilepsia Open*, *5*(3), 354–365. https://doi.org/10.1002/epi4.12414

Knowles, J. K., Helbig, I., Metcalf, C. S., Lubbers, L. S., Isom, L. L., Demarest, S., Goldberg, E. M., George, A. L., Lerche, H., Weckhuysen, S., Whittemore, V., Berkovic, S. F., & Lowenstein, D. H. (2022). Precision medicine for genetic epilepsy on the horizon: Recent advances, present

challenges, and suggestions for continued progress. *Epilepsia*, *63*(10), 2461–2475. https://doi.org/10.1111/epi.17332

Kodera, H., Kato, M., Nord, A. S., Walsh, T., Lee, M., Yamanaka, G., Tohyama, J., Nakamura, K., Nakagawa, E., Ikeda, T., Ben-Zeev, B., Lev, D., Lerman-Sagie, T., Straussberg, R., Tanabe, S., Ueda, K., Amamoto, M., Ohta, S., Nonoda, Y., Nishiyama, K., Tsurusaki, Y., Nakashima, M., Miyake, N., Hayasaka, K., King, M.-C., Matsumoto, N., & Saitsu, H. (2013). Targeted capture and sequencing for detection of mutations causing early onset epileptic encephalopathy. *Epilepsia*, *54*(7), 1262–1269. https://doi.org/10.1111/epi.12203

Koh, H. Y., Smith, L., Wiltrout, K. N., Podury, A., Chourasia, N., D'Gama, A. M., Park, M., Knight, D., Sexton, E. L., Koh, J. J., Oby, B., Pinsky, R., Shao, D. D., French, C. E., Shao, W., Rockowitz, S., Sliz, P., Zhang, B., Mahida, S., El Achkar, C. M., Yuskaitis, C. J., Olson, H. E., Sheidley, B. R., Poduri, A. H., & BCH Neurology Referral and Phenotyping Group. (2023). Utility of Exome Sequencing for Diagnosis in Unexplained Pediatric-Onset Epilepsy. *JAMA Network Open*, *6*(7), e2324380. https://doi.org/10.1001/jamanetworkopen.2023.24380

Koszela, J., Rintala-Dempsey, A., Salzano, G., Pimenta, V., Kamarainen, O., Gabrielsen, M., Parui, A. L., Shaw, G. S., & Walden, H. (2024). A substrate-interacting region of Parkin directs ubiquitination of the mitochondrial GTPase Miro1. *bioRxiv*, 2024.06.03.597144. https://doi.org/10.1101/2024.06.03.597144

Krey, I., Platzer, K., Esterhuizen, A., Berkovic, S. F., Helbig, I., Hildebrand, M. S., Lerche, H., Lowenstein, D., Møller, R. S., Poduri, A., Sadleir, L., Sisodiya, S. M., Weckhuysen, S., Wilmshurst, J. M., Weber, Y., & Lemke, J. R. (2022). Current practice in diagnostic genetic testing of the epilepsies. *Epileptic Disorders: International Epilepsy Journal with Videotape*, *24*(5), 1–22. https://doi.org/10.1684/epd.2022.1448

Krygier, M., Pietruszka, M., Zawadzka, M., Sawicka, A., Lemska, A., Limanówka, M., Żurek, J., Talaśka-Liczbik, W., & Mazurkiewicz-Bełdzińska, M. (2024). Next-generation sequencing testing in children with epilepsy reveals novel clinical, diagnostic and therapeutic implications. *Frontiers in Genetics*, *14*, 1300952. https://doi.org/10.3389/fgene.2023.1300952

Laurie, S., Steyaert, W., De Boer, E., Polavarapu, K., Schuermans, N., Sommer, A. K., Demidov, G., Ellwanger, K., Paramonov, I., Thomas, C., Aretz, S., Baets, J., Benetti, E., Bullich, G.,

Chinnery, P. F., Clayton-Smith, J., Cohen, E., Danis, D., De Sainte Agathe, J.-M., Denommé-Pickon, A.-S., Diaz- Manera, J., Guillot-Noel, L., Haack, T. B., Hanna, M., Hengel, H., Horvath, R., Houlden, H., Jackson, A., Johansson, L., Johari, M., Kamsteeg, E.-J., Kellner, M., Kleefstra, T., Lacombe, D., Lochmüller, H., López-Martín, E., Macaya, A., Marcé-Grau, A., Maver, A., Morsy, H., Muntoni, F., Musacchia, F., Nelson, I., Nigro, V., Olimpio, C., Oliviera, C., Schwabová, J. P., Pauly, M. G., Peterlin, B., Peters, S., Pfundt, R., Piluso, G., Piscia, D., Posada, M., Reich, S., Renieri, A., Ryba, L., Šablauskas, K., Savarese, M., Schöls, L., Schütz, L., Steinke-Lange, V., Stevanin, G., Straub, V., Sturm, M., Swertz, M. A., Tartaglia, M., te Paske, I. B. A. W., Thompson, R., Torella, A., Trainor, C., Udd, B., Van de Vondel, L., van de Warrenburg, B., van Reeuwijk, J., Vandrovocova, J., Vitobello, A., Vos, J., Vynhálková, E., Wijngaard, R., Wilke, C., William, D., Xu, J., Yaldiz, B., Zalatnai, L., Zurek, B., Solve-RD, DITF-GENTURIS, Solve-RD DITF-ITHACA, Solve-RD DITF-UERO-NMD, Solve-RD DITF-RND, Solve-RD consortium, Brookes, A. J., Evangelista, T., Gilissen, C., Graessner, H., Hoogerbrugge, N., Ossowski, S., Riess, O., Schüle, R., Synofzik, M., Verloes, A., Matalonga, L., Brunner, H. G., Lohmann, K., de Voer, R. M., Töpf, A., Vissers, L. E. L. M., Beltran, S., & Hoischen, A. (2025). Genomic reanalysis of a pan-European rare-disease resource yields new diagnoses. *Nature Medicine*, *31*(2), 478–489. https://doi.org/10.1038/s41591-024-03420-w

Lawrence, C. J., Kernohan, K. D., & Dyment, D. A. (2024). Chapter 21—Epilepsy. In G. M. Pastores (Ed.), *Neurogenetics for the Practitioner* (pp. 329–341). Academic Press. https://doi.org/10.1016/B978-0-323-99417-0.00029-X

Leduc-Pessah, H., White-Brown, A., Hartley, T., Pohl, D., & Dyment, D. A. (2022). The Benefit of Multigene Panel Testing for the Diagnosis and Management of the Genetic Epilepsies. *Genes*, *13*(5), 872. https://doi.org/10.3390/genes13050872

Lee, J., Lee, C., Park, W.-Y., & Lee, J. (2020). Genetic Diagnosis of Dravet Syndrome Using Next Generation Sequencing-Based Epilepsy Gene Panel Testing. *Annals of Clinical and Laboratory Science*, *50*(5), 625–637.

Lefèvre, L., Omeiri, H., Drougat, L., Hantel, C., Giraud, M., Val, P., Rodriguez, S., Perlemoine, K., Blugeon, C., Beuschlein, F., Reyniès, A. de, Rizk-Rabin, M., Bertherat, J., & Ragazzon, B. (2015). Combined transcriptome studies identify AFF3 as a mediator of the oncogenic effects of

β-catenin in adrenocortical carcinoma. *Oncogenesis*, *4*(7), e161. https://doi.org/10.1038/oncsis.2015.20

Lemke, J. R., Riesch, E., Scheurenbrand, T., Schubach, M., Wilhelm, C., Steiner, I., Hansen, J., Courage, C., Gallati, S., Bürki, S., Strozzi, S., Simonetti, B. G., Grunt, S., Steinlin, M., Alber, M., Wolff, M., Klopstock, T., Prott, E. C., Lorenz, R., Spaich, C., Rona, S., Lakshminarasimhan, M., Kröll, J., Dorn, T., Krämer, G., Synofzik, M., Becker, F., Weber, Y. G., Lerche, H., Böhm, D., & Biskup, S. (2012). Targeted next generation sequencing as a diagnostic tool in epileptic disorders. *Epilepsia*, *53*(8), 1387–1398. https://doi.org/10.1111/j.1528-1167.2012.03516.x

Lerche, H., Shah, M., Beck, H., Noebels, J., Johnston, D., & Vincent, A. (2012). Ion channels in genetic and acquired forms of epilepsy. *The Journal of Physiology*, *591*(Pt 4), 753. https://doi.org/10.1113/jphysiol.2012.240606

Leung, M. L., Ji, J., Baker, S., Buchan, J. G., Sivakumaran, T. A., Krock, B. L., Hutchins, R., Bayrak-Toydemir, P., Pfeifer, J., Cremona, M. L., Funke, B., & Santani, A. B. (2022). A Framework of Critical Considerations in Clinical Exome Reanalyses by Clinical and Laboratory Standards Institute. *The Journal of Molecular Diagnostics*, *24*(2), 177–188. https://doi.org/10.1016/j.jmoldx.2021.11.004

Li, J., Gao, K., Yan, H., Xiangwei, W., Liu, N., Wang, T., Xu, H., Lin, Z., Xie, H., Wang, J., Wu, Y., & Jiang, Y. (2019). Reanalysis of whole exome sequencing data in patients with epilepsy and intellectual disability/mental retardation. *Gene*, *700*, 168–175. https://doi.org/10.1016/j.gene.2019.03.037

Liang, L. P., Ho, Y. S., & Patel, M. (2000). Mitochondrial superoxide production in kainate-induced hippocampal damage. *Neuroscience*, *101*(3), 563–570. https://doi.org/10.1016/s0306-4522(00)00397-3

Ligasová, A., Frydrych, I., & Koberna, K. (2023). Basic Methods of Cell Cycle Analysis. *International Journal of Molecular Sciences*, *24*(4), 3674. https://doi.org/10.3390/ijms24043674

Liu, J., Xiao, Q., Xiao, J., Niu, C., Li, Y., Zhang, X., Zhou, Z., Shu, G., & Yin, G. (2022). Wnt/β-catenin signalling: Function, biological mechanisms, and therapeutic opportunities. *Signal Transduction and Targeted Therapy*, *7*(1), 1–23. https://doi.org/10.1038/s41392-021-00762-6

Lohmann, K., & Klein, C. (2014). Next Generation Sequencing and the Future of Genetic Diagnosis. *Neurotherapeutics*, *11*(4), 699–707. https://doi.org/10.1007/s13311-014-0288-8

Loviglio, M. N., Beck, C. R., White, J. J., Leleu, M., Harel, T., Guex, N., Niknejad, A., Bi, W., Chen, E. S., Crespo, I., Yan, J., Charng, W.-L., Gu, S., Fang, P., Coba-Akdemir, Z., Shaw, C. A., Jhangiani, S. N., Muzny, D. M., Gibbs, R. A., Rougemont, J., Xenarios, I., Lupski, J. R., & Reymond, A. (2016). Identification of a RAI1-associated disease network through integration of exome sequencing, transcriptomics, and 3D genomics. *Genome Medicine*, *8*(1), 105. https://doi.org/10.1186/s13073-016-0359-z

Lu, D.-F., Wang, Y.-S., Li, C., Wei, G.-J., Chen, R., Dong, D.-M., & Yao, M. (2015). Actinomycin D inhibits cell proliferations and promotes apoptosis in osteosarcoma cells. *International Journal of Clinical and Experimental Medicine*, *8*(2), 1904–1911. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4402766/

Ma, R., Duan, Y., Zhang, L., Qi, X., Zhang, L., Pan, S., Gao, L., Wang, C., & Wang, Y. (2022). SCN1A-Related Epilepsy: Novel Mutations and Rare Phenotypes. *Frontiers in Molecular Neuroscience*, *15*. https://doi.org/10.3389/fnmol.2022.826183

Marshall, G. F., Gonzalez-Sulser, A., & Abbott, C. M. (2021). Modelling epilepsy in the mouse: Challenges and solutions. *Disease Models & Mechanisms*, *14*(3), dmm047449. https://doi.org/10.1242/dmm.047449

Mazumder, S., DuPree, E., & Almasan, A. (2004). A Dual Role of Cyclin E in Cell Proliferation and Apotosis May Provide a Target for Cancer Therapy. *Current Cancer Drug Targets*, *4*(1), 65–75. https://doi.org/10.2174/1568009043481669

McKnight, D., Morales, A., Hatchell, K. E., Bristow, S. L., Bonkowsky, J. L., Perry, M. S., Berg, A. T., Borlot, F., Esplin, E. D., Moretz, C., Angione, K., Ríos-Pohl, L., Nussbaum, R. L., Aradhya, S., & ELEVIATE Consortium. (2022). Genetic Testing to Inform Epilepsy Treatment Management From an International Study of Clinical Practice. *JAMA Neurology*, *79*(12), 1267–1276. https://doi.org/10.1001/jamaneurol.2022.3651

McMillan, H. J., & Campbell, C. (2017). We need a “made in Canada” orphan drug framework. *Canadian Medical Association Journal*, *189*(41), E1274–E1275. https://doi.org/10.1503/cmaj.170195

Miller, I. O., & Sotero de Menezes, M. A. (1993). SCN1A Seizure Disorders. In M. P. Adam, J. Feldman, G. M. Mirzaa, R. A. Pagon, S. E. Wallace, & A. Amemiya (Eds.), *GeneReviews®*. University of Washington, Seattle. http://www.ncbi.nlm.nih.gov/books/NBK1318/

Millichap, J. J., Koh, S., Laux, L. C., & Nordli, D. R. (2009). Child Neurology: Dravet syndrome: When to suspect the diagnosis. *Neurology*, *73*(13). https://doi.org/10.1212/WNL.0b013e3181b9c880

Minardi, R., Licchetta, L., Baroni, M. C., Pippucci, T., Stipa, C., Mostacci, B., Severi, G., Toni, F., Bergonzini, L., Carelli, V., Seri, M., Tinuper, P., & Bisulli, F. (2020). Whole-exome sequencing in adult patients with developmental and epileptic encephalopathy: It is never too late. *Clinical Genetics*, *98*(5), 477–485. https://doi.org/10.1111/cge.13823

Moraes, F., & Góes, A. (2016). A decade of human genome project conclusion: Scientific diffusion about our genome knowledge. *Biochemistry and Molecular Biology Education*, *44*(3), 215–223. https://doi.org/10.1002/bmb.20952

Mroczek, M., Szczęśniak, D., Ziora-Jakutowicz, K., Kacprzak, M., Aleksandrowicz, P., Bednarska-Makaruk, M., & Kotuła, L. (2025). Whole exome sequencing-based testing of adult epilepsy in a Polish population. *Neurologia i Neurochirurgia Polska*, *59*(1), 70–74. https://doi.org/10.5603/pjnns.101922

Myers, K. A., Johnstone, D. L., & Dyment, D. A. (2019). Epilepsy genetics: Current knowledge, applications, and future directions. *Clinical Genetics*, *95*(1), 95–111. https://doi.org/10.1111/cge.13414

Naumova, N., Koliada, A., Kuzub, N., & Vaiserman, A. M. (2023). Chapter 9—Mitochondrial modulators. In V. K. Koltover (Ed.), *Anti-Aging Pharmacology* (pp. 193–226). Academic Press. https://doi.org/10.1016/B978-0-12-823679-6.00012-6

Nunes, A. T., & Annunziata, C. M. (2018). Proteasome Inhibitors: Structure and Function. *Seminars in Oncology*, *44*(6), 377. https://doi.org/10.1053/j.seminoncol.2018.01.004

Olimpio, C., Paramonov, I., Matalonga, L., Laurie, S., Schon, K., Polavarapu, K., Kirschner, J., Schara-Schmidt, U., Lochmüller, H., Chinnery, P. F., & Horvath, R. (n.d.). Increased Diagnostic Yield by Reanalysis of Whole Exome Sequencing Data in Mitochondrial Disease. *Journal of Neuromuscular Diseases*, *11*(4), 767–775. https://doi.org/10.3233/JND-240020

Osmond, M., Hartley, T., Dyment, D. A., Kernohan, K. D., Brudno, M., Buske, O. J., Innes, A. M., Boycott, K. M., & Care4Rare Canada Consortium. (2022). Outcome of over 1500 matches through the Matchmaker Exchange for rare disease gene discovery: The 2-year experience of Care4Rare Canada. *Genetics in Medicine: Official Journal of the American College of Medical Genetics*, *24*(1), 100–108. https://doi.org/10.1016/j.gim.2021.08.014

Ostrander, B. E. P., Butterfield, R. J., Pedersen, B. S., Farrell, A. J., Layer, R. M., Ward, A., Miller, C., DiSera, T., Filloux, F. M., Candee, M. S., Newcomb, T., Bonkowsky, J. L., Marth, G. T., & Quinlan, A. R. (2018). Whole-genome analysis for effective clinical diagnosis and gene discovery in early infantile epileptic encephalopathy. *Npj Genomic Medicine*, *3*(1), 22. https://doi.org/10.1038/s41525-018-0061-8

Ottman, R., Hirose, S., Jain, S., Lerche, H., Lopes-Cendes, I., Noebels, J. L., Serratosa, J., Zara, F., & Scheffer, I. E. (2010). Genetic testing in the epilepsies-Report of the ILAE Genetics Commission. *Epilepsia*, *51*(4), 655–670. https://doi.org/10.1111/j.1528-1167.2009.02429.x

Oyrer, J., Maljevic, S., Scheffer, I. E., Berkovic, S. F., Petrou, S., & Reid, C. A. (2018). Ion Channels in Genetic Epilepsy: From Genes and Mechanisms to Disease-Targeted Therapies. *Pharmacological Reviews*, *70*(1), 142–173. https://doi.org/10.1124/pr.117.014456

Pai, S. G., Carneiro, B. A., Mota, J. M., Costa, R., Leite, C. A., Barroso-Sousa, R., Kaplan, J. B., Chae, Y. K., & Giles, F. J. (2017). Wnt/beta-catenin pathway: Modulating anticancer immune response. *Journal of Hematology & Oncology*, *10*(1), 101. https://doi.org/10.1186/s13045-017-0471-6

Palmer, E. E., Sachdev, R., Macintosh, R., Melo, U. S., Mundlos, S., Righetti, S., Kandula, T., Minoche, A. E., Puttick, C., Gayevskiy, V., Hesson, L., Idrisoglu, S., Shoubridge, C., Thai, M. H. N., Davis, R. L., Drew, A. P., Sampaio, H., Andrews, P. I., Lawson, J., Cardamone, M., Mowat, D., Colley, A., Kummerfeld, S., Dinger, M. E., Cowley, M. J., Roscioli, T., Bye, A., &

Palmer, E. E., Schofield, D., Shrestha, R., Kandula, T., Macintosh, R., Lawson, J. A., Andrews, I., Sampaio, H., Johnson, A. M., Farrar, M. A., Cardamone, M., Mowat, D., Elakis, G., Lo, W., Zhu, Y., Ying, K., Morris, P., Tao, J., Dias, K.-R., Buckley, M., Diner, M. E., Cowley, M. J., Roscioli, T., Kirk, E. P., Bye, A., &Sachdev, R. K. (2018). Integrating exome sequencing into a diagnostic pathway for epileptic encephalopathy: Evidence of clinical utility and cost effectiveness. *Molecular Genetics & Genomic Medicine*, *6*(2), 186–199. https://doi.org/10.1002/mgg3.355

Panayiotopoulos, C. P. (2005). *The Epilepsies: Seizures, Syndromes and Management*. Bladon Medical Publishing. http://www.ncbi.nlm.nih.gov/books/NBK2606/

Panda, S. P., Dhurandhar, Y., & Agrawal, M. (2022). The interplay of epilepsy with impaired mitophagy and autophagy linked dementia (MAD): A review of therapeutic approaches. *Mitochondrion*, *66*, 27–37. https://doi.org/10.1016/j.mito.2022.07.002

Park, J. E., Miller, Z., Jun, Y., Lee, W., & Kim, K. B. (2018). Next-generation proteasome inhibitors for cancer therapy. *Translational Research*, *198*, 1–16. https://doi.org/10.1016/j.trsl.2018.03.002

Pellinen, J., Foster, E. C., Wilmshurst, J. M., Zuberi, S. M., & French, J. (2024). Improving epilepsy diagnosis across the lifespan: Approaches and innovations. *The Lancet Neurology*, *23*(5), 511–521. https://doi.org/10.1016/S1474-4422(24)00079-6

Poduri, A., & Lowenstein, D. (2011). Epilepsy genetics—Past, present, and future. *Current Opinion in Genetics & Development*, *21*(3), 325–332. https://doi.org/10.1016/j.gde.2011.01.005

Poliquin, S., & Kang, J.-Q. (2022). Disruption of the Ubiquitin-Proteasome System and Elevated Endoplasmic Reticulum Stress in Epilepsy. *Biomedicines*, *10*(3), 647. https://doi.org/10.3390/biomedicines10030647

Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., & Vogelstein, B. (1997). A model for p53-induced apoptosis. *Nature*, *389*(6648), 300–305. https://doi.org/10.1038/38525

Pruneda, J. N., Stoll, K. E., Bolton, L. J., Brzovic, P. S., & Klevit, R. E. (2011). Ubiquitin in motion: Structural studies of the ubiquitin-conjugating enzyme∼ubiquitin conjugate. *Biochemistry*, *50*(10), 1624–1633. https://doi.org/10.1021/bi101913m

Quezada, H., Guzmán-Ortiz, A. L., Díaz-Sánchez, H., Valle-Rios, R., & Aguirre-Hernández, J. (2017). Omics-based biomarkers: Current status and potential use in the clinic. *Boletín Médico Del Hospital Infantil de México (English Edition)*, *74*(3), 219–226. https://doi.org/10.1016/j.bmhime.2017.11.030

Ramakrishnan, A.-B., & Cadigan, K. M. (2017). Wnt target genes and where to find them. *F1000Research*, *6*, 746. https://doi.org/10.12688/f1000research.11034.1

Ramm-Pettersen, A., Stabell, K. E., Nakken, K. O., & Selmer, K. K. (2014). Does ketogenic diet improve cognitive function in patients with GLUT1-DS? A 6- to 17-month follow-up study. *Epilepsy & Behavior: E&B*, *39*, 111–115. https://doi.org/10.1016/j.yebeh.2014.08.015

Rastin, C., Schenkel, L. C., & Sadikovic, B. (2023). Complexity in Genetic Epilepsies: A Comprehensive Review. *International Journal of Molecular Sciences*, *24*(19), 14606. https://doi.org/10.3390/ijms241914606

Rawat, K., Gautam, V., Sandhu, A., Bhatia, A., & Saha, L. (2023). Differential Regulation of Wnt/β-catenin Signaling in Acute and Chronic Epilepsy in Repeated Low Dose Lithium-Pilocarpine Rat Model of Status Epilepticus. *Neuroscience*, *535*, 36–49. https://doi.org/10.1016/j.neuroscience.2023.10.019

Rehm, H. L., Alaimo, J. T., Aradhya, S., Bayrak-Toydemir, P., Best, H., Brandon, R., Buchan, J. G., Chao, E. C., Chen, E., Clifford, J., Cohen, A. S. A., Conlin, L. K., Das, S., Davis, K. W., del Gaudio, D., Del Viso, F., DiVincenzo, C., Eisenberg, M., Guidugli, L., Hammer, M. B., Harrison, S. M., Hatchell, K. E., Dyer, L. H., Hoang, H. N., Kelly, M. A., Kelly, J. M., Kluge, M. L., Komala, T., Kruszka, P., Lau, L., Lebo, M. S., Marshall, C. R., McKnight, D., McWalter, K., Meng, Y., Nagan, N., Necklemann, C. S., Neerman, N., Niu, Z., Paolillo, V. K., Paolucci, S. A., Perry, D., Pesaran, T., Radtke, K., Rasmussen, K. J., Retterer, K., Saunders, C. J., Spiteri, E., Stanley, C., Szuto, A., Taft, R. J., Thiffault, I., Thomas, B. C., Thomas-Wilson, A., Thorpe, E., Tidwell, T. J., Towne, M. C., Zouk, H., Marshall, C., Meng, L., Jobanputra, V., Taft, R., Ashley, E., Nakouzi, G., Shen, W., Kingsmore, S., & Rehm, H. (2023). The landscape of reported VUS

in multi-gene panel and genomic testing: Time for a change. *Genetics in Medicine*, *25*(12), 100947. https://doi.org/10.1016/j.gim.2023.100947

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W. W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., Rehm, H. L., & ACMG Laboratory Quality Assurance Committee. (2015). Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine: Official Journal of the American College of Medical Genetics*, *17*(5), 405–424. https://doi.org/10.1038/gim.2015.30

Ruggiero, S. M., Xian, J., & Helbig, I. (2023). The current landscape of epilepsy genetics: Where are we, and where are we going? *Current Opinion in Neurology*, *36*(2), 86–94. https://doi.org/10.1097/WCO.0000000000001141

Saeki, Y. (2017). Ubiquitin recognition by the proteasome. *The Journal of Biochemistry*, *161*(2), 113–124. https://doi.org/10.1093/jb/mvw091

Salinas, V., Martínez, N., Maturo, J. P., Rodriguez-Quiroga, S. A., Zavala, L., Medina, N., Amartino, H., Sfaello, I., Agosta, G., Serafín, E. M., Morón, D. G., Kauffman, M. A., & Vega, P. (2021). Clinical next generation sequencing in developmental and epileptic encephalopathies: Diagnostic relevance of data re-analysis and variants re-interpretation. *European Journal of Medical Genetics*, *64*(12), 104363. https://doi.org/10.1016/j.ejmg.2021.104363

Samanta, D. (2025). A comprehensive review of evolving treatment strategies for Dravet syndrome: Insights from randomized trials, meta-analyses, real-world evidence, and emerging therapeutic approaches. *Epilepsy & Behavior*, *162*, 110171. https://doi.org/10.1016/j.yebeh.2024.110171

Sánchez Fernández, I., Loddenkemper, T., Gaínza-Lein, M., Sheidley, B. R., & Poduri, A. (2019). Diagnostic Yield of Genetic Tests in Epilepsy: A Meta-Analysis and Cost-Effectiveness Study. *Neurology*, *92*(5), e418–e428. https://doi.org/10.1212/WNL.0000000000006850

Sandu, C., Burloiu, C. M., Barca, D. G., Magureanu, S. A., & Craiu, D. C. (2019). Ketogenic Diet in Patients with GLUT1 Deficiency Syndrome. *Mædica*, *14*(2), 93. https://doi.org/10.26574/maedica.2019.14.2.93

Satam, H., Joshi, K., Mangrolia, U., Waghoo, S., Zaidi, G., Rawool, S., Thakare, R. P., Banday, S., Mishra, A. K., Das, G., & Malonia, S. K. (2023). Next-Generation Sequencing Technology: Current Trends and Advancements. *Biology*, *12*(7), 997. https://doi.org/10.3390/biology12070997

Scheffer, I. E., Berkovic, S., Capovilla, G., Connolly, M. B., French, J., Guilhoto, L., Hirsch, E., Jain, S., Mathern, G. W., Moshé, S. L., Nordli, D. R., Perucca, E., Tomson, T., Wiebe, S., Zhang, Y.-H., & Zuberi, S. M. (2017). ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology. *Epilepsia*, *58*(4), 512–521. https://doi.org/10.1111/epi.13709

Semplici, F., Meggio, F., Pinna, L. A., & Oliviero, S. (2002). CK2-dependent phosphorylation of the E2 ubiquitin conjugating enzyme UBC3B induces its interaction with β-TrCP and enhances β-catenin degradation. *Oncogene*, *21*(25), 3978–3987. https://doi.org/10.1038/sj.onc.1205574

Seong, E., Yuan, L., & Arikkath, J. (2015). Cadherins and catenins in dendrite and synapse morphogenesis. *Cell Adhesion & Migration*, *9*(3), 202–213. https://doi.org/10.4161/19336918.2014.994919

Sheidley, B. R., Malinowski, J., Bergner, A. L., Bier, L., Gloss, D. S., Mu, W., Mulhern, M. M., Partack, E. J., & Poduri, A. (2022). Genetic testing for the epilepsies: A systematic review. *Epilepsia*, *63*(2), 375–387. https://doi.org/10.1111/epi.17141

Slavotinek, A., Rego, S., Sahin-Hodoglugil, N., Kvale, M., Lianoglou, B., Yip, T., Hoban, H., Outram, S., Anguiano, B., Chen, F., Michelson, J., Cilio, R. M., Curry, C., Gallagher, R. C., Gardner, M., Kuperman, R., Mendelsohn, B., Sherr, E., Shieh, J., Strober, J., Tam, A., Tenney, J., Weiss, W., Whittle, A., Chin, G., Faubel, A., Prasad, H., Mavura, Y., Van Ziffle, J., Devine, W. P., Hodoglugil, U., Martin, P.-M., Sparks, T. N., Koenig, B., Ackerman, S., Risch, N., Known, P.-Y., & Norton, M. E. (2023). Diagnostic yield of pediatric and prenatal exome sequencing in a diverse population. *Npj Genomic Medicine*, *8*(1), 10. https://doi.org/10.1038/s41525-023-00353-0

Smith, L., Malinowski, J., Ceulemans, S., Peck, K., Walton, N., Sheidley, B. R., & Lippa, N. (2023). Genetic testing and counseling for the unexplained epilepsies: An evidence-based

practice guideline of the National Society of Genetic Counselors. *Journal of Genetic Counseling*, *32*(2), 266–280. https://doi.org/10.1002/jgc4.1646

Sobreira, N., Schiettecatte, F., Valle, D., & Hamosh, A. (2015). GeneMatcher: A matching tool for connecting investigators with an interest in the same gene. *Human Mutation*, *36*(10), 928–930. https://doi.org/10.1002/humu.22844

Song, J., Wang, J., Jozwiak, A. A., Hu, W., Swiderski, P. M., & Chen, Y. (2009). Stability of thioester intermediates in ubiquitin-like modifications. *Protein Science : A Publication of the Protein Society*, *18*(12), 2492–2499. https://doi.org/10.1002/pro.254

Steinlein, O. K., Mulley, J. C., Propping, P., Wallace, R. H., Phillips, H. A., Sutherland, G. R., Scheffer, I. E., & Berkovic, S. F. (1995). A missense mutation in the neuronal nicotinic acetylcholine receptor α4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. *Nature Genetics*, *11*(2), 201–203. https://doi.org/10.1038/ng1095-201

Steinlein, O. K. (2004). Genes and mutations in human idiopathic epilepsy. *Brain and Development*, *26*(4), 213–218. https://doi.org/10.1016/S0387-7604(03)00149-9

Stewart, M. D., Ritterhoff, T., Klevit, R. E., & Brzovic, P. S. (2016). E2 enzymes: More than just middle men. *Cell Research*, *26*(4), 423–440. https://doi.org/10.1038/cr.2016.35

Strzelczyk, A., & Schubert-Bast, S. (2022). A Practical Guide to the Treatment of Dravet Syndrome with Anti-Seizure Medication. *CNS Drugs*, *36*(3), 217–237. https://doi.org/10.1007/s40263-022-00898-1

Tan, T. Y., Lunke, S., Chong, B., Phelan, D., Fanjul-Fernandez, M., Marum, J. E., Kumar, V. S., Stark, Z., Yeung, A., Brown, N. J., Stutterd, C., Delatycki, M. B., Sadedin, S., Martyn, M., Goranitis, I., Thorne, N., Gaff, C. L., & White, S. M. (2019). A head-to-head evaluation of the diagnostic efficacy and costs of trio versus singleton exome sequencing analysis. *European Journal of Human Genetics*, *27*(12), 1791–1799. https://doi.org/10.1038/s41431-019-0471-9

Tanida, I., Ueno, T., & Kominami, E. (2008). LC3 and Autophagy. *Methods in Molecular Biology (Clifton, N.J.)*, *445*, 77–88. https://doi.org/10.1007/978-1-59745-157-4_4

Tellez-Zenteno, J. F., Pondal-Sordo, M., Matijevic, S., & Wiebe, S. (2004). National and Regional Prevalence of Self-reported Epilepsy in Canada. *Epilepsia*, *45*(12), 1623–1629. https://doi.org/10.1111/j.0013-9580.2004.24904.x

Teng, S., Zhen, F., McRae, B. R., Zhu, E., Frankel, W. N., & Peng, Y. (2022). Sensory regulation of absence seizures in a mouse model of Gnb1 encephalopathy. *iScience*, *25*(11), 105488. https://doi.org/10.1016/j.isci.2022.105488

Thomas, R. H., & Berkovic, S. F. (2014). The hidden genetics of epilepsy—A clinically important new paradigm. *Nature Reviews Neurology*, *10*(5), 283–292. https://doi.org/10.1038/nrneurol.2014.62

Thul, P. J., & Lindskog, C. (2017). The human protein atlas: A spatial map of the human proteome. *Protein Science : A Publication of the Protein Society*, *27*(1), 233. https://doi.org/10.1002/pro.3307

Tissue expression of UBE2R2—Summary—The Human Protein Atlas. (n.d.). Retrieved December 2, 2023, from https://www.proteinatlas.org/ENSG00000107341-UBE2R2/tissue

Torii, S., Kasai, S., Yoshida, T., Yasumoto, K., & Shimizu, S. (2020). Mitochondrial E3 Ubiquitin Ligase Parkin: Relationships with Other Causal Proteins in Familial Parkinson's Disease and Its Substrate-Involved Mouse Experimental Models. *International Journal of Molecular Sciences*, *21*(4), 1202. https://doi.org/10.3390/ijms21041202

Ube2r2 ubiquitin-conjugating enzyme E2R 2 [Mus musculus (house mouse)]—Gene—NCBI. (n.d.). Retrieved December 6, 2024, from https://www-ncbi-nlm-nih-gov.proxy.bib.uottawa.ca/gene/67615

Vadlamudi, L., Milne, R. L., Lawrence, K., Heron, S. E., Eckhaus, J., Keay, D., Connellan, M., Torn-Broers, Y., Howell, R. A., Mulley, J. C., Scheffer, I. E., Dibbens, L. M., Hopper, J. L., & Berkovic, S. F. (2014). Genetics of epilepsy: The testimony of twins in the molecular era. *Neurology*, *83*(12), 1042. https://doi.org/10.1212/WNL.0000000000000790

Valassina, N., Brusco, S., Salamone, A., Serra, L., Luoni, M., Giannelli, S., Bido, S., Massimino, L., Ungaro, F., Mazzara, P. G., D'Adamo, P., Lignani, G., Broccoli, V., & Colasante, G. (2022). Scn1a gene reactivation after symptom onset rescues pathological phenotypes in a mouse model

of Dravet syndrome. *Nature Communications*, *13*(1), 161. https://doi.org/10.1038/s41467-021-27837-w

van der Geest, M. A., Maeckelberghe, E. L. M., van Gijn, M. E., Lucassen, A. M., Swertz, M. A., van Langen, I. M., & Plantinga, M. (2024). Systematic reanalysis of genomic data by diagnostic laboratories: A scoping review of ethical, economic, legal and (psycho)social implications. *European Journal of Human Genetics*, *32*(5), 489–497. https://doi.org/10.1038/s41431-023-01529-z

van Slobbe, M., van Haeringen, A., Vissers, L. E. L. M., Bijlsma, E. K., Rutten, J. W., Suerink, M., Nibbeling, E. A. R., Ruivenkamp, C. A. L., & Koene, S. (2024). Reanalysis of whole-exome sequencing (WES) data of children with neurodevelopmental disorders in a standard patient care context. *European Journal of Pediatrics*, *183*(1), 345–355. https://doi.org/10.1007/s00431-023-05279-4

Voisin, N., Schnur, R. E., Douzgou, S., Hiatt, S. M., Rustad, C. F., Brown, N. J., Earl, D. L., Keren, B., Levchenko, O., Geuer, S., Verheyen, S., Johnson, D., Zarate, Y. A., Hančárová, M., Amor, D. J., Bebin, E. M., Blatterer, J., Brusco, A., Cappuccio, G., Charrow, J., Chatron, N., Cooper, G. M., Courtin, T., Dadali, E., Delafontaine, J., Giudice, E. D., Doco, M., Douglas, G., Eisenkölbl, A., Funari, T., Giannuzzi, G., Gruber-Sedlmayr, U., Guex, N., Heron, D., Holla, Ø. L., Hurst, A. C. E., Juuusola, J., Kronn, D., Lavrov, A., Lee, C., Lorrain, S., Merckoll, E., Mikhaleva, A., Norman, J., Pradervand, S., Prchalová, D., Rhodes, L., Sanders, V. R., Sedláček, Z., Seebacher, H. A., Sellars, E. A., Sirchia, F., Takenouchi, T., Tanaka, A. K., Taska-Tench, H., Tønne, E., Tveten, K., Vitiello, G., Vlčková, M., Uehara, T., Nava, C., Yalcin, B., Kosaki, K., Donnai, D., Mundlos, S., Brunetti-Pierri, N., Chung, W. K., & Reymond, A. (2021). Variants in the degron of AFF3 are associated with intellectual disability, mesomelic dysplasia, horseshoe kidney, and epileptic encephalopathy. *American Journal of Human Genetics*, *108*(5), 857–873. https://doi.org/10.1016/j.ajhg.2021.04.001

Wang, D., Pascual, J. M., & De Vivo, D. (1993). Glucose Transporter Type 1 Deficiency Syndrome. In M. P. Adam, J. Feldman, G. M. Mirzaa, R. A. Pagon, S. E. Wallace, & A. Amemiya (Eds.), *GeneReviews®*. University of Washington, Seattle. http://www.ncbi.nlm.nih.gov/books/NBK1430/

Wang, F., Zhao, M., Jiang, Y., Xia, S., Sun, D., Zhou, D., & Dong, Z. (2023). LncRNA UBE2R2-AS1, as prognostic marker, promotes cell proliferation and EMT in prostate cancer. *Histology and Histopathology*, *38*(6), 637–645. https://doi.org/10.14670/HH-18-505

Wang, J., Lin, Z.-J., Liu, L., Xu, H.-Q., Shi, Y.-W., Yi, Y.-H., He, N., & Liao, W.-P. (2017). Epilepsy-associated genes. *Seizure*, *44*, 11–20. https://doi.org/10.1016/j.seizure.2016.11.030

Wang, J., Zhang, F., Luo, Z., Zhang, H., Yu, C., & Xu, Z. (2024). VPS13D affects epileptic seizures by regulating mitochondrial fission and autophagy in epileptic rats. *Genes & Diseases*, *11*(6), 101266. https://doi.org/10.1016/j.gendis.2024.101266

Wang, Q., Xue, H., Yue, Y., Hao, S., Huang, S.-H., & Zhang, Z. (2022). Role of mitophagy in the neurodegenerative diseases and its pharmacological advances: A review. *Frontiers in Molecular Neuroscience*, *15*. https://doi.org/10.3389/fnmol.2022.1014251

Wang, S., Xia, W., Qiu, M., Wang, X., Jiang, F., Yin, R., & Xu, L. (2016). Atlas on substrate recognition subunits of CRL2 E3 ligases. *Oncotarget*, *7*(29), 46707. https://doi.org/10.18632/oncotarget.8732

Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A., & Van Eldik, L. J. (2014). Using mice to model Alzheimer's dementia: An overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Frontiers in Genetics*, *5*. https://doi.org/10.3389/fgene.2014.00088

Wijk, S. J. L., & Timmers, H. T. M. (2010). The family of ubiquitin-conjugating enzymes (E2s): Deciding between life and death of proteins. *The FASEB Journal*, *24*(4), 981–993. https://doi.org/10.1096/fj.09-136259

Wijk, S. J. L. van, Vries, S. J. de, Kemmeren, P., Huang, A., Boelens, R., Bonvin, A. M. J. J., & Timmers, H. T. M. (2009). A comprehensive framework of E2–RING E3 interactions of the human ubiquitin–proteasome system. *Molecular Systems Biology*, *5*, 295. https://doi.org/10.1038/msb.2009.55

Wilson, J. H., & Hunt, T. (Eds.). (2002). *Molecular biology of the cell. prob, 2002: A problems approach / John Wilson & Tim Hunt* (4. ed). Garland.

Wilson, P. C., Love-Gregory, L., Corliss, M., McNulty, S., Heusel, J. W., & Gaut, J. P. (2020). Beyond Panel-Based Testing: Exome Analysis Increases Sensitivity for Diagnosis of Genetic Kidney Disease. *Kidney360*, *1*(8), 772–780. https://doi.org/10.34067/KID.0001342020

Xu, J., Xiao, M., Huang, Z., Chen, Z., & Lin, J. (2024). UBE2R2-AS1, as a prognostic marker of gastric cancer, promotes the malignant phenotype of gastric cancer cells. *Histology and Histopathology*, *39*(6), 739–745. https://doi.org/10.14670/HH-18-677

Xue, Y., Ankala, A., Wilcox, W. R., & Hegde, M. R. (2015). Solving the molecular diagnostic testing conundrum for Mendelian disorders in the era of next-generation sequencing: Single-gene, gene panel, or exome/genome sequencing. *Genetics in Medicine*, *17*(6), 444–451. https://doi.org/10.1038/gim.2014.122

Yang, X., Pan, W., Xu, G., & Chen, L. (2020). Mitophagy: A crucial modulator in the pathogenesis of chronic diseases. *Clinica Chimica Acta*, *502*, 245–254. https://doi.org/10.1016/j.cca.2019.11.008

Yozawitz, E., & Moshé, S. L. (2022). The influence of genetics on epilepsy syndromes in infancy and childhood. *Acta Epileptologica*, *4*(1), 41. https://doi.org/10.1186/s42494-022-00110-3

Zhang, L., Dai, L., & Li, D. (2021). Mitophagy in neurological disorders. *Journal of Neuroinflammation*, *18*(1), 297. https://doi.org/10.1186/s12974-021-02334-5

Zhang, L., Jin, Y., Yang, H., Li, Y., Wang, C., Shi, Y., & Wang, Y. (2019). SMYD3 promotes epithelial ovarian cancer metastasis by downregulating p53 protein stability and promoting p53 ubiquitination. *Carcinogenesis*, bgz078. https://doi.org/10.1093/carcin/bgz078

Zhong, F., Gan, Y., Song, J., Zhang, W., Yuan, S., Qin, Z., Wu, J., Lü, Y., & Yu, W. (2022). The inhibition of PGAM5 suppresses seizures in a kainate-induced epilepsy model via mitophagy reduction. *Frontiers in Molecular Neuroscience*, *15*, 1047801. https://doi.org/10.3389/fnmol.2022.1047801

Zhu, J., & Tsai, N.-P. (2020). Ubiquitination and E3 Ubiquitin Ligases in Rare Neurological Diseases with Comorbid Epilepsy. *Neuroscience*, *428*, 90–99. https://doi.org/10.1016/j.neuroscience.2019.12.030