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Elucidating Genetic Causes of Dystonia by Large-Scale Next-Generation Sequencing

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Abstract

Dystonia is a neurological movement disorder characterized by abnormal postures or movements caused by involuntary muscle contractions, affecting approximately 3 million people worldwide. A hallmark of the disease is its pronounced clinical and etiological heterogeneity. Dystonia can occur in isolation or in combination with other neurological or systemic features. This variability complicates diagnosis, efforts to uncover its pathophysiology, and the development of effective treatments. Dystonia has a significant hereditary component, with about 25% of patients having affected relatives. However, despite the identification of over 400 dystonia-associated genes, especially due to advancements in next-generation sequencing, the etiology in most patients remains elusive, particularly in isolated and focal dystonia, the largest yet least-studied patient group. Genetic testing is complicated by the highly heterogeneous nature of the disease and the expanding list of implicated and candidate genes, many of which lack independent validation. Given dystonia's rarity and genetic complexity, large cohorts are indispensable for comprehensively unraveling its genetic spectrum.

This thesis investigated genetic causes of dystonia using a multi-dimensional approach, combining systematic literature review with analyses of genetic and clinical data from more than 2,500 unique patients, most of whom presented with isolated focal dystonia. Objective I analyzed genotype-phenotype correlations in three recently described isolated dystonia genes, revealing broad phenotypic spectra and clinical overlap with other genetic forms despite gene-specific trends and highlighting the essential role of genetic testing for establishing a diagnosis (Thomsen et al., 2023, *Movements Disorders*). Objective II categorized genetic forms of dystonia based on their underlying molecular mechanisms, describing seven key cellular pathways and illustrating how biologically defined grouping can help organize genetic heterogeneity (Thomsen et al., 2024, *Annual Review of Pathology*). In Objective III, targeted gene panel sequencing was applied to *n*=1,207 dystonia versus *n*=1,036 Parkinson's disease patients, identifying a significantly higher burden of rare variants in dystonia genes and yielding genetic diagnoses in 4.0% of dystonia patients. Additionally, analysis of DNA methylation patterns (episignature) proved effective in the classification of rare variants in the *KMT2B* gene, enabling a distinction between pathogenic and benign changes (Thomsen et al., 2024, *Movement Disorders*). In Objective IV, exome sequencing of a large dystonia cohort (*n*=1,924), including previously unsolved patients from the gene panel analysis, yielded a diagnosis in 8.1% of patients, provided supporting evidence for several dystonia candidate genes, and expanded the known phenotypic spectrum of some genes (Thomsen et al., in press, *Annals of Clinical and Translational Neurology*). Lastly, in Objective V, optical genome mapping was applied to a patient with a strong suspicion of a genetic etiology but negative exome, and revealed a homozygous *KIF1C* deletion, which was functionally

validated by transcriptome analysis, demonstrating the added diagnostic value of such methods (Thomsen et al., manuscript prepared for submission).

Altogether, this work presents an extensive genetic characterization of the largest multicenter dystonia cohort to date. It highlights the diagnostic value of comprehensive sequencing strategies, the utility of integrating functional data, and the potential of organizing genetic heterogeneity through biological classification. The generated datasets provide a foundation for future gene discovery, improved genetic variant interpretation, and the development of mechanism-based therapies.

Zusammenfassung

Dystonie ist eine neurologische Bewegungsstörung, die durch unwillkürliche Muskelkontraktionen verursacht wird und sich in abnormalen Haltungen oder Bewegungen äußert. Weltweit sind schätzungsweise drei Millionen Menschen betroffen. Ein zentrales Merkmal der Erkrankung ist ihre ausgeprägte klinische und ätiologische Heterogenität. Dystonie kann isoliert oder in Kombination mit weiteren neurologischen oder systemischen Symptomen auftreten. Diese Variabilität erschwert nicht nur die Diagnosestellung, sondern auch das Verständnis der zugrundeliegenden Pathomechanismen und die Entwicklung wirksamer Therapien. Etwa 25 % der Patient:innen weisen eine positive Familienanamnese auf, was auf eine erhebliche hereditäre Komponente hinweist. Trotz der Identifikation von über 400 Dystonie-assoziierten Genen – insbesondere durch Fortschritte in der *Next-Generation*-Sequenzierung – bleibt die Ätiologie bei den meisten Betroffenen unklar, insbesondere bei isolierter fokaler Dystonie, der größten und gleichzeitig am wenigsten untersuchten Patientengruppe. Die genetische Diagnostik wird durch die hohe Heterogenität der Erkrankung sowie durch die stetig wachsende Zahl an gesicherten und potenziellen Krankheitsgenen erschwert, von denen viele bislang nicht unabhängig bestätigt wurden. Angesichts der Seltenheit und genetischen Komplexität von Dystonie sind große Patientenkohorten unerlässlich, um das genetische Spektrum umfassend zu charakterisieren.

In dieser Arbeit wurden genetische Ursachen von Dystonie durch einen multidimensionalen Ansatz untersucht, der eine systematische Literaturauswertung mit der Analyse genetischer und klinischer Daten von über 2.500 individuellen Patient:innen kombinierte, überwiegend mit isolierter fokaler Dystonie. Ziel I befasste sich mit Genotyp-Phänotyp-Korrelationen bei drei kürzlich beschriebenen isolierten Dystoniegenen. Diese Analysen zeigten breite phänotypische Spektren und Überschneidungen mit anderen genetischen Formen, trotz gen-spezifischer Trends, und unterstreichen die zentrale Bedeutung genetischer Testung für die Diagnosestellung (Thomsen et al., 2023, *Movement Disorders*). Ziel II klassifizierte genetische Dystonieformen anhand ihrer zugrunde liegenden molekularen Mechanismen. Es wurden sieben zentrale zelluläre Signalwege beschrieben und aufgezeigt, wie eine biologische Gruppierung helfen kann, die ausgeprägte genetische Heterogenität zu strukturieren (Thomsen et al., 2024, *Annual Review of Pathology*). In Ziel III wurde eine *Gene-Panel*-Sequenzierung bei 1.207 Dystonie- und 1.036 Parkinson-Patient:innen durchgeführt. Dabei zeigte sich eine signifikant höhere Belastung mit seltenen Varianten in bekannten Dystoniegenen in der Dystoniegruppe; bei 4,0 % konnte eine molekulare Diagnose gestellt werden. Zusätzlich erwies sich die Analyse von DNA-Methylierungsmustern (Episignatur) als hilfreich für die Klassifikation seltener Varianten im

KMT2B-Gen in pathogene und benigne Veränderungen (Thomsen et al., 2024, *Movement Disorders*). Ziel IV umfasste die Exom-Sequenzierung einer großen Kohorte von 1.924 Dystonie-Patient:innen, darunter viele diagnostisch ungelöste Patient:innen aus der *Gene-Panel*-Untersuchung. Hierdurch konnten bei 8,1 % molekulare Diagnosen gestellt, mehrere Kandidatengene gestützt und das phänotypische Spektrum bekannter Gene erweitert werden (Thomsen et al., im Druck, *Annals of Clinical and Translational Neurology*). In Ziel V wurde bei einem Patienten mit starkem klinischen Verdacht auf eine genetische Ursache und negativer Exomdiagnostik eine homozygote *KIF1C*-Deletion mittels *Optical Genome Mapping* detektiert und durch Transkriptomanalyse funktionell validiert – ein Beispiel für den diagnostischen Mehrwert fortschrittlicher genetischer Methoden (Thomsen et al., Manuskript vorbereitet zur Einreichung).

Insgesamt stellt diese Arbeit eine umfassende und vielschichtige genetische Charakterisierung der bisher größten multizentrischen Dystonie-Kohorte dar. Sie unterstreicht den diagnostischen Nutzen umfassender Sequenzierstrategien, die Bedeutung funktioneller Daten sowie das Potenzial biologischer Klassifikation zur Strukturierung genetischer Heterogenität. Die gewonnenen Datensätze bilden eine wertvolle Grundlage für zukünftige Genentdeckungen, eine verbesserte Varianteninterpretation und die Entwicklung Mechanismus-basierter Therapien.

Contents

1 Introduction

1.1 Dystonia – a Highly Heterogeneous Movement Disorder

Dystonia is a clinically and etiologically heterogeneous neurological movement disorder. For much of medical history, dystonia was considered a psychiatric condition without a clearly identifiable organic cause. In 1911, the German neurologist Hermann Oppenheim introduced the term *dystonia musculorum deformans* to describe adolescents with fluctuating muscle tone and spasms, proposing a neurological and possibly hereditary basis (Klein & Fahn, 2013; Oppenheim, 1911). Today, dystonia is defined as "sustained or intermittent muscle contractions causing abnormal, often repetitive movements, postures, or both" (Albanese et al., 2013). These movements are typically patterned, twisting, and may be tremulous, and are often initiated or worsened by voluntary action (Albanese et al., 2013). Dystonia can refer either to a clinical sign occurring in the context of another neurological condition or to a disease itself in which dystonia is the primary or sole feature. Its etiology is diverse, encompassing acquired, inherited, and idiopathic causes, with genetic factors playing an increasingly recognized role (Albanese et al., 2025). Dystonia was long considered a rare disorder (defined in the EU as affecting fewer than 1 in 2,000 individuals, i.e., <50/100,000). However, recent epidemiological studies suggest a higher prevalence of isolated dystonia, with estimates up to 52.7 per 100,000 individuals (Dressler et al., 2022), although the actual prevalence may be even higher due to under- or misdiagnosis.

Dystonia presents with considerable clinical variability. While some individuals develop severe forms early in life, often accompanied by additional neurological features, the majority of patients experience adult-onset dystonia confined to one body region, such as the neck or the hands. This clinical diversity has posed significant challenges for diagnosis and treatment development and decisions.

1.1.1 Classification of Dystonia

To better capture this heterogeneity, and building on earlier classification systems, an international expert group developed a now widely adopted consensus classification, structured along two axes: clinical characteristics (Axis I) and etiology (Axis II) (Albanese et al., 2013). Axis I describes the observable features of dystonia, including age at onset, body distribution, temporal pattern, and associated features. Body distribution distinguishes between focal dystonia (affecting a single body region), multifocal dystonia (two or more non-contiguous regions), segmental dystonia (two or more contiguous regions), and generalized dystonia (involving the

trunk and at least two other regions). The temporal pattern refers to both the overall disease course (static or progressive) and the variability of symptoms, which may be persistent, action-specific, fluctuating, or paroxysmal. Based on associated features, dystonia can be classified as isolated, where dystonia is the only symptom, or combined with other movement disorders such as myoclonus, parkinsonism or chorea. Dystonia can also occur as part of complex syndromes that include other prominent neurological or systemic manifestations, such as developmental delay, deafness, epileptic encephalopathy, hepatic dysfunction, and others (Lohmann & Klein, 2020; Thomsen et al., 2024a).

Axis II addresses the underlying etiology and integrates information about nervous system pathology and causative factors. This includes evidence of neurodegeneration, static structural brain lesions, or the absence of anatomical abnormalities. It further distinguishes between inherited forms, acquired causes (e.g., due to perinatal brain injury or drug exposure), and idiopathic cases, where no cause is identified – the most common form in clinical practice. Importantly, Axis II is considered dynamic and should be updated as new genetic and biological discoveries are made.

1.1.2 Treatment Options for Dystonia

Although recent genetic and molecular discoveries have advanced our understanding of dystonia, their translation into effective therapies remains limited (Thomsen et al., 2024a). Current management is largely symptomatic and based on several treatment pillars, including botulinum toxin injections, pharmacological therapies, physical therapy, and, in selected cases, surgery (Balint et al., 2018). The choice of treatment depends on clinical features, such as symptom distribution and severity, as well as the underlying etiology. If a specific cause is known, for example, in metabolic or autoimmune forms, etiology-directed therapy should be pursued (Jinnah et al., 2018). In patients with severe, drug-refractory dystonia, deep brain stimulation (DBS) offers an effective alternative. Notably, the effectiveness of DBS in hereditary dystonia can vary depending on the causative gene (Sarva et al., 2024). To enable the development of targeted therapies, a deeper understanding of the genetic architecture, underlying molecular mechanisms, and their relation to treatment response is urgently needed.

1.2 Genetic Causes of Dystonia

A subset of dystonia patients can be explained by monogenic causes. The heritability is estimated at approximately 25%, as around a quarter of patients have other affected relatives (Waddy et al., 1991). Despite this considerable heritability and the identification of several causal genes, the majority of patients remain without a genetic diagnosis, particularly those with later-onset,

isolated focal dystonia, which is by far the most common form and is therefore of high clinical relevance (Ahn et al., 2023; Thomsen et al., 2024a; Zech et al., 2020).

Knowing the genetic basis of a dystonia has translational importance in terms of prognosis, family planning, treatment decisions, and, in the future, also for patient stratification and prioritization in clinical trials (Pozojevic et al., 2021). However, genetic testing remains challenging due to the highly heterogeneous nature of the disease, the rapidly growing list of dystonia-linked genes, and the reliance on clinical diagnosis due to the absence of an established biomarker.

1.2.1 Genes Linked to Monogenic Dystonias

Although familial clustering of dystonia was already recognized in the early 20th century (Klein & Fahn, 2013; Oppenheim, 1911), the first dystonia-associated gene was not identified until 1994. Pathogenic variants in *GCH1*, encoding GTP cyclohydrolase 1, were discovered in patients with dopa-responsive dystonia through linkage analysis and subsequent prioritization of the gene based on its role in dopamine biosynthesis (Ichinose et al., 1994). A few years later, a three-base-pair deletion in *TOR1A*, encoding Torsin A, was identified as a cause of isolated dystonia, likewise through linkage analysis in a large family (Ozelius et al., 1997). In the following years, genetic linkage studies and candidate gene approaches led to the discovery of many additional dystonia-associated genes. Since 2011, next-generation sequencing (NGS) has significantly accelerated gene discovery in dystonia, enabling the identification of numerous genes such as *GNAL* (Fuchs et al., 2013), *KMT2B* (Zech et al., 2016), and *VPS16* (Cai et al., 2016; Steel et al., 2020).

Just as the general classification of dystonia reflects its clinical and etiological heterogeneity, a system has been developed to categorize and name its genetic forms, which evolved alongside genetic discoveries. Initially based on chromosomal loci (DYT1, DYT2, etc.) that had been linked to a familial disorder with unidentified causative genes, the nomenclature was revised in 2016 by the Task Force for the Nomenclature of Genetic Movement Disorders of the Movement Disorder Society (MDS) to reflect confirmed monogenic causes and their predominant phenotypes (Marras et al., 2016). In this system, genes are designated with a movement disorder prefix followed by the name of the causal gene (e.g., DYT-TOR1A for dystonia caused by pathogenic variants in the *TOR1A* gene) and require a well-established genotype-phenotype link.

Presently, the MDS Task Force recognizes 53 established dystonia genes (**Figure 1**), based on their association with frequent or predominant dystonia phenotypes and excluding unconfirmed gene-dystonia associations (Lange et al., 2022; Thomsen et al., 2024a). This list includes 10 genes causing isolated, 10 genes causing combined, and 33 genes causing complex forms of dystonia,

as well as 7 genes primarily linked to other disorders in which dystonia can be a prominent feature. Another large group of genes, exemplified by six prominent examples in **Figure 1**, is associated with neurodevelopmental disorders that often include dystonia. With ongoing NGS efforts, the number of confirmed and candidate dystonia genes continues to grow, although many of them await replication by independent studies (Atasu et al., 2024; Keller Sarmiento & Mencacci, 2021; Lange et al., 2022). Altogether, this results in over 400 potential dystonia-causing genes (Thomsen et al., 2024a).

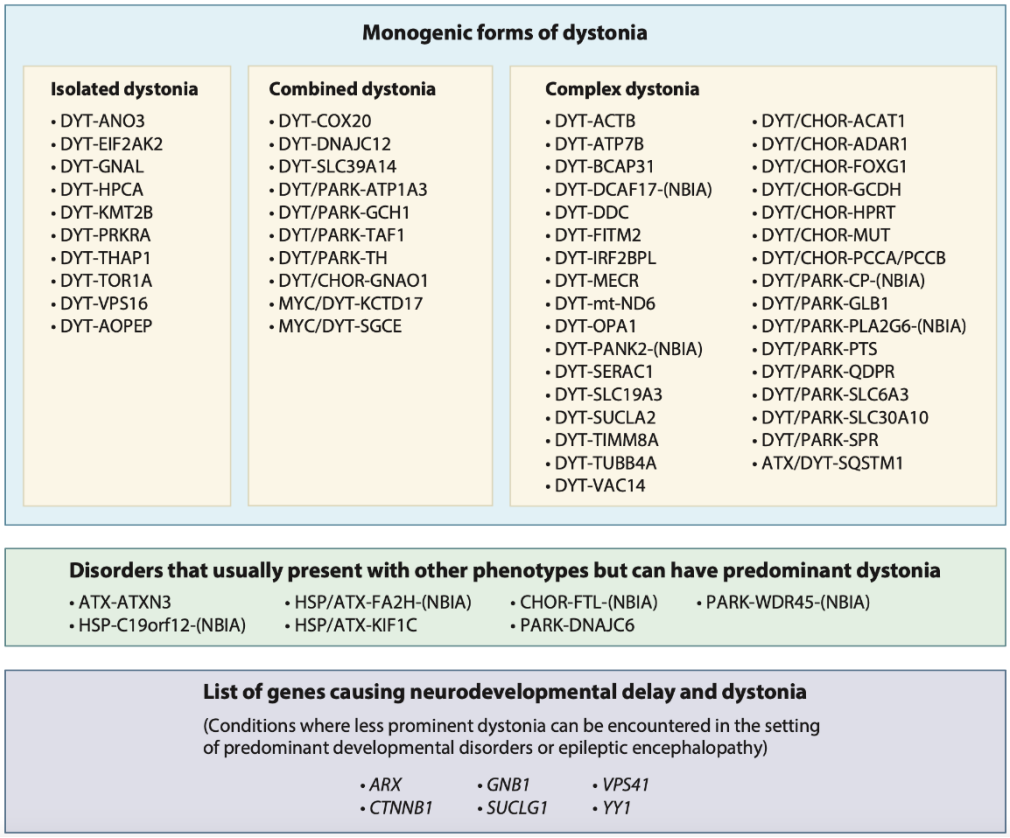

Figure 1. Overview of confirmed monogenic forms of dystonia. For genes causing neurodevelopmental delay and dystonia, the figure shows selected examples. Additional genes in this large group can be found on the OMIM® webpage (https://www.omim.org). Abbreviations: ATX, ataxia; CHOR, chorea; DYT, dystonia; HSP, hereditary spastic paraplegia; NBIA, neurodegeneration with brain iron accumulation; PARK, Parkinson's disease.
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1.2.2 Genotype-Phenotype Relationships

The different dystonia genes are associated with gene-specific clinical trends, such as a typical age at onset, commonly affected body regions, or frequently observed accompanying symptoms (Lange et al., 2021). For example, *GNAL* variant carriers typically present with adult-onset

cervical dystonia, often as an isolated feature, whereas *KMT2B*-related dystonia usually begins in childhood, is generalized in over 85% of cases, almost always involves the lower limbs, and frequently includes developmental delay (Lange et al., 2021).

Despite these gene-specific trends, there is substantial clinical overlap across different genetic forms (Lange et al., 2021). In addition, variable expressivity, where individuals with the same pathogenic variant show differing severity or symptoms, and pleiotropy, where a single gene can cause multiple distinct clinical syndromes, further complicate the genotype-phenotype correlation (Thomsen et al., 2024a). This is demonstrated by the increasing number of genes initially linked to other neurological conditions – such as epilepsy, developmental delay, or ataxia – that are now recognized to cause dystonia as a prominent or even isolated manifestation (Keller Sarmiento & Mencacci, 2021; Svorenova et al., 2022).

Taken together, these complexities emphasize that while clinical clues may inform diagnostic prioritization, genetic testing remains essential to establish a definitive diagnosis. Still, systematic genotype-phenotype investigations are valuable for identifying recurrent patterns, supporting variant interpretation, and informing clinical decision-making.

1.3 Genetic Testing Strategies in Dystonia

Choosing a genetic test involves balancing the likelihood for a diagnosis, cost, and the clinical presentation of the patient (Kernohan & Boycott, 2024). Sanger sequencing of single genes was widely used in the past, particularly before the advent of NGS technologies in the 2010s. Today, the utility of single-gene testing in dystonia diagnostics is limited, as the clinical and genetic heterogeneity rarely allows for confident prioritization of a single gene. However, it may still be appropriate in selected cases, such as *SGCE* in familial myoclonus-dystonia or *GCH1* in dopa-responsive dystonia, or in specific populations with known founder mutations, e.g., the recurrent GAG-deletion in *TOR1A* in Ashkenazi Jewish patients (Pozojevic et al., 2021). In addition, Sanger sequencing remains useful for screening patient cohorts for variants in newly described dystonia genes (Pott et al., 2021), or for validating variants identified by NGS, which can occasionally yield false-positive results (Mu et al., 2016).

Due to the pronounced heterogeneity of dystonia, genome-wide approaches enabled by NGS technologies have proven more effective. These include gene panels, exome sequencing, and whole genome sequencing, among others. While all are based on parallel sequencing of millions of short DNA fragments (typically 100-150bp in length), they differ in read depth and genomic regions covered (Kernohan & Boycott, 2024), as illustrated in **Figure 2**. Gene panels target a predefined set of genes and are available, e.g., for commonly implicated dystonia genes. In

contrast, exome and genome sequencing analyze all coding regions or the entire genome, respectively, offering a key advantage: as the field of dystonia genetics evolves rapidly, such datasets can be reanalyzed to search for novel or previously overlooked disease-associated genes (Laurie et al., 2025). Another NGS-based approach is RNA sequencing, which captures all transcribed mRNA in a tissue or cell type. Unlike DNA-based approaches, it provides functional insights into the effects of genomic variants if they have a direct effect on the transcriptional level, e.g., by altering splicing patterns or causing nonsense-mediated decay that leads to reduced transcript levels. Its diagnostic utility in rare genetic diseases has recently been demonstrated, particularly in cases unsolved after exome sequencing (Yépez et al., 2022).

The diagnostic utility of NGS approaches in dystonia has been demonstrated by several studies, most of which were small-scale (median cohort size: n=88) and primarily based on gene panel or exome sequencing (Wirth et al., 2025). Reported diagnostic yields varied substantially depending on patient selection criteria and study type, ranging from 9% to 41.7% (Wirth et al., 2025), while rates can be as low as 1% in subgroups with late-onset, isolated focal dystonia (Zech et al., 2020)

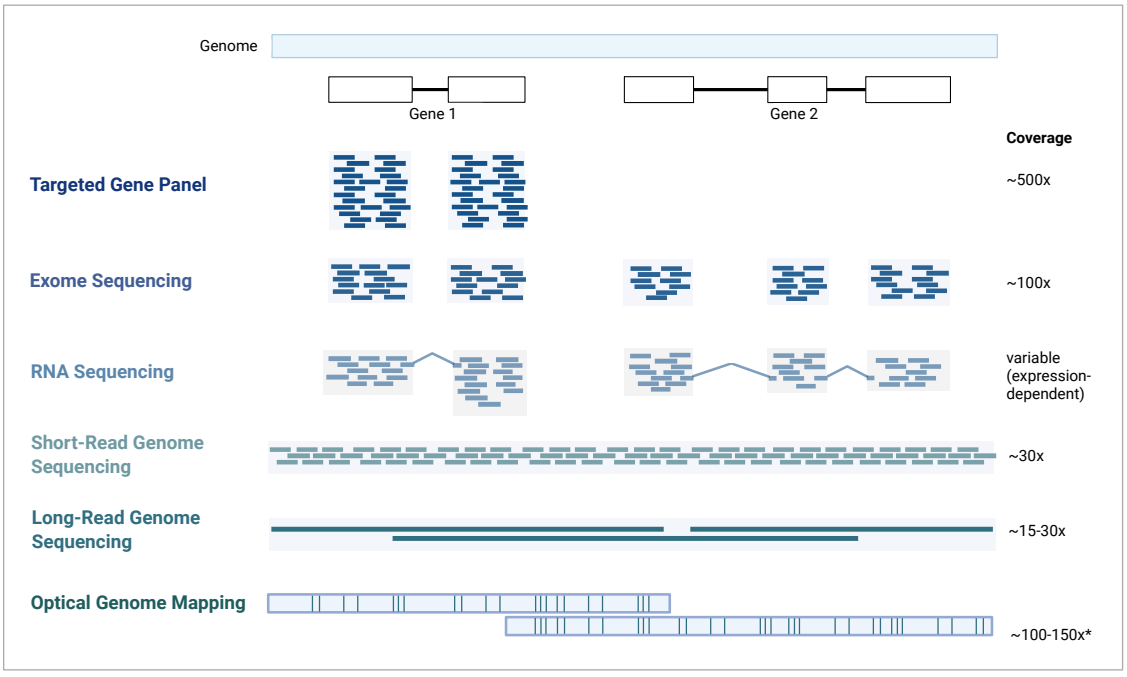

Figure 2. Schematic overview of selected genetic testing methods. The figure illustrates selected genetic testing approaches, highlighting differences in sequencing read type and genomic regions covered. Short-read–based methods include targeted gene panels (covering selected disease genes), exome sequencing (covering all coding exons), RNA sequencing (capturing transcribed mRNA and enabling, e.g., splice analysis through split reads (shown as connecting arcs)), and short-read genome sequencing (covering the entire genome). In contrast, long-read genome sequencing spans large continuous DNA segments and enables comprehensive coverage of genomic regions. Optical genome mapping (OGM), a non-sequencing high-resolution cytogenetics method, also targets the full genome and supports the detection of large structural rearrangements.*OGM coverage refers to effective molecule coverage, not base-level read depth as in sequencing. *Figure created with BioRender.com.*

– the most common form observed in epidemiological studies (Bailey et al., 2022). Since the various genetic forms of dystonia are extremely rare, larger studies are needed to better understand the relevance and diversity of genetic factors.

Despite the increasing application of NGS, most genetic studies in dystonia have focused on patients of European ancestry (Lange et al., 2021; Zech et al., 2020). This lack of diversity limits the generalizability of genetic findings and complicates variant interpretation for patients from underrepresented populations. Moreover, broader genomic representation has proven to be essential for disease gene discovery and for advancing our understanding of genetic contributors to disease (Williamson & Fatumo, 2024). Emerging studies in dystonia from regions such as India and the Middle East have begun to uncover distinct variant spectra and population-specific mutations (Alakkas et al., 2025; Dhar et al., 2024). However, large-scale genomic data from many global populations – particularly in Africa, Asia, and Latin America – remain scarce. Limited representation in genetic research may also contribute to disparities in access to diagnostics and advanced therapies. Improving diversity in dystonia research is therefore essential to achieve a more complete understanding of the disorder's genetic architecture.

Additionally, although NGS has greatly accelerated gene discovery and diagnostic yield, it has inherent limitations. Certain genomic regions, such as GC-rich, low-complexity, or highly homologous sequences, are difficult to capture accurately (Kernohan & Boycott, 2024). Structural variant (SV) detection is possible with advanced bioinformatics, but remains challenging due to high false-positive rates and limited sensitivity for more complex SVs. Emerging technologies help overcome many of these limitations, though at higher cost. Long-read sequencing platforms (e.g., Oxford Nanopore or PacBio) generate reads of 10-50kb, enabling improved SV detection **(Figure 2)**. Another promising approach is optical genome mapping (OGM), a high-resolution cytogenetic method that fluorescently labels long DNA molecules and subsequently linearizes them in nanochannels to detect SVs from 500bp up to whole chromosome rearrangements **(Figure 2)**. OGM has already led to several successful diagnoses in rare diseases (Dremsek et al., 2021). In dystonia, however, these technologies have rarely been applied.

1.3.1 Interpretation of Genetic Data

One of the major challenges in genetic testing is interpreting the large number of genetic variants identified. Exome sequencing yields approximately 20,000 coding variants per individual, while genome sequencing identifies up to 4 million variants (Gaynor et al., 2024), most of which are benign and represent normal human variation. Distinguishing truly disease-causing variants requires careful and systematic assessment. Guidelines from the American College of Medical

Genetics and Genomics (ACMG) and the Association for Molecular Pathology provide a structured framework for this, incorporating evidence from segregation analysis, *in silico* predictions, population databases, and functional studies (Richards et al., 2015). Variants are classified into five categories (benign, likely benign, uncertain significance, likely pathogenic, and pathogenic) based on the strength and combination of these different criteria.

Strong evidence for pathogenicity may include null variants (such as stop-gain or frameshift) in a gene where loss of function is a known disease mechanism, or experimental studies demonstrating a deleterious functional effect. Moderate evidence can come from a variant's absence or extremely low frequency in control databases such as gnomAD, its segregation with disease in multiple affected family members, or its location within a mutational hotspot or functional domain of the protein. Supporting evidence may include damaging predictions from *in silico* prediction tools or a phenotype highly specific for the gene in question (Richards et al., 2015).

However, the majority of detected rare variants remain classified as variants of uncertain significance (VUS) due to limited (functional) information on the variant as well as insufficient or conflicting evidence for either a pathogenic or benign role – a common issue in clinical genetics (Chen et al., 2023). Ongoing efforts in functional characterization, population genetics, and international data sharing are essential to refine variant classification and improve diagnostic accuracy. Especially with regard to novel disease genes, replicating findings in independent patients and families remains a critical step toward confirming their disease association.

1.3.2 Functional Approaches to Variant Interpretation

For a few dystonia-associated genes, gene-specific functional assays have been developed to assess the impact of genetic variants. *THAP1* encodes a transcription factor whose activity can be quantified using a luciferase reporter assay (Lohmann et al., 2012). Variants in *GNAL*, which encodes a stimulatory G-protein alpha subunit, can be evaluated using a bioluminescence resonance energy transfer (BRET) assay that tests their ability to form functional G-protein coupled receptors (Fuchs et al., 2013). For *KMT2B*, recent studies have identified a unique DNA methylation pattern, or "episignature", in peripheral blood that provides a readout of KMT2B loss of function (Ciolfi et al., 2021; Lee et al., 2022; Mirza-Schreiber et al., 2022). This episignature is characterized by CpG hypermethylation at specific genomic locations and reflects the loss of histone H3 lysine 4 (H3K4) methylation typically mediated by functional KMT2B protein. While such assays provide important tools for variant validation, they are currently not available for

most dystonia genes and may be technically demanding and time-consuming, as is the case for the *THAP1* and *GNAL* assays.

1.4 Molecular Mechanisms of Genetic Dystonias

The discovery of monogenic causes of dystonia – particularly the rapid increase in gene identifications since NGS application – has significantly advanced our understanding of the disorder's molecular underpinnings. While all dystonias share excessive muscle activity caused by dysfunction within the brain's motor network as their underlying biology (Jinnah & DeFazio, 2023), genetic findings have revealed that, on a molecular level, dystonia can result from dysfunction in a wide range of cellular processes, reflecting the diverse biological roles of the respective gene products. With each newly discovered gene, our understanding of the underlying molecular processes continues to grow.

A well-established example is disrupted striatal dopamine signaling, which has long been recognized as a central mechanism in dystonia, as the first gene linked to the disorder, *GCH1*, plays a central role in dopamine biosynthesis (Ichinose et al., 1994). Since then, additional isolated and combined dystonia genes, such as *GNAL* (Fuchs et al., 2013), *GNAO1* (Nakamura et al., 2013), and *ADCY5* (Carapito et al., 2015), have been identified that affect pre- and postsynaptic components of striatal dopamine pathways. A second key mechanism revealed through gene discoveries is transcriptional dysregulation, particularly during neurodevelopment, as illustrated by two of the most common isolated dystonia genes, *THAP1* and *KMT2B*. Both genes regulate transcriptional programs essential for neuronal maturation during development (Barbagiovanni et al., 2018; Yellajoshyula et al., 2017). Another notable example is the identification of *EIF2AK2* variants as a cause of isolated dystonia, advancing our understanding of the cellular stress response, particularly via the eIF2α pathway – a pathway that is also affected in other isolated dystonia forms, such as DYT-PRKRA (Kuipers et al., 2021). Additional mechanisms have been implicated, including disrupted calcium signaling and synaptic transmission (e.g., *HPCA, ATP1A3*) (Charlesworth et al., 2015; de Carvalho Aguiar et al., 2004) and impaired autophagy and lysosomal function (*VPS16*, *VPS41*) (Steel et al., 2020).

While these mechanisms are supported by genetic and functional data, their interconnections and shared downstream consequences remain incompletely understood. Gaining a more comprehensive understanding of dystonia's genetic landscape is therefore critical to identifying and connecting such pathways. A central question in dystonia research has been whether genetically and clinically heterogeneous forms should be lumped together based on a shared pathophysiology, or whether they should be split into distinct biological subgroups defined by

underlying molecular mechanisms (Jinnah & DeFazio, 2023). Identifying such biological subgroups could support a mechanism-based classification and inform more targeted therapeutic strategies.

An example of how the identification of shared disease mechanisms may lead to improved patient care can be found in a related movement disorder – Parkinson's disease (PD). Among the most frequently implicated genes in monogenic PD is *LRRK2*, with disease-associated variants often leading to increased kinase activity of the LRRK2 protein. Recently, pathogenic variants in *RAB32*, encoding a Rab GTPase, were linked to PD and were also shown to increase LRRK2 kinase activity (Gustavsson et al., 2024); indicating that genetically distinct forms share the same pathogenic mechanism. Inhibitors targeting LRRK2 kinase activity are currently in clinical trials (Hu et al., 2023), and it has been proposed that such treatments might also benefit *RAB32*-associated PD patients. Thus, identifying shared molecular biology is a key step towards targeted treatments.

To address the question of shared molecular mechanisms in dystonia in greater depth, Objective II of this thesis provides a literature review of molecular mechanisms underlying monogenic forms of dystonia.

1.5 Objectives

The overarching aim of this work is to advance the understanding of genetic dystonia by analyzing large-scale sequencing data to assess gene and variant frequencies, genotype-phenotype relationships, and the diagnostic utility of different sequencing strategies, as well as by reviewing the molecular basis of monogenic dystonias. Specifically, the following objectives were addressed and are illustrated in **Figure 3**:

Objective I: To investigate genotype-phenotype relationships in newly described dystonia genes (*AOPEP*, *EIF2AK2*, *VPS16*) by systematically analyzing the published literature.

Objective II: To group known genetic forms of dystonia into molecular categories based on the biological functions of the underlying genes.

Objective III: To analyze the frequency of pathogenic variants in the seven most common isolated and combined dystonia genes in a large dystonia sample (n=1,207) compared to Parkinson's disease patients (n=1,036) using gene panel sequencing.

Objective IV: To analyze the prevalence of pathogenic variants and associated phenotypes across all reported dystonia-related genes (*n*=406) in a large patient sample (*n*=1,924) using exome sequencing.

Objective V: To identify the genetic cause of a complex movement disorder in a patient not resolved by exome sequencing, using a multi-omics approach including whole genome sequencing, RNA sequencing, and optical genome mapping.

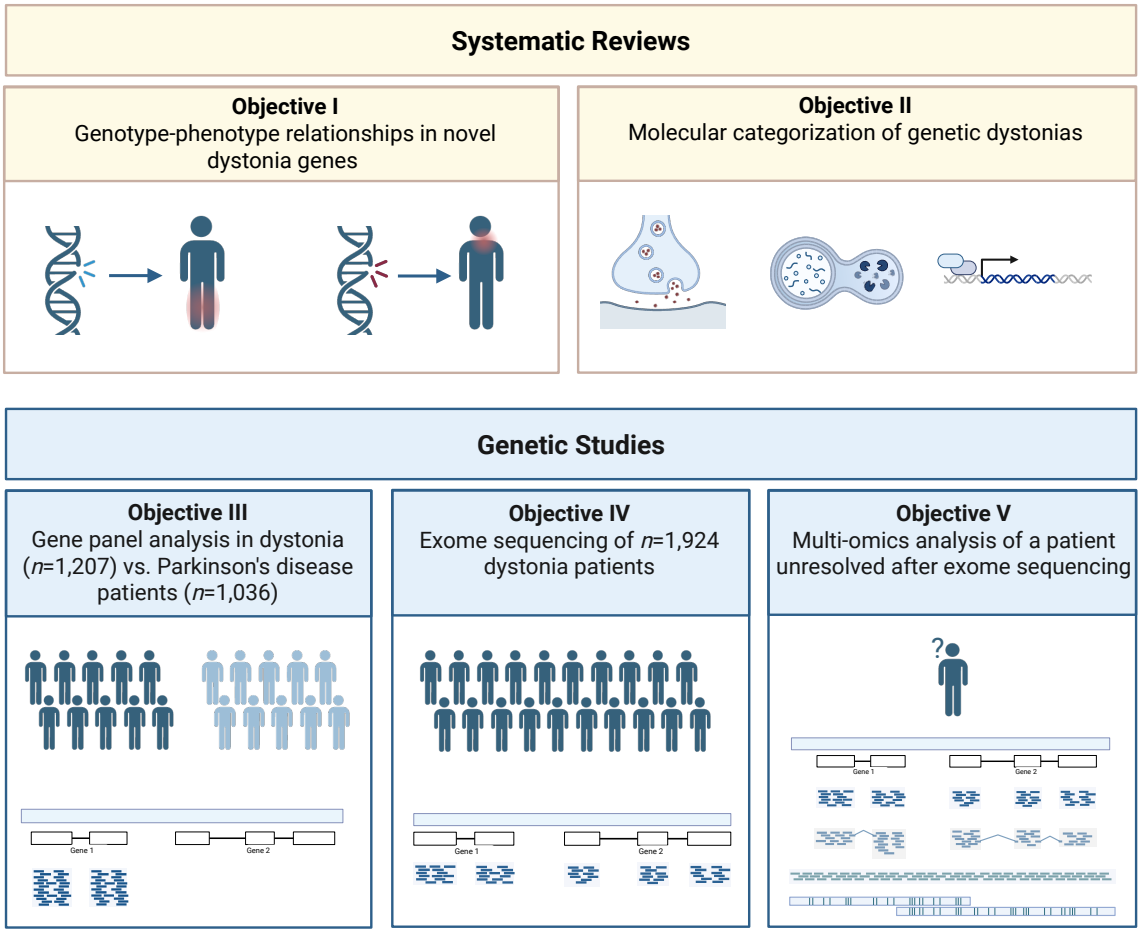

Figure 3. Schematic overview of the five objectives addressed in this thesis, spanning systematic reviews (Objectives I–II) and genetic studies (Objectives III–V). Details on each objective are provided in the main text. *Figure created with BioRender.com.*

2 Material and Methods

This chapter provides an overview of the methods used in this thesis, including patient cohorts, sequencing strategies, data preprocessing, analysis and interpretation, online resources and software tools, and wet-lab work. Detailed methodological descriptions are available in the respective publications.

2.1 Literature Research (Objectives I, II, and IV)

Systematic literature research was conducted with PubMed using defined search terms to generate a comprehensive dystonia gene list and to review relevant publications for variant interpretation and functional pathway analysis. For genotype-phenotype correlations in isolated dystonia genes, demographic, clinical, and genetic data from the literature were extracted using the standardized data extraction protocol from MDSGene (https://www.mdsgene.org/methods).

2.2 Patient Samples (Objectives III-V)

Patient samples for this thesis were mainly recruited within the framework of two large dystonia registries: The **German Dystonia Registry (DysTract)** (https://www.isms.uni-luebeck.de/forschung-1/deutsches-dystonie-register) and the international **Dystonia Coalition** (https://www.dystoniacoalition.org/). DysTract includes approximately 2,500 dystonia patient samples from various centers in Germany, including Lübeck. The Dystonia Coalition comprises over 3,200 samples from sites across North America and Europe. Demographic, clinical, and family history data were collected using a standardized protocol. Both registries predominantly include patients with isolated focal dystonia. Outside of these two cohorts, additional samples from international collaborators (e.g., from Malaysia and South Korea) were included. Written informed consent was obtained from all participants prior to genetic testing, and the study was approved by the ethics committee at the University of Lübeck.

2.3 Genomic Methods (Objectives III-V)

In total, sequencing-based analyses were performed for over 2,500 unique dystonia patients. For all applications, DNA or RNA was extracted from peripheral blood.

A total of 1,207 samples (mainly from DysTract) were selected for **targeted gene panel sequencing**, which was conducted at Centogene (Rostock, Germany) using Illumina NextSeq technology.

Exome sequencing was performed for 1,950 samples from both DysTract and the Dystonia Coalition, including about 550 unsolved patients from gene panel sequencing. Sequencing was carried out at the Competence Centre for Genomic Analysis (CCGA) in Kiel, Germany, using Illumina NovaSeq 6000.

Methylation profiling was conducted for 95 patients with uncertain variants in *KMT2B*, using the Illumina MethylationEPIC BeadChip following bisulfite conversion. Sample processing and array scanning were performed at CCGA.

A subset of unsolved patients from exome sequencing underwent **RNA sequencing** and **whole genome sequencing**, both performed at CCGA. One patient for whom both methods contributed to the genetic diagnosis is presented in this thesis.

Optical genome mapping (OGM) of one exome-negative patient was performed by Mirja Thomsen at the Institute of Neurogenetics, Lübeck, using Bionano Genomics technology. This involved library preparation and imaging on the Bionano Saphyr system.

2.4 Raw Data Processing (Objectives III-V)

Gene panel data were processed by Centogene (Rostock, Germany) using internal pipelines, including alignment to the reference genome, variant calling, and annotation. Exome and genome data were processed by the Systems Biology Group at the University of Lübeck (Prof. Hauke Busch; team: Fabian Ott, Marius Möller, Saad Abdelwakeel), following GATK Best Practices, including read trimming, alignment, variant calling, and annotation. Processing was performed on the Lübeck High Performance Compute Infrastructure "OmicsCluster". Methylation array data were also processed by the Systems Biology Group, including quality control, normalization, and M-value calculation. RNA sequencing data were processed by Dr. Vicente Yépez (Technical University of Munich), including read trimming, alignment, quality control, and detection of aberrant splicing and expression (DROP pipeline). OGM data were processed using the Bionano Solve pipeline v3.8, including *de novo* assembly and structural variant (SV) calling.

2.5 Data Analysis (Objectives III-V)

Preprocessed data were further analyzed using **R v4.3.2** by Mirja Thomsen. A range of packages were applied for data handling, cohort-based variant filtering, statistical analysis, and visualization (**Table 1**).

For **variant interpretation**, a structured filtering strategy was developed and applied based on read quality, population frequency, functional annotations, and relevant literature. Classification

of variants followed the ACMG guidelines, supported by online databases such as gnomAD, ClinVar, Varsome, OMIM, and more (**Table 2**).

SV calls identified by OGM were analyzed on **Bionano Access Server v1.8** and filtered based on gene overlap, inheritance patterns, SV type, and rarity by Mirja Thomsen. Among others, she was trained for this during her participation in a Solvathon at Radboud University in Nijmegen, The Netherlands.

Visual inspection of candidate variants – including single nucleotide variants, small insertions and deletions, and SVs – was performed using the Integrative Genomics Viewer (IGV), also allowing for assessment of RNA-seq-based splicing or expression effects.

Table 1. R packages used in the analysis

R package	Purpose
maftools	Filtering and summarizing variant data across the cohort
dplyr	Data wrangling (filtering, grouping, transformation)
baseR, stats	Basic statistical analysis (e.g., summary statistics, Fisher's exact test)
pROC	ROC curve analysis and AUC calculation
ggplot2, ggpubr	Data visualization

Table 2. Online resources used for variant annotation and interpretation

Resource	Purpose	URL
PubMed	Literature search	https://pubmed.ncbi.nlm.nih.gov
gnomAD	Population allele frequencies and variant constraint metrics	https://gnomad.broadinstitute.org
MDSGene	Clinical and genetic data on movement disorder genes	https://www.mdsgene.org
ClinVar	Clinical significance and interpretation of variants	https://www.ncbi.nlm.nih.gov/clinvar/
VarSome	Variant annotation and ACMG classification support	https://varsome.com
GTex	Gene expression across human tissues	https://gtexportal.org
OMIM	Gene-disease associations	https://www.omim.org

Resource	Purpose	URL
SpliceAI	Prediction of splice-altering effects of variants	https://ci-spliceai.com
CADD	Prediction of deleteriousness of variants	https://cadd.gs.washington.edu/
MetaDome	Domain-based tolerance landscape for missense variants	https://stuart.radboudumc.nl/metadome/

2.6 Wet-lab validation (Objectives III and IV)

Variants of interest were validated by Sanger sequencing using patient DNA at the Institute of Neurogenetics, Lübeck. Primers were designed using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), followed by PCR amplification, separation and quality control upon agarose gel electrophoresis, Sanger sequencing, and capillary electrophoresis.

3 Results with Related Publications

3.1 Objective I: Genotype-Phenotype Relationships in Novel Dystonia Genes

A hallmark of dystonia is its enormous clinical and genetic heterogeneity. The growing number of publications related to novel disease-causing genetic variants in dystonia patients makes it increasingly difficult to interpret these results and apply them in clinical practice. Further, the phenotypic overlap of subtypes complicates accurate diagnosis and choice of treatment. To aid movement disorder specialists in this challenge, a comprehensive online resource was developed: the International Parkinson and Movement Disorder Society (MDS) Genetic Mutation Database, short: MDSGene (Lill et al., 2016). The database gives a comprehensive overview of different genetic movement disorders, including dystonia, and offers descriptive summary statistics on demographic, genetic, and clinical data from the published literature. In addition, systematic literature reviews highlight key findings such as characteristic phenotypic features of specific genetic forms and the most promising treatment options, and they help guide clinicians in making genetic testing decisions (Lange et al., 2021).

Since the first MDSGene review on isolated dystonia genes (Lange et al., 2021), pathogenic variants in three additional genes, namely, *VPS16* (Steel et al., 2020)*, EIF2AK2* (Kuipers et al., 2021), and *AOPEP* (Zech et al., 2022), have been shown to cause isolated dystonia. Therefore, MDSGene's standardized data extraction protocol was applied to investigate the genotype-phenotype relationships in these newly described dystonia genes (Objective I).

The study covering this objective is titled "MDSGene: Extending the list of isolated dystonia genes by *VPS16*, *EIF2AK2*, and *AOPEP"*. The data were briefly summarized in a letter format for publication in *Movement Disorders*, and all data were uploaded on the MDSGene website (https://www.mdsgene.org). Mirja Thomsen conducted the literature search, systematically extracted genetic data, performed the statistical analyses, and prepared Figure I-1. The first draft of the manuscript was written by Mirja Thomsen and supervised and revised by Katja Lohmann.

Similar to the first MDSGene review on isolated dystonia genes (Lange et al., 2021), it was found that the novel genes each have a broad phenotypic spectrum and that there is significant phenotypic overlap between the genetic forms. Even though it is not possible to define clinical "red flags" that allow diagnosing a specific genetic cause, there are certain phenotypic characteristics. However, the only way to arrive at a conclusion about the underlying genetic cause remains genetic testing.

MDSGene: Extending the list of isolated dystonia genes by *VPS16*, *EIF2AK2*, and *AOPEP*

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Following MDSGene's protocol, seven genes linked to isolated dystonia were recently reviewed (Lange et al., 2021). Since then, pathogenic variants in three additional genes, *VPS16* (Steel et al., 2020), *EIF2AK2* (Kuipers et al., 2021; Magrinelli et al., 2022), and *AOPEP* (Garavaglia et al., 2022; Zech et al., 2022), have unequivocally been shown to cause isolated dystonia (Lange et al., 2022). In this letter, we summarize phenotypic and genetic data from 15 publications reporting on 56 dystonia patients carrying pathogenic variants in these genes (see supporting information methods). All data are available on the MDSGene website (www.mdsgene.org); key data are illustrated in **Figure I-1** and the supporting information.

Variants in *VPS16* are the most frequent novel cause of isolated dystonia (Steel et al., 2020). To date, 33 mutation carriers from 25 families were reported (median age at onset [AAO]: 13.5 years). The most frequent site of onset was the neck (13/33). Over the course of the disease, limb involvement was prominent (29/33), but also axial (25/33), cervical (25/33), and laryngeal dystonia (24/33) were common. Dystonia generalized in 26/33 cases. The majority (22/33) of patients had isolated dystonia, whereas 4 of 33 had dystonia combined with myoclonus, and 7 of 33 featured a more complex phenotype (co-occurring with other (non)neurological presentations). DYT-VPS16 is usually inherited in an autosomal dominant fashion. Family history was positive in 12 of 25 index patients. Negative family history was linked to inheritance from an unaffected parent (seven patients) or *de novo* occurrence (one patient). Fifteen of the 18 reported pathogenic variants are truncating.

In *EIF2AK2*, 14 heterozygous mutation carriers from seven families have been reported with a median AAO of 6 years. Symptoms most frequently started in the limbs (10/14), with subsequent generalization in 11 of 14 patients. Limb involvement was characteristic (13/14). Dystonia was reported to be isolated in 11 of 14 and complex in 3 of 14 patients. DYT-EIF2AK2 follows an autosomal dominant mode of transmission; family history was positive in 3 of 7 index patients. Interestingly, 11 of 14 patients carried a recurrent missense mutation

(NM_001135651.3:c.388G>A;p.Gly130Arg), which arose *de novo* at least twice (Kuipers et al., 2021; Magrinelli et al., 2022).

Finally, 11 *AOPEP* biallelic mutation carriers from nine (including three consanguineous) families have been reported (median AAO: 20 years). Symptoms often started in the hands (5/11). With disease progression, the arms were most frequently affected (10/11). Dystonia was mostly generalized (8/11) and isolated (9/11). DYT-AOPEP follows a recessive inheritance pattern. Family history was positive in 2 of 9 index patients; 15 of 18 variants are truncating.

Regarding treatment, mixed responses to deep brain stimulation (DBS) have been described, warranting further evaluation: DBS significantly alleviated symptoms in 5 of 11 DYT-VPS16 and both DYT-AOPEP patients, while 3 of 6 DYT-EIF2AK2 patients only moderately benefited.

To summarize, there is significant phenotypic overlap between these three novel dystonia forms; for example, most patients presented with isolated dystonia, but ~20% of patients had combined (*AOPEP*) or complex (*VPS16, EIF2AK2*) dystonia. Common generalization and limb involvement are also shared with other forms of isolated dystonia, particularly DYT-TOR1A and DYT-KMT2B. Similar to DYT-THAP1, laryngeal involvement appears to be frequent in the three novel forms. DYT-VPS16 and DYT-AOPEP stand out for relatively common cervical involvement, comparable with DYT-GNAL.

Despite the described phenotypes and patterns, it is currently not possible to establish a diagnosis on clinical grounds alone. Hence, these three genes should be included in genetic testing panels for hereditary dystonia.

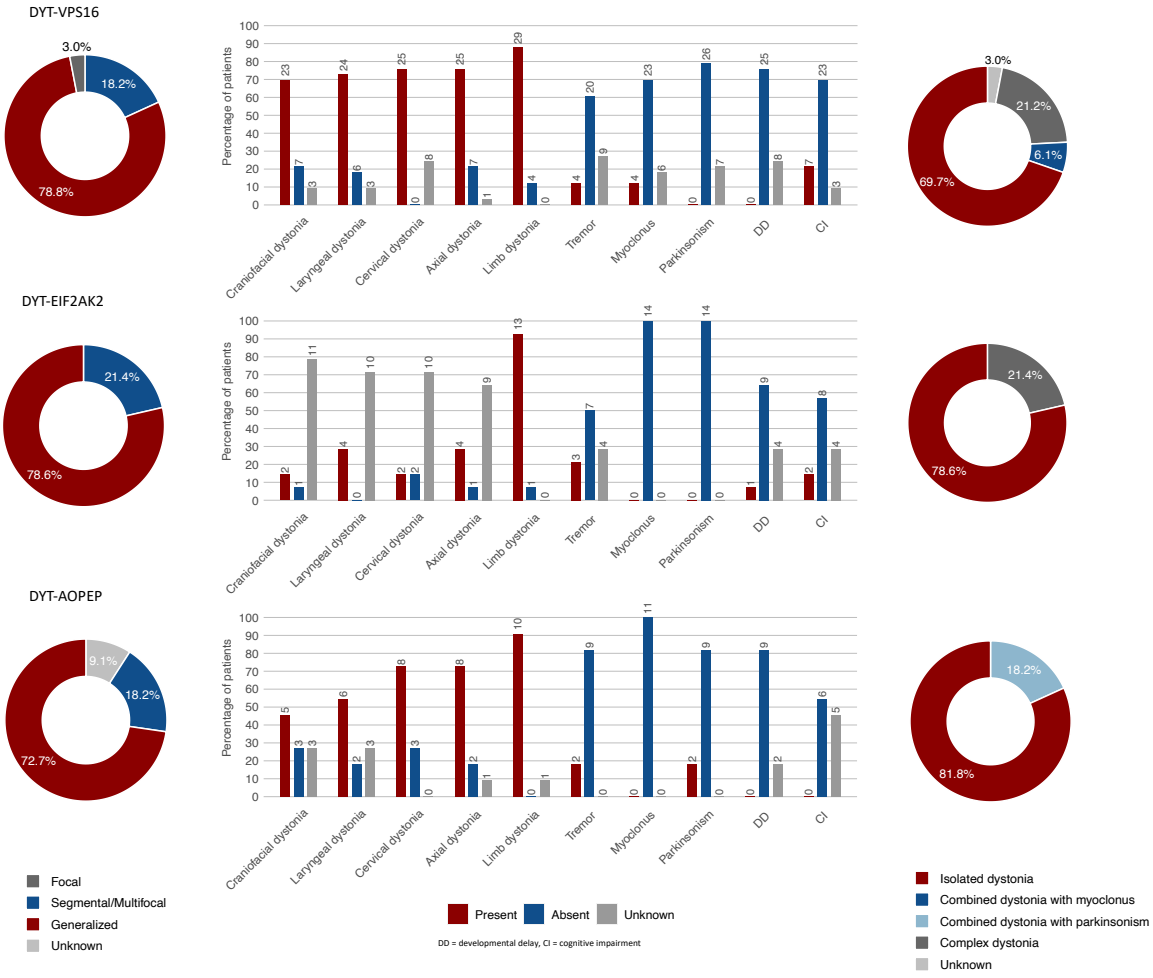

Figure I-1. Characteristics of the three novel isolated dystonia genes. Distribution of dystonia (left panel), signs and symptoms (middle panel), and dystonia categories (right panel) in carriers of pathogenic variants in *VPS16, EIF2AK2,* and *AOPEP*. Circles in the left panel indicate the percentage of patients with generalized (red), multifocal/segmental (blue), focal (dark gray), or unknown (light gray) distribution of dystonia. Bar diagrams in the middle panel show the presence (red), absence (blue), or missing information (gray) of dystonia in different body parts as well as other main movement disorder phenotypes. Circles in the right panel indicate the percentage of patients presenting with isolated dystonia (red), dystonia combined with myoclonus (dark blue) or parkinsonism (light blue), complex dystonia (dark gray; co-occurring with other neurologic or systemic presentations), or unknown category (light gray).

ACKNOWLEDGMENTS

We thank Natascha Bahr for creating Supplementary Figure 2, and Harutyun Madoev for maintaining and expanding the MDSGene Website.

3.2 Objective II: Molecular Categorization of Genetic Dystonias

Gaining a better understanding of the pathogenesis of dystonia is essential for the development of targeted and effective treatments – the ultimate goal of research. A major obstacle in dystonia research has been the condition's pronounced heterogeneity, which is also reflected in the wide range of underlying etiologies. From a therapeutic perspective, it has become clear that there may not be a "one-size-fits-all" treatment, as some patients respond to certain available therapies while others do not (Koptielow et al., 2024). Therefore, a central question in dystonia research is whether all dystonia forms share a common underlying disease mechanism or whether each subtype has a distinct etiopathogenesis (Jinnah & DeFazio, 2023) - with the clinical resemblance (i.e., excessive involuntary muscle activity) being only superficial.

While the discovery of genetic causes has advanced our understanding of dystonia pathogenesis, the relationships between the respective proteins and pathways often remain unclear, as a variety of seemingly unrelated cellular processes (such as the endoplasmic reticulum stress response or gene regulation during neurodevelopment) have been implicated in dystonia pathogenesis. A key research goal is to identify pathways and therapeutic targets that are shared across genetically diverse forms of dystonia, as they may converge on common downstream pathophysiological processes. Targeting such shared mechanisms could overcome the need to develop individualized therapeutics for each rare dystonia subtype. The aim of this work was to evaluate the literature on genetic forms of dystonia and related functional studies in order to identify such pathophysiological groups and provide a comprehensive summary of the current state of knowledge (Objective II).

The review article covering this objective is titled "Genetics and Pathogenesis of Dystonia*"* and was published in *Annual Review of Pathology*. Mirja Thomsen authored the chapter "Common themes: shared pathophysiology and disease mechanisms" and prepared Figures II-2 and II-3, which were redrawn by the journal to match its publication style. The work was supervised and revised by Katja Lohmann.

This review concludes that it is unlikely that a single driver molecule or mechanism underlies all dystonia types. Instead, distinct primary mechanisms appear to trigger similar downstream effects, converging on the cellular or anatomical level and ultimately leading to the clinical phenotype of dystonia. As a result, different forms of dystonia can be categorized into "molecular groups", which may facilitate both the development of targeted treatment and the design of clinical trials.

Genetics and Pathogenesis of Dystonia

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Note: Only part of the article, namely, the chapter "Common themes: shared pathophysiology and disease mechanisms" along with the abstract and conclusions, is printed here.

ABSTRACT

Dystonia is a clinically and genetically highly heterogeneous neurological disorder characterized by abnormal movements and postures caused by involuntary sustained or intermittent muscle contractions. A number of groundbreaking genetic and molecular insights have recently been gained. While they enable genetic testing and counseling, their translation into new therapies is still limited. However, we are beginning to understand shared pathophysiological pathways and molecular mechanisms. It has become clear that dystonia results from a dysfunctional network involving the basal ganglia, cerebellum, thalamus, and cortex. On the molecular level, more than a handful of, often intertwined, pathways have been linked to pathogenic variants in dystonia genes, including gene transcription during neurodevelopment (e.g., *KMT2B*, *THAP1*), calcium homeostasis (e.g., *ANO3*, *HPCA*), striatal dopamine signaling (e.g., *GNAL*), endoplasmic reticulum stress response (e.g., *EIF2AK2*, *PRKRA*, *TOR1A*), autophagy (e.g., *VPS16*), and others. Thus, different forms of dystonia can be molecularly grouped, which may facilitate treatment development in the future.

COMMON THEMES: SHARED PATHOPHYSIOLOGY AND DISEASE MECHANISMS

Pathophysiology

The planning, coordination, and execution of movement involve a complex interaction of different brain areas (**Figure II-2**). In this, the motor cortex is the final output area that integrates incoming signals and sends information to the spinal cord and muscles. While in other movement disorders, specific brain regions could be linked to disease pathogenesis, e.g., the basal ganglia in Parkinson's disease or the cerebellum in cerebellar ataxias, the anatomical basis of dystonia is still debated, as several brain regions have been implicated in the disease (Sharma, 2019).

Neuropathological studies can be a powerful tool to elucidate the underlying mechanism of disease; however, in dystonia research, they have had minimal impact on our understanding because they have not shown any consistent structural brain abnormalities (Sharma, 2019). This may be due to several reasons, including the small number of studies, lack of systematic quantitative assessment, and, maybe most important, the substantial phenotypic and pathological heterogeneity in dystonia. While combined and complex dystonia forms often show neurodegeneration or developmental alterations in the cerebellum or basal ganglia, most studies on isolated dystonia have not revealed evidence of neuronal loss or overt neuroanatomical changes.

Early CT- and MRI-based imaging studies suggested a basal ganglia origin of dystonia. These studies investigated patients with acquired dystonia and revealed that focal lesions in the putamen (a part of the striatum) or GPi most commonly caused dystonia (Bhatia & Marsden, 1994). This first model proposed that dystonia develops due to a hyperfunction of the direct pathway and a hypofunction of the indirect pathway, leading to reduced inhibition of the thalamus by the GPi and ultimately increased excitation of the motor cortex (DeLong, 1990). However, additional lesion studies revealed that the thalamus, cortex, cerebellum, and brainstem are also frequently affected in acquired dystonia (Neychev et al., 2011). Furthermore, imaging studies in patients with genetic and idiopathic dystonia have highlighted subtle abnormalities in all these brain regions even in the absence of apparent structural lesions (Jinnah et al., 2017). Even though the implicated areas are similar across studies, the results are inconsistent even for specific dystonia subtypes, and most studies don't detect any abnormalities in discrete anatomical regions. However, the spatial and temporal pattern of brain activity (determined by FDG-PET) in the putamen, GPi, cerebellum, and motor cortex was shown to differ in dystonia patients compared to controls (Poston & Eidelberg, 2012), suggesting that these structures are linked together in a network, and their abnormal connectivity underlies dystonia.

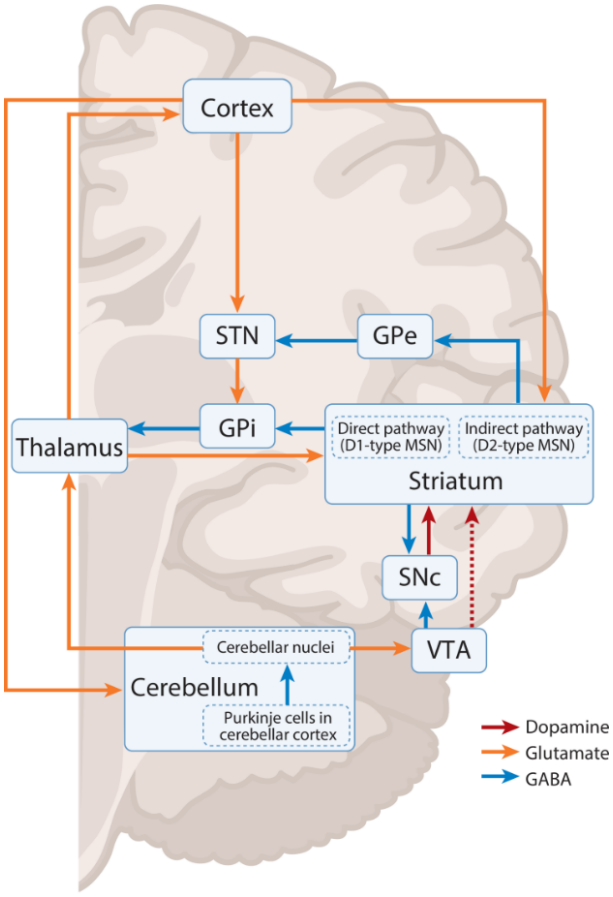

Figure II-2. Schematic representation of brain circuits involved in dystonia. Dystonia is considered to arise from a dysfunctional brain network involving the cortico-cerebello-thalamo-cortical and cortico-striato-thalamo- cortical loops, which interact with each other in producing and modulating movement. The basal ganglia pathways have a well-established role in motor control: The striatum receives modulatory dopaminergic input from the substantia nigra pars compacta (SNc), which can stimulate direct or indirect pathway medium spiny neurons (MSNs). The indirect pathway [dopamine receptor 2 (D2)-type MSN] relays through the globus pallidus externus (GPe) and subthalamic nucleus (STN), leading to the excitation of globus pallidus internus (GPi) neurons (by the excitatory neurotransmitter glutamate), which inhibit thalamic neurons [by the inhibitory neurotransmitter gamma-aminobutyric acid (GABA)] and reduce movement. In contrast, stimulation of the direct pathway [dopamine receptor 1 (D1)-type MSN] inhibits GPi neurons, consequently removing inhibitory input into the thalamus and facilitating movement through excitatory stimulation of the cortex. In addition, the so-called hyperdirect pathway includes excitatory projection directly from the cortex to STN, but its role in dystonia is less clear. Moreover, the involvement of the cerebellum in dystonia has been increasingly considered. GABAergic Purkinje cells of the cerebellar cortex project on downstream deep cerebellar nuclei, which, in turn, have direct connections to the basal ganglia via the thalamus, which is considered the primary hub structure of the two pathways. Moreover, GABAergic neurons in the ventral tegmental area (VTA) send inhibitory input to SNc dopaminergic neurons and thereby alter dopaminergic input into the striatum. Additionally, the VTA contains dopaminergic neurons that project directly to the striatum but are believed to play a more minor role in motor function (dashed red line). Importantly, no consensus exists on how exactly the different circuits are altered in dystonic versus healthy conditions. Dystonic movements may be caused by both pathological activation and suppression of the same pathway, and the relevance of each network component remains to be elucidated and may be distinct for different disease subtypes. For clarity reasons, the structures belonging to the basal ganglia (STN, GPe, GPi, striatum, and SNc) and the VTA are not located at the correct anatomical positions with respect to the coronal brain section in the background. *Figure adapted from images created with BioRender.com.*

Generally, interventional studies have a greater potential to establish a causal link, so much of the research has also focused on animal models. In rodents, selective lesions in the striatum can cause dystonia (Fernagut et al., 2002; Stefanova et al., 2003). Moreover, activation of excitatory glutamate receptors in the cerebellum can induce dystonia (Pizoli et al., 2002), and DBS of deep cerebellar nuclei or activation of inhibitory GABA receptors in the cerebellum alleviates dystonia (Calderon et al., 2011; White & Sillitoe, 2017). Besides these phenotypic models, many genetic animal models have been developed to study the disease mechanism and have provided evidence that dystonia may be caused by abnormal cerebellar Purkinje cell or striatal neuron firing (Jinnah et al., 2017). However, a considerable limitation of the available genetic models is that most do not replicate the overt motor features found in human patients, impeding the establishment of cause and effect. Nevertheless, they have substantially contributed to our understanding of the molecular pathogenesis of many inherited dystonia forms (see next chapter), demonstrating that the utility of a specific model largely depends on the research question. In humans, interventional studies are problematic for obvious regions; however, the effectiveness of GPi-DBS in many dystonia forms (but not others) has provided evidence for the involvement of the basal ganglia in at least certain subtypes.

The body of evidence suggests that dystonia does not result from the dysfunction of a single brain area but from a dysfunctional network involving the cortico-striato-thalamo-cortical as well as the cortico-cerebello-thalamo-cortical loops (**Figure II-2**) (Balint et al., 2018; Jinnah et al., 2017; Latorre et al., 2020; Morigaki et al., 2021). Traditionally, these two brain circuits were considered to be separated from each other, while currently, a more direct interaction is considered to play a critical role in dystonia (Morigaki et al., 2021). In this, direct connections between the cerebellum and basal ganglia have been increasingly demonstrated (Yoshida et al., 2022). While the network model proposes that all dystonia subtypes arise from a dysfunctional interplay of basal ganglia, cerebellum, thalamus, and cortex, the exact relevance of each component remains to be elucidated and might be distinct for each dystonia subtype.

Molecular Mechanisms

While the discovery of genetic causes has led to a better understanding of dystonia pathogenesis, the relationships between the respective proteins and pathways often remain unclear. A central goal is to identify pathways and therapeutic targets shared by genetically distinct forms of dystonia, as apparently unrelated genetic forms could converge on shared downstream pathophysiological processes. Treatments targeting pathophysiological clusters would overcome the need to develop individualized therapeutics for each individual, and often extremely rare, dystonia subtype. Here we address in detail seven pathways affected by monogenic forms of

dystonia, including striatal dopamine signaling, gene transcription and neurodevelopment, calcium signaling and synaptic transmission, cellular stress response, endoplasmic reticulum (ER) and nuclear envelope (NE) function, cytoskeleton, and autophagy and lysosomal function. Other pathways such as mitochondrial dysfunction (other than that related to calcium homeostasis) and metal accumulation such as in Wilson's disease (copper) or neurodegeneration with brain iron accumulation (NBIA) are not discussed in detail here.

Striatal dopamine signaling. There has been much evidence indicating that defects in dopamine signaling in striatal neurons play a central role in dystonia pathogenesis. For instance, dystonia is often observed in patients with Parkinson's disease (Shetty et al., 2019), which is associated with a loss of dopaminergic neurons in the *substantia nigra* (SNc), and dystonia is a frequent side effect in patients treated with anti-dopaminergic agents (Ribot et al., 2019).

The striatum is the main input station of the basal ganglia and is mainly made up of medium spiny neurons (MSNs). MSNs receive excitatory glutamatergic input from the cortex and thalamus and modulatory dopaminergic input from the midbrain, especially the SNc (**Figure II-2**). They are classified into D1-type dopamine receptor(D1R)-expressing (part of the direct pathway) and D2-type dopamine receptor(D2R)-expressing (part of the indirect pathway) neurons that are generally believed to exert opposite effects on movement control. The abovementioned traditional model of the imbalance between the direct and indirect pathway resulting in dystonia has been reassessed, and it is now believed that the spatial and temporal patterns of pathway activity (and, therefore, GPi activity) are more important (Balint et al., 2018).

Dystonia-linked genes affect striatal dopamine signaling at three stages: dopamine synthesis, cycling, and signaling (**Figure II-3**). Pathogenic variants in *GCH1, SPR, PTS, TH,* and *QDPR* are linked to DRD. These genes are highly expressed in SNc dopaminergic neurons and are involved in dopamine synthesis or metabolism. Additionally, dopa decarboxylase deficiency (due to biallelic variants in *DDC*) causes complex dystonia and biallelic variants in *DNAJC12*, encoding a heat shock protein that increases TH stability, have been linked to a neurodevelopmental syndrome with prominent dystonia and parkinsonism (Lange et al., 2022). Furthermore, loss of function (LOF) variants in *SLC18A2* (Keller Sarmiento & Mencacci, 2021), encoding a transporter for dopamine packing in synaptic vesicles, and *SLC6A3* (Marras et al., 2016), encoding a transporter for reuptake of synaptic dopamine, have been associated with autosomal recessive dystonia-parkinsonism. Importantly, the above-mentioned genetic defects leading to reduced dopamine synthesis and release are treatable with pharmacotherapy (levodopa or dopamine agonists) or even viral vector-based gene therapy that has yielded promising results in

DYT-DDC patients and a knockout (KO) mouse model for DYT/PARK-SLC6A3 that replicates the human phenotype (Mastrangelo et al., 2023).

The first clue of an important link between dopamine signaling and non-DRD came from the discovery of heterozygous pathogenic variants in *GNAL* (encoding G-Protein G(Olf) Subunit Alpha; Gαolf). Gαolf is coupled to D1R. Upon dopamine binding, Gαolf dissociates from the beta-gamma subunit and activates adenylate cyclase type 5 (AC5), resulting in the production of the second messenger cAMP and increased neuronal activity (Hervé, 2011). Thus, pathogenic variants in *GNAL,* which are mostly LOF alleles (Fuchs et al., 2013; Kumar et al., 2014), are expected to lead to an uncoupling of D1R activation and cAMP production, resulting in reduced direct pathway activity. Of note, Gαolf is also coupled to adenosine receptor 2A, which has a less defined role in motor function but is evidenced to co-localize with D2R and modulate indirect pathway activity (Mori, 2020). Interestingly, pathogenic variants in *ADCY5*, the gene encoding AC5, are also associated with mixed movement disorders, including dystonia (Lange et al., 2022). In contrast to *GNAL*, variants in *ADCY5* were shown to be gain of function (GOF), leading to increased cAMP production in cell-based assays (Doyle et al., 2019). Therefore, dystonic movements may be caused by both pathological activation and suppression of the direct pathway, further supporting that the simple model of decreased thalamus inhibition is not suitable.

Additional components of dopamine receptor signaling have been implicated in dystonia: Patients with heterozygous *GNAO1* variants (encoding G-Protein G(o) Subunit Alpha, Gαo) can present with predominant or isolated dystonia (Lange et al., 2022; Wirth et al., 2022). Gαo is coupled to D2R and, upon dopamine binding, inhibits AC5 activity and cAMP production, influencing the excitability of indirect pathway neurons. Phenotypic outcomes likely depend on the specific pathogenic variant (Wirth et al., 2022). Additionally, heterozygous variants in *GNB1*, a gene linked to neurodevelopmental delay and dystonia, may impact striatal dopamine signaling. The gene encodes the ß1-subunit of G-protein G(olf) that interacts with the *GNAL* gene product Gαolf. Pathogenic *GNB1* variants may reduce the association with Gαolf and reduce coupling to D1R (Lohmann et al., 2017). Furthermore, the beta-gamma subunit also signals to various downstream effectors, e.g., inhibiting presynaptic voltage-gated calcium and potassium channels, thereby affecting neurotransmitter release (Khan et al., 2013). Additionally, variants in dopamine receptors we receptors were directly linked to movement disorders when, for instance, a variant in *DRD2* (encoding D2R) was reported to cause childhood-onset chorea with prominent dystonia in a large pedigree (van der Weijden et al., 2021).

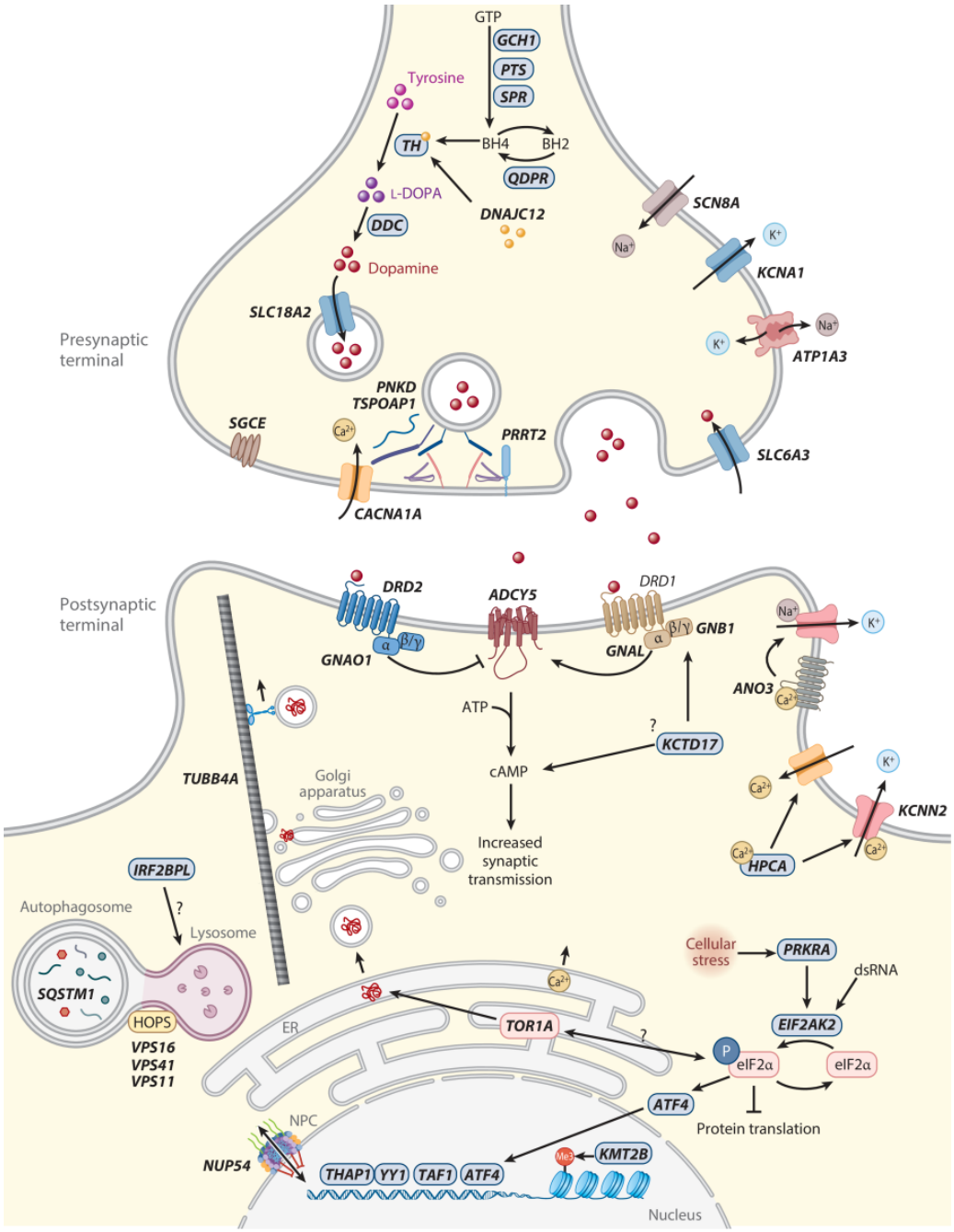

Figure II-3. Molecular mechanism of dystonia genes. Genes implicated in dystonia (boldface) are involved in several neuronal cell functions, and many are functionally connected through mutual regulation. Molecular functions include dopamine synthesis and processing (*GCH1*, *PTS*, *SPR*, *QDPR*, *TH*, *DNAJC12*, *DDC*, *SLC18A2*, and *SLC6A3*), ion channels or pumps regulating neuronal excitability (*SCN8A*, *KCNA1*, *ATP1A3*, *CACNA1A*, and *KCNN2*), synaptic vesicle release (*TSPOAP1*, *PNKD*, and *PRRT2*), G protein–coupled receptor signaling (*ADCY5*, *DRD2*, *GNAL*, *GNB1*, and *GNAO1*), cellular stress response (*PRKRA*, *EIF2AK2*, *TOR1A*, and *ATF4*), calcium homeostasis (*ANO3*, *HPCA*, and *TOR1A*), autophagy and lysosomal function (*VPS16*, *VPS41*, *VPS11*, *SQSTM1*, and *IRF2BPL*), regulation of gene transcription (*KMT2B*, *THAP1*, *YY1*, *TAF1*, and *ATF4*), protein processing and trafficking (*TOR1A*, *TUBB4A*, and *NUP54*), and synapse assembly and structure (*SGCE* and *TUBB4A*). The figure shows a presynaptic dopaminergic neuron and postsynaptic medium spiny neuron (expressing D1-type or D2-type dopamine receptors) as an example, but dystonia genes may also primarily function in other brain cells, for instance, in the cerebellum. Details about the function of the represented genes and biological consequences due to dystonia-causing genetic variants can be found in the main text. Abbreviations: ER, endoplasmic reticulum; HOPS, homotypic fusion and protein sorting complex; NPC, nuclear pore complex. *Figure adapted from images created with BioRender.com*

Pathogenic variants in other DYT-genes, although without obvious functional connection, were also shown to be associated with altered striatal dopaminergic neurotransmission. For DYT-TOR1A, the most prevalent genetic form of isolated dystonia, knockin rodent models carrying the recurrent trinucleotide GAG-deletion demonstrated alterations in both pre- and postsynaptic dopamine signaling, i.e., impaired dopamine release (Downs et al., 2021) and deficient D2R signaling (Napolitano et al., 2010). For DYT-THAP1, mice with conditional knockout (cKO) of *THAP1* in the central nervous system (CNS) displayed impaired D2R signaling and differential expression of genes involved in dopamine signaling, including *DRD2, ADCY5, GNAL*, and *SLC6A3* (Frederick et al., 2021). Additionally, functional work in Drosophila has revealed that the homologs of *KCTD17* and *HPCA* disrupt dopaminergic postsynaptic pathways (K.-F. Chen et al., 2019; Q. Li et al., 2017; Pfeiffenberger & Allada, 2012).

A recent systems biology approach (Mencacci et al., 2020) further supported the central role of dopaminergic signaling in dystonia. The study aimed to identify the cellular specificity of all currently known DYT-genes and predict their functional relationships in the adult brain. Dystonia genes were overall enriched in striatal MSNs (in particular *ADCY5, GNAL, ANO3, KCTD17,* and *HPCA*), and these genes were associated with synaptic transmission, especially with postsynaptic structures. These findings suggest that especially the postsynaptic function of MSNs may be critical in dystonia pathogenesis. Remarkably, none of the non-DRD genes were specific to dopaminergic neurons or co-clustered with the DRD genes, supporting the view of DRDs as a distinct subgroup from both a clinical and biological perspective.

Gene transcription and neurodevelopment. Pathogenic variants in *THAP1* and *KMT2B* are frequent causes of isolated dystonia (Lange et al., 2021), and the gene products are involved in the regulation of gene expression as transcription factors or histone methyltransferases, indicating that aberrant transcriptional regulation is a molecular mechanism in dystonia pathogenesis. *THAP1* encodes the 'THAP domain-containing protein 1', a zinc-finger transcription factor that regulates gene transcription. Most dystonia-causing missense variants are located within the DNA binding domain (Lange et al., 2021) and alter the sequence-specific DNA binding ability of the protein (Campagne et al., 2012), leading to reduced transcriptional activity (Lohmann et al., 2012). Other variants hinder the protein from being localized to the nucleus (Osmanovic et al., 2011) or disrupt the dimerization of THAP1 with itself or cofactors (Hollstein et al., 2017; Sengel et al., 2011) - in any case resulting in LOF and altered gene transcription of target genes, some of which have been identified in transcriptome studies and may include other dystonia-related genes (Baumann et al., 2021; Diaw et al., 2022; Frederick et al., 2019). A recent study in iPSC-derived MSNs from DYT-THAP1 patients further registered a reduced expression of GABA-A receptor

alpha2 subunit, proposing that loss of THAP1 leads to reduced GABAergic synaptic transmission in the basal ganglia (Staege et al., 2021).

Insights from rodent models have shed further light on the consequences of loss of THAP1 function: *THAP1*-cKO in the murine CNS provided evidence that the protein is essential for myelination and CNS maturation, as its conditional deletion delays maturation of the oligodendrocyte lineage and leads to persistent motor deficits (Yellajoshyula et al., 2017). Transcriptional alterations consequential to the loss of THAP1 function were greater at younger ages, further indicating that *THAP1* is particularly important during neurodevelopment (Ruiz et al., 2015). In the striatum and cerebellum, significant changes in gene expression particularly affected genes involved in regulating neuronal growth, synaptic transmission, gliosis, cytokine signaling, and myelination. Intriguingly, among the differentially expressed genes were other dystonia-causing genes, including *GCH1, TH, SGCE, ANO3, GNAL,* and *TUBB4A* in the striatum and *KMT2B* in the cerebellum (Frederick et al., 2019), linking several DYT-genes on the molecular level. An interaction of *THAP1* and *TOR1A* has already been reported in earlier studies: THAP1 targets the *TOR1A* promotor in vitro to suppress its transcription, and this suppression is decreased in *THAP1*-mutated cells (Gavarini et al., 2010; Kaiser et al., 2010).

KMT2B (histone-lysine N-methyltransferase 2B) encodes an epigenetic "writer" involved in transcriptional regulation through methylation of a lysin residue (K4) of the histone 3 (H3) protein, a component of the DNA-packing chromatin. H3K4 methylation is associated with active transcription, specifically important for transcriptional consistency and stability during cell division (Benayoun et al., 2014; Muramoto et al., 2010). Like *THAP1, KMT2B* has the highest brain regional expression in the cerebellum and plays an essential role in normal development and maturation of brain circuits involved in motor control. It is involved in the differentiation of embryonic stem cells (Glaser et al., 2009) and regulates only a specific set of genes rather than being responsible for the overall levels of H3K4 methylation in the genome (Meyer et al., 2017). Very recently, this specificity was further elucidated with the discovery of a unique DNA methylation pattern in peripheral blood from DYT-KMT2B patients that has been described by three independent studies (Ciolfi et al., 2021; Lee et al., 2022; Mirza-Schreiber et al., 2022) and differed from epigenetic alterations made by other KMT2 methyltransferases. This so-called „episignature" is based on CpG-methylation, an epigenetic mechanism known to be inversely correlated to H3K4 methylation (Meissner et al., 2008). Loss of KMT2B function was associated with hypermethylation of these CpG-sites, consequential to loss of H3K4 methylation, indicating that haploinsufficiency is likely the disease mechanism underlying DYT-KMT2B. This is in line with the observation that most mutations found in patients are truncating LOF variants, but also,

some missense variants have been shown to affect enzyme activity with the same molecular consequence (Mirza-Schreiber et al., 2022).

Remarkably, a recent study in mouse embryonic stem cells revealed that it is not the methylation of H3K4 itself that underlies KMT2B's transcriptional regulation. Instead, while binding to the target gene's promotor region, KMT2B functions as a repellent for other histone methyltransferases and the DNA methylation machinery that is associated with silent transcription (Douillet et al., 2020). In other words, KMT2B prevents active suppression. This raises the possibility that *KMT2B*-dependent transcription, which is reduced in DYT-KMT2B patients, could potentially be rescued by inhibition of other epigenetic enzymes.

Intriguingly, an interaction between *KMT2B, THAP1,* and *TOR1A* has been suggested: In fibroblasts from *KMT2B* mutation carriers, reduced levels of *TOR1A* and *THAP1* mRNA were detected, indicating that KMT2B may be an upstream regulator of other DYT-genes (Meyer et al., 2017).

Another link between transcriptional regulation and dystonia pathogenesis was made with the discovery of *YY1* variants as a cause of complex neurological syndromes with dystonic features (Ferng et al., 2022; Gabriele et al., 2017). *YY1* (yin and yang 1) encodes a zinc-finger transcription factor with the highest brain regional expression in the cerebellum. Like THAP1, the protein has a known key role in neurodevelopment and myelination, particularly important for maturation of the oligodendrocyte lineage. In fact, it has been suggested that the proteins encoded by *YY1* and *THAP1* interact as co-regulators of the same genes, as loss of THAP1 function also reduces the DNA occupancy of YY1 (Yellajoshyula et al., 2017, 2022).

Aberrant transcriptional regulation is also likely the molecular basis of *TAF1*-related X-linked dystonia-parkinsonism (XDP) (Domingo et al., 2016). Downregulation of *TAF1* in XDP patients compared to controls has been shown in post-mortem striatum tissue (Makino et al., 2007) and in iPSC-derived neural stem cells at early stages (Ito et al., 2016). Transcriptional changes following *TAF1* knockout particularly affect genes involved in neurodevelopment and synaptic transmission and histone genes (Gudmundsson et al., 2019). *TAF1* (TATA-Box Binding Protein Associated Factor 1) encodes a transcription factor which is part of the transcription factor IID complex (TFIID) involved in RNA polymerase II-mediated transcription. It is ubiquitously expressed but also has the highest brain regional expression in the cerebellum, and brain-specific isoforms exist. During normal development in mice, *TAF1* expression is extremely elevated in the embryonic stage and decreased and maintained at stable levels from postnatal week three onwards (Jambaldorj et al., 2012), proposing an essential function of TAF1 during embryogenesis.

Notably, while coding variants in *TAF1* lead to a severe neurodevelopmental disorder (O'Rawe et al., 2015), an intronic SVA insertion is the cause of XDP (Aneichyk et al., 2018), which may have a milder impact on the protein's level and function and thus downstream transcriptional changes.

These findings reveal that transcriptional alterations occurring during early development underlie certain subtypes of hereditary dystonia, supporting the view of dystonia as a neurodevelopmental circuit disorder. Even though these alterations bring about multiple different effects, they seem to converge in aberrant signal transmission in striatal and cerebellar neurons, with several different DYT-genes being connected on the molecular level through mutual regulation.

Calcium signaling and synaptic transmission. Calcium (Ca^{2+}) ions are considered key signaling molecules in the cell (Groenendyk et al., 2021). In neurons, they regulate neurotransmitter release, neuronal excitability, and synapse formation (Kinoshita et al., 2022). The precise temporal and spatial regulation of responses to even subtle changes in intracellular Ca^{2+} levels is of high importance in modulating synaptic vesicle release and neuronal activity. These changes arise from either Ca^{2+} influx from the extracellular space or the release of Ca^{2+} from the ER (Karagas & Venkatachalam, 2019), the primary Ca^{2+} storage in the cell. Further, a functional interaction between the ER and mitochondria modulates cell bioenergetics and functionality since Ca^{2+} released by the ER is taken up by mitochondria, where it regulates the activity of transporters, enzymes, and proteins involved in organelles' metabolism (Rossi et al., 2019). Notably, several forms of complex dystonia are associated with genes linked to mitochondrial function (e.g., *COX20*, *SERAC1, SUCLA2, TIMM8A*) and result from defects in energy homeostasis but are reviewed elsewhere (Ghaoui & Sue, 2018). Furthermore, several proteins involved in synaptic transmission, specifically, membrane depolarization and repolarization by ion currents, vesicle release, and calcium homeostasis, have been implicated in dystonia (**Figure II-3**):

Pathogenic variants *ATP1A3* underlie rapid-onset dystonia-parkinsonism (RDP). The gene encodes the neuron-specific α3 subunit of the Na^+/K^+-ATPase (NKA α3) pump, which generates electrochemical ion gradients essential for maintaining and restoring resting membrane potentials, initiating action potentials, and neurotransmitter release. Heterozygous *ATP1A3* variants may result in reduced catalytic activity and a failure to generate the pump currents (Ng et al., 2021). In rodents, simultaneous pharmacological blockage of the pump in both the cerebellum and basal ganglia replicated the features of RDP (Calderon et al., 2011), whereby blocking in the basal ganglia alone resulted in parkinsonism and, on the other hand, cerebellar blockage resulted in dystonic movements. Furthermore, the authors observed cerebellar hyperactivity during dystonic

movements that could be alleviated by acute perfusion of GABA into the cerebellum. This aberrant cerebellar activity was found to adversely affect basal ganglia function, supporting earlier findings that cerebellar output nuclei alter the neuronal firing rates and dopamine levels in the basal ganglia. Recently, the first human in vivo evidence in RDP patients showed that Na^+ predominantly accumulated inside cerebellar cells due to pump deficiency (Prasuhn et al., 2022). Another hint implicating the cerebellum as the primary instigator in this disease is that while other neurons can upregulate other NKA isoforms to compensate for dysfunctional NKA α3, cerebellar Purkinje cells lack this option due to exclusive expression of this specific isoform (Murata et al., 2020). Additionally, NKA α3 plays a vital role in regulating intracellular Ca^{2+} levels since any alterations in Na^+ also affect Ca^{2+}. It has been shown that blockage of NKA α3 leads to an increase in both Na^+ and Ca^{2+} (Kinoshita et al., 2022), which is believed to result in abnormally high neuronal firing rates.

Moreover, ion channels influencing neuronal excitability and implicated in movement disorders including dystonic features are encoded by *KCNN2 (*small conductance calcium-activated potassium channel, SK2) (Balint et al., 2020) and *KCNA1* (voltage-gated potassium channel, Kv1.1) (Manville et al., 2022). As potassium channels, they contribute to repolarization after action potentials by generating an efflux of K^+ ions, thereby dampening neuronal firing rates. For *KCNN2*, this is coupled to changes in intracellular Ca^{2+} levels. Disease-related LOF variants lead to increased neuronal excitability, affecting primarily cerebellar neurons (Balint et al., 2020; Choi & Choi, 2016).

Very recently, biallelic LOF and missense variants in *TSPOAP1* have been linked to complex and isolated dystonia, respectively (Mencacci et al., 2021). The gene encodes RIMBP1, a central component of the presynaptic active zone that determines the precise localization of presynaptic voltage-gated calcium channels (VGCCs), thus ensuring tight coupling between incoming action potentials and Ca^{2+}-dependent vesicle release. Missense variants were shown to lead to abnormally increased synaptic transmission in cerebellar Purkinje cells, likely due to recruiting more VGCCs to the active zone. In contrast, LOF variants are expected to reduce the density of VGCCs in the active zone, proposing that both decreased and increased cerebellar synaptic transmission can underlie dystonia. Interestingly, variants in *CACNA1A*, encoding a VGCC, have recently been linked to prominent dystonia (Keller Sarmiento & Mencacci, 2021; Lipman et al., 2022). As with *TSPOAP1*, both LOF and GOF were identified in patients, leading to irregular firing of cerebellar Purkinje cells. Moreover, *CACNA1A* null mice develop dystonia associated with cerebellar atrophy (Fletcher et al., 2001)**.**

Other hints for aberrant vesicle release underlying dystonic phenotypes are pathogenic variants in *PNKD* and *PRRT2*, both associated with paroxysomal dyskinesias with prominent dystonia in a subset of patients. Both genes encode proteins that negatively regulate presynaptic vesicle release, and disease-related LOF variants lead to increased synaptic transmission, especially within cerebellar Purkinje cells (F. Chen et al., 2022; Tan et al., 2018). In *PRRT2*-related disease, defective coupling of presynaptic Ca^{2+} influx and vesicle release was suggested to lie at the core of the disease (Harvey et al., 2021). Additionally, PRRT2 was shown to negatively regulate SCN8A (Fruscione et al., 2018) – pathogenic variants in this gene cause a similar phenotype including dystonia. *SCN8A* encodes a voltage-gated sodium channel essential for the rapid membrane depolarization as part of action potentials, and mice harboring *SCN8A* pathogenic variants exhibit abnormal sodium currents in cerebellar Purkinje cells and cortical pyramidal neurons (Hamann et al., 2003).

HPCA, a gene linked to isolated dystonia (Lange et al., 2021) encodes the neuron-specific calcium-binding protein hippocalcin, mainly expressed in striatal MSNs**.** Upon cytosolic Ca^{2+} binding, the protein undergoes a conformational change leading to translocation from the cytosol to the plasma membrane**.** This translocation was shown to result in K^+ currents that control neuronal spike frequency by slow afterhyperpolarization (Andrade et al., 2012). Also, hippocalcin is evidenced to directly interact with VGCCs (Helassa et al., 2017), and loss of this interaction in *HPCA* knockdown neurons led to an uncoupling of depolarization and Ca^{2+} influx (Charlesworth et al., 2015). Consequently, Ca^{2+} influx through N-type calcium channels increases, resulting in increased vesicle release. Different pathogenic variants either prevent hippocalcin's translocation and the accompanied K^+ currents or lead to increased Ca^{2+} influx and vesicle release. Either way, the result is hyperexcitability of striatal neurons (Osypenko et al., 2019).

Moreover, involvement of impaired calcium homeostasis and synaptic transmission in dystonia is supported by pathogenic variants in *ANO3* and *KCTD17*, although the gene products have less defined functions. *ANO3* encodes Anoctamin-3, belongs to a family of calcium-activated chloride channels, and is, most highly expressed in striatal neurons (Charlesworth et al., 2012). While the name suggests the involvement of chloride channels in dystonia pathogenesis, it has now been demonstrated that ANO3 does not have ion channel activity (Kim et al., 2022). Instead, evidenced functions of the protein include direct interaction with sodium-activated potassium channels, which are involved in resting membrane potential maintenance and, therefore, neuronal excitability (Kim et al., 2022). ANO3 dampens excitability by increasing the sodium sensitivity of the channel. In line with this, *ANO3*-KO rats show a significant reduction of K(Na)-currents accompanied by a decreased threshold for action potential firing leading to increased neuronal

firing (Huang et al., 2013). A second function of ANO3 is that of a lipid scramblase that dissipates the asymmetrical distribution of phospholipids in the plasma membrane in response to small increases in intracellular Ca^{2+} levels - a critical process in apoptosis and modifying synaptic connections (Kim et al., 2022). However, how this function is linked to dystonia remains unclear. Lastly, ANO3 may act as a Ca^{2+} sensor that regulates Ca^{2+} homeostasis in neurons. Cell studies have demonstrated clear abnormalities in endoplasmic-reticulum(ER)-dependent Ca^{2+} signaling in *ANO3*-mutated cells. It is suggested that ANO3 influences Ca^{2+} signaling by reducing the Ca^{2+} pool inside the ER (Charlesworth et al., 2012). While it remains to be investigated how mutant ANO3 impairs signal transduction, it is conceivable that it leads to abnormal striatal neuron excitability.

KCTD17 encodes a member of the potassium channel tetramerization domain proteins. However, other than the name suggests, the protein is not predicted to form transmembrane domains but was shown to be distributed in the cytosol, dendritic projections, and synapses (Q. Li et al., 2017). Expression studies showed that *KCTD17, ANO3,* and *HPCA* belong to the same putaminal co-expression network (see above), suggesting that all three proteins function mainly in the striatum and are functionally interconnected. It has recently been demonstrated that KCTD17 may modulate neuronal excitability by regulating cAMP production and G-protein ß-subunit levels (Muntean et al., 2022). In addition, fibroblasts carrying pathogenic *KCTD17* variants displayed abnormalities in Ca^{2+} release from the ER in response to different stimuli (Mencacci et al., 2015). Similar to ANO3, this is believed to be due to a reduced Ca^{2+} pool in the ER.

Finally, other dystonia forms have been linked to perturbed Ca^{2+} homeostasis: *TOR1A* knockin mice displayed increased amplitudes of Ca^{2+} currents after depolarization in striatal neurons (Iwabuchi et al., 2013), and iPSC-derived MSNs from *SGCE*-mutation carriers displayed elevated basal intracellular Ca^{2+} levels compared to controls (Kutschenko et al., 2021).

The exact cellular mechanism by which abnormalities in Ca^{2+} homeostasis can lead to dystonia or if they are even a consequence is poorly understood. However, it can be inferred that impaired Ca^{2+} handling leads to altered cellular sensitivity to Ca^{2+}, which in turn induces rapid neuronal repolarization after action potentials. This would enable faster firing rates and manifest in hyperexcitability of the affected brain regions – as stated above, affecting primarily cerebellar (*ATP1A3, KCNA1, CACNA1A, TSPOAP1, PNKD, PRRT2, SCN8A, KCNN2*) or striatal (*HPCA, ANO3, KCTD17*) neurons. Regarding treatment, the involvement of dysregulated Ca^{2+} responses raises exciting possibilities, as selective calcium-channel blockers and modulators are abundant in the drug market.

Cellular stress response. Another major area of functional convergence among DYT-genes is the regulation of cellular stress response. The eIF2a (eukaryotic initiation factor 2 alpha) pathway is an essential and conserved component of the integrated stress response (ISR). It is activated by various types of stress stimuli, e.g., viral infections, unfolded proteins in the ER, hypoxia, and nutrient deprivation, in order to restore cellular homeostasis (Pakos-Zebrucka et al., 2016). The core event of the pathway is the phosphorylation of eIF2a by one of four members of the eIF2a kinase family, leading to a decrease in global protein synthesis and induction of selected genes that either promote cellular recovery and restore homeostasis or induce apoptosis (**Figure II-3**). In this, transient eIF2a phosphorylation is generally favorable for cellular survival, while prolonged phosphorylation due to exposure to severe stress is pro-apoptotic (Donnelly et al., 2013).

Pathogenic variants in two key players of this process, namely, *PRKRA* and *EIF2AK2*, have been linked, although rarely, with isolated dystonia (Lange et al., 2021; Thomsen et al., 2023). *EIF2AK2* (Eukaryotic translation initiation factor 2 alpha kinase, also referred to as PKA or PKR) is one of the kinases responsible for eIF2a phosphorylation. It is activated by either double-stranded RNA (due to viral infections) or through the binding of PRKRA in response to cellular stress (Garcia-Ortega et al., 2017). All pathogenic *EIF2AK2* variants reported thus far are located within one of the double-stranded RNA binding motifs of the protein, which are also the site of interaction with PRKRA (Thomsen et al., 2023). Functional studies in DYT-EIF2AK2 patients' fibroblasts revealed that the mutated protein led to prolonged phosphorylation of eIF2a in response to stress and, therefore, to persistent activation of the eIF2a pathway (Kuipers et al., 2021). It is suggested that this might lead to a failure to activate survival mechanisms, resulting in enhanced cellular death or consistent cellular abnormalities.

As introduced above, PRKRA (interferon-inducible double-stranded RNA-dependent Protein Kinase Activator A) is an important regulator of EIF2AK2. The most common dystonia-causing *PRKRA* variant (p.P222L) was shown to lead to prolonged and enhanced activation of EIF2AK2 in response to ER stress in patient cells, pointing to a GOF mechanism (Vaughn et al., 2015). The intensified activation was due to altered binding kinetics of the mutated PRKRA protein, reflected by an increased affinity of the PRKRA-EIF2AK2 interaction. A subsequent study confirmed the same effect for other dystonia-associated *PRKRA* variants (Burnett et al., 2020). Therefore, mutated PRKRA seems to increase cellular susceptibility to ER stress, disabling the cells to cope with cellular stress and restore homeostasis. Furthermore, brain imaging findings in DYT-PRKRA patients suggested nigrostriatal neurodegeneration (Lemmon et al., 2013; Pinto et al., 2020), indicative of enhanced cellular death consequential to the intensified stress response.

Nevertheless, because neurodegeneration is usually not observed in dystonia, future studies are needed to investigate whether apoptosis is indeed a cellular consequence in DYT-EIF2AK2 and DYT-PRKRA.

Pathogenic variants in a third gene that is part of the eIF2a pathway, *ATF4*, were reported in patients with cervical dystonia (Rittiner et al., 2016); however, pending confirmation by additional studies. ATF4 is a transcription factor and an effector of EIF2AK2.

In addition, stress-induced eIF2α signaling has been reported to be significantly dysregulated in other forms of dystonia, i.e., DYT-TOR1A (Beauvais et al., 2018; Rittiner et al., 2016), DYT-THAP1 (Zakirova et al., 2018), and XDP (Aneichyk et al., 2018). Furthermore, cellular stress response is tightly linked to Ca^{2+} signaling, as Ca^{2+} ions released from the ER are involved in integrating and regulating cellular stress-coping mechanisms in response to various stress stimuli (Groenendyk et al., 2021).

The discovery of aberrant eIF2α signaling underlying certain forms of dystonia brings about exciting therapeutic possibilities: A recent study discovered that the flavonoid luteolin was sufficient at disrupting the PRKRA-EIF2AK2 interaction. Consequently, the increased sensitivity of patient cells to ER stress was rescued, and the cells were protected from ER-stress-induced apoptosis (Burnett et al., 2020). Hence, a pharmacological inhibitor that disrupts this interaction in the brain could benefit DYT-EIF2AK2 and DYT-PRKRA patients. Furthermore, the implication of eIF2α signaling in dystonia pathogenesis suggests that response to viral infections (which trigger the ISR) may be a potential risk factor for disease development in patients harboring genetic variants associated with reduced penetrance. An important future question will be to determine where in the brain selective vulnerability to altered eIF2α signaling occurs and how pathogenic variants in the abovementioned ubiquitously expressed genes manifest in dystonia.

Endoplasmic reticulum and nuclear envelope function. The function and stress response of the ER is also tightly linked to *TOR1A* – the main dystonia gene (Lange et al., 2021), in which a 3-base-pair deletion leading to the deletion of a glutamate residue (ΔE) is the most frequent pathogenic variant. *TOR1A* encodes TorsinA, a protein belonging to the AAA+ family that uses ATP hydrolysis for several cellular functions, including protein trafficking, refolding, and degradation (Hanson & Whiteheart, 2005). Studies have demonstrated that expression of ΔE TorsinA is associated with abnormalities in protein folding and trafficking (Gonzalez-Alegre, 2019), affecting, e.g., D2R processing in striatal neurons (see above). Additionally, a role for TorsinA in the maintenance of ER protein homeostasis and, thus, prevention of ER stress has

been demonstrated. In this line, ΔE TorsinA has been linked to increased ER stress (Bragg et al., 2004) and sensitivity to ER stress (Nery et al., 2011). TorsinA is ubiquitously expressed in all tissues, while the DYT-TOR1A phenotype is restricted to the nervous system. It is evidenced that this may be due to the homologous protein TOR1B, whose expression rescues phenotypes caused by TOR1A dysfunction in cells, except in neurons where *TOR1B* is not expressed (Jungwirth et al., 2010).

Wild-type TorsinA cycles between the ER and the contiguous nuclear envelope (NE), with the bulk of protein detected in the ER (Goodchild & Dauer, 2004). In contrast, ΔE TorsinA predominantly co-localizes with NE markers and disrupts the normal NE membrane structure, resulting in the formation of NE-derived bleb-like structures and trafficking defects (Jokhi et al., 2013; Naismith et al., 2004). In the heterozygous state, ΔE TorsinA also recruits wild-type TorsinA to the NE, causing a dominant-negative effect (Goodchild & Dauer, 2004). This mislocalization of ΔE TorsinA was utilized to study which cellular pathways are modifiers of the pathologic phenotype observed in DYT-TOR1A. Interestingly, the eIF2α signaling pathway was the top hit (Rittiner et al., 2016), and the knockdown of each of the four eIF2α kinases (including EIF2AK2) was shown to worsen the mislocalization significantly. This suggests that decreased eIF2α signaling might play a central role in the disease mechanism of DYT-TOR1A (in contrast to DYT-EIF2AK2 and DYT-PRKRA, where increased eIF2α signaling is the underlying pathomechanism).

Very recently, the role of the nuclear core complex (NPC) and a subset of its components, the phenylalanine-glycine-rich region-containing nucleoporins (FG-NUPs), has been highlighted in the etiology of DYT-TOR1A (Prophet et al., 2022). NPCs form channels in the NE that control the bidirectional transport of proteins, mRNA, and other macromolecules between the cytoplasm and nucleus. The abnormal bleb-like structures observed in *TOR1A*-mutated cells were shown to result in NPC biogenesis deficits and, therefore, deficient nuclear transport. Furthermore, these structures contain non-functional FG-NUP-condensates that lead to the sequestration of protein quality-control network components, ultimately triggering ER stress. It is proposed that the vulnerability window associated with penetrance of *TOR1A*-related dystonia may be explained by the transient nature of "blebs" and depends on the effectiveness of cells in coping with nuclear-transport defects and proteotoxicity. Additionally, biallelic variants in one of the nucleoporin genes itself, *NUP54*, were recently linked to early-onset complex dystonia with striatal lesions (Harrer et al., 2023a). Intriguingly, abnormal NEs were also a reported feature of cerebellar Purkinje cells of *SGCE*-KO mice (Yokoi et al., 2012), suggesting that nucleoporins may have broader mechanistic implications across different dystonia subtypes.

A role for TorsinA in NPC-independent transport across the NE has also been demonstrated. In contrast to the canonical mRNA export through NPCs, mRNAs can be part of large ribonucleoprotein complexes that exit the nucleus via NE budding and are transported to specific cellular locations. This process, in which TorsinA is a vital mediator, is particularly essential for synapse formation and plasticity. In the absence of TorsinA, ribonucleoproteins are sequestered in the perinuclear space of the NE and don't reach their synaptic target, impairing proper synaptic protein synthesis and, thus, synaptic terminal development (Jokhi et al., 2013).

In summary, the diverse functions attributed to TorsinA (and to the ER/NE system), including protein processing, trafficking of biomolecules, and regulation of stress response, seem to be vital for neurodevelopment and synaptic plasticity and function. Pathogenic *TOR1A* variants, in particular the recurrent GAG-deletion, impair these functions, resulting in multiple downstream consequences like abnormal receptor composition and defective synaptic physiology. It is proposed that the basal ganglia are the brain structure mostly affected by TorsinA dysfunction (Gonzalez-Alegre, 2019). Further studies are required to explore which mechanisms are dystonia-causing and which are merely subclinical consequences. As eIF2α signaling and nuclear transport dysfunction are also implicated in other dystonia subtypes, they are likely relevant in the disease pathogenesis.

Autophagy and lysosomal function. Evidence suggests an involvement of the endolysosomal and autophagic systems in dystonia pathogenesis. For instance, many lysosomal storage disorders, in which lysosomal dysfunction leads to the accumulation of various substrates in cells, can present with prominent complex dystonia (Niemann-Pick Type C, GM1 Gangliosidosis). Patients affected by these diseases often display characteristic pathologic imaging findings affecting the striatum (Phua et al., 2020).

The first clue of an important link between autophagy and lysosomal function and isolated dystonia came from the discovery of mutations in the *VPS16* gene (Cai et al., 2016). It encodes 'Vacuolar protein sorting-associated protein 16 homolog', a part of the homotypic fusion and protein sorting complex (HOPS) comprising six proteins (VPS11, VPS16, VPS18, VPS33A, VPS39, and VPS41). The HOPS complex mediates the fusion of late endosomes and autophagosomes with lysosomes and, therefore, plays a fundamental role in removing misfolded or aggregated proteins and damaged cellular organelles (Ostrowicz et al., 2010). *VPS16* has the highest brain regional expression in the cerebellum but is also expressed in many other brain regions. Almost all identified disease-causing variants lead to a loss of protein function (Thomsen et al., 2023). Electron microscopy studies in cultured patients' fibroblasts revealed vacuolar

changes consistent with lysosomal dysfunction and indicative of an impaired function of the HOPS complex (Steel et al., 2020). This observation also confirmed two earlier studies: abnormal accumulation of vacuoles was demonstrated in a Drosophila *VPS16* knockdown model (Pulipparacharuvil et al., 2005), and depletion of *VPS16* in a human cell line study resulted in an impairment of endosomal-lysosomal function (Wartosch et al., 2015).

Biallelic variants in two additional HOPS complex components, i.e., *VPS11* and *VPS41*, have been linked to complex neurodevelopmental disorders with dystonia (Monfrini et al., 2021a; Sanderson et al., 2021; Steel et al., 2020). Functional studies in patient-derived fibroblasts carrying variants in *VPS16, VPS11,* and *VPS41* display comparable abnormalities of the lysosomal and autophagic compartments (Monfrini et al., 2021b; Steel et al., 2020). It is postulated that this may hinder key cellular processes within the neural networks involved in motor control, ultimately manifesting in dystonic movements (Steel et al., 2020). While disease-related variants in *VPS16, VPS11,* and *VPS41* display overlapping abnormalities on the cellular level, imaging findings suggest some differences in the pathophysiology of the disorders. For example, brain MRI of all investigated patients with VPS41-related disease demonstrated progressive cerebellar atrophy. On the other hand, patients with VPS11-associated dystonia, as well as some DYT-VPS16 patients, showed subtle basal ganglia changes (Monfrini et al., 2021b). Hence, the brain structures primarily affected by the disorders may be distinct, supporting the view that dystonia is a network disorder without one unifying causative mechanism or brain area.

Interestingly, for some patients with *VPS16* and *VPS41* variants, imaging findings were indicative of brain iron accumulation (Monfrini et al., 2021b). Complex dystonia is one of the most frequent clinical presentations of neurodegeneration with brain iron accumulation (NBIA), a group of genetic disorders displaying iron accumulation in the basal ganglia (e.g., linked to *DCAF17, PANK2, CP*) that is also tightly linked to lysosomal dysfunction (reviewed in Hinarejos et al., 2020).

In addition to the discussed HOPS-associated disorders, other dystonias have been linked to pathogenic variants in endo-lysosomal and autophagic pathways. This includes *IRF2BPL*, linked to complex dystonia and encoding a zinc-finger transcription factor that may also function as an ubiquitin ligase (Higashimori et al., 2018). Electron microscopy studies in cultured patients' fibroblasts confirmed extensive abnormalities consistent with lysosomal dysfunction (Ginevrino et al., 2020). Additionally, it is suggested from imaging findings that nigrostriatal degeneration may contribute to the disorder (Prilop et al., 2020).

Another example is *SQSTM1*, which encodes an autophagy receptor (Sequestosome 1/ p62) that targets ubiquitinated cargos to the autophagosome and, therefore, plays a vital role in autophagy regulation. Biallelic LOF variants were reported in several unrelated cases with childhood-onset neurodegeneration presenting with dystonia, ataxia, cognitive decline, and gaze palsy (Haack et al., 2016; Muto et al., 2018; Zúñiga-Ramírez et al., 2019). Functional work in cultured patients' fibroblasts demonstrated clear abnormalities in autophagy flux and mitophagy (autophagy of mitochondria). Intriguingly, *SQSTM1* is known to be part of an autophagy gene transcription program that is induced in response to ER stress via the eIF2a-signaling pathway (B'chir et al., 2013). It is suggested that the eIF2α-ATF4 pathway is the major regulatory pathway that induces the transcriptional activation of a large number of autophagy genes in response to cellular stress. Thus, these findings link two cellular pathways involved in dystonia pathogenesis, integrated stress response and autophagy, on the molecular level.

Given the substantial relationship between the discussed dystonia forms and lysosome-associated disorders, possible therapeutic approaches may be deduced from lysosome-associated disorders, including autophagy inducers, small-molecule chaperones, or substrate-reducing molecules, which are already under study (Bonam et al., 2019; Marques & Saftig, 2019).

Cytoskeleton. Some forms of dystonia indicate that abnormalities in the cytoskeleton can underlie dystonic phenotypes. A relevant example is the ε-sarcoglycan gene *(SGCE),* linked to myoclonus-dystonia (MD). The protein belongs to the sarcoglycan family comprising six different transmembrane glycoproteins (α, β, γ, δ, ε, and ζ), which are best known for their function in muscular tissue where they form part of the dystrophin-glycoprotein complex (DGC) that links the cytoskeleton to the extracellular matrix (Ozawa et al., 2005).

Two major hypotheses on the disease mechanism of MYC/DYT-SGCE have been proposed. First, since *SGCE* belongs to the imprinted genes, which are generally known to be involved in multiple developmental processes, it is believed to be crucial during brain development. In line with this, *SGCE* was shown to be ubiquitously and highly expressed during embryonic development in rodents. Thereafter, the expression dramatically declined but was preserved in neurons, with high levels in cerebellar Purkinje cells (Xiao & LeDoux, 2003). In the human brain, the major brain-specific isoform (11b) is also mainly expressed in the cerebellum and moderately expressed in the striatum (Ritz et al., 2011). Hence, the expression pattern of the gene suggests that the disease has at least a neurodevelopmental component (like, e.g., DYT-THAP1 and DYT-KMT2B).

Second, abnormal synaptic transmission and Ca^{2+} homeostasis have been implicated in SGCE-related disease: The brain-specific isoform was shown to be enriched in presynaptic structures

(Nishiyama et al., 2004) and interact with postsynaptic scaffolding proteins, suggesting that ε-sarcoglycan plays an essential role in synapse assembly and function (Cazurro-Gutiérrez et al., 2021). Furthermore, ε-sarcoglycan may be part of DGC-like complexes in the brain. As loss of dystrophin is known to cause increased activity of Ca^{2+} channels in neurons, it is hypothesized that loss of ε-sarcoglycan could likewise result in neuronal membrane damage and Ca^{2+} accumulation (Menozzi et al., 2019). A recent study in iPSC-MSNs confirmed elevated basal intracellular Ca^{2+} levels in patient-derived cells compared to controls (Kutschenko et al., 2021). Even though it is tempting to speculate that the pathophysiological changes in *SGCE*-related MD arise from a DGC dysfunction in the brain, it remains to be investigated whether ε-sarcoglycan is genuinely a part of DGC-like complexes in the brain and how the whole DGC function would be affected by a loss of this protein. ε-sarcoglycan might also have independent and brain-specific functions (Cazurro-Gutiérrez et al., 2021).

Animal models have provided evidence for the involvement of both the basal ganglia and cerebellum in MYC/DYT-SGCE. For instance, *SGCE*-KO mice showed increased striatal dopamine levels and reduced pre- and postsynaptic striatal D2R levels, suggesting a possible role of ε-sarcoglycan in stabilizing synaptic membranes of dopaminergic neurons (Zhang et al., 2012). On the other hand, mice with cerebellar *SGCE*-cKO demonstrated aberrant firing of cerebellar Purkinje cells and deep cerebellar nuclei neurons (Washburn et al., 2019). Interestingly, the authors observed that cerebellar knockout produced dystonia and myoclonus, whereas knockout in the basal ganglia resulted in only subtle motor defects, suggesting that the cerebellum may be the primary instigator. Furthermore, a recent mouse model carrying a heterozygous LOF allele and mimicking the MD phenotype has provided evidence that ε-sarcoglycan may act as an inhibitor of synaptogenesis, as the protein loss resulted in excessive formation of excitatory synapses. This is also supported by the fact that pharmacologically enhancing GABA transmission alleviated symptoms (J. Li et al., 2021).

Another example where pathogenic changes in cytoskeletal proteins underlie dystonia is *TUBB4A*-related disease. *TUBB4A* encodes the brain-specific β-tubulin isotype 'β-tubulin 4A' with high cerebellar expression, and pathogenic variants can cause a broad spectrum of diseases, including "whispering dystonia" (also known as DYT4) (Erro et al., 2015; Lohmann et al., 2013). β-tubulins form heterodimers with α-tubulins that are arranged into microtubules – the largest cytoskeletal filament in cells. Microtubules are essential for maintaining and changing cell morphology, axon extension, and assembly of mitotic spindles during cell division and serve as tracks for intracellular transport. Notably, TUBB4A depletion did not cause a phenotype in mice, pointing to dominant-acting mutant tubulin (Fertuzinhos et al., 2022). Moreover, it was shown

that mutant TUBB4A proteins are integrated into the microtubule network (Vulinovic et al., 2018).

It is postulated that different cell types are differentially susceptible to tubulin perturbations, depending on the expression ratio of other ß-tubulin isoforms and, therefore, the degree of the dominant-negative effect of mutant TUBB4A. In this, oligodendrocytes seem to be particularly affected. Dystonia-causing variants were shown to be associated with aberrant microtubule growth dynamics in oligodendrocytes, supporting a GOF mechanism (Krajka et al., 2022). It is postulated that this may disrupt cell proliferation, resulting in impaired arborization and myelination of neurons, ultimately leading to reduced signal transduction. Notably, impaired oligodendrocyte maturation with the consequence of myelination deficits seems to be a convergent mechanism, as this is also an observed feature in THAP1- and YY1-related dystonia (see above).

Additionally, an iPSC study demonstrated functional impairment of microtubule-associated transport in iPSC-derived neurons from DYT-TUBB4A patients (Vulinovic et al., 2018), which could affect essential cargos, deliveries of mitochondria, and lysosomal function.

Another hereditary dystonia form involving a cytoskeletal protein is *ACTB*-associated complex dystonia-deafness. *ACTB* encodes the ubiquitously expressed and highly conserved β-actin that forms cytoskeletal filaments essential for cell migration, cell morphology, mitosis, and intracellular transport. Dystonia-causing variants were shown to destabilize actin filaments which may lead to various cellular defects (Hundt et al., 2014). Notably, imaging findings were indicative of striatal neuron dysfunction and GPi-DBS successfully improved dystonia in patients (Skogseid et al., 2018; Straccia et al., 2022).

Even though the exact mechanism by which abnormalities in cytoskeletal proteins induce dystonic phenotypes is poorly understood, it is becoming increasingly evident that they may be accompanied by cellular changes frequently observed across dystonia forms. These include impaired calcium homeostasis, synapse assembly and function, and myelination deficits, resulting in aberrant neuronal firing.

CONCLUSIONS

We gain the most mechanistic insight into the pathogenesis of dystonia by understanding the action of genes and their encoded products mutated in monogenic forms of dystonia - a list that is constantly growing. Considering the currently known isolated dystonia genes, disease mechanisms are diverse and affect gene transcription during neurodevelopment (*KMT2B,*

THAP1), calcium homeostasis (*HPCA, TOR1A, ANO3*), striatal dopamine signaling (*GNAL*), ER stress response (*PRKRA, EIF2AK2, TOR1A*), and autophagy (*VPS16*). Relationships between the associated disease pathways remain difficult to understand, but as demonstrated, several connections have been identified that link the distinct molecular pathways implicated in dystonia. For instance, transcription factors regulate other dystonia genes, and impaired ER function and calcium homeostasis may induce a stress response, autophagy, and aberrant synaptic function.

Intriguingly, even though the molecular origin of pathogenesis might differ between dystonia subtypes, convergence on the cellular and anatomical level has become increasingly evident. Several different molecular defects eventually lead to aberrant activity of striatal or cerebellar neurons, disturbing the brain circuits involved in motor control. The current state of research suggests that it is unlikely that one driver molecule or mechanism exists. Instead, the distinct mechanisms trigger the same downstream effects, eventually leading to the clinical phenotype of dystonia. As a consequence, different forms of dystonia can be categorized into "molecular groups", which will assist with treatment development and application.

An important future question will be whether the same molecular pathways are also affected in idiopathic dystonia patients, especially the ones with late-onset focal dystonia, the most common subtype. Therefore, large genomic efforts are warranted to further elucidate the genetic basis of this patient group.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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3.3 Objective III: Large-Scale Gene Panel Analysis of the Most Common Dystonia Genes

To date, pathogenic variants in at least ten genes have been associated with forms of isolated dystonia (Lange et al., 2022; Thomsen et al., 2023), some of which have been described in only a few families. In addition, many genes have been associated with the diverse group of combined dystonia (Lange et al., 2022), including *GCH1* and *SGCE* (Pérez-Dueñas et al., 2022), which play a major role in dopa-responsive dystonia and myoclonus-dystonia, respectively. However, the phenotypic and genetic spectrum of these genes, as well as the frequency of pathogenic variants, remain incompletely understood, both in patients with dystonia and in those with other, sometimes co-occurring, movement disorders such as Parkinson's disease (PD). For a rare disorder like dystonia, large-scale screening efforts are essential to deepen our understanding of its genetic architecture and genotype-phenotype relationships; however, such efforts have rarely been undertaken, as available sample sizes are typically small (Wirth et al., 2025).

To address this, a large patient sample (n=1,207 dystonia and n=1,036 PD patients) was screened using targeted gene capture sequencing of the seven most common isolated and combined dystonia genes (*TOR1A*, *THAP1*, *GNAL*, *KMT2B*, *PRKRA*, *GCH1*, and *SGCE*) to evaluate the frequency and role of rare variants in these genes (Objective III). Additionally, to support the interpretation of *KMT2B* variants, the DNA methylation pattern in blood was analyzed to assess the characteristic "episignature" that has been described in cells with KMT2B loss of function.

The study covering this objective is titled "Large-scale screening: Phenotypic and mutational spectrum in isolated and combined dystonia genes" and was published in *Movement Disorders*. Mirja Thomsen and Katrin Marth performed the evaluation of genetic variants, including pathogenicity scoring. Mirja Thomsen conducted all statistical analyses and prepared all figures and tables, including the graphical abstract. After data pre-processing by Fabian Ott, she also performed the normalization, analysis, and interpretation of the methylation data. The first draft of the manuscript was written by Mirja Thomsen and supervised and revised by Katja Lohmann.

By targeted gene sequencing, a molecular diagnosis could be established in 4.0% of patients, including novel dystonia-causing variants. The overall frequency of rare variants was significantly higher in dystonia patients compared to PD patients, highlighting their role as dystonia genes. Episignature analysis of *KMT2B* variants emphasized the importance of functional studies for variant interpretation, and an overview of these results has been added to the MDSGene website for researchers and diagnostic laboratories. This large-scale dataset, along with functional data for *KMT2B* variants, will serve as a guide in future variant interpretation.

Large-scale screening: Phenotypic and mutational spectrum in isolated and combined dystonia genes

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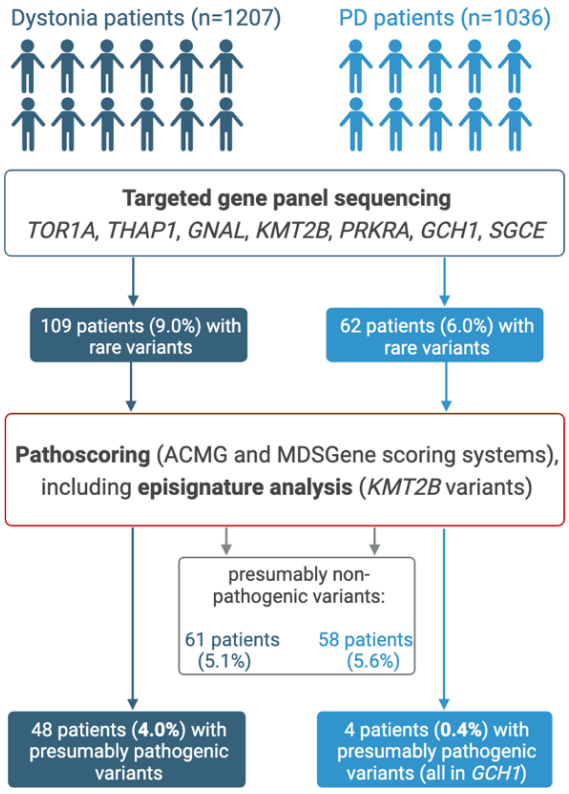

Graphical abstract

ABSTRACT

Background: Pathogenic variants in several genes have been linked to genetic forms of isolated or combined dystonia. The phenotypic and genetic spectrum and the frequency of pathogenic variants in these genes have not yet been fully elucidated, neither in patients with dystonia nor with other, sometimes co-occurring movement disorders such as Parkinson's disease (PD).

Objectives: To screen >2,000 patients with dystonia or PD for rare variants in known dystonia-causing genes.

Methods: We screened 1,207 dystonia patients from Germany (DysTract consortium), Spain, and South Korea, and 1,036 PD patients from Germany for pathogenic variants using a next-generation sequencing gene panel. The impact on DNA methylation of *KMT2B* variants was evaluated by analyzing the gene's characteristic episignature.

Results: We identified 171 carriers (109 with dystonia [9.0%]; 62 with PD [6.0%]) of 131 rare variants (minor allele frequency <0.005). Thereof, 52 patients (48 dystonia [4.0%]; four PD [0.4%, all with *GCH1* variants]) carried 33 different (likely) pathogenic variants, of which 17

were not previously reported. Pathogenic biallelic variants in *PRKRA* were not found. Episignature analysis of 48 *KMT2B* variants revealed that only two of these should be considered (likely) pathogenic.

Conclusion: This study confirms pathogenic variants in *GCH1*, *GNAL*, *KMT2B*, *SGCE*, *THAP1*, and *TOR1A* as relevant causes in dystonia and expands the mutational spectrum. Of note, likely pathogenic variants only in *GCH1* were also found among PD patients. For DYT-KMT2B, the recently described episignature served as a reliable readout to determine the functional effect of newly identified variants.

INTRODUCTION

Dystonia is a rare movement disorder characterized by abnormal movements and postures that are caused by involuntary sustained or intermittent muscle contractions (Albanese et al., 2013). The clinical presentation of dystonia is highly heterogeneous, including various ages at onset (AAO), body distribution of symptoms, and associated features (Albanese et al., 2013). Dystonia can be isolated, combined with another movement disorder such as parkinsonism or myoclonus, or part of a complex neurological or systemic disorder with extracerebral features (Albanese et al., 2013).

Monogenic forms, i.e., due to pathogenic variants in a single gene, can explain a fraction of dystonia patients, often with early disease onset (<20 years) and a non-focal presentation (Zech et al., 2020). To date, pathogenic variants in at least ten genes have been linked to forms of isolated dystonia (Lange et al., 2022; Thomsen et al., 2023), of which pathogenic variants in *TOR1A, THAP1, GNAL, KMT2B, ANO3*, and *PRKRA* have been reported in >25 patients each (Lange et al., 2021). Pathogenic variants in four additional genes causing isolated dystonia, i.e., *AOPEP, EIF2AK2, HPCA*, and *VPS16,* have been confirmed more recently and, to date, were only reported in less than 10 families each, except for *VPS16* variants, which have been reported in at least 25 families (Lange et al., 2021; Thomsen et al., 2023). For *HPCA*, for instance, disease-causing variants have only been reported in a handful of families (Lange et al., 2021). Further, many genes have been linked to the diverse group of combined dystonia (Lange et al., 2022), of which *GCH1* and *SGCE* (Pérez-Dueñas et al., 2022) play a major role in dopa-responsive dystonia and myoclonus-dystonia, respectively (see also www.mdsgene.org). Of note, pathogenic *GCH1* variants have also been implicated in the pathogenesis of Parkinson's disease (PD) (Mencacci et al., 2014), and a recent systematic literature review revealed that about 10% of patients with *GCH1* mutations present with isolated parkinsonism (Weissbach et al., 2022).

A challenge in genetic testing is the interpretation of variants as disease-causing (pathogenic) or not (benign). Different recommendations have been developed for this, e.g., the standards and guidelines of the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015) or the pathogenicity scoring applied within MDSGene (Kasten et al., 2018). They both use a weighted score combining evidence from recurrence/family studies (segregation or *de novo* occurrence), *in silico* prediction (for instance, the CADD score (Rentzsch et al., 2019)), variant frequency in public databases such as the genome aggregation server (gnomAD, https://gnomad.broadinstitute.org/), and functional studies. However, the latter are often not available, for instance, for *TOR1A,* or labour-intensive such as for *THAP1* (Lohmann et al., 2012) and *GNAL* (Fuchs et al., 2013). The situation might be different for *KMT2B*, a large gene with many rare missense variants, the interpretation of which is particularly challenging. Recently, a characteristic so-called "episignature" for KMT2B loss of function was described that is based on aberrant CpG methylation and can be used as a functional readout to evaluate the effect of variants on the protein's function (Mirza-Schreiber et al., 2022).

The phenotypic and mutational spectrum of dystonia-linked genes is constantly expanding, and newly identified variants require careful evaluation. In this study, we aimed to evaluate the frequency and role of rare variants in seven of the most common isolated and combined dystonia genes, i.e., *TOR1A, THAP1, GNAL, KMT2B, PRKRA, GCH1,* and *SGCE*, by screening more than 2,000 patients with dystonia or PD. The overall frequency of rare variants was significantly higher in dystonia patients compared to PD patients, underlining their role as dystonia genes. Further, this large-scale dataset and the functional evaluation of *KMT2B* variants will guide future variant interpretation.

MATERIALS AND METHODS

Study population

We included a total of 1,207 patients with dystonia. These patients were recruited in Germany (*n*=1014, within the DysTRACT consortium, a large research-based registry of patients with a diagnosis of dystonia (https://www.isms.uni-luebeck.de/en/research/dystract/), in Spain (*n*=92), in South Korea (*n*=75), or at several other sites (*n*=26) (Supplementary Table S1). All patients were examined by movement disorder specialists. Dystonia patients had a median age of 57 years (interquartile range (IQR), 45-68), a median AAO of 36 years (IQR, 21-49), and included 564 males (46.7%) and 643 females (53.3%). Most of the enrolled dystonia patients (726/1207, 60.1%) presented with focal dystonia, 229/1207 (19.0%) had segmental or multifocal, and 125/1207 (10.4%) had generalized dystonia (Supplementary Table S1). Of the patients with focal

dystonia, most had cervical dystonia (293/726, 40.4%), upper limb dystonia (80/726, 11.0%), blepharospasm (63/726, 8.7%), or musician's dystonia (225/726, 31.0%). As a disease control group, we included 1,036 PD patients from Germany with a median age of 71 years (IQR, 60-78), a median AAO of 61 years (IQR, 52-69.75), of whom 641 were male (61.9%) and 376 were female (36.3%, information missing for 19) (Supplementary Table S1). The study was approved by the local Ethics Committee of the University of Lübeck, Germany, and written informed consent was obtained from all participants prior to the genetic tests.

Genetic analysis

We performed a next-generation sequencing-based gene panel analysis including all coding exons of *TOR1A* (NM_000113), *GNAL* (NM_182978), *THAP1* (NM_018105), *KMT2B* (NM_014727), *PRKRA* (NM_003690), *GCH1* (NM_000161), and *SGCE* (NM_003919). Sequencing was carried out between 2016 and 2021 in a total of eight batches containing 51-780 samples each at Centogene (Rostock, Germany). Genomic DNA was enzymatically fragmented, and regions of interest were enriched using DNA capture probes (Twist Biosciences, custom design). The final indexed libraries were sequenced on an Illumina platform (NextSeq), with a sequencing quality parameter of 99.5% coverage of the targeted regions and a minimum read depth of 100x. Bioinformatic pipeline for mapping, variant calling, and annotation has been described elsewhere (Almeida et al., 2022).

ANO3 variants were previously tested in a subset of patients (*n*=729) using the same panel, and results were reported elsewhere (Olschewski et al., 2019). For a few patients (*n*=6), a genetic diagnosis was already previously found by gene-specific Sanger sequencing. However, these patients were still included in this gene panel study, also to rule out a second genetic cause. Notably, there was no enrichment for patients with a known genetic diagnosis in this study.

Sanger sequencing was performed for validation of rare (minor allele frequency [MAF] <0.005), presumably protein-changing variants.

For assessing the pathogenicity of detected variants, two different published scoring systems were used: the one used for MDSGene (www.mdsgene.org/methods) and the standards and guidelines from the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015), despite its known limitations (Agaoglu et al., 2022; Liu et al., 2018).

Episignature analysis for *KMT2B* variants

To assess the functional effect of *KMT2B* variants, the DYT-KMT2B-specific methylation pattern ("episignature") in peripheral blood, comprising 113 specific CpG sites, was analyzed as

described (Mirza-Schreiber et al., 2022), using the Illumina MethylationEPIC BeadChip. The mean of the normalized methylation levels (mean(z)) and the coefficient of variation (CV = SD/|mean|) were used as quantifiers (Supplementary Methods). For normalization, we used either 17 DYT-SGCE patients or 38 unaffected individuals as controls and performed the calculations 1) using all 113 sites and 2) using 103 sites that were left after data cleaning following best practices (Supplementary Methods).

RESULTS

Through gene panel analysis and subsequent Sanger sequencing, 171 carriers (109 with dystonia [9.0%], 62 with PD [6.0%]) of 131 different heterozygous, rare, protein-changing variants were detected, of which the majority (*n*=111) were not previously reported (not listed in MDSGene after systematic literature research; see Supplementary Table S2). After classification of these variants by using the MDSGene and ACMG scoring systems, 77 variants were considered as (likely) benign (Supplementary Table S2), 33 as (likely) pathogenic (**Table III-1**), and 21 were left as variants of uncertain significance ("VUS", **Table III-2**). The 33 presumably pathogenic variants were detected in 52 patients, of whom 48 had dystonia (48/1207, 4.0%) and four had PD (4/1036, 0.4%).

The additive frequency of rare, presumably pathogenic variants in all seven tested genes was significantly higher in dystonia patients than in PD patients (Fisher's exact test, $p<0.00001$, respectively). Notably, the frequency of rare, presumably benign variants and VUS did not significantly differ between the two groups (Fisher's exact test, $p=0.5723$), underlining the relevance of presumably pathogenic variants only. Excluding variants in *GCH1*, which have a known role also in PD, the frequency of presumably pathogenic variants was also significantly higher in dystonia patients (Fisher's exact test, $p<0.00001$, respectively).

Among the dystonia patients, 48 carriers of presumably disease-causing variants were identified in the heterozygous state in *TOR1A* (*n*=11, 0.9%), *GCH1* (*n*=11, 0.9%), *THAP1* (*n*=11, 0.9%), *SGCE* (*n*=8, 0.7%), *GNAL* (*n*=5, 0.4%), and *KMT2B* (*n*=2, 0.2%) (**Figure III-1**). Of note, no carriers of biallelic pathogenic *PRKRA* variants were found. The dystonia patients with (likely) pathogenic variants had a median AAO of 12 years (IQR, 7-17) and included 26 males (54.2%) and 22 females (45.8%). Compared to the overall sex distribution in the dystonia sample (564/1207 males = 46.7%, 643 females/1207 = 53.3%), there was no significant predominance of one sex in the group of carriers of presumably disease-causing variants (Fisher's exact test, $p=0.3049$). The small number of patients with likely disease-causing variants per gene did not allow us to search for statistical differences in sex distribution for each genetic subtype.

Table III-1: Overview of carriers of (likely) pathogenic variants in *TOR1A*, *THAP1*, *GNAL*, *KMT2B*, *GCH1*, and *SGCE*.

	Gene	Patient ID	cDNA change	Protein change	CADD score v1.6	GnomAD exomes frequency v2.1.1	Novel	Pathoscoring		Origin	Age (years)	Age at onset (years)	Sex (m/f)	Family history	Dystonia type	Affected region
								ACMG	MDSGene							
Dystonia patients	*TOR1A*	L-3837	c.907_909del	p.Glu303del	22.3	0.000115	no	P	DP	DEU	43	12	m	negative	generalized	n.a.
		L-4004								DEU	40	7	m	negative	generalized	neck, limbs, trunk,
		L-4591								DEU	35	13	f	positive	focal	hand
		L-7404								DEU	23	10	f	negative	generalized	n.a.
		L-11062								KOR	25	9	m	negative	generalized	n.a.
		L-11514								DEU	57	25	m	negative	generalized	n.a.
		L-11542								DEU	53	11	m	negative	generalized	n.a.
		L-11584								ESP	22	10	m	positive	focal	right leg
		L-11627								ESP	24	12	m	negative	generalized	n.a.
		L-13343								DEU	45	4	m	positive	generalized	n.a.
		L-4286	c.40_45del	p.Ala14_Pro15del	22.3	n.a.	no	LP	PrP	DEU	80	30	f	positive	focal	neck
	THAP1	L-8923	c.16T>C	p.Ser6Pro	29.7	n.a.	no	LP	PrP	DEU	55	18	m	negative	segmental	neck, oromandibular
		L-11557	c.292G>T	p.Glu98*	36.0	n.a.	yes	P	PrP	ESP	77	10	m	positive	generalized	n.a.
		L-11640								ESP	51	15	f	negative	segmental	oromandibular
		L-2257	c.474del	p.Lys158Asnfs*23	26.5	n.a.	no	P	PrP	DEU	78	8	m	negative	generalized	n.a.
		L-11577	c.61T>G	p.Lys24Glu	29.8	n.a.	yes	LP	PoP	ESP	26	16	m	negative	generalized	n.a.
		L-13633	c.62C>T	p.Ser21Phe	32.0	n.a.	no	LP	PrP	DEU	29	7	f	negative	generalized	n.a.
		L-14814								DEU	37	4	f	positive	generalized	n.a.
		L-4155	c.68A>C	p.His23Pro	32.0	n.a.	no	P	PrP	DEU	43	9	m	positive	focal	hand
		L-3841	c.70A>G	p.Lys24Glu	31.0	n.a.	no	LP	PrP	DEU	42	14	f	n.a.	multifocal	neck, hand, foot
		L-11501	c.71+2T>C		33.0	n.a.	yes	LP	PrP	DEU	55	1	f	negative	generalized	n.a.
		L-7807									56	30	f	negative	generalized	orofacial, neck, limbs
	GNAL	L-13315	c.1060_1065del	p.Phe354_Leu355del	21.1	n.a.	yes	LP	PrP	DEU	62	46	f	negative	segmental	face, shoulder, hand, neck
		L-12521	c.1264dup	p.Tyr422Leufs*3	34.0	n.a.	yes	LP	PrP	DEU	52	16	m	n.a.	focal	neck
		L-4486	c.868G>A	p.Gly290Ser	33.0	n.a.	yes	LP	PoP	DEU	64	37	m	negative	focal	neck
		L-11929	c.1115T>G	p.Ile372Ser	29.6	n.a.	yes	LP	PrP	DEU	63	40	f	positive	generalized	neck, limbs
		L-7606								DEU	50	32	m	negative	segmental	neck, oromandibular

	Gene	Patient ID	cDNA change	Protein change	CADD score v1.6	GnomAD exomes frequency v2.1.1	Novel	Pathoscoring		Origin	Age (years)	Age at onset (years)	Sex (m/f)	Family history	Dystonia type	Affected region
								ACMG	MDSGene							
	KMT2B	L-8941	c.3568_3577del	p.Leu1190Serfs*162	42.0	n.a.	no	P	DP	AFG	33	7	f	negative	generalized	lower limbs, trunk, neck
		L-13774	c.3400C>T	p.Gln1134*	33.0	n.a.	yes	P	PrP	DEU	39	27	f	negative	multifocal	n.a.
	GCH1	L-3773	c.181G>T	p.Glu61*	38.0	n.a.	no	P	DP	DEU	68	7	f	positive	generalized	n.a.
		L-12163	c.229T>C	p.Ser77Pro	25.0	n.a.	yes	LP	PoP	DEU	34	15	f	negative	generalized	neck, feet, trunk
		L-858	c.262C>G	p.Arg88Gly	32.0	0.000004	no	LP	PrP	DEU	40	16	f	positive	generalized	n.a.
		L-12641	c.283C>T	p.Pro95Ser	31.0	n.a.	no	LP	PrP	DEU	64	2	m	negative	multifocal	neck, lower limbs
		L-11635	c.323G>T	p.Gly108Val	29.2	n.a.	yes	LP	PoP	ESP	73	18	f	negative	generalized	especially cervical region
		L-14616	c.4G>A #	p.Glu2Lys	23.7	n.a.	yes	LP	PrP	DEU	55	38	m	negative	segmental	face, hand, neck
		L-11143	c.638_641del (!)	p.Lys213fs	14.7	0.000016	yes	LP	PoP	KOR	17	14	f	negative	segmental	n.a.
		L-11944	c.680C>T	p.Thr227Ile	28.7	n.a.	yes	LP	PrP	DEU	87	6	f	positive	generalized	limbs, neck, trunk
		L-8340								DEU	82	6	m	positive	multifocal	right hand, foot
		L-5967	c.745A>G	p.Arg249Gly	22.7	n.a.	yes	LP	PoP	DEU	49	38	m	negative	segmental	Right hand, arm
		L-14447	c.671A>G #	p.Lys224Arg	21.3	0.000386	no	VUS	PoP	DEU	25	8	m	negative	generalized	face, neck, trunk, limbs
	SGCE	L-11895	c.109+1G>T		35.0	n.a.	no	LP	PrP	DEU	74	34	f	negative	myoclonus-dystonia	n.a.
		L-6808	c.1291_1297dup	p.Gly433fs	33.0	n.a.	yes	P	PrP	DEU	39	15	m	n.a.	myoclonus-dystonia	limbs
		L-2354	c.289C>T	p.Arg97*	36.0	0.0000089	no	P	PrP	DEU	76	2	m	negative	myoclonus-dystonia	n.a.
		L-4151	c.304C>T	p.Arg102*	38.0	n.a.	no	P	PrP	DEU	81	5	m	positive	myoclonus-dystonia	n.a.
		L-4168								DEU	69	0.1	f	positive	myoclonus-dystonia	n.a.
		L-8162								DEU	51	3	m	negative	myoclonus-dystonia	neck, hand, upper limbs
		L-6589	c.418G>T	p.Glu140*	38.0	n.a.	yes	LP	PrP	DEU	36	15	m	negative	myoclonus-dystonia	neck, trunk, limbs
		L-12173	c.771_772del	p.Cys258fs	32.0	n.a.	no	P	PrP	DEU	61	4	f	positive	myoclonus-dystonia	n.a.
PD patients	*GCH1*	L-11358	c.4G>A #	p.Glu2Lys	23.7	n.a.	yes	LP	PrP	DEU	43	36	m	n.a.	no dystonia	
		L-9896	c.586G>T	p.Ala196Ser	23.4	0.0000398	yes	LP	PoP	DEU	69	48	m	negative	no dystonia	
		L-11656	c.671A>G #	p.Lys224Arg	21.3	0.000386	no	LP	PoP	DEU	75	70	f	n.a.	no dystonia	
		L-5970								DEU	68	60	m	negative	no dystonia	

PD= Parkinson's disease, LP= likely pathogenic, P= pathogenic, PoP= possibly pathogenic, PrP= probably pathogenic, DP= definitely pathogenic, n.a.= not available, m= male, f= female; Novel variant means a variant that has not previously been reported in a dystonia patient. For the country of origin, the standard ISO Country code is listed.

#variant found in both PD and dystonia patients (!)The nomenclature of this variant is based on NM_001024070

Table III-2: Overview of carriers of variants of uncertain significance in *TOR1A*, *THAP1*, *GNAL*, *KMT2B*, *GCH1*, and *SGCE*.

	Gene	Patient ID	cDNA change	Protein change	CADD score v1.6	GnomAD exomes frequency v2.1.1	Novel	Pathoscoring		Origin	Age (years)	Age at onset (years)	Sex (m/f)	Family history	Dystonia type	Affected region
								ACMG	MDSGene							
Dystonia patients	*TOR1A*	L-7938	c.331G>C	p.Val111Leu	22.0	0.000095	yes	VUS	PoP	DEU	30	21	m	positive	segmental	neck
		L-3584	c.719T>C	p.Leu240Ser	27.1	0.000028	yes	VUS	PoP	DEU	84	44	f	negative	focal	neck
	GNAL	L-12036	c.74C>A	p.Pro25Gln	14.5	n.a.	yes	VUS	B	DEU	56	50	m	negative	multifocal	shoulders
		L-8257	c.313A>C	p.Ile105Leu	25.7	n.a.	yes	VUS	PoP	DEU	53	23	f	negative	multifocal	face, shoulder, neck, limbs
		L-3811	c.580T>G	p.Tyr194Asp	29.5	n.a.	yes	VUS	PoP	DEU	49	38	f	negativ	focal	blepharospasm
	KMT2B	L-12226	c.1550G>A	p.Ser517Asn	22.2	0.000004	yes	VUS	PoP	DEU	36	20	m	n.a.	segmental	n.a.
		L-12253	c.4573G>A	p.Gly1525Arg	34.0	0.000065	yes	VUS	PoP	DEU	47	7	f	n.a.	segmental	n.a.
		L-12626	c.6475C>G	p.Pro2159Ala	12.5	0.000010	yes	VUS	B	DEU	58	15	f	n.a.	focal	cranial
		L-12035	c.5108T>C	p.Leu1703Pro	24.6	n.a.	yes	VUS	PoP	DEU	69	63	f	negative	focal	neck
	GCH1	L-11878	c.509+3A>G #		13.2	0.0000955	yes	VUS	PoP	DEU	54	38	f	positive	segmental	n.a.
	SGCE	L-11614	c.936C>A	p.Asp312Glu	23.3	n.a.	yes	VUS	PoP	DEU	57	18	m	n.a.	generalized	n.a.
		L-12406	c.158C>T	p.Ser53Leu	23.4	0.000004	no	VUS	PoP	DEU	60	10	m	negative	focal	neck
		L-13044	c.277G>T	p.Gly93Cys	32.0	0.000012	yes	VUS	PoP	DEU	81	5	m	positive	myoclonus-dystonia	n.a.
		L-3624	c.391>G #	p.Ile131Val	18.0	0.000182	yes	VUS	PrP	DEU	20	3	f	n.a.	generalized	trunk, neck, limbs, tongue
		L-14626								DEU	41	33	m	n.a.	generalized	n.a.
PD patients	*TOR1A*	L-11950	c.741C>A	p.Asn247Lys	21.9	n.a.	yes	VUS	PoP	DEU	59	55	f	n.a.	no dystonia	
	KMT2B	L-11741	c.2822C>G	p.Ser941Cys	23.4	n.a.	yes	VUS	PoP	DEU	75	65	m	n.a.	no dystonia	
		L-11843	c.2843C>T	p.Thr948Ile	19.8	n.a.	yes	VUS	PoP	DEU	71	59	f	n.a.	no dystonia	
	GCH1	L-5282	c.202C>T	p.Leu68Phe	26.8	n.a.	yes	VUS	PoP	DEU	68	59	f	negative	no dystonia	
		L-7781	c.509+3A>G #		13.2	0.0000955	yes	VUS	PoP	DEU	82	n.a.	m	n.a.	no dystonia	
	SGCE	L-5503	c.1235C>T	p.Pro412Leu	21.4	n.a.	yes	VUS	PrP	DEU	70	56	f	n.a.	no dystonia	
		L-11375								DEU	74	43	m	positive	no dystonia	
		L-10938	c.1138A>T	p.Ile380Leu	24.2	n.a.	yes	VUS	PoP	DEU	56	54	f	n.a.	no dystonia	
		L-6057	c.391A>G #	p.Ile131Val	18.0	0.000182	yes	VUS	PrP	DEU	60	60	m	negative	no dystonia	
		L-8365								DEU	61	n.a.	m	n.a.	no dystonia	
		L-11924	c.502A>C	p.Asn168His	24.9	n.a.	yes	VUS	PoP	DEU	41	2	m	positive	no dystonia	

PD= Parkinson's disease, LP= likely pathogenic, P= pathogenic, PoP= possibly pathogenic, PrP= probably pathogenic, DP= definitely pathogenic, n.a.= not available, m= male, f= female; Novel variant means a variant that has not previously been reported in a dystonia patient. For the country of origin, the standard ISO Country code is listed. #variant found in both PD and dystonia patients

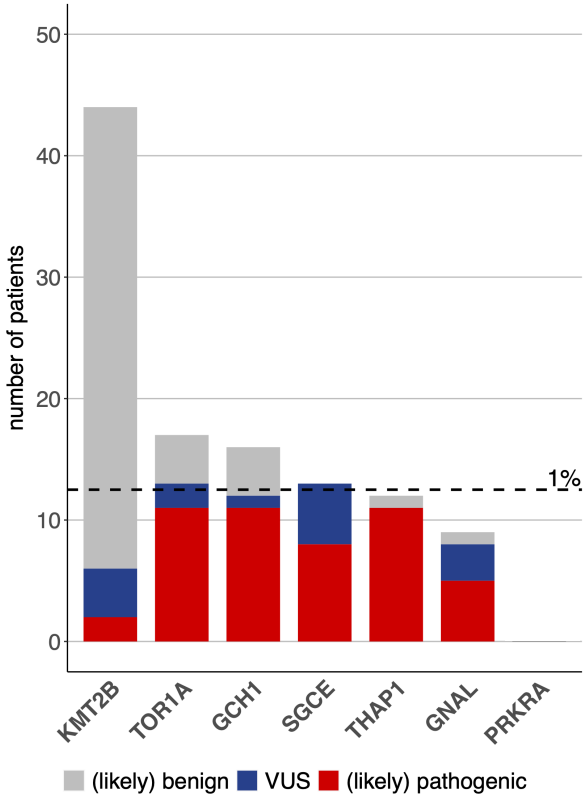

Figure III-1: Prevalence of rare variants in our dystonia sample (*n*=1,207) according to the predicted pathogenicity. The absolute number of identified carriers of rare (minor allele frequency [MAF] <0.005), variants in *TOR1A*, *SGCE*, *GCH1*, *THAP1*, *GNAL*, *KMT2B*, and *PRKRA* is displayed. VUS: variant of uncertain significance. Of note, for the four VUSs in *KMT2B*, testing of the episignature was not possible due to lack of DNA.

Family history was positive in 16/48 cases (33.3%, four unknown). 23 patients presented with generalized (23/48, 47.9%), eleven with multifocal or segmental (22.9%), six with focal dystonia (12.5%), eight with myoclonus-dystonia (16.7%, all *SGCE*-linked), and for one patient information was missing.

Specifically, ten patients originating from South Korea, Germany, and Spain that mainly presented with early-onset generalized dystonia carried the known dystonia-causing GAG deletion in *TOR1A*. Additionally, one previously reported patient with adult-onset cervical dystonia carried a 6-bp deletion (c.40_45delGCGCCG, p.Ala14_Pro15del) in *TOR1A* (Vulinovic et al., 2014). Pathogenic or likely pathogenic variants in *THAP1* were detected in eleven patients, including three recurrent variants (c.292G>T:p.Glu98*, c.62C>T:p.Ser21Phe, and c.71+2T>C) that were found in two unrelated patients each and are absent in gnomAD. Seven out of eleven DYT-THAP1 patients had generalized dystonia, and ten had an AAO below 18 years. For *GNAL*, five German patients with four different, not previously described, likely pathogenic variants were identified that all had adolescence to adulthood disease onset and presented with cervical dystonia

(focal in 2/5 patients). One *GNAL* variant occurred recurrently (c.1115T>G, p.Ile372Ser) in our dystonia patients but is absent from gnomAD controls. After pathogenicity scoring and functional evaluation (episignature) of rare *KMT2B* variants, two variants were classified as pathogenic (see below). For the combined dystonia-parkinsonism gene *GCH1*, eleven dystonia patients were found to carry (likely) pathogenic variants. Ten different, mainly missense variants were identified, of which one, not previously published variant, occurred in two German siblings with dopa-responsive dystonia (c.680C>T, p.Thr227Ile). The identified DYT-GCH1 patients presented with generalized (6/11) or segmental/multifocal (5/11) dystonia that started in childhood in most cases (9/11, information missing for one). In eight patients, the affected body sites at last examination included the neck. Six different variants were detected in *SGCE*, including a truncating variant (c.304C>T, p.Arg102*) in three independent German patients that is absent from control databases. All eight DYT-SGCE patients presented with myoclonus-dystonia.

The median AAO and percentage of patients with generalized dystonia for each genetic subtype are displayed in **Figure III-2**. In both our data and the MDSGene database, the latest median AAO was observed for DYT-GNAL patients (37.0 and 38.0 years, respectively), and DYT-SGCE patients had the earliest disease manifestations (4.5 and 4.0 years, respectively). In MDSGene, at least half of the patients with *TOR1A*, *THAP1*, *KMT2B*, and *GCH1* variants developed generalized dystonia, while in our data, percentages were even higher (for *TOR1A*, *THAP1*, and *GCH1*). Of note, comparison of *KMT2B* data is complicated by the extremely small sample size (n=2). In both data sets, *GNAL* mutation carriers rarely showed generalization (0% and 9.6%, respectively).

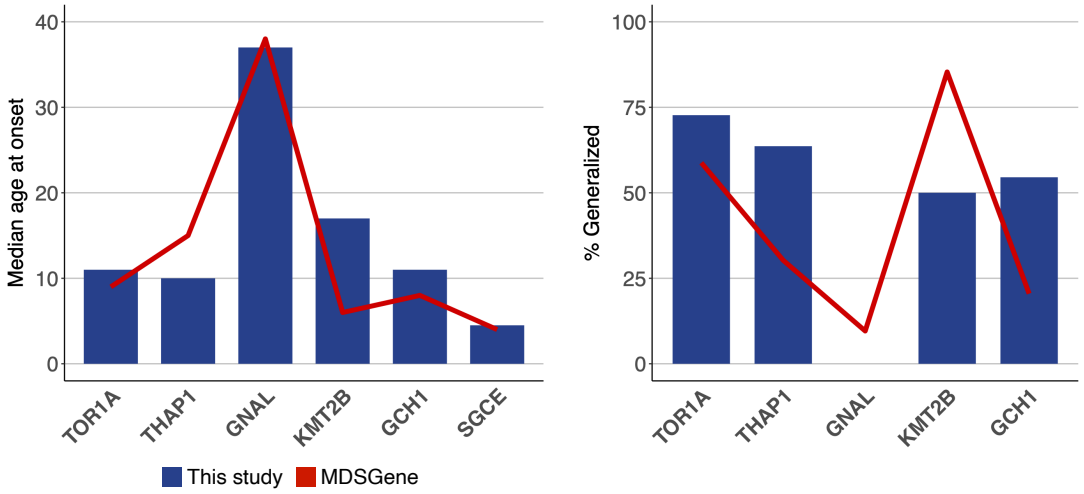

Figure III-2: Comparison of median age at onset (left) and percentage of patients with generalized dystonia (right) between the MDSGene data and our study. Of note, information on the body distribution of dystonia is usually not available for DYT-SGCE patients (only on the presence of myoclonus and dystonia), thus, *SGCE* is not displayed in the right panel.

Altogether, 16 of the here identified, presumably dystonia-causing variants have not previously been reported.

Among the PD patients, four different variants were classified as likely pathogenic, all in *GCH1* (**Table III-1**). The four PD patients had no sign of dystonia (current median age: 68.5 years, IQR: 61.75-70.5). Notably, two variants (c.4G>A:p.Glu2Lys and c.671A>G:p.Lys224Arg) were detected in both dystonia and PD patients in our sample.

Episignature analysis for *KMT2B* variants

To assess the functional effect of rare *KMT2B* variants, the DYT-KMT2B-specific methylation pattern ("episignature") in patients' blood was analyzed. Two of the 48 tested variants (c.3400C>T, p.Gln1134* and c.3568_3577delCTGAGTGTGC, p.Leu1190Serfs*162) were shown to result in strong hypermethylation and showed mean(z) and CV values characteristic of loss of KMT2B function (**Figure III-3,** Supplementary Table S3), which was interpreted as positive functional evidence during pathogenicity scoring. The p.Gln1134* variant (mean(z) = 4.18, CV = 2.06) was found in a 39-year-old German patient with a developmental disorder and dysmorphic features who developed multifocal dystonia at the age of 27 years. Family history was negative, but no family members were available to test if the variant arose *de novo*. The p.Leu1190Serfs*162 (mean(z) = 3.77, CV = 1.93) variant was found in a previously reported patient (Klein et al., 2019) with generalized dystonia and mild intellectual disability and occurred *de novo*. All missense and in-frame indel variants showed values indicative of benign variants. Repeating the calculation with I) all 113 sites of the published episignature (Mirza-Schreiber et al., 2022) or II) unaffected individuals as controls instead of DYT-SGCE patients yielded

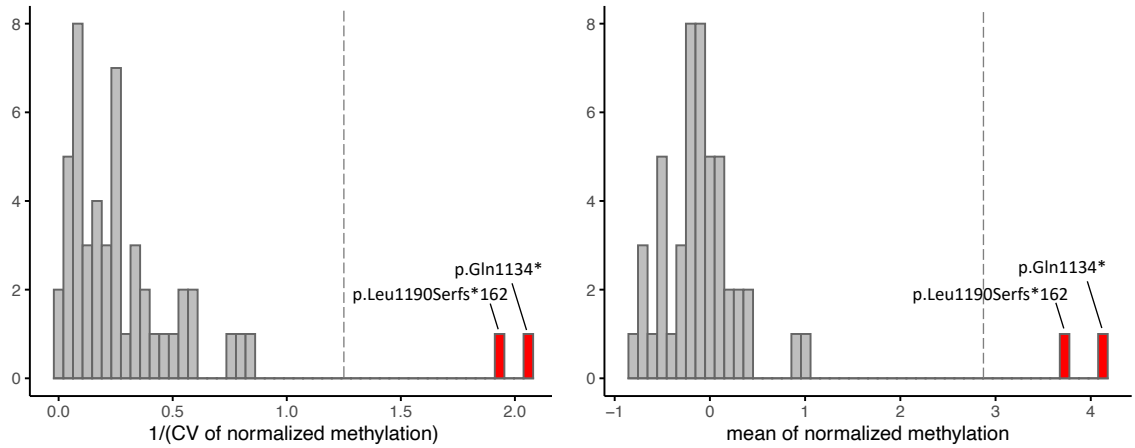

Figure III-3: Histogram of individual mean and coefficient of variation (CV) of the episignature's normalized methylation levels. The dashed vertical lines represent the maximum observed values in 162 non-*KMT2B* samples as described in Mirza-Schreiber et al. 2022. Only the two truncating variants show values indicative of a loss of KMT2B function.

comparable results, rating only the two abovementioned truncating variants as pathogenic (Supplementary Table S3). In total, 26 out of 48 tested *KMT2B* variants were reclassified through episignature analysis (mostly from VUS to likely benign). An overview of the results of testing of the episignature has also been added to the MDSGene website at https://www.mdsgene.org.

DISCUSSION

Here, we report on the role and frequency of variants in the isolated and combined dystonia genes *TOR1A*, *THAP1*, *GNAL, KMT2B, PRKRA, SGCE,* and *GCH1* in a large dystonia sample (*n*=1,207) as well as in a disease control group with PD (*n*=1,036). In total, 33 different presumably pathogenic variants were identified, one of which was only found in the disease control group (in *GCH1*) and two found in both PD and dystonia patients (in *GCH1*). Additionally, we report 21 rare VUS and 77 variants that were considered (likely) benign after careful evaluation. Among the dystonia patients, pathogenic variants in *TOR1A*, *THAP1*, and *GCH1* were most frequent (0.9% each), followed by *SGCE* (0.7%), *GNAL* (0.4%), and *KMT2B* (0.2%). We did not identify any carrier of a biallelic variant in the dystonia gene *PRKRA*, confirming that this genetic subtype is extremely rare (Lange et al., 2021), especially outside of Brazil, where most reported patients originate from and where the prevalence was estimated to be around 5% in isolated dystonia patients (Dos Santos et al., 2018).

The frequency of rare, presumably pathogenic variants was significantly higher in dystonia patients compared to PD patients, confirming the overall enrichment of variants in the investigated genes among dystonia patients and underlining their role as dystonia genes. A potential molecular diagnosis was established in 48/1207 (4.0%) dystonia patients. These patients had a median AAO of 12 years (IQR, 7-17), and 48% presented with generalized dystonia. Compared to the median AAO and body distribution in all dystonia patients (36 years (IQR, 21-49), 10% generalized dystonia), this confirms the observation that it is more likely to identify a monogenic cause in patients with an earlier AAO and generalized body distribution of symptoms (Zech et al., 2021b). An exome sequencing study (Zech et al., 2020) of smaller size (*n*=1,100, including 764 dystonia patients) identified diagnostic variants in 19% of included dystonia patients. However, most of these patients had additional neurological symptoms. Among isolated dystonia patients, the diagnostic yield was only 3.9%, comparable to the one in this study which mainly included isolated dystonia patients, underscoring that the diagnostic yield largely depends on the patient selection criteria. This is also reflected by the variable outcomes of other, smaller next-generation sequencing studies in dystonia (including 16-189 cases) with overall diagnostic yields of 11.7-37.5% (Gorcenco et al., 2020).

We identified 11 dystonia patients (0.9%) carrying two different variants in *TOR1A* that were classified as (likely) pathogenic (p.Glu303del, p.Ala14_Pro15del). While the pathogenic nature of the p.Ala14_Pro15del variant is supported by only one functional study (Vulinovic et al., 2014), numerous studies have proven the pathogenicity of the recurrent GAG deletion, mainly characterized by mislocalization of the mutant torsinA from the endoplasmic reticulum to the nuclear envelope and altered nuclear envelope morphology (Hettich et al., 2014). DYT-TOR1A is the most prevalent monogenic form of isolated dystonia, and, to date, at least 680 dystonia patients (about 98% of reported DYT-TOR1A patients) have been described to carry the p.Glu303del variant (Lange et al., 2021). The majority had childhood disease onset (about 70%) and developed generalized dystonia (about 60%). In this study, ten carriers were identified that mainly (8/10) presented with early-onset generalized dystonia (median AAO = 10.5 years, IQR: 9.25-12), in keeping with previous observations. Nevertheless, the phenotypic spectrum of reported variant carriers is broad, which is also reflected in our study, as two patients only developed focal dystonia affecting one leg or hand, respectively.

We identified eleven dystonia patients (0.9%) carrying eight presumably pathogenic variants in *THAP1*, three of which have not been reported before, including a nonsense mutation (p.Glu98*) that occurred in two unrelated Spanish dystonia patients but is absent from control databases. Another nonsense mutation (p.Glu97*) was previously described in seven unrelated dystonia patients (Camargo et al., 2014; da Silva-Junior et al., 2014), providing good evidence for the pathogenicity of this variant. The p.Ser21Phe variant was reported in two unrelated dystonia patients (da Silva-Junior et al., 2014; Paudel et al., 2016) and was also identified in two of our patients with generalized dystonia. Additionally, we found a novel missense variant at the same amino acid position (p.Ser21Ala) in one patient with generalized dystonia, suggesting that this variant also has a pathological role. Additionally, we report a novel splice site variant (c.71+2T>C), predicted to lead to a splice donor site loss by spliceAI (https://ci-spliceai.com/), that was detected in two independent patients with generalized dystonia. For two of five previously described variants, positive functional evidence was reported (p.His23Pro and p.Lys24Glu) (Lohmann et al., 2012) and was taken into account in the pathogenicity scoring. Mutations in *THAP1* are a cause of childhood- or adolescent-onset dystonia with a mixed phenotype (Lange et al., 2021), which is reflected in our DYT-THAP1 patients that have a median AAO of 10 years (IQR, 7.5-15.5) and show focal, segmental, and generalized body distributions of symptoms.

For *GNAL*, we identified five dystonia patients (0.4%) with four different variants that have not previously been described and are absent from control databases (p.Phe354_Leu355del,

p.Ile372Ser, p.Tyr422Leufs*3, p.Gly290Ser). Fitting with previous observations (Lange et al., 2021), these include missense as well as nonsense mutations, and patients mainly presented with adult-onset cervical dystonia. One variant occurred recurrently (c.1115T>G, p.Ile371Ser) in our patients, supporting its role in the development of dystonia. Notably, one of the variant carriers (L-11929) presented with generalized dystonia in combination with chorea, which has not been reported in *GNAL*-related disease before and might expand the phenotypic spectrum. Future functional tests will reveal if the here-identified, novel variants are indeed disease-causing.

Mutations in *KMT2B* as a cause of dystonia were first described in 2016/2017 (Meyer et al., 2017; Zech et al., 2016). Since then, at least 68 different, mainly truncating mutations have been described (Lange et al., 2021). As *KMT2B* is a large gene with 37 exons, we detected many rare variants and demonstrated that functionally evaluating their effect on DNA methylation is a powerful and important tool for interpretation. *KMT2B* encodes the lysine-specific histone methyltransferase 2B and thus links disordered chromatin states to the disease mechanism of dystonia. Since histone methylation is inversely correlated to CpG-methylation, DNA methylation analysis can be used to evaluate the effect of sequence variants. More specifically, loss of KMT2B function was found to result in hypermethylation at 113 specific CpG sites ("episignature") (Mirza-Schreiber et al., 2022). After episignature analysis, the majority of rare variants were reclassified as (likely) benign. Only the two truncating variants (p.Gln1134* and p.Leu1190Serfs*162) were shown to result in strong hypermethylation and showed mean(z) and CV values characteristic of loss of KMT2B function. Thus, the frequency of pathogenic *KMT2B* variants in our study (2/1207, 0.2%) is much lower than in a previous study (12/764, 1.6%) (Zech et al., 2020). This might be due to the fact that the here-investigated patients mainly had isolated, focal dystonia and that DYT-KMT2B is mostly generalized and often accompanied by additional features (Lange et al., 2021). In line with this, the two carriers of pathogenic *KMT2B* variants in this study presented with dystonia and a developmental disorder. However, it is also possible that the number of true pathogenic mutations would have been lower in previous studies if functional analysis had been performed. We propose that missense variants in particular should be functionally evaluated to allow correct variant interpretation.

Missense and truncating variants in *GCH1* are frequent causes of dopa-responsive dystonia. While about 70% of patients with pathogenic *GCH1* variants present with isolated dystonia, only about 10% of carriers have a pure parkinsonism phenotype (Weissbach et al., 2022), in accordance with the distribution in our study (11/15 isolated dystonia, 4/15 pure PD). The eleven dystonia patients with a presumably pathogenic *GCH1* variant carried two truncating and eight missense variants. Their median AAO of 11 years is slightly above the reported eight years, and the

prevalent occurrence of generalized or multifocal dystonia fits previous observations (Weissbach et al., 2022). Six of the identified, likely pathogenic variants were not previously reported, including a novel missense variant p.Thr227Ile found in two German siblings with childhood-onset dopa-responsive dystonia. Additionally, four PD patients were found to carry novel likely pathogenic *GCH1* variants, of which two (p.Glu2Lys and p.Lys224Arg) were also detected in a dystonia patient, suggesting that these variants may manifest as either PD or dystonia.

Variants in *SGCE* are mainly linked to childhood-onset dystonia in combination with myoclonus. In line with this, all eight of the here-identified carriers of (likely) pathogenic *SGCE* variants presented with myoclonus-dystonia, and the median age at onset was 4.5 years. As reported in the literature, the majority of the here detected variants (5/6) are predicted to have a truncating effect on the protein. This includes the p.Arg102* variant that was found in three independent German patients and is absent from control databases, supporting its role in the pathogenesis of myoclonus-dystonia.

Lastly, a total of 21 variants were classified as VUS (**Table III-2**) as they are not supported by enough evidence to consider them disease-causing. However, the identification of additional patients or functional testing may reclassify these variants and clarify their role in the development of dystonia.

One limitation of our study design using gene panel sequencing is that it captures only a limited number of genes and cannot be easily adjusted for novel discoveries. Presumably, our study's diagnostic yield would have been higher if newly discovered dystonia genes, e.g., *VPS16*, had been included. Notably, a subset of the patients (n=114) included here were screened for *VPS16* variants by Sanger sequencing, and indeed, one carrier of a clearly pathogenic, truncating variant was found (Pott et al., 2021). Other, more recently reported genes for isolated dystonia, such as *EIF2AK2* (Kuipers et al., 2021), *AOPEP* (Zech et al., 2022), and *EIF4A2* (Harrer et al., 2023b), or combined dystonia, such as *KCTD17* among many others (Keller Sarmiento & Mencacci, 2021; Mencacci et al., 2015), have not yet been targeted. The best way to screen dystonia patients comprehensively is to perform exome or even genome sequencing. Notably, these methods are much more expensive and are currently underway for a subset of the dystonia patients from DysTract.

For a rare disorder like dystonia, large screening efforts are inevitable to gain a deeper understanding of the underlying genetic architecture as well as the genotype-phenotype relationships; however, this has been rarely done, as sample sizes for this rare disorder are usually very small. We here screened >1,200 dystonia patients using targeted gene capture sequencing of

seven dystonia genes that enabled us to establish a presumptive molecular diagnosis in 4.0% of patients, most of whom had early-onset generalized dystonia, emphasizing that this patient group should be prioritized for genetic testing. Other patients in the DysTract and Dystonia Coalition sample were shown to be carriers of pathogenic variants in *ANO3* or *VPS16* by other means (Olschewski et al., 2019; Pott et al., 2021). We were able to confirm previously described pathogenic variants by providing additional patients and also discover novel, presumably dystonia-causing variants in the dystonia genes *TOR1A, THAP1, GNAL, KMT2B, GCH1,* and *SGCE*, thus, expanding their mutational spectrum. In addition, application of the episignature analysis for *KMT2B* variants demonstrated the importance of functional studies for the interpretation of sequence variants and provides meaningful interpretation for almost 50 such variants. Thus, this large-scale dataset and the functional evaluation of *KMT2B* variants will guide future variant interpretation.

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3.4 Objective IV: Exome Sequencing of *n*=1,924 Dystonia Patients

While gene panel sequencing has the advantages of being relatively cost-effective and covering a custom selection of genes considered most relevant for the disease in question, it has important shortcomings – most notably, that it cannot be easily adapted to new gene discoveries, especially not retrospectively, and therefore cannot be reanalyzed over time. A method that overcomes these limitations, while still being cost-effective compared to other techniques, is exome sequencing, in which the coding regions of the genome are sequenced (Kernohan & Boycott, 2024).

While some genetic dystonia forms are relatively common (e.g., DYT-TOR1A or DYT-THAP1) and are effectively identified by dystonia panels, most forms are extremely rare. Moreover, a large number of genes are now associated with dystonia, and the list of new candidate genes is constantly growing. Exome sequencing is therefore likely the most effective strategy to arrive at a genetic diagnosis for patients. Therefore, exome sequencing was performed in a large sample of dystonia patients (*n*=1,924 after quality control), including about 550 patients unresolved after gene panel sequencing (see Objective III), in order to increase the diagnostic yield and analyze the prevalence of pathogenic variants and associated phenotypes across the exome. To this end, a systematic literature search was conducted to generate a list of all reported dystonia-related genes (*n*=406), and rare variants in these genes were evaluated in detail.

The study covering this objective is titled “Genetic Diversity and Expanded Phenotypes in Dystonia: Insights from Large-Scale Exome Sequencing” and has been accepted in *Annals of Clinical and Translational Neurology*. Mirja Thomsen conducted the literature search to compile the list of dystonia-related genes. Following data pre-processing by Fabian Ott, she extracted, filtered, and evaluated all rare genetic variants in these genes and carried out the normalization and interpretation of the methylation data. She performed part of the wet-lab work for variant validation and supervised the rest, including the co-supervision Bachelor’s and Master’s students. All statistical analyses, as well as the preparation of tables and figures, were carried out by her. The first draft of the manuscript was written by Mirja Thomsen and revised by Katja Lohmann.

This study comprehensively examined the genetic landscape in a large dystonia sample, representing the most extensive next-generation sequencing study conducted in dystonia at that time. Despite pre-screening for many known variants, a molecular diagnosis was established in 8.1% of index patients. The strongest predictors of a genetic diagnosis were the presence of generalized dystonia and age at onset. This work highlights the utility of exome sequencing, provides further evidence for several candidate genes, and extends the phenotypic spectrum of some genes to include prominent, sometimes isolated dystonia.

Genetic Diversity and Expanded Phenotypes in Dystonia: Insights from Large-Scale Exome Sequencing

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ABSTRACT

Objective: Dystonia is one of the most prevalent movement disorders, characterized by significant clinical and etiological heterogeneity. Despite considerable heritability (~25%), the etiology in most patients remains elusive. Moreover, understanding correlations between clinical manifestation and genetic variants has become increasingly complex.

Methods: Exome sequencing was conducted on 1,924 genetically unsolved, mainly late-onset isolated dystonia patients, recruited primarily from two dystonia registries (DysTract and the Dystonia Coalition). Rare variants in genes previously linked to dystonia (*n*=406) were examined, confirmed via Sanger sequencing, and analyzed for segregation when possible.

Results: We identified 137 distinct likely pathogenic/ pathogenic variants (according to ACMG criteria) across 51 genes in 163/1,924 patients, including 153/1,895 index patients (diagnostic yield 8.1%). The strongest predictors of a genetic diagnosis were generalized dystonia (28.6% yield) and age at onset (20.4% yield in patients with onset <30 years). Notably, 56.2% of these variants were novel, with recurrent variants in *EIF2AK2*, *VPS16*, *KCNMA1*, and *SLC2A1*. Additionally, 321 index patients (16.9%) harbored variants of uncertain significance in 102 genes. The most frequently implicated genes included *VPS16*, *THAP1*, *GCH1*, *SGCE*, *GNAL*, and *KMT2B*. Presumably pathogenic variants in less well-established dystonia genes were also found, including *KCNMA1*, *KIF1A*, and *ZMYND11*. At least six variants (in *ADCY5*, *GNB1*, *IR2BPL, KCNN2*, *KMT2B*, and *VPS16*) occurred *de novo,* supporting pathogenicity.

Interpretation: This study provides valuable insights into the genetic landscape of dystonia, underscores the utility of exome sequencing for diagnosis, substantiates several candidate genes,

and expands the phenotypic spectrum of some genes to include prominent, sometimes isolated dystonia.

INTRODUCTION

Dystonia is one of the most prevalent movement disorders, characterized by involuntary muscle contractions leading to abnormal postures and repetitive movements (Albanese et al., 2013). Its clinical manifestations span a broad spectrum, from isolated cases to those combined with other movement disorders like parkinsonism, myoclonus, or chorea, to patients with additional neurological or systemic features (Albanese et al., 2013).

An essential aspect of establishing a dystonia diagnosis and appropriate treatment lies in genetic testing. However, despite the disease's considerable heritability, with about 25% of patients having affected relatives (Waddy et al., 1991), and the identification of several causal genes, a significant proportion of patients remain genetically unsolved, particularly those with later-onset focal dystonia - the most common form observed in epidemiological studies (Charlesworth et al., 2013; Zech et al., 2020). Genetic testing is complicated by the highly heterogeneous nature of the disease (Ahn et al., 2023; Powis et al., 2020; Zech et al., 2020), the ever-expanding list of dystonia-linked genes, and the reliance on clinical diagnosis due to the absence of an established biomarker. Presently, the Movement Disorder Society Task Force for the Nomenclature of Genetic Movement Disorders (MDS Nomenclature Task Force) recognizes 53 established dystonia genes (Lange et al., 2022). Additionally, numerous genes have been linked to dystonia as a feature of other neurological disorders. With ongoing sequencing efforts, the list of unconfirmed dystonia candidate genes continues to grow, with many awaiting replication by independent studies (Atasu et al., 2024; Keller Sarmiento & Mencacci, 2021; Lange et al., 2022; Zech et al., 2020), altogether resulting in over 400 potential dystonia-linked genes (Thomsen et al., 2024a).

The phenotypic presentations of dystonia are diverse, with considerable overlap between the different genetic forms (Lange et al., 2021; Thomsen et al., 2023). Instances of classical phenotypes expanding over time or a single gene causing multiple distinct phenotypes (pleiotropy) further highlight this complexity. In this regard, an increasing number of genes, previously associated with other neurological conditions, including developmental delay, epileptic encephalopathy, and ataxia, are now recognized to play a significant role in patients with prominent, sometimes isolated dystonia (Keller Sarmiento & Mencacci, 2021; Svorenova et al., 2022).

As the phenotypic and mutational spectrum of dystonia expands, newly identified genetic variants require thorough evaluation to establish a causal gene-disease link. Guidelines from the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015), incorporating information from segregation analysis, *in silico* predictions, and population databases, among others, are instrumental in this task. For some genes, specific functional assays have been developed to assess the impact of variants on protein function, such as aberrant CpG methylation serving as a functional readout for evaluating variants in the dystonia gene *KMT2B* (Mirza-Schreiber et al., 2022). The most compelling evidence for novel dystonia genes, however, will always come from replicating the findings in independent patients.

Given dystonia's rarity and genetic complexity, large cohorts are indispensable for comprehensively unraveling its genetic spectrum. To achieve this, we performed exome sequencing on 1,924 dystonia patients. In contrast to other dystonia cohorts typically enriched with early-onset, symptom-complex patients, this cohort is unique in that it predominantly consists of adult-onset isolated dystonia patients, corresponding to the composition of patients seen in epidemiological studies. We describe detailed phenotypic and genetic findings in over 400 genes previously linked to dystonia, with the aim of facilitating future data aggregation, advancing clinical variant interpretation, improving genotype-phenotype correlations, and contributing to the validation of emerging dystonia genes and their associated phenotypes.

METHODS

Study population

A total of 1,967 samples were included in the exome sequencing study, comprising 1,950 dystonia patients and 17 unaffected family members. One sample failed sequencing, and 25 samples were removed after relationship analysis (Supplementary Methods) revealed that those sample IDs referred to identical patients recruited twice from different sites. Altogether, this yielded a total number of 1,924 patients with dystonia without a previous genetic diagnosis who were included in the final cohort.

The vast majority of samples were recruited in the framework of two large dystonia registries, DysTract (https://www.isms.uni-luebeck.de/en/research/dystract/) and the Dystonia Coalition (https://www.dystoniacoalition.org/). These samples had undergone genetic pre-screening, comprising: 1) Hot-spot screening for known pathogenic variants in dystonia genes in about 80% of the samples using the Global Screening Array (GSA V01, Illumina; with custom content) and 2) Gene panel sequencing in about a third of the samples, comprising the genes *ANO3*, *GCH1*, *GNAL*, *KMT2B*, *PRKR*A, *SGCE*, *THAP1*, and *TOR1A* (results published in Olschewski et al.,

2019 and Thomsen et al., 2024b). Patients with negative pre-screening, early disease onset, positive family history, or multisite involvement were prioritized when selecting samples from the dystonia registries. Patient samples were collected in movement disorder clinics across Europe, North America, and Australia. Additionally, 72 samples from patients of Asian ethnicity (primarily from Malaysia) were included. The total sample consisted of diverse ethnic backgrounds, including White (*n*=1,787), Asian (*n*=72), African American (*n*=32), Latino (*n*=18), Ashkenazi Jewish (*n*=7), American Indian (*n*=5), and Mixed (*n*=3) individuals. Patients were phenotyped by movement disorder specialists, and secondary causes, including acquired dystonia, were excluded. Written informed consent was obtained from all participants prior to genetic testing, and the study was approved by the ethics committee at the University of Lübeck (04-180).

Sequencing and data processing

Genomic DNA was extracted from peripheral blood samples, and exome sequencing was performed at the Competence Centre for Genomic Analysis in Kiel, Germany, using Illumina NovaSeq. Sequencing reads were preprocessed and aligned to the Hg38 reference genome. Variant calling and annotation were conducted using DeepVariant, GLnexus, and VEP, with further details provided in the Supplementary Methods.

Variant filtering

A comprehensive list of dystonia-linked genes was generated by systematically querying the PubMed database (search term: dystoni* [TITLE/ABSTRACT] AND (gene* OR genetic* OR mutation* OR mutated OR varia*) AND "english"[Language]) until December 2024, resulting in 406 distinct genes that have been linked to dystonic symptoms in the literature (Supplementary Table 1). We utilized this phenotype-driven candidate gene list to identify genetic causes in our dystonia sample.

We filtered out variants with the following criteria: (a) low variant allele frequency (<20%), (b) low sequencing depth (<10 reads), (c) synonymous variants, (d) minor allele frequency >0.05% in the gnomAD population database, (e) variants classified as benign or likely benign in ClinVar, and (f) single heterozygous variants in recessive disease genes. Variants with an allele frequency of 0.25-0.75 were considered heterozygous. For established dystonia genes that have been assigned a DYT prefix by the MDS Nomenclature Task Force (Lange et al., 2022), the remaining variants underwent detailed individual evaluation using ACMG standards and guidelines (Richards et al., 2015). For genes not classified as established dystonia genes, additional filtering was applied before individual evaluation by ACMG standards: excluding single heterozygous loss-of-function (LOF) variants in genes with good tolerance for such variants (gnomAD pLI-

score = 0), missense variants classified as likely benign by the alpha missense score (Cheng et al., 2023), and variants with a low CADD Score (<10) (Rentzsch et al., 2019).

All likely pathogenic or pathogenic variants were considered disease-causing and underwent validation through Sanger sequencing. Segregation analysis was performed if family members' DNA was available.

Relevant literature was reviewed to assess phenotypic and genetic information for each gene. Phenotype-genotype relationships were categorized as consistent, partially consistent, quite inconsistent, or unclear/unknown. The unclear/unknown category applied to cases with limited clinical information or candidate genes lacking an established gene-phenotype relationship.

Statistical analysis

Statistical analysis and visualization were performed in R version 4.3.2 using base R functions for statistical tests and data manipulation, the pROC package for generating and analyzing ROC curves, and the ggplot2 package for visualizations. For calculating diagnostic yield, patients were grouped based on clinical criteria, including age at onset (AAO: <30 years, 30-50 years, and >50 years), dystonia subtypes (focal, segmental/multifocal, generalized), family history (positive or negative), and the presence of additional features versus isolated dystonia. These groupings, except AAO, were also used for ROC analysis and statistical comparisons using Fisher's exact test. AAO was treated as a continuous variable for the Mann-Whitney U test comparing resolved and unresolved patients and ROC curve analysis. This approach was chosen to capture clinically relevant differences. Missing data were handled by excluding these patients. All statistical tests were two-sided, and a p-value < 0.05 was considered statistically significant.

Episignature analysis for *KMT2B* variants

For patients with rare variants in *KMT2B* and a sufficient amount of DNA available (n=45), the functional effect was assessed by analyzing the disease-specific methylation pattern ("episignature") in peripheral blood, using the Illumina MethylationEPIC BeadChip. The mean of the normalized methylation levels (mean(z)) and the coefficient of variation (CV = SD/ |mean|) were used as quantifiers (Supplementary Methods).

RESULTS

Patient characteristics and diagnostic yield

There were 1,895 index patients and 29 affected family members among the 1,924 included unique dystonia patients. Key patient characteristics are summarized in **Table IV-1**. Over half of the patients (54.3%) had focal dystonia, with cervical dystonia being the most common subtype

(n=550, 28.6%). About 93% of all patients had isolated dystonia, while in 130 patients (6.8%), additional neurological (other than tremor) or systemic features were reported, such as bradykinesia (*n*=41), ataxia (*n*=35), myoclonus (*n*=35), or neurodevelopmental features (*n*=12).

Table IV-1 Patient characteristics in the investigated dystonia sample by diagnostic outcome

	All patients	**Patients with diagnostic variant**	**Patients with VUS**	**Yet-unsolved patients**
Total patients	1,924	163	323	1,438
Index patients	1,895	153 (8.1%)	321 (16.9%)	1,421 (75.0%)
Age at examination	54 years (43-65)	46 years (32.5-59.5)	55 years (45-65)	55 years (44-66)
Age at onset (median, IQR)	33 years (22.5-43.5)	19 years (5.25-32.75)	34 years (24-44)	34 years (24-44)
Sex				
Males	776 (40.3%)	70 (42.9%)	121 (37.5%)	585 (40.7%)
Females	1,148 (59.7%)	93 (57.1%)	202 (65.5%)	853 (59.3%)
Family history[a]				
Positive	561 (29.6%)	51 (33.3%)	73 (22.7%)	437 (30.9%)
Negative	1,233 (65.1%)	93 (60.8%)	187 (58.3%)	953 (66.9%)
NA	101 (5.3%)	9 (5.9%)	61 (19.0%)	31 (2.2%)
Type of dystonia				
Generalized	236 (12.3%)	73 (44.8%)	30 (9.3%)	133 (9.2%)
Segmental/multifocal	549 (28.5%)	36 (22.1%)	92 (28.5%)	421 (29.3%)
Focal	1,045 (54.3%)	53 (32.5%)	193 (59.8%)	799 (55.6%)
NA	94 (4.9%)	1 (0.6%)	8 (2.5%)	85 (5.9%)
Isolated dystonia	1,794 (93.2%)	112 (68.7%)	308 (95.4%)	1,374 (95.5%)
Additional neurological features reported (other than tremor)	130 (6.8%)	51 (31.3%)	15 (4.6%)	64 (4.5%)

IQR: interquartile range, NA: information not available, VUS: variant of uncertain significance
[a]calculated for index patients only

Exome sequencing revealed likely pathogenic or pathogenic variants in dystonia-linked genes in 163 patients, including 153 out of 1,895 index patients (8.1% diagnostic yield), with an additional 321 index patients (16.9%) having variants of uncertain significance (VUS) in dystonia-linked genes (**Figure IV-1**, **Figure IV-2**, and **Figure IV-3**, Supplementary Table 2 and 3).

Compared to unsolved patients and those with VUS combined, patients with a genetic diagnosis had a significantly earlier AAO (Mann-Whitney U Test, $p<0.0001$), higher prevalence of generalized dystonia (Fisher's exact test, $p<0.0001$), positive family history (Fisher's exact test, $p=0.043$), and additional clinical features (Fisher's exact test, $p<0.0001$). There were no significant differences between patients with VUS and unsolved patients regarding these characteristics.

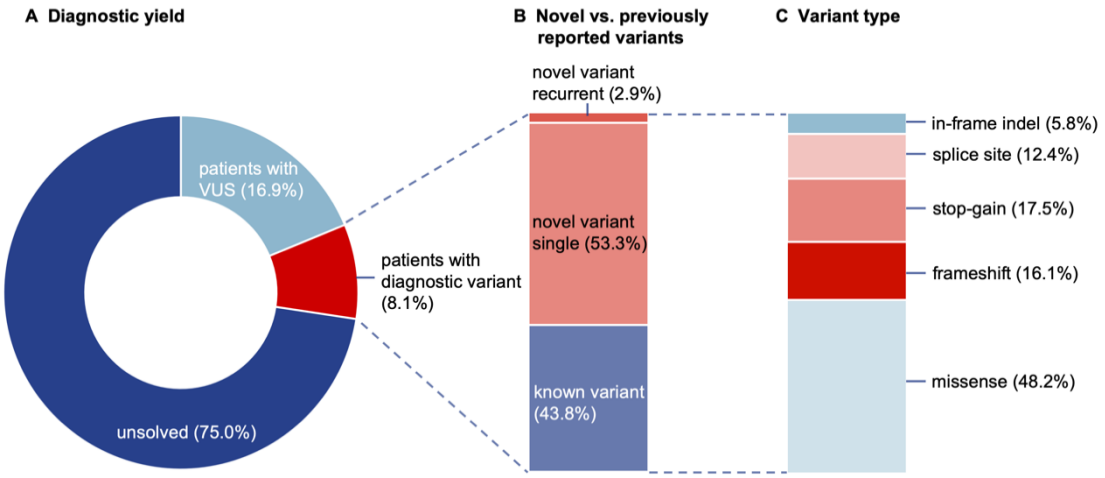


Figure IV-1. Overview of exome sequencing results. (A) Proportion of index patients for whom a diagnostic variant was identified, who carry a variant of uncertain significance (VUS), and who remain unsolved after searching for pathogenic variants in dystonia-linked genes. **(B)** Proportion of diagnostic variants that were previously reported (known variant), not previously reported and detected in a single patient or pedigree (novel variant single), and not previously reported and found recurrently in at least two unrelated dystonia patients (novel variant recurrent). **(C)** Distribution of diagnostic variants by variant type.

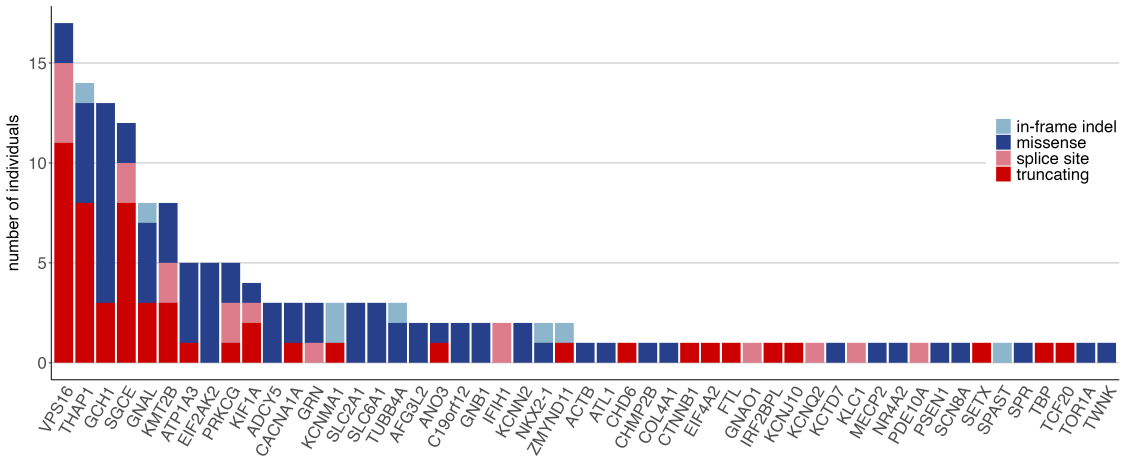


Figure IV-2. Genetic landscape in our dystonia sample (*n*=1,895 index patients) identified by exome sequencing. The number of individuals harboring a presumably pathogenic variant in genes previously linked to dystonia is shown, representing 51 distinct genetic forms and a total diagnostic yield of 8.1%. Truncating variants include stop-gain and frameshift variants.

The diagnostic yield varied substantially across index patient groups, with the highest yield in patients with additional features (36.3%), followed by those with generalized dystonia (29.3%) and those with an AAO < 30 years (20.4%) (**Figure IV-4A**). ROC curves were generated to evaluate the predictive performance of four variables—AAO, positive family history, generalized dystonia, and additional features—along with a combined model incorporating all four variables (**Figure IV-4B**). The area under the curve (AUC), which measures predictive accuracy, was 0.68

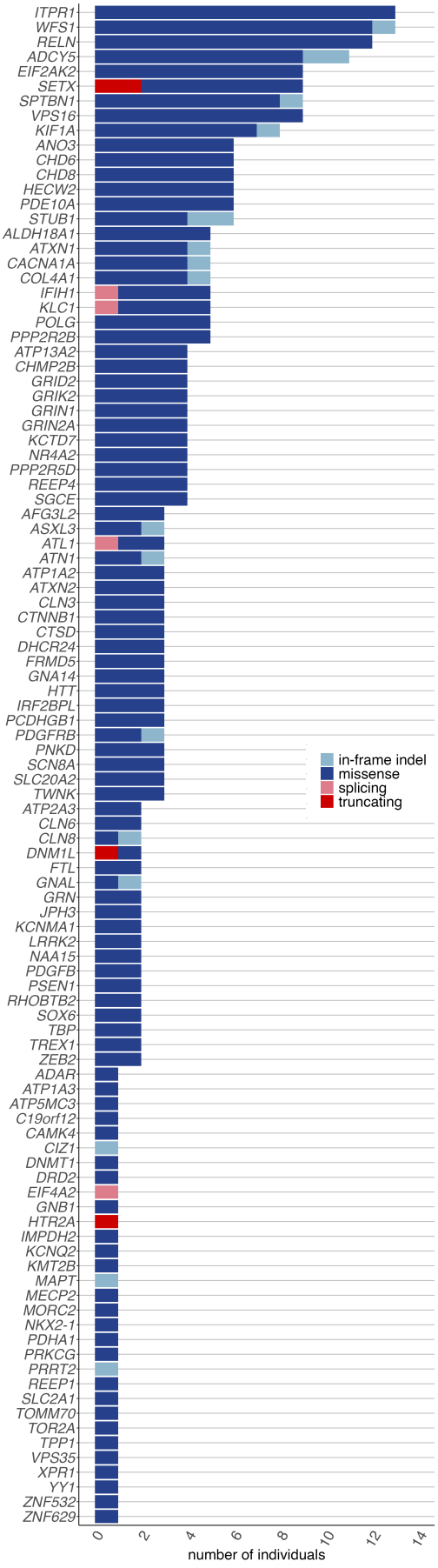


Figure IV-3. Variants of uncertain significance in our dystonia sample (*n*=1,895 index patients) identified by exome sequencing. The number of individuals harboring variants of uncertain significance in genes previously linked to dystonia is shown. This represents 329 distinct variants in 102 genes found in 321 index patients (16.9%). Truncating variants include stop-gain and frameshift variants.

for AAO, 0.67 for generalized dystonia, 0.62 for additional features, and 0.53 for family history. The combined model, incorporating all four predictors, achieved an AUC of 0.76, indicating the best predictive performance, with AAO and generalized dystonia being the strongest individual predictors.

Genetic findings

Among the index patients with a genetic diagnosis (*n*=153, 8.1%), 137 distinct variants were identified in 51 genes previously linked to dystonic syndromes (Supplementary Table 2**, Figure IV-2**). The majority (131/137, 95.6%) of variants were found in the heterozygous state in genes known to be linked to dominant inheritance. One variant in *SLC6A1* was identified in the mosaic state (variant allele frequency = 0.2 and clearly reduced peak in Sanger sequences). One variant (in *MECP2*) was found in the hemizygous state in a male patient, associated with X-linked inheritance, and four variants (two in *GCH1* and one each in *SETX* and *SPR*) were biallelic and linked to autosomal recessive disorders. At least six variants occurred *de novo* (in *ADCY5*, *GNB1*, *IR2BPL, KCNN2*, *KMT2B*, and *VPS16*) (**Supplementary Figure**

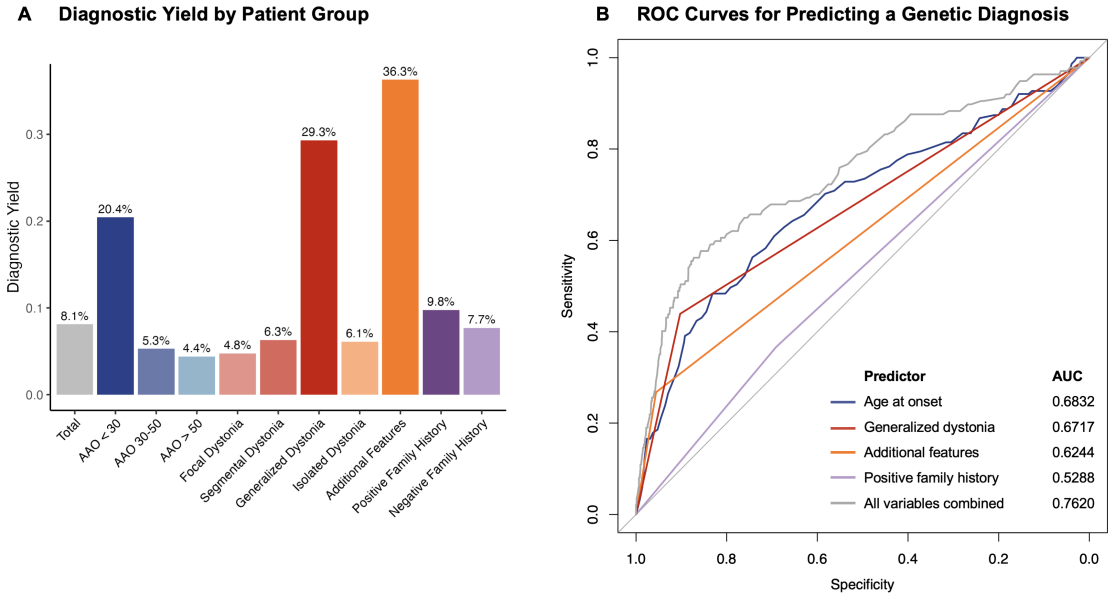
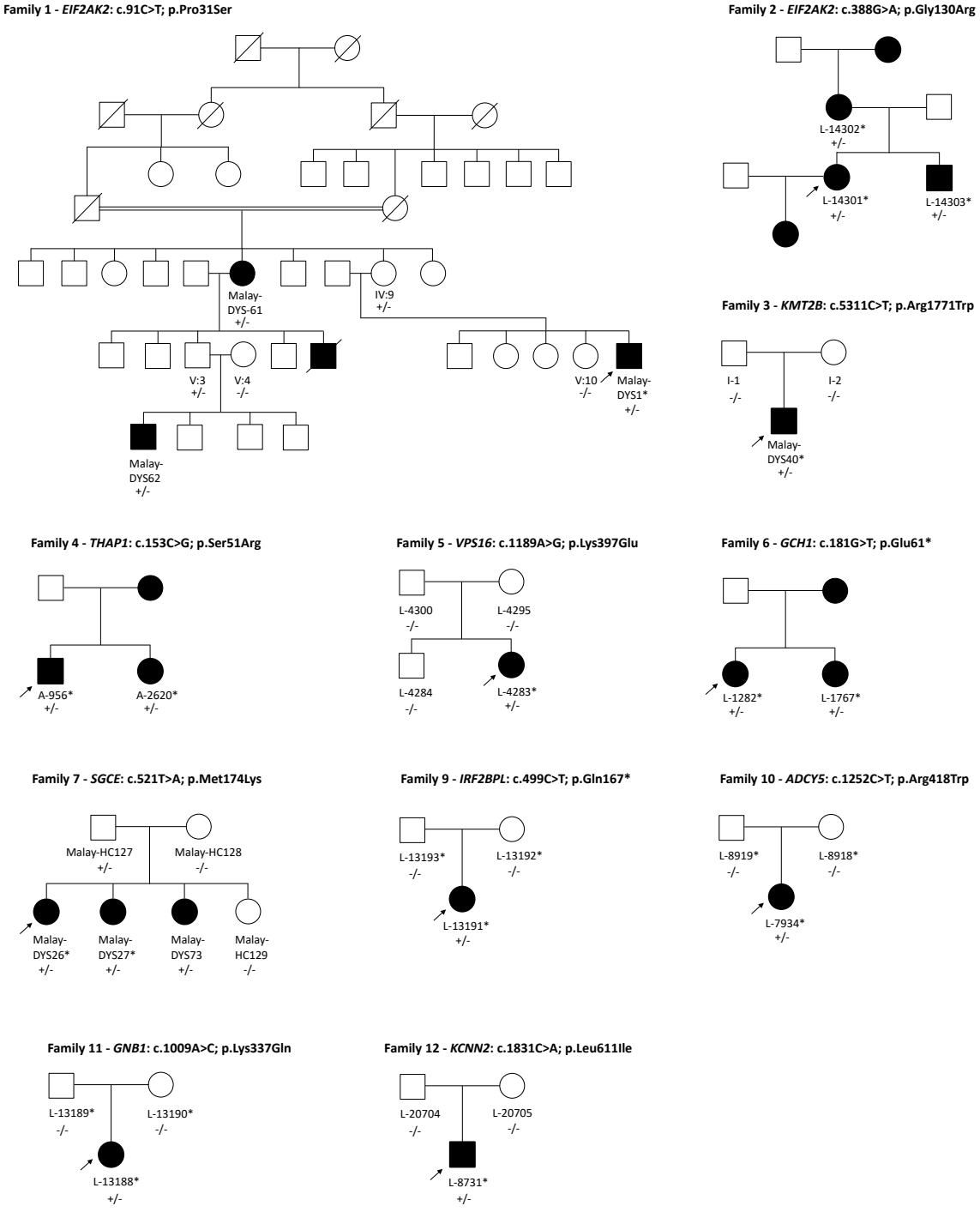


Figure IV-4. Diagnostic yield across patient groups and predictive performance of clinical factors for genetic diagnosis. (A) Bar plot showing the diagnostic yield (%) across different patient groups, considering index patients only. The segmental dystonia group includes patients with multifocal dystonia. **(B)** ROC curves illustrating the performance of individual predictors and a combined model for genetic diagnosis. The predictors include age at onset, generalized dystonia, additional features, positive family history, and a combined model incorporating all four predictors. Each curve shows the balance between sensitivity (true positive rate) and 1-specificity (false positive rate), with the Area Under the Curve (AUC) indicating predictive performance.

IV-S1). Over half of the identified presumably pathogenic variants (77/137; 56.2%) were not previously reported in ClinVar or MDSGene. Among these, four recurred in at least two unrelated index patients (in *EIF2AK2*, *KCNMA1*, *SLC2A1*, and *VPS16*; see below), supporting pathogenicity. Details about the variants, pathogenicity scoring, and clinical information can be found in Supplementary Table 2.

A wide spectrum of genetic causes was identified, encompassing established dystonia genes as defined by the MDS Nomenclature Task Force in 103 out of 163 diagnoses, movement disorder or developmental delay genes that frequently include dystonia as a phenotype (9/163 and 4/163 diagnoses, respectively), genes typically linked to other neurological disorders in which dystonia has been rarely reported (41/163 diagnoses), and dystonia candidate genes (7/163 diagnoses) (Supplementary Table 2). Genotype-phenotype relationships were consistent with the literature in most patients (111/163; 68.1%), partially consistent in 14 (8.6%), quite inconsistent in 25 (15.3%), and unclear/unknown in 14 (8.6%) (Supplementary Table 2). Notably, all variants in established dystonia genes demonstrated at least partial genotype-phenotype consistency.



Supplementary Figure IV-S1. Pedigrees of families in which exome sequencing identified a disease-causing variant, and DNA from family members was available for segregation analysis. Squares represent males, and circles represent females. Filled symbols indicate individuals with dystonia, and individuals with a diagonal line through their symbol are deceased. Index patients are marked with arrows. Mutation status is indicated as follows: "+" denotes the pathogenic variant, while "–" denotes the wildtype allele. Individuals marked with an asterisk underwent exome sequencing, whereas those with an ID without an asterisk were tested for the respective variant only by Sanger sequencing.

Meanwhile, cases classified as "quite inconsistent" were mostly focal (*n*=15) or segmental/multifocal (*n*=8) dystonia and involved variants in genes typically associated with other disorders where dystonia is rarely reported, such as *C19orf12*, *GRN*, *KIF1A*, and *PRKCG*.

Findings in established dystonia genes

The most frequently implicated genes included the well-established isolated and combined dystonia genes *VPS16* (*n*=17 index patients), *THAP1* (*n*=14), *GCH1* (*n*=13), *SGCE* (*n*=12), *GNAL* (*n*=8), and *KMT2B* (*n*=8). Other dystonia genes with likely pathogenic or pathogenic variants were *EIF2AK2* (*n*=5 index patients), *ANO3* (*n*=2), *EIF4A2* (*n*=1), and *TOR1A* (*n*=1) for genes causing isolated dystonia. Of these, the genes *VPS16*, *EIF2AK2*, and *EIF4A2*, which were relatively recently associated with dystonia, were not included in the pre-screening process. The frequencies of these genetic forms in our dystonia sample were 0.9% (17/1,895 index patients) for *VPS16*, 0.3% (5/1,895) for *EIF2AK2* and 0.05% (1/1,895) for *EIF4A2*. Additionally, likely pathogenic or pathogenic variants were found in *ATP1A3* (*n*=5) and *GNAO1* (*n*=1) for genes causing combined dystonia, as well as *ACTB* (*n*=1), *IRF2BPL* (*n*=1), *SPR* (*n*=1), and *TUBB4A* (*n*=3) for genes linked to dystonia with other neurological or systemic features (Supplementary Table 2). Notably, 43 out of 84 variants (51.2%) in established dystonia genes were not previously reported, including two novel recurrent variants (*EIF2AK2*:p.Pro31Ser and *VPS16*:p.Ile484Thrfs*70).

For six variants, segregation analysis revealed that the identified variant co-segregated with the dystonia phenotype in the affected families, supporting its pathogenic role (*EIF2AK2*:p.Pro31Ser, *EIF2AK2*:p.Gly130Arg, *THAP1*:p.Ser51Arg, *GCH1*:p.Glu61*, *SGCE*:p.Met174Lys, *SGCE*:c.233-1G>A:p.?) (**Supplementary Figure IV-S1**). Additionally, three variants were confirmed to be *de novo* (*KMT2B*:p.Arg1771Trp, *VPS16*:p.Lys397Glu, and *IRF2BPL*:p.Gln167*).

Of note, for several established dystonia genes, no carrier of a disease-causing variant was identified in our large patient group (e.g., in *AOPEP*, *HPCA*, *PRKRA*, and *TH*).

Interestingly, one patient with early-onset generalized dystonia harbored two known likely pathogenic variants in different genes (*TOR1A*:p.Arg288Gln and *SPAST*:p.Glu418del).

For 32 rare variants in *KMT2B*, identified in 45 patients, the disease-specific methylation pattern (“episignature”) in patients’ blood was analyzed to assess their functional effect. Two of the tested variants were shown to result in strong hypermethylation and showed mean(z) and CV values characteristic of loss of KMT2B function (Supplementary Table 4), which was interpreted as

positive functional evidence during pathogenicity scoring. Notably, these two variants were canonical splice site variants (c.4779+1G>A and c.3789+1G>A), not previously reported, and associated with childhood-onset generalized dystonia (Supplementary Table 2). Family history was negative in both patients; however, family members were not available to test whether the variants arose *de novo*.

Findings in movement disorder genes that frequently include dystonia

Variants in movement disorder genes frequently comprising dystonia as a phenotype included recurrent variants in *ADCY5*, *C19orf12*, and *SLC2A1*. Two unrelated patients with early-onset mixed movement disorders including generalized dystonia carried the same pathogenic variant in *ADCY5* (p.Arg418Trp), proven *de novo* in one case. A known missense variant in *C19orf12* (p.Gly58Arg) was found in two unrelated patients with adult-onset focal dystonia (cervical dystonia and blepharospasm), both without additional features. Two unrelated patients with adult-onset cervical dystonia, one also involving the upper limbs and shoulders, carried a novel *SLC2A1* variant (p.Gln25Lys). Additionally, a known pathogenic variant in *FTL* (p.Glu58*) was identified in a patient with isolated adult-onset cervical dystonia.

Findings in neurodevelopmental delay genes that frequently include dystonia

Presumably pathogenic variants in neurodevelopmental disorder genes that frequently include dystonia as a phenotype comprised a novel nonsense variant in *CTNNB1* (p.Gln4*) in a patient with isolated cervical dystonia, a recurrent *de novo* missense variant in *GNB1* (p.Lys337Gln) in two unrelated patients with infancy-onset generalized dystonia - one of whom had a complex phenotype including global developmental delay, hypotonia, myoclonus, and vertical supranuclear gaze palsy, and was recently published (Reyes et al., 2023) - and a known hemizygous missense variant in *MECP2* (p.Arg97Cys) in a male patient with cervical and truncal dystonia, along with developmental delay (Supplementary Table 2).

Findings in genes usually linked to non-dystonia phenotypes

We identified 41 dystonia patients with likely pathogenic or pathogenic variants in 23 uncommonly dystonia-linked genes. Of these, seven showed consistent genotype-phenotype relationships, four partial consistency, 21 were quite inconsistent, and nine were unclear/unknown (Supplementary Table 2). While 13 variants had been reported in the context of other phenotypes, 24 were previously undescribed.

Genes with pathogenic variants in several affected individuals included genes usually associated with spinocerebellar ataxia (*PRKCG* (*n*=5), *CACNA1A* (*n*=3), and *AFG3L2* (*n*=2)), spastic

paraplegia (*KIF1A* (*n*=4)), frontotemporal dementia (*GRN* (*n*=3)), paroxysmal movement disorders (*KCNMA1* (*n*=3)), and others *(SLC6A1* (*n*=3), *IFIH1* (*n*=2), and *NKX2-1* (*n*=2)), while only one carrier of a presumably pathogenic variant was found in the remaining 14 uncommon dystonia genes (in *ATL1*, *CHMP2B*, *COL4A1*, *KCNJ10*, *KCNQ2*, *KCTD7*, *PDE10*, *PSEN1*, *SCN8A*, *SETX*, *SPAST*, *TBP*, *TCF20*, and *TWNK*).

Notably, four variants were recurrently found in at least two unrelated patients (*AFG3L2*:p.Arg280Trp, *GRN*:p.Cys139Arg, *IFIH1*:c.454-1G>T, *KCNMA1*: p.Ser11_Ser12delinsGly). Except for one patient with the *AFG3L2* variant, these showed quite inconsistent or unclear genotype-phenotype relationships, i.e., in our study, were identified in patients with isolated, persistent dystonia, but are otherwise usually reported in conjunction with other phenotypes.

Findings in dystonia candidate genes

We also investigated dystonia candidate genes, uncovering seven novel likely pathogenic variants that were all absent from control databases and predicted to be deleterious (Supplementary Table 2). This included two missense variants in *KCNN2*, with one (p.Leu611Ile) occurring *de novo* in a patient with infancy-onset generalized myoclonus-dystonia who also showed signs of bradykinesia and ataxia.

Two variants were identified in *ZMYND11*, including a stop-gain variant (p.Arg495*) in a patient with infancy-onset generalized dystonia and an in-frame insertion (p.His315dup) in a patient with cervical dystonia, tremor, and polyneuropathy with unknown AAO. One variant each was identified in the genes *CHD6* (p.Glu2697Thrfs*29 in a patient with adolescence-onset generalized dystonia with myoclonus and ataxia), *KLC1* (c.*1+1G>A in a patient with adult-onset cervical dystonia), and *NR4A2* (p.Gln273Arg in a patient with adult-onset cervical dystonia). For *KLC1* and *NR4A2*, five and four additional patients, respectively, with similar phenotypes of adult-onset focal dystonia, carried VUS (Supplementary Table 3**, Figure IV-3**).

No carriers of presumably pathogenic variants in other dystonia candidate genes were detected among our 1,924 patients, including the recently proposed genes *ATP5F1B*, *ACBD6*, and *SPTBN1*. However, several VUS were identified in candidate genes, including *SPTBN1*, where nine patients were found to carry rare missense and in-frame indels. They all shared an adult-onset focal or segmental dystonia phenotype involving the upper body (Supplementary Table 3).

While the clinical relevance of the identified VUS remains uncertain, the observed clustering of rare variants in these genes among patients with similar phenotypes may warrant further investigation.

DISCUSSION

This study comprehensively examined the genetic landscape of dystonia in 1,924 patients, the most extensive next-generation sequencing study conducted in dystonia patients to date. Importantly, this sample predominantly consists of adult-onset isolated dystonia patients, the most frequent but yet understudied form of dystonia. Despite negative pre-screening for many known pathogenic variants, the diagnostic yield of the present study was 8.1%, with an additional 16.9% of index patients harboring VUS. Patients with diagnostic variants had a significantly lower median AAO and a higher prevalence of generalized dystonia, additional neurological or systemic features, and positive family history compared to those without a diagnosis. ROC curve analysis indicated that AAO and the presence of generalized dystonia were the strongest predictors of a genetic diagnosis, with diagnostic yields of 28.6% in generalized dystonia and 20.4% in AAO <30. These findings align with prior studies and highlight the importance of considering exome sequencing in these patient groups (Zech et al., 2020; Zech et al., 2021b). Diagnostic yields in dystonia vary widely based on patient selection, ranging from 11.7% to 37.5% (Gorcenco et al., 2020). For instance, one study in 764 dystonia patients reported a 19% yield overall; however, the yield reached almost 50% in patients with early-onset (<20 years), generalized, non-isolated dystonia, while it was as low as 1% in patients with late-onset, isolated focal dystonia (Zech et al., 2020).

Our findings provide crucial insights into the relevance of genetic forms of dystonia and their corresponding phenotypes and will aid future variant interpretation and clinical diagnostics. Presumably pathogenic variants include 77 (56.2%) novel variants, with recurrent variants in *EIF2AK2*, *VPS16*, *KCNMA1*, and *SLC2A1*, and novel variant types such as two splice site variants in *KMT2B*. For *KMT2B*, we conducted episignature analysis, which has proven to reliably distinguish between benign and pathogenic variants (Mirza-Schreiber et al., 2022). An overview of the results of episignature testing has also been added to the MDSGene website (https://www.mdsgene.org).

Genotype-phenotype relationships and phenotypic expansions

This study identified diverse genetic causes of dystonia, with presumably pathogenic variants in 51 distinct genes, highlighting the genetic heterogeneity of dystonia. Most detected variants aligned with the phenotypic spectrum reported in the literature (Supplementary Table 2), though

some unusual findings were also observed (Supplementary Table 2, Supplementary Discussion). As new discoveries unfold, understanding the correlations between clinical manifestations and genetic variants has become increasingly complex due to mechanisms such as variable expressivity, incomplete penetrance, and genetic pleiotropy. Indeed, our study revealed instances where the genotype-phenotype correlation was inconsistent with previous reports. This included variants in *AFG3L2*, *ATL1*, *C19orf12*, *CHMP2B*, *COL4A1*, *GRN*, *KCNMA1*, *KCTD7*, *KIF1A*, *PDE10A*, *PRKCG*, *PSEN1*, *SCN8A*, *SLC2A1*, and *SLC6A1*. For some of these, further investigations are warranted to evaluate their true pathogenicity.

Noteworthy are three patients with *GRN* variants which were previously described in frontotemporal dementia patients (Gómez-Tortosa et al., 2013; Masellis et al., 2006). However, all our patients had adult-onset focal dystonia (cervical or upper limb) without additional features (age at examination: 25-76 years). This suggests that dystonia may occur without or prior to cognitive and behavioral symptoms due to *GRN* variants.

Further, we identified three patients with novel variants in *KCNMA1*, including a recurrent in-frame deletion and a frameshift variant, all absent in gnomAD v4. *KCNMA1* is typically associated with paroxysmal non-kinesigenic dyskinesia, developmental delay, and seizures, with LOF being a known disease mechanism (Miller et al., 2021). Our patients presented with childhood- or adult-onset focal persistent dystonia (cervical or upper limb) without additional features, except for tremor and left eye ophthalmoplegia in one patient. Although movement disorders are common in *KCNMA1*-related disease, particularly in LOF variants (Miller et al., 2021), patients with isolated dystonia have not yet been reported.

We also identified four likely pathogenic variants in *KIF1A*: two truncating, one splice site, and one missense variant affecting the kinesin motor domain, where the majority of pathogenic variants are found (Nemani et al., 2020). *KIF1A* is typically associated with spastic paraplegia, but our patients presented with isolated upper body dystonia (cranial, cervical, or upper limbs), with two patients exhibiting tremor and one muscle atrophy at the shoulders. While dystonia is not uncommon in *KIF1A*-related diseases, this is the first report of patients without spasticity, potentially expanding the phenotypic spectrum.

Another interesting gene was *CACNA1A*, which has been linked to several diseases, including episodic ataxia, cerebellar ataxia, and developmental and epileptic encephalopathy. Recent reports have shown that dystonia can also be primary and/or generalized in cases with *CACNA1A* variants (Alshareet et al., 2024; Indelicato & Boesch, 2021; Rinaldi et al., 2024). In our study, we identified three patients with likely pathogenic variants, all having isolated dystonia affecting

cranial or cervical muscles, with disease onset in their 40s. This confirms that variants in *CACNA1A* are also linked to isolated dystonia.

Dystonia candidate genes

Screening our data for proposed dystonia candidate genes identified presumably pathogenic variants in *CHD6*, *KCNN2*, *KLC1*, *NR4A2*, and *ZMYND11*, but not in others, e.g., *ATP5F1B*, *ACBD6*, *CIZ1*, *SHQ1* and *SPTBN1*.

Variants in *KCNN2* were initially linked to neurodevelopmental disorders, sometimes with movement disorders (Mochel et al., 2020), and later associated with tremulous myoclonus-dystonia. (Balint et al., 2020). We identified two patients with likely pathogenic missense variants. The first had infancy-onset generalized dystonia, myoclonus, ataxia, and bradykinesia – symptoms previously reported in *KCNN2*-related disease (Mochel et al., 2020) - and carried a *de novo* variant, supporting its pathogenicity and confirming its role in myoclonus-dystonia. The second variant (p.Ser376Leu), located in the protein's highly missense-intolerant ion channel domain (Wiel et al., 2019) which harbors other pathogenic variants (Balint et al., 2020; Mochel et al., 2020), was found in a patient with isolated cervical dystonia since age 27 years, potentially broadening the phenotypic spectrum of *KCNN2*.

In 2020, *CHD6*, *KLC1*, and *ZMYND11* were linked to early-onset dystonia combined with developmental delay or intellectual disability (Zech et al., 2020). Here, we identified variants in these genes with different phenotypes. This included a novel frameshift variant in *CHD6* in a patient with adolescence-onset generalized dystonia and myoclonus, as well as a novel splice-site variant in *KLC1* in a patient with late-onset cervical dystonia without additional features. For *ZMYND11*, we observed a stop-gain variant in a patient with infancy-onset isolated generalized dystonia and a single amino acid duplication in a patient with cervical dystonia, tremor, and polyneuropathy (AAO unknown).

Although no pathogenic variants were identified in the recently proposed candidate gene *SPTBN1*, we discovered nine patients with rare missense or in-frame VUS. All these carriers had adolescence or adult onset (median AAO: 40 years, range: 16-46) focal or segmental dystonia, predominantly affecting the neck or upper extremities. Notably, the initial report described a splice site variant in a patient with adolescent-onset segmental dystonia and developmental delay (Zech et al., 2020).

Furthermore, we identified a novel, likely pathogenic missense variant in *NR4A2*, a gene recently associated with levodopa-responsive dystonia and developmental delay (Winter et al., 2021;

Wirth et al., 2020). Our patient presented with cervical dystonia with no additional features reported, and information on levodopa responsiveness was unavailable. Additionally, four patients harbored missense variants in *NR4A2* classified as VUS, all located within the ligand-binding domain at missense-intolerant locations (Wiel et al., 2019). These patients all presented with adult-onset focal dystonia affecting the neck or upper limbs, with AAO ranging from 27 to 44 years.

Notably, our data support a role of pathogenic variants in *CHD6*, *KLC1*, *NR4A2,* and *ZMYND11* in dystonia even without neurodevelopmental features, while the relevance of identified VUS in several candidate genes remains unclear.

Limitations

Our study has some limitations. First, exome sequencing may miss non-coding variants and structural variations. Additional genome sequencing might increase the diagnostic yield by 5-10% (Fellner et al., 2024). Second, the exclusion of patients with known pathogenic variants likely biased the sample towards rarer or novel genetic causes, limiting generalizability of prevalence and observed genotype-phenotype relationships. This is underlined by the absence of the most frequent dystonia variant, p.E303del in *TOR1A*, which was excluded by pre-screening. Third, the classification of VUS remains challenging, and such variants should be treated with caution until further supporting evidence, such as segregation or functional data, becomes available. Access to family members for segregation analysis is often limited and functional studies are currently available only for a small subset of genes, including *KMT2B*. Additionally, clinical phenotyping has inherent limitations: variability in expert assessments can lead to misdiagnosis, and within this large cohort, some patients may have alternative explanations for their symptoms. While AI-guided tools like video analysis or machine-learning diagnostics may reduce this variability in the future, they are not yet implemented in clinical practice. Lastly, missing clinical information is a challenge. Despite recontacting the referring clinicians for all patients with presumably pathogenic variants to review the phenotype and family history, some data was unavailable (indicated as “not mentioned” under additional symptoms in Supplementary Table S2). This is especially a caveat for variants in genes not typically linked to dystonia and inconsistent phenotype.

CONCLUSION AND OUTLOOK

Taken together, our study demonstrates the usefulness of exome sequencing to elucidate the molecular basis in a heterogeneous disorder like dystonia. Given the large number of genes and variants linked to dystonia, diagnostic gene-specific approaches or panels are often impractical,

making comprehensive screening methods like exome or genome sequencing the most efficient path to diagnosis. These unbiased screening strategies can be reanalyzed at any time to include novel disease genes, increasing the diagnostic yield in the long term (Laurie et al., 2025). To aid future variant interpretation, we therefore also provide a list of all (*n*=321) index patients with VUS in dystonia-linked genes (Supplementary Table 3), which may be reclassified as likely pathogenic as more carriers are identified. This is particularly relevant for candidate genes like *CHD6*, *KLC1, NR4A2,* and *SPTBN1*, in which several VUS were identified. Furthermore, this exome data set can be utilized for novel disease gene discovery through gene burden analysis and exome-wide filtering of single patients or families for deleterious variants.

Finding a genetic diagnosis is invaluable for the patients and their families as it facilitates clinical management, treatment decisions, and genetic counseling, provides prognostic information, and offers crucial insights for the development of targeted therapies.

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DATA AVAILABILITY

Raw data were generated at the Competence Centre for Genomic Analysis in Kiel, Germany, and are available from the corresponding author on request.

3.5 Objective V: Multi-Omics Analysis of a Patient Unsolved After Exome Sequencing

In Objectives III and IV, gene panel and exome sequencing were applied to investigate large dystonia patient cohorts. While these approaches enabled a molecular diagnosis in a subset of patients, many remained genetically unsolved, including individuals with strong clinical indicators of a genetic cause, such as early disease onset, positive family history, or a severe phenotype. This may be due to the inherent limitations of the applied short-read sequencing techniques, which only cover part of the genome and focused on the detection of small nucleotide variants (SNVs) and insertions or deletions (indels). Although most currently known disease-causing genetic variants are located in coding regions, certain types, such as complex structural variants or regulatory non-coding changes, may be missed by these methods.

While whole genome sequencing (WGS) covers the entire genome and can detect non-coding and structural variants to some extent, advanced methods like optical genome mapping (OGM) have proven particularly effective in identifying complex structural changes and hold promise for uncovering overlooked contributors to disease (Schrauwen et al., 2024). Furthermore, integrating other technologies, such as transcriptome analysis, may guide variant interpretation (Yépez et al., 2022), whereby "multi-omics" refers to the integrated analysis of multiple molecular layers.

To explore the diagnostic potential of such methods, OGM, WGS and RNA sequencing were applied in a patient with an inconclusive exome result but strong clinical suspicion of a genetic disorder. The study covering this objective, "Multi-omics characterization of a *KIF1C* structural variant in a patient with a complex movement disorder partially responsive to deep brain stimulation", has been prepared for publication. Mirja Thomsen performed the genetic analyses, including OGM wet-lab work, filtering and analysis of OGM data, leading to the identification of a homozygous *KIF1C* deletion, and interpretation of pre-processed exome, genome, and transcriptome data. She also prepared Figures V-2 and V-3. CNV calling from exome data was performed by Saad Abdelwakeel; transcriptome data processing and statistical analysis by Vicente Yépez. The first draft of the manuscript was written by Mirja Thomsen (genetic part) and Max Borsche (clinical part) and revised by Katja Lohmann and Norbert Brüggemann.

OGM successfully led to a genetic diagnosis of a homozygous 2-exon deletion in *KIF1C*, which had initially been missed by exome sequencing, as it focused solely on SNVs and small indels. Transcriptome analysis revealed markedly reduced *KIF1C* mRNA expression and complete absence of normal splicing, highlighting the utility of a multi-omics approach for functional variant interpretation.

Multi-omics characterization of a *KIF1C* structural variant in a patient with a complex movement disorder partially responsive to deep brain stimulation

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Manuscript prepared for publication

ABSTRACT

Introduction: Biallelic *KIF1C* variants are associated with a spectrum of neurological phenotypes, including spasticity, cerebellar ataxia, chorea, and dystonia. Most reported patients involve single-nucleotide variants, whereas copy number variations (CNVs) have only been described once. The therapeutic potential of deep brain stimulation (DBS) in *KIF1C*-related disease has not yet been evaluated.

Methods: We report a patient presenting with spasticity, ataxia, dystonia, and head tremor, in whom prior exome sequencing was inconclusive. A multi-omics approach was applied, including optical genome mapping (OGM), genome sequencing, and transcriptome sequencing. The clinical effect of DBS was assessed.

Results: OGM revealed a homozygous *KIF1C* exon 17–18 deletion, retrospectively detectable in exome and genome data but initially missed by sequence analysis. Transcriptome sequencing demonstrated significantly reduced *KIF1C* expression and absence of normally spliced transcripts, consistent with a loss-of-function mechanism. DBS led to partial clinical improvement, with notable reduction of dystonia and head tremor.

Conclusion: Structural variants may be an underrecognized cause of complex movement disorders involving spasticity, ataxia, and dystonia. Diagnostic methods specifically targeting structural variation, such as OGM or CNV analysis of short-read data, can be essential for diagnosis. Transcriptome data adds valuable functional insight. This report also suggests that DBS may offer symptomatic benefit in *KIF1C*-associated disease, particularly for dystonia.

INTRODUCTION

The recent development of molecular genetic methods has opened a new era of diagnosing rare neurogenetic diseases, enabling the identification of causes in previously molecularly undiagnosable patients (Kernohan & Boycott, 2024). Of these methods, short-read exome and genome sequencing are particularly effective in detecting single nucleotide variants (SNVs) and small insertions or deletions (indels), while advanced bioinformatic tools have improved the identification of other variant types, such as copy number variants (CNVs), albeit with limitations (Kernohan & Boycott, 2024; Moreno-Cabrera et al., 2020; Whitford et al., 2019). Further, Optical Genome Mapping (OGM), a technique that uses ultra-long, fluorescently labeled DNA molecules, is specifically used to detect structural variants (SVs) without sequencing (Dremsek et al., 2021). Furthermore, the integration of other omics technologies, such as transcriptome analysis, may guide variant interpretation (Kernohan & Boycott, 2024).

These developments may also impact studies on hereditary ataxias and hereditary spastic paraplegias (HSP) (Synofzik & Schüle, 2017) that are characterized by a plethora of genetic alterations and complex phenotypes with varying clinical and genetic overlap (Parodi et al., 2018). For example, biallelic variants in the *KIF1C* (*Kinesin Family Member 1C*) gene cause an autosomal recessively inherited complex movement disorder, referred to as spastic ataxia type 2 (SPAX2) or HSP/ATX-KIF1C (Dor et al., 2014; Lange et al., 2022; Novarino et al., 2014). *KIF1C* encodes a microtubule-dependent motor protein essential for intracellular transport (Abid Ali et al., 2025; Nagel et al., 2022; Saji et al., 2024). Only about 25 patients with biallelic *KIF1C* variants have been reported thus far, including truncating (Dor et al., 2014; Novarino et al., 2014; Yücel-Yılmaz et al., 2018), splice site (Laurie et al., 2025; Novarino et al., 2014), and missense variants (Caballero Oteyza et al., 2014; Dor et al., 2014), as well as one four-exon deletion (Novarino et al., 2014).

Here we characterize a biallelic 2-exon deletion in a patient with an infantile-onset, complex movement disorder partially responding to deep brain stimulation (DBS), identified through OGM and further analyzed using exome, genome, and transcriptome data.

METHODS

After consenting to the research project, the patient underwent video-taped neurological examinations and biomaterial collection. The Ethics committee of the University of Lübeck approved the study, according to the Declaration of Helsinki.

Optical Genome Mapping (OGM)

Ultra-high-molecular-weight (UHMW) genomic DNA (gDNA) was extracted from fibroblasts using the Bionano Prep SP-G2 and labeled using the DLS-G2 Protocol. The labeled DNA was loaded onto a Bionano Saphyr Chip, linearized in nanochannels, and imaged with the Saphyr instrument. The run yielded 606.75Gbp of fragments >150kbp with 169× effective coverage, 90.4% map rate, N50 of 347.29kbp, and label density of 14.47/100kbp. *De novo* assembly was performed with Bionano Solve (v3.8), and SVs were analyzed in Bionano Access (v1.8) using the T2T-CHM13 reference genome.

Exome and Genome Sequencing

Genomic DNA was extracted from peripheral blood using the QIAamp DNA Mini Kit (Qiagen). Exome and genome sequencing were performed at the Competence Centre for Genomic Analysis (CCGA), Kiel, Germany. For exome sequencing, the Illumina DNA Prep with Enrichment kit and IDT xGen Exome v2 baits were used. Whole genome sequencing libraries were prepared using the Illumina DNA Prep Kit. Sequencing was performed on an Illumina NovaSeq 6000 with 150bp paired-end reads, achieving ~150× coverage for exomes and ~50× coverage for genomes. Reads were aligned to the hg38 reference genome using BWA-mem2.

RNA Sequencing

Total RNA was extracted from peripheral blood using the PaxGene Blood RNA kit (Qiagen). Library preparation was done with the Illumina Stranded Total RNA Kit, and sequencing was performed on an Illumina NovaSeq 6000 at CCGA, Kiel, Germany.

RNA-seq data were processed using the nf-core/rnaseq pipeline v3.15.1 with the option --aligner star_rsem. Reads were trimmed with Trim Galore v0.6.7 and aligned to the GENCODE GRCh38 primary assembly (release 44) using STAR v2.7.10a with --twopassMode BASIC. Gene-level counts were obtained using the summarizeOverlaps function from the GenomicAlignments R package. Quality control was performed with Qualimap (v2.3), RSeQC (v5.0.2), SAMtools (v1.2), and results were summarized using MultiQC (v1.24.1). Genes with a 95th percentile FPKM < 1 were excluded from analysis.

Aberrant gene expression was detected using OUTRIDER v1.20.1, with significance defined as $p \leq 10^{-5}$. Aberrant splicing was analyzed using FRASER 2.0 v1.99.4, excluding junctions with <20 reads in all samples or insufficient splice site coverage in ≥75% of samples. Splicing outlier genes were defined by Holm-adjusted *p*-values ≤ 0.1, and outlier junctions required FDR ≤ 0.1 and an absolute ΔJaccard index > 0.1. For outlier detection, the index sample was analyzed together with 51 additional blood RNA-seq samples (mainly from dystonia patients) that were processed and sequenced in the same way. To increase statistical power, 347 blood RNA-seq samples from the Solve-RD cohort were included in the analysis.

RESULTS

We report a White male patient with a predominantly hyperkinetic and progressive movement disorder manifesting immediately after birth with flaccid muscular tone. At the age of 2 years, abnormal head posturing and tremor were noticed and increased over the following 6 years, particularly when writing. Afterwards, the symptoms were more slowly progressive. Upon neurological examination in his early twenties, he exhibited head tremor, dystonia predominantly of the neck and trunk, cerebellar ataxia with severe intention tremor and dysarthria, spastic paraparesis, and myoclonic jerks. Clinical features also include mild horizontal saccadic pursuit, intermittent nystagmus, as well as proximal weakness of both legs (MRC 3). Intake of alcohol resulted in an improvement, especially of myoclonic jerks. The patient had no cognitive impairment and successfully graduated from law school. The family history was negative, but his parents were consanguineous, being second-degree cousins. MRI showed symmetric white matter T2-hyperintensities and bilateral hypointensities of the globus pallidus (**Figure V-1 A-D**). Propanolol (40mg per day at the age of 20 years) had a transient effect on tremor and myoclonus, whereas baclofen (dosage unknown), L-Dopa (300mg), and carbamazepine (300mg) did not lead to relevant clinical improvement. Trihexyphenidyl in a daily dosage of 15mg had a moderate effect on the tremor and cervical dystonia.

Although the patient suffered from a multisystemic neurological syndrome, a treatment attempt with DBS was initiated upon thorough consultation targeting dystonia, tremor, and myoclonus. Therefore, we decided to target both the globus pallidus internus (GPi) and nucleus ventralis intermedius (ViM) bilaterally at the age of 25 years, before the genetic cause was determined. DBS improved cervical and trunk dystonia and myoclonic jerks subjectively by 80% ten months post-surgery, with improved head control and purposeful grasping, relevantly ameliorating the patient's quality of life. Dysarthria deteriorated slightly at 19-month follow-up and improved again at 31-month follow-up, while the other symptoms remained stable over this period. The optimal clinical effect was achieved with GPi stimulation, allowing ViM stimulation to remain

discontinued at the last follow-up. The patient was last evaluated at the age of 34, nine years after DBS surgery, demonstrating a persistent marked improvement in cervical dystonia and myoclonus, while the tremor of the upper extremities showed a moderate response. A slight worsening of the gait disturbance was observed. The patient lives independently and is employed in a regular administrative position. Trihexyphenidyl had an additional symptomatic effect, after a dosage increase of 6-10mg/day at 19-month follow-up and 15 mg/day at the latest follow-up.

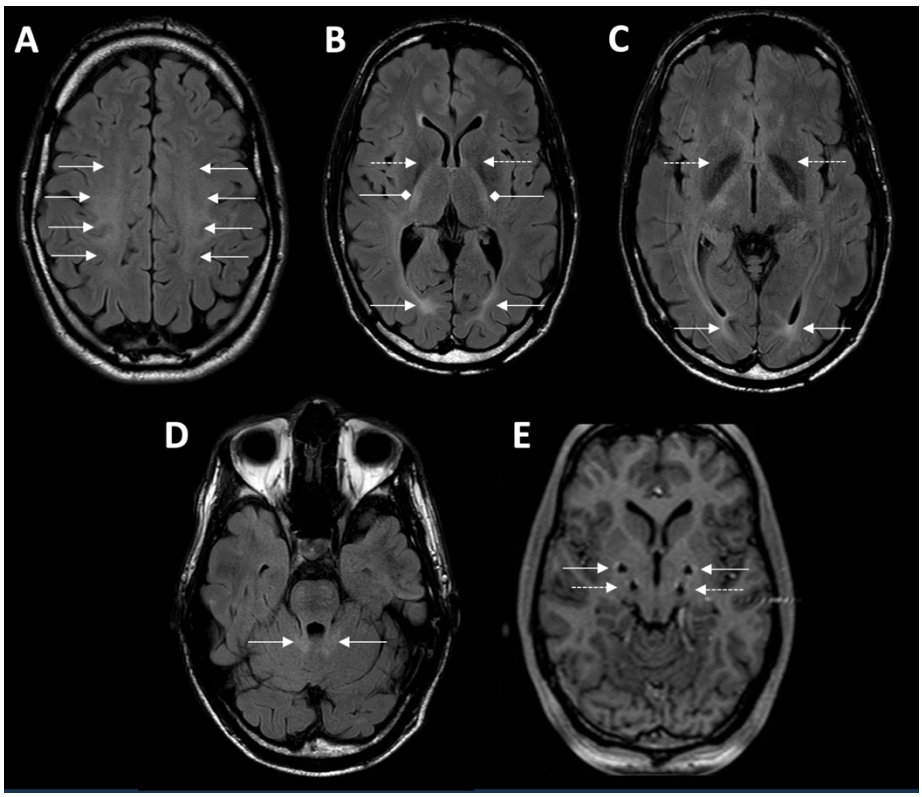

Figure V-1. Magnetic resonance imaging (MRI). (A-D) MRI of the patient at the age of 27 years. Axial T2-weighted (fluid-attenuated inversion recovery) images are shown. Imaging demonstrated symmetrical T2-hyperintense cerebral demyelination involving central (white arrows in A) and occipital (white arrows B and C) white matter, the internal capsule (white square-ended arrows in B), and the cerebellar peduncles (white arrows in D). Moreover, symmetric T2 hypointensities in the globus pallidus were observed (white dotted-line arrows in B and C). **(E)** Post-surgery MR imaging of the four deep brain stimulation electrodes bilaterally in the globus pallidus internus (GPi, white arrows) and nucleus ventralis intermedius (ViM, white dotted-line arrows).

Identification of a Homozygous *KIF1C* Deletion

Using OGM, a homozygous deletion of an estimated 4,245bp within a 13,665bp region at chr17, encompassing exons 17-23 of the *KIF1C* gene (NM_006612.6), was identified in the patient (**Figure V-2A**). Prior clinical trio-exome sequencing, which had focused on SNVs and small indels had been negative. To refine the exact location of the deletion, exome sequencing reads were inspected using the Integrative Genomics Viewer (IGV), revealing an absence of sequencing

coverage for exons 17-18 in the patient, while surrounding exons were adequately covered (**Figure V-2B**). Exome data from the unaffected mother showed reduced coverage of these exons (~50%) compared to a control sample, consistent with a heterozygous deletion. Further examination of genome sequencing data generated in parallel with OGM confirmed the precise breakpoints at chr17:5,013,289–5,017,563 (hg38; NM_006612.6:c.1492-1666del), corresponding to a 4,275bp deletion (**Figure V-2C**). This deletion is absent in the gnomAD CNVs 4.1.0 database.

Reduced *KIF1C* Expression and Aberrant Splicing

RNA sequencing confirmed *KIF1C* disruption, identifying it as an expression outlier in the patient (p-value = 5.19×10^{-6}, z-score = -4.99, fold change = 0.73) (**Figure V-3A**), indicating significantly reduced mRNA expression (by 27% compared to the mean of other analyzed transcriptomes). IGV analysis of RNA sequencing data revealed no expression of exons 17-18 (**Figure V-3B**). Additionally, *KIF1C* was identified as a splicing outlier (p-value = 2.50×10^{-8}, adjusted p-value = 0.012), with a complete absence of normal splicing. Further, IGV inspection revealed several split reads in which exon 16 was directly spliced to exon 19 (**Figure V-3B**).

On the protein level (NP_006603.2), skipping of exons 17-18 is predicted to result in the loss of amino acids 498-555 and introduces a frameshift afterwards, creating a premature stop codon at position 570 (p.Gly498_Gln555delfs*570).

Additionally, the *MRPS28* gene, encoding Mitochondrial Ribosomal Protein S28, was identified as an expression outlier in the patient (p-value = 5.43×10^{-6}, z-score = -13.56, fold change = 0.07) (**Figure V-3A**). However, no SNVs, indels, or SVs based on genome sequencing and OGM data were found that could account for the reduced expression. Moreover, the signal was based on an unusual read distribution lacking clear splice junctions and including reads across intronic regions, suggesting a technical artifact (e.g., misalignment or RNA degradation).

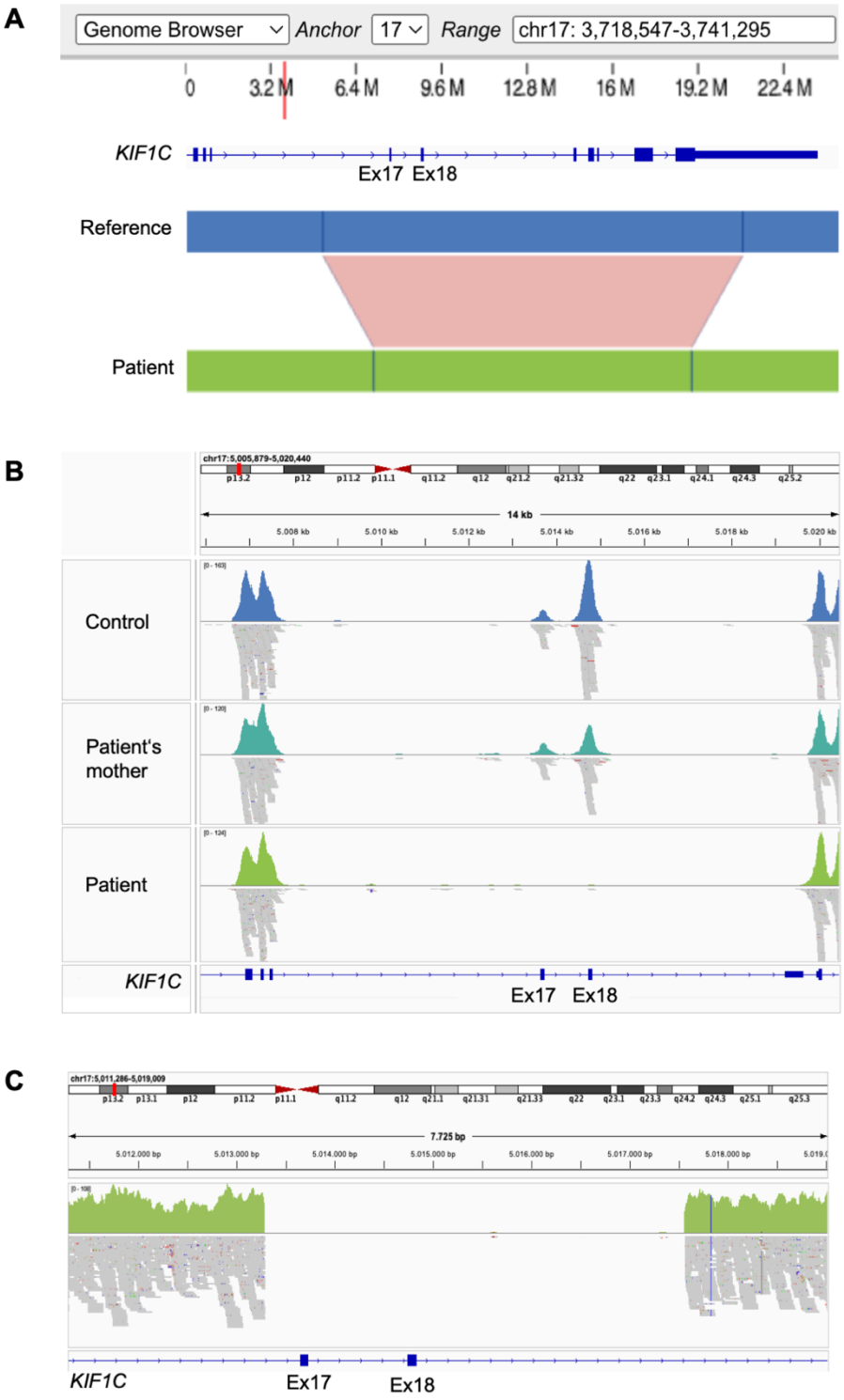

Figure V-2. Results from DNA analysis. (A) Optical Genome Mapping detected a homozygous deletion at chr17:4,901,429–4,915,093 (T2T reference genome; corresponding to chr17:5,011,105–5,024,768 in the hg38 reference) in the patient, which overlaps the *KIF1C* gene. **(B)** Integrative Genomics Viewer (IGV) snapshot of exome sequencing coverage for *KIF1C*. The patient shows no reads covering exons 17-18, consistent with a homozygous deletion. The mother shows reduced coverage (~50%) of these two exons compared to a control sample (upper panel), indicative of a heterozygous deletion. **(C)** IGV snapshot of genome sequencing reads revealing a homozygous 4,275bp deletion with precise breakpoints (hg38; chr17:5,013,289–5,017,563).

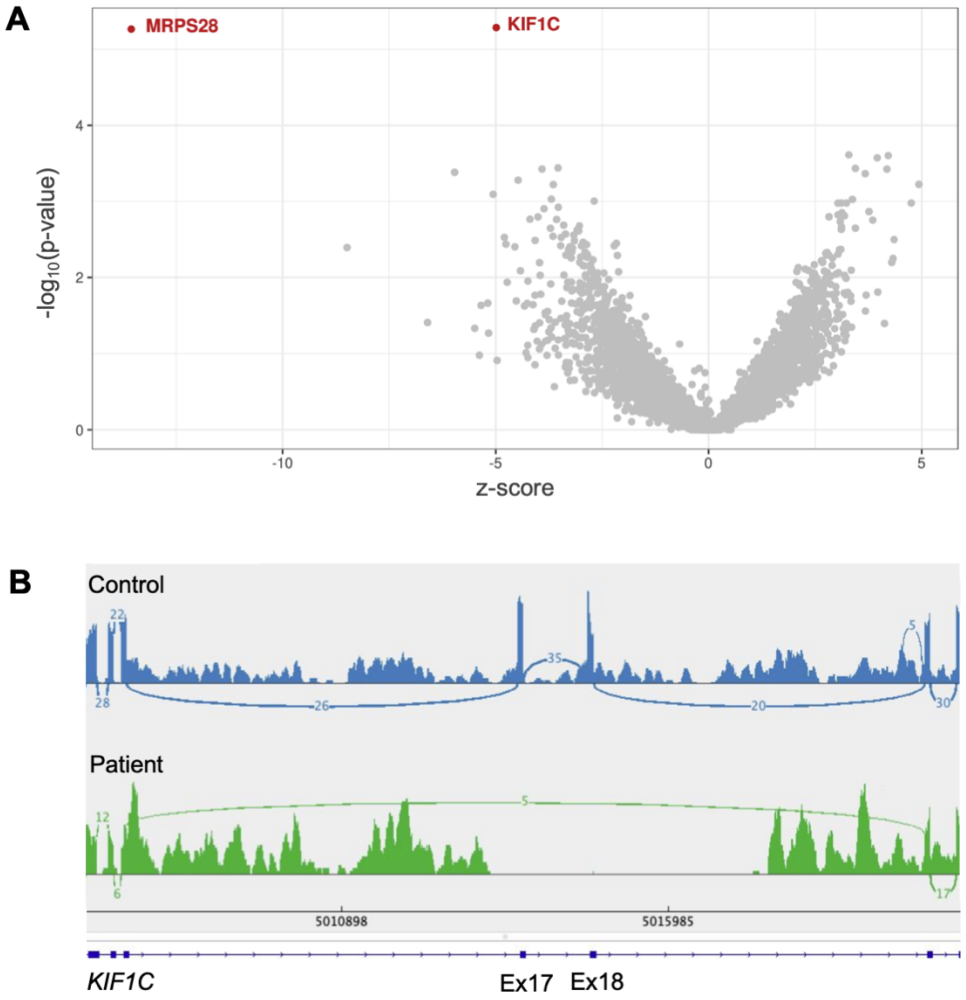

Figure V-3. RNA sequencing results. (A) Volcano plot from OUTRIDER-based expression analysis, revealing *KIF1C* as a significantly underexpressed gene in the patient. The x-axis shows the z-score of gene expression, and the y-axis indicated the -$\log_{10}$(*p*-value). **(B)** Sashimi plot of the *KIF1C* transcript from patient RNA sequencing data shows absent expression of exons 17 and 18. Splice junction reads indicate exon skipping, with exon 16 being directly spliced to exon 19. Numbers above the arcs represent the number of junction-spanning reads supporting each splicing event.

DISCUSSION

Here, we describe a biallelic 2-exon deletion in *KIF1C* in a patient with an infantile-onset, complex movement disorder, partially responsive to DBS. The structural variant was first identified through OGM, but complementary tools were required to determine the precise location. The genetic diagnosis initially escaped detection through exome sequencing, as the analysis focused on SNVs and small indels. Although CNV calling was not initially performed on exome data, advanced CNV-calling tools, such as ExomeDepth, later also revealed the homozygous deletion in the patient, but not the heterozygous one in the mother – likely because it only comprised 2 exons. These findings underscore the need for broader application of

comprehensive CNV detection tools in patients with complex syndromes, including ataxia and spasticity.

Genetic analyses suggest that the exons 17-18 deletion results in partial nonsense-mediated mRNA decay, as evidenced by lower *KIF1C* expression levels in the patient, based on transcriptome analysis. Further, the deletion is expected to introduce a frameshift and a premature stop codon, likely leading to a complete absence of functional KIF1C protein. If any protein were produced, it would be missing more than half of its sequence (amino acids 498-1,103), encompassing crucial domains involved in autoinhibition and interaction with binding partners (Siddiqui et al., 2019).

Previously reported disease-causing variants include missense variants in the kinesin motor domain, splice site, and truncating variants, as well as a deletion of exons 14-18 in a Moroccan family (Novarino et al., 2014) – a deletion overlapping with the one found in our patient. Identifying another SV in *KIF1C* highlights the need to consider this variant type in genetic diagnostics, particularly when only a heterozygous pathogenic SNV is found, as it may be combined with a heterozygous CNV.

While ataxia and spasticity are the core symptoms in *KIF1C*-associated disease, our patient's additional clinical features, i.e., dystonia, head titubation/tremor (Caballero Oteyza et al., 2014; Dor et al., 2014), and characteristic distribution of leukoencephalopathy (Caballero Oteyza et al., 2014; Dor et al., 2014), conform with the few *KIF1C*-linked disease patients previously published. Importantly, there is no report on DBS in *KIF1C*-related disease yet, probably because DBS cannot be expected to have an ameliorating effect on cerebellar ataxia or spasticity. Nevertheless, our patient underwent DBS before the genetic diagnosis was made to improve the patient’s prominent dystonic and myoclonic symptoms (by bilateral electrodes in the GPi) and tremor (by bilateral electrodes in the ViM). He benefited from DBS, although the therapeutic response was difficult to anticipate given the complex phenotype and neuroimaging abnormalities.

In conclusion, the clinical benefit observed after DBS highlights a potential symptomatic treatment option in *KIF1C*-linked disease, particularly in patients presenting with dystonia, myoclonus, and head tremor. At the same time, this report underscores the importance of including CNV analysis in the genetic workup of complex movement disorders, as well as the value of a multi-omics approach – in this case, additional transcriptome analysis that provided insights into the deletion’s effects on mRNA expression and splicing.

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4 Discussion and Conclusion

In this thesis, hereditary dystonia was investigated through a multi-dimensional approach combining analyses of genetic and clinical data and systematic literature reviews. Literature reviews served as the basis to explore genotype-phenotype correlations and to newly propose seven converging molecular pathways based on shared mechanisms across genetic forms. A variety of genetic methods were applied to a total of over 2,500 dystonia patients. A molecular diagnosis was established in 212 individuals, based on the in-depth interpretation of more than 3,000 rare variants in dystonia-associated genes, including functional assessment via methylation analysis in 95 patients with variants in *KMT2B*. One particularly complex patient was solved after a diagnostic journey of more than 15 years, using a combined multi-omics approach.

Specifically, in Objective I, genotype–phenotype correlations in three novel isolated dystonia genes were systematically analyzed, revealing a broad phenotypic spectrum and clinical overlap with other genetic forms despite gene-specific trends, and highlighting that genetic testing remains the only reliable tool to determine the underlying cause (Thomsen et al., 2023, *Movement Disorders*). In Objective II, a comprehensive review classified genetic dystonia forms based on underlying molecular mechanisms, suggesting that biological groups with distinct primary triggers may converge on shared downstream pathways at the cellular or anatomical level (Thomsen et al., 2024, *Annual Review of Pathology*). In Objective III, targeted gene panel sequencing in dystonia versus Parkinson's disease patients revealed a significantly higher burden of rare variants in common dystonia genes and enabled molecular diagnoses in 4.0% of dystonia patients, including novel disease variants. Additionally, episignature analysis proved valuable for the classification of rare *KMT2B* variants, helping to distinguish pathogenic from benign changes (Thomsen et al., 2024, *Movement Disorders*). In Objective IV, exome sequencing in a large dystonia cohort – including previously unsolved cases from gene panel analysis – led to a molecular diagnosis in 8.1% of index patients, provided evidence for several dystonia candidate genes, and expanded the known phenotypic spectrum of some genes (Thomsen et al., in press, *Annals of Clinical and Translational Neurology*). Lastly, in Objective V, in a patient with a strong suspicion of a genetic etiology but negative exome, a homozygous *KIF1C* deletion was identified through optical genome mapping (OGM) and functionally validated by transcriptome analysis, demonstrating the added diagnostic value of such methods (Thomsen et al., manuscript prepared for submission).

4.1 Diagnostic Value of Broad and Functional Genomic Testing in Dystonia

4.1.1 Yield of Different Genetic Testing Strategies

Different genetic methods were applied in this work, varying in terms of genomic regions covered, types of detectable variants, and associated costs. These factors must be carefully considered and balanced when choosing an appropriate genetic testing strategy (Kernohan & Boycott, 2024). Direct comparison of their diagnostic value is challenging, as the yield strongly depends on the characteristics of the tested patient sample.

In line with a previous study (Zech et al., 2020), this thesis confirmed that age at onset (AAO) and generalized dystonia are strong predictors of a genetic diagnosis (Objective IV). Additionally, a genetic diagnosis was significantly more likely in patients presenting with additional features or a positive family history. For this reason, many dystonia cohorts investigated in genetic studies are enriched for such patients to increase the probability of a genetic diagnosis. The dystonia sample studied in this thesis (for both gene panel and exome sequencing) is unique in that it predominantly consisted of patients with later onset, focal, and isolated dystonia. Notably, the 8.1% overall diagnostic yield achieved – and a yield of over 5% among patients with late-onset isolated focal dystonia – is quite high compared to a previous exome sequencing study of 764 dystonia patients, in which the yield in this subgroup was only 1%, despite a total yield of 19% (Zech et al., 2020). This clearly illustrated that diagnostic yield is highly dependent on the characteristics of the tested population.

The diagnostic yield from exome sequencing (8.1%) was about twice as high as that of gene panel analysis (4.0%), even though the exome sequencing included around 550 individuals who had already tested negative in panel-based screening – thus lowering the expected yield. Without the inclusion of pre-screened samples, the diagnostic rate from exome sequencing would have been even higher. While some gene panel studies have achieved much higher yields (e.g., 18.5% in a small cohort of mostly early-onset isolated dystonia using a broad panel of 148 genes (Ma et al., 2018), these results are highly dependent on patient selection and panel design. The advantage of exome sequencing becomes even more apparent when considering the number of different genes identified. While the gene panel applied in this work targeted seven common dystonia genes, disease-causing variants detected through exome sequencing were found in 51 distinct genes – most of which would not have been captured by a fixed panel. Since it is not possible to anticipate which genes will be implicated in a given patient group, panels are of limited utility in a genetically heterogeneous disorder like dystonia, even though they are more cost-effective. Even a comprehensive panel covering all currently known dystonia genes would fail to include newly

discovered genes. Some genetic diagnoses in this work involved such genes, e.g., a patient with a pathogenic *EIF4A2* variant, a gene that had only recently been associated with dystonia in a publication to which the here presented exome sequencing study contributed a family (Harrer et al., 2023b).

To assess the added value of OGM in cases where exome sequencing remains inconclusive despite strong clinical suspicion of a genetic etiology, a number of unsolved patients was further investigated. One illustrative case, in which this approach successfully led to a diagnosis, is presented in this work (Objective V). In this individual, who presented with a complex movement disorder including prominent dystonia, but also spasticity, chorea, and ataxia, OGM immediately identified a homozygous multi-exon deletion in the *KIF1C* gene. This finding illustrates the utility of this relatively novel technology, which has also proven successful in other rare disease patients (Orellana et al., 2025). OGM is based on high-resolution imaging of ultra-long, fluorescently labeled DNA molecules and enables genome-wide detection of structural variants (SVs) ranging from ~500bp up to entire chromosomes, including complex rearrangements (Dremsek et al., 2021). It is not a sequencing method but rather a labeling and imaging technique that does not rely on amplification or alignment to a reference genome. As such, it can more accurately resolve repetitive or complex genomic regions compared to short-read sequencing approaches. One current limitation to the broad application of OGM in clinical diagnostics is the relatively high cost. In addition, OGM requires fresh blood samples and a specialized DNA extraction protocol to obtain ultra-high molecular weight DNA, which limits its applicability to archived DNA samples.

Notably, when copy number variant (CNV) calling using ExomeDepth was later applied to the exome data, the same homozygous deletion could also be detected, underscoring that advanced bioinformatic tools should routinely be employed on short-read sequencing data to maximize diagnostic yield. Indeed, a previous study in dystonia patients showed that applying CNV analysis to exome data increased the yield by 1.5% (Zech et al., 2021a). However, CNV detection from short-read data remains limited in accuracy and is prone to false positives (Kernohan & Boycott, 2024). In this case, while the homozygous deletion was correctly identified by ExomeDepth, the heterozygous deletion in the mother was missed, likely due to its small size, as heterozygous CNVs spanning only 1-2 exons are frequently undetected in exome data (Moreno-Cabrera et al., 2020). Furthermore, complex SVs, such as combinations of deletions, inversions, or translocations, are typically not captured by exome-based analyses at all. Given that many dystonia-related genes follow autosomal-dominant inheritance and may involve small heterozygous CNVs or complex structural rearrangements, technologies analyzing long DNA molecules, such as OGM or long-read sequencing, may be essential in selected cases for the

identification of clinically relevant SVs in dystonia. This has also been demonstrated in a recent study of 47 unresolved neurodevelopmental disorder families, in which OGM identified pathogenic SVs in 5 cases (10.6%) that had been missed by standard sequencing methods (Schrauwen et al., 2024). Interestingly, these included SVs affecting only 1-4 exons that escaped detection even with CNV-calling algorithms applied to exome data.

Another sequencing approach that has been shown to increase the diagnostic yield in dystonia is whole-genome sequencing (WGS). A recent study involving 305 dystonia patients identified 37 additional diagnoses (12.1%) through WGS, uncovering disease-causing variants that had been missed by exome sequencing (Zech et al., 2025). This increased yield was primarily due to the more uniform coverage of the genome, improved detection of SVs and CNVs, and the detection of known disease-related intronic variants. As a follow-up to the work of the present thesis, WGS data has already been generated for 276 unsolved dystonia patients and is currently being analyzed to increase the diagnostic yield. Long-read sequencing may further improve the yield by enabling the detection of small variants, (complex) SVs, and repeat expansions in a single test. A recent review highlighted its potential to overcome current limitations in dystonia diagnostics and called for its broader integration into routine workflows (Wirth et al., 2025). In line with this, Mirja Thomsen contributed to the Solve-RD "Solvathons" (https://solve-rd.eu), during which short- and long-read genome sequencing, transcriptomics, OGM, and methylation data from unsolved rare disease patients were jointly analyzed. Several diagnoses were made through this multi-omics approach, including repeat expansions and complex SVs – variant types often missed by standard short-read approaches (Yépez et al., in press, *Nature Genetics*).

4.1.2 Integrating Functional Information

Transcriptome analysis is an emerging and powerful tool that adds another layer of information and has proven valuable for the interpretation of genomic variants (Yépez et al., 2022). In this work, RNA sequencing provided insights into the functional consequences of the identified deletion in *KIF1C* (Objective V). It revealed significantly reduced gene expression, consistent with (partial) nonsense-mediated decay, as well as abnormal splicing resulting in a frameshift that is unlikely to produce any functional protein. Beyond aiding in the interpretation of detected genomic variants, transcriptome sequencing can also be applied as a "RNA-first" approach to identify disease-causing variants that would otherwise be missed, e.g., deep intronic variants with little prior annotation (Ludwig et al., 2024). Beyond the described case, transcriptome analysis has already been conducted in 50 additional dystonia patients unresolved after exome sequencing – several of whom carry uncertain candidate variants – and is expected to yield further diagnoses.

Similarly, proteomics provides yet another layer of molecular information and is increasingly being explored as a complementary tool in genetic diagnostics. In a recent study of 76 genetically unsolved dystonia patients, two proteomics-based strategies were applied: one aimed at interpreting VUS identified through WGS, the other followed a "proteome-first" approach, using protein expression profiles to guide variant discovery. Together, these approaches led to six additional diagnoses (Zech et al., 2025), underscoring the diagnostic potential of proteomics.

Another functional approach applied in this thesis was episignature analysis for rare variants in the dystonia gene *KMT2B*. Since the KMT2B protein plays a key role in histone methylation – an epigenetic modification inversely correlated with DNA methylation – assessing DNA methylation at specific genomic loci offers a feasible and informative method for evaluating the pathogenicity of *KMT2B* variants (Mirza-Schreiber et al., 2022). This so-called episignature analysis was performed for variants detected through both gene panel and exome sequencing and enabled a clear distinction between pathogenic and benign variants (Objective III and IV). As a result, a molecular diagnosis could be confirmed in a total of four patients, while a non-contributory role of *KMT2B* could be established in 91 others. This is particularly important for *KMT2B*, as a large number of rare missense variants were identified (see **Figure III-1**), the majority of which were found to be benign. All results were integrated into the MDSGene database (https://www.mdsgene.org) to support the broader research and clinical community in *KMT2B* variant interpretation.

Importantly, such an episignature may also be identifiable for additional genes involved in dystonia. To date, about 60 disease-specific episignatures in peripheral blood have been documented across Mendelian and predominantly neurodevelopmental disorders (Levy et al., 2022). While some of the implicated genes are directly linked to epigenetic regulation, such as *KMT2B*, others do not have a known or direct epigenetic function but still have characteristic effects on DNA methylation patterns. A major limitation in the context of dystonia is the rarity of each genetic form, which makes it challenging to collect a sufficient number of samples to reliably define gene-specific methylation changes.

4.1.3 Integrating Familial and Segregation Data

Interestingly, although a positive family history was significantly more frequent among patients with a genetic diagnosis, its predictive value for identifying a causative variant was neglectable (AUC from ROC analysis: 0.53). The exome sequencing sample included 24 families with at least two sequenced members, of which 11 could be solved (46%). This illustrates that the availability of DNA from additional family members, whether affected or unaffected, can substantially facilitate diagnosis. This aligns with a previous observation that establishing diagnoses in patients

who have at least one relative sequenced is significantly more successful (Zech et al., 2025). Particularly in trios (an affected child and both unaffected parents), a genetic diagnosis was often achieved rapidly. This applied to six trios in the dataset, in which *de novo* variants were identified in *ADCY5*, *GNB1*, *KCNN2*, *KMT2B*, *IRF2BPL*, and *VPS16* (**Figure IV-S1**). Importantly, half of these variants (in *KMT2B*, *VPS16*, and *KCNN2*) would have remained classified as VUS without parental DNA analysis. Moreover, these findings provide key supporting evidence for the disease association of those variants: clearly pathogenic missense variants in *VPS16* and *KMT2B* had previously been rarely described (Lange et al., 2021; Thomsen et al., 2023), and *KCNN2* had been considered a candidate gene with limited independent validation (Lange et al., 2022). In summary, although sequencing family members comes at a significant increase in cost, it can be a highly valuable tool for establishing a genetic diagnosis in dystonia.

4.1.4 Integrating Genetic Findings into Clinical Care

Another important aspect demonstrating the value of a genetic diagnosis is the identification of actionable genetic causes, i.e., gene defects for which promising treatments are available or currently emerging. Although this applies to only a limited number of genetic forms of dystonia to date, several examples are directly relevant to the patients identified in this work. One such example is dopa-responsive dystonia caused by pathogenic variants in *GCH1* or *SPR* (Weissbach et al., 2022), identified in 24 and 1 individuals, respectively, through gene panel or exome sequencing. Additionally, novel genetic forms such as dystonia caused by variants in *NR4A2* have been linked to levodopa responsiveness (Winter et al., 2021), offering a potential treatment option for one of the patients described here. Beyond pharmacological approaches, several genetic dystonia forms are known to respond particularly well to DBS, including DYT-TOR1A, DYT-KMT2B, and DYT/MYC-SGCE (Sarva et al., 2024), affecting 10, 12, and 26 patients in this work, respectively. As highlighted in Objective I, patients with DYT-VPS16 may also benefit from DBS, and one of the here identified DYT-VPS16 patients has already undergone DBS with good clinical response (Objective IV, Supplementary Table S2). Moreover, patients with variants in *TOR1A*, *THAP1*, and *KMT2B* may respond favorably to anticholinergic treatment (Lange et al., 2021). Other genetic diagnoses can guide alternative treatment strategies, such as in *ADCY5*-related movement disorders, where the use of theophylline has recently shown benefit in a small number of patients (Taenzler et al., 2025). Together, these findings illustrate how genetic information can already inform treatment decisions in selected cases, and how continued research may allow more patients with a confirmed genetic diagnosis to benefit from emerging targeted therapies.

4.2 The Molecular Diversity of Dystonia is High – but Organizable

4.2.1 Genetic Heterogeneity

Sequencing large cohorts of dystonia patients, as done in this thesis, provides valuable insights into the relevance of individual genetic forms in this highly heterogenous disorder. This genetic heterogeneity in dystonia is evident both at the gene and the variant level.

In our predominantly isolated dystonia sample, a few genes were considerably more frequent than others: *VPS16*, *THAP1*, *GCH1*, and *SGCE* were each identified in more than 10 patients through exome sequencing. Notably, although *TOR1A* is typically the most frequently implicated gene in isolated dystonia, it was excluded due to pre-screening. In gene panel analysis, *TOR1A*, *THAP1*, *GCH1*, and *SGCE* were also the most abundant forms. In contrast, most genetic forms were extremely rare. Overall, 51 different genes were implicated in our exome sequencing cohort, with about half of them detected in only a single patient among the about 2,000 screened ones, demonstrating the immense genetic heterogeneity. Similarly, another recent sequencing study found that every second genetic diagnosis involved a gene not previously implicated in that cohort (Zech et al., 2025). Interestingly, the same study demonstrated that the number of causally implicated genes increases almost linearly with the number of sequenced individuals. For example, sequencing of 708 families revealed 77 distinct genetic forms, while sequencing of 1,825 families identified 205. When placed in the context of this trend, our observation of 51 distinct genetic forms in 1,895 index patients lies substantially below the expected gene count, likely reflecting differences in the composition of the patient cohort. Their cohort included nearly 70% of patients with coexisting features such as other movement disorders or developmental delay, whereas over 90% of the patients analyzed in this study presented with isolated dystonia. This suggests that isolated dystonia is genetically less diverse than combined forms, possibly reflecting both biological and diagnostic factors, including the lower diagnostic yield in isolated cases.

Comprehensive and large-scale analysis also highlighted the relevance of recently described, but still insufficiently characterized, genetic forms of dystonia. The three genes that were phenotypically characterized in Objective I (*VPS16*, *EIF2AK2*, and *AOPEP*) were not included in the gene panel analysis, as they were identified after the project had been initiated. Exome sequencing revealed that *AOPEP*-related dystonia is extremely rare, with no carriers identified in the entire sample. In contrast, *VPS16* and *EIF2AK2* were found in 0.9% and 0.3% of patients, respectively – frequencies that are low but typical for monogenic forms of dystonia, which are individually rare. Nevertheless, the identification of 17 *VPS16* and 5 *EIF2AK2* carriers further confirms their relevance as genetic causes of dystonia.

Beyond gene-level heterogeneity, dystonia also shows remarkable variant-level diversity. While few dystonia-causing variants are recurrent – such as the GAG deletion in *TOR1A,* which has been identified in several hundred patients (Lange et al., 2021) – many disease-associated variants are unique. This was observed in both the gene panel and exome sequencing data, in which at least half of the disease-implicated variants had not been previously reported. Additionally, only 7 out of 32 variants from gene panel analysis and 15 out of 137 from exome sequencing were found recurrently in at least two unrelated patients. This high proportion of unique variants significantly complicates variant interpretation and underscores the need for comprehensive databases, functional studies, and international data sharing to better assess the pathogenicity of rare and novel findings.

4.2.2 Organizing Genetic Heterogeneity by Molecular Mechanisms

Beyond individual gene associations, functional and biological categorization can help to structure the complexity of genetic dystonia. As outlined in Objective II, the pronounced heterogeneity observed in hereditary dystonia can be organized into molecular groups that share common underlying mechanisms. These different mechanisms are often interconnected and converge at the cellular or anatomical level, ultimately disrupting the finely regulated motor network of the brain and leading to dystonic symptoms. The seven molecular categories used to classify dystonia-associated genes, as described in the review of Objective II (Thomsen et al., 2024a), are:

- Striatal dopamine signaling
- Gene transcription and neurodevelopment
- Calcium signaling and synaptic transmission
- Cellular stress response
- Endoplasmic reticulum (ER) and nuclear envelope function
- Cytoskeleton
- Autophagy and lysosomal function.

As illustrated in **Figure 4**, the genetic forms identified in our exome sequencing cohort could be assigned to these molecular groups to stratify patients and characterize the most frequently affected biological pathways in this patient population. The largest group was regulation of gene expression and neurodevelopment, comprising 34 patients and including genes such as *THAP1* and *KMT2B*. This was followed by striatal dopamine signaling (29 patients, e.g., with variant in *GNAL* or *GCH1*), calcium signaling and synaptic transmission (27 patients, e.g., *ATP1A3*, *CACNA1A*, and *KCNMA1*), autophagy and lysosomal function (23 patients, e.g., *VPS16* and *GRN*), and cytoskeleton and intracellular transport (23 patients, e.g. *SGCE* and *KIF1A*).

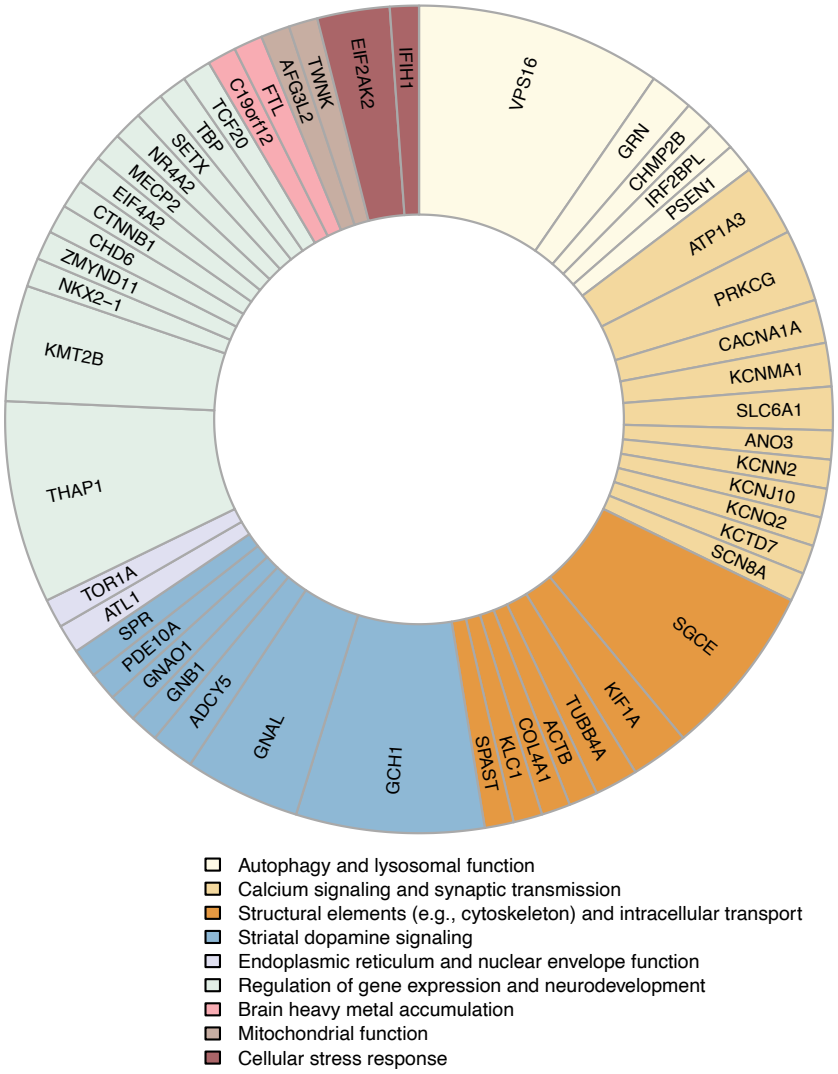

Figure 4. Overview of molecular pathways implicated in the exome sequencing cohort. Genes with identified pathogenic variants are grouped according to their associated molecular pathways. The size of each segment reflects the number of patients carrying a variant in the respective gene. Colors indicate the molecular pathway assigned to each gene (see legend).

The identification of novel dystonia-associated genes may help to further refine or expand these molecular groups. In this thesis, several previously proposed candidate genes could be substantiated by the identification of additional patients, thereby supporting the involvement of the connected pathways in dystonia. These include *KCNN2* (calcium signaling and synaptic transmission), *ZMYND11, CHD6*, and *NR4A2* (gene expression and neurodevelopment), and *KLC1* (intracellular transport along microtubule).

Such a biological framework for classifying genetic dystonia forms is of high translational value, as it opens up the possibility for mechanism-based therapies targeting pathophysiological clusters, rather than developing individualized treatments for each, often extremely rare dystonia subtype. For instance, in the context of dysregulated calcium homeostasis and altered synaptic signaling,

selective calcium channel blockers and modulators, which are abundant in the drug market, may be tested and benefit several genetic forms from this group.

Notably, the molecular classification proposed in the review article included in this thesis (Objective II) initiated the international meeting *Dystonia Pathogenesis: An Integrative Approach* (Dystonia Medical Research Foundation, 2024), at which Mirja Thomsen presented the classification approach as an invited speaker. At this meeting, the outlined ideas were further discussed by experts from the field, and new collaborations were established to advance a more integrated understanding of dystonia pathogenesis.

While the discovery of genetic dystonia forms and their molecular grouping represent an important step toward understanding the disease mechanism of dystonia and navigating its heterogeneity, it is important to emphasize that the majority of dystonias remain idiopathic, i.e., with no known underlying cause. Future research will need to determine to what extent the molecular mechanisms defined in monogenic forms also apply to idiopathic patients.

4.2.3 Genotype-Phenotype Correlations: Trends, Overlaps, and Expanding Spectra

Just as the genetic causes of dystonia are highly heterogeneous, the phenotypes associated with individual genes are also variable, despite gene-specific trends. In Objective I, a systematic review of three novel dystonia genes (*VPS16*, *EIF2AK2*, and *AOPEP*) showed recognizable trends, e.g., *EIF2AK2*-related dystonia typically begins in childhood and affects the limbs, although these trends overlap with patterns seen in other genetic forms. These observed trends, as well as previously reported genotype-phenotype correlations for seven isolated dystonia genes (Lange et al., 2021), were generally supported by our panel and exome data. For example, DYT-GNAL has a reported median AAO of 38 years, compared to 37 years (panel) and 45.4 years (exome) in our data. For DYT-SGCE, the median AAO was 4 years in the literature, 4.5 years in our panel cohort, and 8.5 years in exome data. Similarly, for *EIF2AK*2, the literature median AAO was 9.5 years and 6 years in our patients; generalized dystonia was reported in 80% and 85% of cases, respectively.

Despite the overall consistency with published trends, several atypical findings were noted. For instance, one DYT-EIF2AK2 patient presented with bradykinesia and rigidity – symptoms not previously associated with this gene. For DYT-VPS16, one patient had neurodevelopmental delay, and another showed bradykinesia and resting tremor, both novel features for this gene. Additional patients will help determine whether these features are part of the extended phenotype or isolated exceptions.

Three phenomena complicate genotype-phenotype correlation in dystonia: variable expressivity, reduced penetrance, and pleiotropy. Variable expressivity refers to the same genetic cause leading to different phenotypic expressions, e.g., in terms of AAO, severity, or additional features. Reduced penetrance describes that not all individuals carrying a pathogenic variant will develop symptoms. A striking example of this is the common 3-base-pair deletion in *TOR1A*: Up to 70% of carriers remain asymptomatic, while others range from mild focal dystonia to severe childhood-onset generalized dystonia (Lange et al., 2021). Reduced penetrance was also observed in a DYT-EIF2AK2 family from the exome cohort, in which an unaffected male transmitted the pathogenic variant to his son, who developed dystonia (**Figure IV-S1**). Pleiotropy refers to a gene causing multiple distinct syndromes. An example from the field of dystonia is *TUBB4A* – variants in which have been shown to cause isolated dystonia, isolated hypomyelination, or hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) (Balint & Bhatia, 2015). Another example is *GCH1*: while typically associated with childhood-onset isolated dystonia, it can also cause combined dystonia-parkinsonism or late-onset isolated parkinsonism (Weissbach et al., 2022). In line with this, *GCH1* variants were identified in both dystonia and Parkinson's disease patients in our panel cohort, whereas the other six dystonia genes were only implicated in dystonia.

In light of these phenomena, it is not surprising that we observed phenotypic expressions that deviated from the published spectrum and identified presumably pathogenic variants in genes not previously associated with isolated dystonia, such as in *KCNMA1*, *KIF1A*, and *GRN*. This reflects a growing trend in the field, where genes initially linked to other neurological conditions, such as developmental delay, epileptic encephalopathy, or ataxia, are increasingly recognized in patients with prominent and sometimes even isolated dystonia (Ahn et al., 2023; Keller Sarmiento & Mencacci, 2021). In this context, we identified a *de novo* frameshift variant in the *ERF* gene in one of the patients from the exome cohort – a gene that has so far only been associated with craniosynostosis. Through collaboration, a second patient with similar dystonic phenotype and a frameshift variant in *ERF* was found, suggesting that *ERF* may have pleiotropic effects and a possible role in dystonia (Thomsen et al., manuscript in preparation).

Taken together, the observed variability suggests that additional genetic, epigenetic, or environmental factors are likely involved in disease manifestation.

4.3 Outlook: Closing the Diagnostic Gap

The main focus of the future perspectives emerging from this thesis is to close the diagnostic gap in dystonia. Achieving this will contribute to a better understanding of the underlying molecular mechanisms and biological subtypes, ultimately enabling the development of more effective and targeted treatments. The most compelling evidence for the existence of this diagnostic gap is that many patients with affected relatives still lack a genetic diagnosis. This study confirmed previous findings that a positive family history is not yet a reliable predictor of identifying a genetic cause (Zech et al., 2021b). This is particularly noteworthy, as one would typically expect a positive family history to strongly indicate heritability. The discrepancy suggests that a substantial number of genetic contributors to dystonia remain undetected by current testing approaches and that additional, yet-unidentified genetic factors contribute to the disease. In some patients, this may involve more complex inheritance models, such as digenic or oligogenic mechanisms, where the combined effect of variants in different genes results in disease manifestation. In addition, a large proportion of patients from genetic studies harbor VUS: in our exome cohort, 16.9% (321 patients) carried VUS in dystonia-associated genes. Closing the diagnostic gap therefore involves both identifying novel genetic causes and resolving uncertain findings. Several approaches may support this effort, including overcoming technical limitations (see chapter 4.1.1), integrating functional analysis (see chapter 4.1.2), as well as leveraging independent cohorts and international data sharing.

Some findings from this thesis illustrate the value of such collaborative efforts. For example, a novel *EIF2AK2* missense variant identified in our cohort remained a VUS until a parallel study from India (Dhar et al., 2024) reported it as likely pathogenic in another dystonia patient, supporting its reclassification. Similarly, a *GNB1* variant in our cohort, previously listed as a VUS in ClinVar, could be classified as pathogenic due to its *de novo* origin in our case. This also enabled a diagnosis in another patient in our cohort for whom segregation data was unavailable. Likewise, several studies have shown that reanalysis of sequencing data over time increases the diagnostic yield as new evidence emerges (Laurie et al., 2025; Zech et al., 2025). The datasets generated in this thesis represent a valuable resource for such efforts, and sharing them may help resolve VUS in other cohorts as well.

Beyond variant reclassification, these data will also support the identification of novel dystonia-associated genes. Large cohorts enable gene burden analysis, which assesses whether rare, likely deleterious variants in a gene are enriched in patients compared to controls. This approach helped identify *VPS16* as a dystonia gene, followed by international case-finding that confirmed its relevance (Steel et al., 2020). Similarly, replication of candidate genes identified through family-based analyses remains a critical step and depends on international data sharing.

In summary, by analyzing more than 2,500 unique dystonia patients using diverse genetic approaches, this work provides a comprehensive dataset offering key insights into the diagnostic utility of genomic testing, the genetic heterogeneity of dystonia and its organization into biologically meaningful molecular groups, and the evolving spectrum of genotype–phenotype correlations. It also lays important groundwork for novel gene discoveries and emphasizes the importance of comprehensive approaches to improve diagnostic yield to support future precision medicine.

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