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**Delineating the genotypic and phenotypic spectrum of *HECW2*-related neurodevelopmental disorders**

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**Abstract**

**Background:** Variants in *HECW2* have recently been reported to cause a neurodevelopmental disorder with hypotonia, seizures, and impaired language; however, only six variants have been reported and the clinical characteristics have only broadly been defined.

**Methods:** Molecular and clinical data were collected from clinical and research cohorts. Massive parallel sequencing was performed and identified individuals with a *HECW2*-related neurodevelopmental disorder.

**Results:** We identified 13 novel missense variants in *HECW2* in 22 unpublished cases, of which 18 were confirmed to have a *de novo* variant. In addition, we reviewed the genotypes and phenotypes of previously reported and new cases with *HECW2* variants (N=35 cases). All variants identified are missense, and the majority of likely pathogenic and pathogenic variants are located in or near the C-terminal HECT domain (88.2%). We identified several clustered variants and four recurrent variants [p.(Arg1191Gln);p.(Asn1199Lys);p.(Phe1327Ser);p.(Arg1330Trp)]. Two variants, [p.(Arg1191Gln);p.(Arg1330Trp)], accounted for 22.9% and 20% of cases respectively. Clinical characterization suggests complete penetrance for hypotonia with or without spasticity (100%), developmental delay/intellectual disability (100%) and developmental language disorder (100%). Other common features are behavioral problems (88.9%), vision problems (83.9%), motor coordination/movement (75%) and gastrointestinal issues (70%). Seizures were present in 61.3% of individuals. Genotype-phenotype analysis shows that HECT domain variants are more frequently associated with cortical visual impairment and gastrointestinal issues. Seizures were only observed in individuals with variants in or near the HECT domain.

**Conclusion:** We provide a comprehensive review and expansion of the genotypic and phenotypic spectrum of *HECW2* disorders, aiding future molecular and clinical diagnosis and management.

**Key Words:** HECW2, neurodevelopment, intellectual disability

## Introduction

Neurodevelopmental disorders (NDDs) affect >3% of children worldwide and can often pose a significant burden on both the individual and their family. NDDs with intellectual disability (ID) are characterized by cognitive impairment and challenges with adaptive behavior [1]. Genetic factors have been shown to play an important role in the etiology of NDDs, especially in more severe NDDs [2, 3]. Family-based sequencing approaches have demonstrated that undiagnosed cases of ID are largely caused by *de novo* variants in genes and/or pathways which genetically overlap with other NDDs [4–7].

HECW2, also known as NEDL2, is a member of the Nedd4 family of mammalian E3 ubiquitin ligases involved in regulation of p73 stability [8, 9]. Several members of Nedd4 are involved in cell signaling pathways that directly affect proliferation, migration and differentiation through glial cell line- derived neurotrophic factor (GNDF)/Ret signaling [8, 10]. HECW2 combines directly with p73, a member of the p53 family that is predominantly expressed in adult brain, heart and lungs and plays a significant role in both adult and embryonal neurogenesis [8, 9, 11]. Additionally, p73 knockout mice show developmental defects of the central nervous system, embryonal and adult neurogenesis and display a reduction in cortical thickness due to the loss of mature cortical neurons [11]. Animal models of HECW2 show severe abnormalities in early development. Hecw2 null mice die within two weeks after birth with progressive bowel motility defect and a decreased number of enteric neurons [10]. Knockdown of *hecw2a* in zebrafish led to early morphological abnormalities of the brain, spinal cord, eyes, the body trunk and tail [12].

Recently, independent studies have identified a total of six *de novo* heterozygous likely pathogenic and pathogenic missense variants in *HECW2* in 13 families with ID/developmental delay, hypotonia, seizures and other variable features [12–18]. In this study, we include 22 new cases and expand the genotypic and phenotypic spectrum associated with *HECW2*-related neurodevelopmental disorders.

## Materials and methods

### Patient recruitment and clinical assessment

Written informed consent was obtained from the parents/legal guardian, and all studies were approved by the Ethics Committee or Institutional Review Board of all contributing centers. Patients and their clinical data were gathered from several clinical diagnostic and research laboratories, either through exchange between laboratories or through GeneMatcher [19]. All

affected individuals were evaluated by a child neurologist and/or a clinical geneticist. Detailed genetic data were obtained from each participating lab, and clinical information was gathered from clinicians [13–18].

**Sequencing analysis**

Genomic DNA was obtained from the affected individuals and parents (if possible). In all cases, next-generation sequencing was performed in the affected individuals and available parental samples. Exome sequencing was performed for all individuals in either in a clinical or research setting, with the exception of cases 3 and 4 who had targeted sequencing of a panel of genes for ID and case 12 who had genome sequencing. In short, exonic regions and flanking splice junctions were captured and paired-end sequencing was performed on an Illumina instrument (Illumina Inc, San Diego, CA, USA). For case 12, PCR-free whole genome sequencing libraries were prepared using Kapa Hyper Prep (Roche Holding AG, Basel, Switzerland) followed by sequencing on an Illumina HiSeq4000 with paired-end reads (Illumina Inc, San Diego, CA, USA). After removing low-quality reads, the filtered reads were aligned to the human reference genome. Further processing and variant calling of single nucleotide variants (SNVs) and insertion/deletions (InDels) were performed using a “best practices” workflow. Variants were annotated and filtered based on variant minor allele frequency based on databases such as the Genome Aggregation Database (gnomAD) and The Trans-Omics for Precision Medicine (TOPMed) Bravo database [20, 21], variant location and predicted effect on protein function. *De novo* variants identified in patients 3 and 6 were confirmed by Sanger sequencing as they were identified in a research setting. *HECW2* variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines [22]. Variants reported in this manuscript were classified as pathogenic/likely pathogenic. In addition, variants of uncertain significance were also included for affected individuals in which this was the primary suspected causal variant, but for which there was not enough evidence to classify the variant as likely pathogenic (such as absence of a parental genotype).

**Homology modeling**

The amino acid sequence of HECW2 (isoform 1) was retrieved from the UniProt database (identifier: Q9P2P5). Three-dimensional modelling techniques were utilized for the construction of wild-type and mutant HECW2 using the MODELLER program [23]. The crystal structure of WWP1 (PDB ID: 6J1X) [24] was used as a template for the construction of wild-type (WT) and mutant (MT: p.Arg1191Gln, p.Asn1199Lys, p.Phe1327Ser, p.Arg1330Trp) models of HECW2.

PYMOL was used for structural visualization and image processing (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

### HECW2 genotype and phenotype analysis

A literature search for *HECW2* was performed to retrieve all published cases with likely pathogenic and pathogenic *HECW2* variants reported to be implicated in a neurodevelopmental disorder with available clinical details. All variants in *HECW2* were annotated using ANNOVAR [25]. Bioinformatic prediction scores were annotated from dbnsfp35a and dbscSNV1.1 to evaluate missense and splice site variants respectively [26, 27], including Combined Annotation Dependent Depletion (CADD) and Genomic Evolutionary Rate Profiling (GERP++) scores [28, 29]. A Fisher's exact test was done to test differences between groups.

## Results

### Genetic spectrum of *HECW2* variation

We identified 22 novel cases with a neurodevelopmental disorder associated with 15 different heterozygous missense variants in *HECW2* (Table 1; Supplementary Table 1). In 18 cases, the variant was confirmed to be *de novo*, including one case (case 10) with a mosaic variant in the proband (Table 1). Thirteen of the variants identified were novel variants and two variants [p.(Arg1191Gln) and p.(Arg1330Trp)] have previously been published and are thus recurrent [13–18]. When considering all *HECW2* variants (N=19) and all cases reported to date (N=35), we identified four variants present in at least two cases [p.(Arg1191Gln), p.(Asn1199Lys), p.(Phe1327Ser) and p.(Arg1330Trp)]. Two missense variants [p.(Arg1191Gln) (N=8/35) and p.(Arg1330Trp), (N=7/35)] were present in 22.9% and 20% of cases respectively [13–18]. In addition, we identified two amino acid (E1445 and R1495) each with two different mutations, and a clustering of mutations at T1503, S1504, and S1505 each in a single patient (Table 1).

All variants identified in this study (N=13) and those previously published were missense variants (Table 1; N=19 total), with one missense variant p.(Tyr863His) which may impact pre-mRNA splicing as well and lead to a loss of the wild-type 3' acceptor of exon 12 (based on Human Splicing Finder and dbscSNV; Table 1), however SpliceAI did not predict a significant change [30]. All variants were absent from gnomAD and TopMed databases (Supplementary table 1) [20].

All variants had a CADD score > 20 and were located at positions conserved between species (Table 1).

The majority of all variants identified in *HECW2* so far (N=19) are present in the C-terminal area of the protein (18/19; 94.7%; Figure 1), and particularly in the C-terminal HECT domain of HECW2 (11/19; 57.9%). Nearly all likely pathogenic or pathogenic variants (15/17; 88.2%) are located in or near the C-terminal HECT domain. Three variants were present in the region of interaction with *TP73* (3/19; 15.8%) and one in the coiled-coil domain (1/19; 5.3%) (Figure 1).

All variants were deposited into ClinVar under accession numbers: SCV001519450, SCV001519452-SCV001519463, SCV000741462 and SCV001444913.

**Phenotypic spectrum of *HECW2* variation**

We report 22 cases (10 females and 12 males) with variants in *HECW2* (median age = 4.8 years [22 months - 27 years]) (Table 2; Supplementary Table 2). All our cases presented with developmental delay and mild to severe intellectual disability along with several other features including hypotonia. We analyzed the prevalence of clinical features of our 22 cases together with 13 previously published cases (Table 2; Supplementary table 2) [12–18]. Clinical features mentioned below are based on individuals with variants classified as pathogenic and likely pathogenic variants only (N= 33).

Developmental delay and delayed or absent speech were prevalent in all patients assessed (100%). Intellectual disability was present in all individuals old enough to assess (100%). Of all individuals 5 years old or older, 46.2% (6/13) could talk and 58.3% (7/12) could walk, although often with limited capacity. All our patients exhibited hypotonia with or without spastic rigidity (100%). Axial hypotonia was common, with considerable additional problems with movement/motor coordination (75%). Stereotypies such as hand flapping and rocking were common (57.1%), and chorea, choreoathetosis, an ataxic gait, dyspraxia and dystonia were also observed.

Behavioral abnormalities were described in 88.9% of cases, including autism spectrum disorder (ASD) following DSM-5 criteria in five cases and autistic features in 16 cases. One or more ASD-like features included self-stimulatory behaviors, attachment and social interaction abnormalities, self-injurious behaviors, inappropriate distress when frustrated, sensory aversions and

indifference/insensitivity to pain were present a majority of individuals. Hyperactivity, impulse control, episodes of inconsolability and occasional aggressive behaviors were also observed.

Optic neuropathy, cortical visual impairment (CVI) and ocular movement disorders were commonly noted (83.9%). CVI was noted in 53.8% of patients with visual abnormalities, though foveal hypoplasia and optic nerve coloboma and atrophy/hypoplasia were also seen. A range of ocular movement abnormalities were described, including esotropia, exotropia, dissociated vertical deviation, supranuclear gaze palsy, nystagmus and abnormalities of ocular fixation and conjugate movements.

Esophageal-gastrointestinal dysmotility and/or dysphagia (70%) was reported in the form of gastroesophageal reflux disease (GERD), gastroparesis, and constipation with eight cases requiring gastrotomy tube.

Seizures, previously recognized as a main feature of *HECW2*-related neurodevelopmental disorders, were present in 61.3% of cases. EEG abnormalities were observed in 69.6% of cases when assessed (N=23). Abnormal brain MRI findings were present in 53.1% of available cases, often demonstrating global decreased parenchymal volume. There was considerable variation in additional findings, which included cortical dysplasia of the left temporal lobe, poor development of anterior temporal lobes and a thin corpus callosum. In three out of four cases with serial brain MRIs available, progressive parenchymal volume loss was noted over time. These three cases were all reported to have seizures, with one case described with a normal MRI before seizure onset [17]. Some of patients have microcephaly (20%) and short stature (25%). The median head circumference was -0.51 SD and height was -0.66 SD for available cases.

Facial dysmorphisms were observed in 56.7% of the cases, commonly with prominent forehead and nasal bridge, deep-set eyes, midface retrusion, and high-arched palate. Disorders or difficulties of sleep were found in 42.9%, and were mainly represented by insomnia, with one case of sleep apnea and a case of twins exhibiting somnambulism features. Less common manifestations of *HECW2*-related neurodevelopmental disorder were skeletal abnormalities (32.1%). Decreased bone mineralization was observed most often followed by pectus excavatum, pes planus, brachydactyly of hands and feet, and hindfoot and pronation deformities of the feet. Joint involvement was comprised of contractures (arthrogryposis) and joint hypermobility. Failure to thrive was reported in 28% of cases.

Cardiac abnormalities were reported in three patients, each with a unique presentation. Case 2, a toddler male, had sinus bradycardia; case 19, middle childhood male, had a small patent foramen ovale with left to right atrial shunting in addition to an incomplete right bundle branch block; and case 1, a middle childhood female, had cardiomyopathy.

**Structural analysis of normal and mutant HECW2**

The three-dimensional structures of the wild-type (WT) and mutant (MT) HECW2 proteins were built using homology modeling techniques. HECW2 isoform 1 consists of 1572 amino acids (AA) and includes a C2 domain (yellow), a WW1 and WW2 domain (green) and a C-terminal HECT domain (cyan) (Figure 1A). We modeled 1089–1566AA of WT and MT HECW2 to inspect the 3D location of the C-terminal variants (Figure 1B) and the potential effect of these two recurrent variants on HECW2 (p.Arg1191Gln, p.Asn1199Lys, p.Phe1327S and p.Arg1330Trp; Figure 1C-E). Figure 1B shows that all HECT domain variants cluster three-dimensionally with the four variants adjacent to the HECT domain. Homology modelling of HECW2 with variants p.Arg1191Gln, p.Asn1199Lys, p.Phe1327S and p.Arg1330Trp displayed some degree of difference in overall conformation compared to WT with a root mean square deviation (RMSD) value of 0.72 Å, 1.06 Å, 1.12 Å and 0.693 Å, respectively (Figure 1C). Moreover, the surface electrostatic potential displays a difference in charge distribution of these variants as compared to WT as shown in figure 1D-E.

**Genotype-phenotype correlation**

As mentioned above, the majority of variants identified in HECW2 so far were present in the C-terminal area (Figure 1). One variant however was identified in the N-terminal region [p.(Ile138Val) in case 10]. This case presents with a milder phenotype without seizures (Supplementary Table 1); however, was found to be mosaic for the variant which may explain his milder phenotype. Cases 7 [p.(Ala940Thr)] and 16 [p.(Tyr863His)], with variants that are located mid-protein between the WW domains, also had a milder phenotype without seizures. All patients with a severe phenotype including seizures/epilepsy had a variant in or adjacent to the HECT domain (Figure 1B; Suppl Table 2). Seizures/epilepsy were present in 65.5% (19/29) of individuals with a HECT domain or adjacent HECT variant (Figure 1B) and absent (0/4) from individuals with variants in other locations of HECW2 (p=0.025).

We next assessed 18 individuals with likely pathogenic and pathogenic variants in the HECT domain and 15 individuals with likely pathogenic and pathogenic variants outside the HECT

domain. There were a few significant differences noted between these two groups: Individuals with HECT domain variants had a significantly higher frequency of CVI (68.8 % [11/16] HECT versus 20% [3/15] non-HECT; p=0.011). Gastrointestinal issues were also more common in the HECT group (87.5%; 14/16) than the non-HECT group (50%; 7/14) [p=0.046], with a larger percentage being fed via gastrostomy tube in the HECT domain variant group (37.5% vs 21.4%).

Recurrent variant [p.(Arg1330Trp)], which was identified in seven cases (20%), was associated with severe ID, developmental delay, CVI and gastrointestinal (GI) disorders. Seizures and brain MRI abnormalities were reported in five of the seven [p.(Arg1330Trp)] cases with available information, and two cases had reported intractable seizures. EEG was abnormal in all cases assessed (N=4). Six cases were non-verbal or severely limited in their speech, and one was too young to assess. Five of six cases with available information presented with CVI and GI abnormalities. Additionally, four of six of these patients had movement disorders.

Another recurrent variant [p.(Arg1191Gln); 22.9% (8 cases)] revealed considerable phenotypic variability. Four of seven patients had seizures with at least one with severe intractable generalized seizures. Most patients with this variant were non-verbal, with a speech capability of a limited number of words in two individuals who were more than five years old. Five patients demonstrated movement disorders. Six patients with the variant [p.(Arg1191Gln)] reported visual disorders, ranging from strabismus (N=3), to optic neuropathies (N=2) and CVI (N=2).

## Discussion

In this study, we further delineate the genotypic and phenotypic spectrum of *HECW2*-related neurodevelopmental disorder. Heterozygous missense variants in *HECW2* have been associated with an autosomal dominant neurodevelopmental disorder with hypotonia, seizures and absent language (NDHSAL; OMIM #617268). By comparing published cases (N=13) and novel cases (N=22), we show that developmental delay, intellectual disability, abnormal muscle tone and speech delay are common features of the disorder, and that seizures and other associated features are variable.

HECW2 (alias NEDL2) is expressed in all regions of the adult and fetal brain and facilitates neurogenesis and neurodevelopment [13, 14]. We identified 13 novel variants in *HECW2*, bringing the total number of reported variants to 19. All are missense variants. HEWC2 is intolerant to

missense variants (z=3.305) [20] and subRVIS (Residual Variation Intolerance Score) demonstrates this to be true for the majority of the protein (C2 domain and everything C-terminal of this domain, with exception of the WW domains) [31]. The C-terminal HECT domain and its vicinity are associated with the highest mutation burden in all cases (31/35 cases). HECW2 is a HECT (homologous to E6-AP carboxy terminus)-type E3 ubiquitin ligase, with the C-terminal HECT domain containing the catalytic cysteine residue (p.1540) required for substrate ubiquitination [32]. As no nonsense or frameshift variants have been described, nor variants in the catalytic cysteine residue (p.1540), this suggests that variants might show reduced function (but not completely abolished) or act through a gain-of-function mechanism. This hypothesis is supported by the fact that *HECW2* is highly intolerable of haploinsufficiency and complete loss-of-function (pLI = 1) [20].

There are 16 large deletions in ClinVar encompassing *HECW2* [33]. The majority (N=13) are deletions encompassing several genes in this genomic region, referred to as chr2q32-q33 microdeletion syndrome, of which haploinsufficiency of *SATB2* is an established mechanism of disease. Although chr2q32-q33 microdeletion syndrome and *HECW2*-neurodevelopmental disease have several clinical features in common, these overlapping features are consistent with *SATB2* haploinsufficiency [34, 35]. In addition, two deletions covering *HECW2* and other genes (but not *SATB2*) have been described with limited clinical information. Only one deletion encompassing *HECW2* in a single patient with schizophrenia has been described [36]. Therefore, it is unclear whether heterozygous deletions of *HECW2* support loss of function as the mechanism of *de novo* missense variants in *HECW2*.

A gain-of-function mechanism is however supported by the clustering of recurrent missense variants in genes in neurodevelopmental disorders [37]. We identified specific protein domains implicated in *HECW2* neurodevelopmental disease. For example, we identified four recurrent variants [p.(Arg1191Gln); p.(Asn1199Lys); p.(Phe1327Ser); p.(Arg1330Trp)]. Two variants [p.(Arg1191Gln) and p.(Arg1330Trp)] were mutated in an exceptionally high percentage of cases (22.9% and 20% respectively). In addition, we found two locations E1445 and R1495, mutated in two cases each, and 3 variants present at positions T1503, S1504, S1505, showing clustered protein locations in the HECT domain. With three-dimensional modeling we show that all HECT domain variants and the variants adjacent to the HECT domain cluster closely together in two areas (Figure 1B), suggesting these specific areas are important in protein function and *HECW2*-neurodevelopmental disease. We also demonstrate that the four recurrent variants, p.(Arg1191Gln); p.(Asn1199Lys); p.(Phe1327Ser); p.(Arg1330Trp), show a difference in protein

surface property (electrostatic surface potential) and conformation (RMSD value) compared to WT. This suggests these variants can affect the protein function, stability, enzymatic function or the binding of partner biomolecules.

Furthermore, for other neurodevelopmental disorders caused by rare variants in E3 ubiquitin ligases, such as *HUWE1* and *NEDDL4*, the majority of pathogenic variants are also found in the HECT domain and are missense [38, 39]. For HUWE1, both loss-of-function and gain-of-function mechanisms have been implicated in neurodevelopmental disease, each leading to distinct types of ID [40]. The mechanisms of gain-of function effects through missense variants are not fully elucidated; however, increased protein levels or increased auto-ubiquitination have been described for certain missense mutations in and near the HECT domain *in vitro* [41, 42]. Missense variants in the HECT domain of NEDD4L are believed to alter conformation and subsequently trigger auto-ubiquitination followed by proteasomal degradation [38].

Clinical analysis of *HECW2*-related neurodevelopmental disorders in this study and existing literature suggests that intellectual disability, developmental delay and hypotonia are consistent features in all cases. Language and/or speech impairments were identified in all patients. Hypotonia is central with greater involvement of the musculature of the trunk.

Brain MRI was normal in 46.9% of cases when assessed. In affected individuals with an abnormal MRI, abnormalities were regularly consistent with global decreased parenchymal volume. The etiology of this is unclear, but this is likely mainly due to an abnormal neurogenesis, as evidenced by the presence of cortical dysplasia, hypoplasia of the corpus callosum and anterior temporal lobes, and other brain malformations in several cases. Abnormal neurogenesis in *HECW2*-related neurodevelopmental disorders is also supported by studies in animal models. Prior studies of p73 knockout mice, a direct interaction partner of *Hecw2*, demonstrated poor differentiation of oligodendrocytes and defects in both embryonal and adult neurogenesis [11]. Data from *Hecw2a* knock down in zebrafish, which display early brain developmental defects, also suggests a motor neuron migration defect [12]. The latter can cause malformations of cortical development, such as cortical dysplasia, which predisposes patients to seizures [12, 43]. Focal cortical dysplasia of unilateral or bilateral temporal lobes has been directly described in our cohort. Seizures were identified in 61.3% of patients with variable presentation.

Behavioral abnormalities in *HECW2* neurodevelopmental disorder patients are common (88.9%) and encompass similar manifestations. Compulsive, unusual and repetitive movements are

observed in patients with behavioral and movement abnormalities. Hyperactivity, impaired impulse control, and limited tolerance for frustration, inappropriate laughter, self-injurious or self-stimulatory behaviors and/or unusual repetitive movements are all common. We show that five cases with *HECW2*-related neurodevelopmental disease also qualify for a diagnosis of ASD. Other cases were too young for evaluation and/or with abnormalities of social interactions and self-stimulatory, restricted, and repetitive behaviors. Currently, the Gene4denovo database, an integrated database of *de novo* variations in humans, contains 29 *de novo HECW2* variants of unknown significance in cases labeled as autism [44], of which 28 are non-coding.

Gastrointestinal issues were seen in 70% of patients. Existing literature has associated reduced expression of *HECW2* with congenital aganglionic megacolon (Hirschsprung's disease), that can present with delayed meconium passage, constipation, feeding changes and in severe cases necrotizing enterocolitis [45, 46], due to underdevelopment of the nerve cells in the large intestine (colon). Hirschsprung's disease is caused by variants in RET, and its ligand GDNF is regulated by HECW2 [10]. In addition, mice lacking Hecw2 exhibit decreased numbers of enteric neurons, progressive bowel dysmotility and intestinal hypoganglionosis [46]. Given this, a neurologic etiology of enteric system dysfunction may contribute to GI issues in *HECW2* neurodevelopmental disease.

Repetitive movements and stereotypies were commonly seen in *HECW2*-related neurodevelopmental disease (57.1%). Non-specific chorea and choreoathetosis were also observed.

Last, consistent and comprehensive genotype-phenotype correlation is difficult, given the small number of individuals described. HECT domain and closely adjacent HECT variants, including recurrent variant p.(Arg1330Trp), seem to be associated with a more severe phenotype including seizures. HECT domain variants are more highly associated with CVI and severe GI issues compared to any non-HECT variants. Finally, individuals with the recurrent p.(Arg1191Gln) variant show a variable presentation of features, which suggests that environmental factors during fetal development or the genomic background might be important in the determination of disease presentation.

In conclusion, we expand the phenotypic spectrum of *HECW2*-related neurodevelopmental disease and we show that developmental delay/intellectual disability, hypotonia and speech delay are consistent features. Vision problems, particularly cortical visual impairment, behavioral and

GI issues are also common features of the condition. Finally, we report that pathogenic variants are clustered in and near the HECT domain of the protein with the recurrence of several variants.

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**Competing interests**

LF, KW, IMW, FZ, EAN, and ET are employees of GeneDx, Inc. All other co-authors have no conflicts of interest related to the work in this manuscript.

**Ethics approval statement**

The study was approved by the Columbia University IRB (IRB-AAAS3433).

**Contributorship statement**

AA, HK, PD, IS: Data analysis, data curation, literature review and first draft preparation; AN: Homology modeling; IS, WC: Study set-up, supervision and manuscript revision; LF, KW, IMW, MRZR, CF, TS, MR, KB, SW, AZ, MB, AC, CSH, AA, TC, SH, EFM, CH, JAH, DL, FT, VKM, MC, SML, IJ, EAN, FZ, MH, BK, ET: clinical and genetic data organization; All authors have read and revised the manuscript.

**Data sharing statement**

HECW2 variants reported in this study were deposited into ClinVar under accession numbers: SCV001519450, SCV001519452- SCV001519463, SCV000741462 and SCV001444913 (https://www.ncbi.nlm.nih.gov/clinvar/). There are no conditions to reuse the data.

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## Figure and Table legends

**Figure 1: Location, homology modeling and structural analysis of HECW2 variants.** (A). Isoform 1 HECW2 C2 domain: AA 167–301 (yellow); WW1 domain: AA 807–840 (green); WW2 domain: AA 985–1018 (green); HECT domain: AA 1237–1572 (cyan). Novel variants identified in this study are indicated above HECW2; variants previously published are indicated in the bottom. Variants highlighted in blue were found in ≥ 2 independent cases, with the number of individuals found per variant indicated in parenthesis. *may impact splicing as well. All variants are classified as likely pathogenic or pathogenic, with exception of **two VUS found in two novel cases. (B) Three-dimensional (3D) structures of WT HECW2 predicted using the MODELLER program and visualized with the PyMOL software. The location of the variants here and previously identified are mapped on the WT structure of HECW2 and the residues are shown as a stick model. (C) Superposed protein structures of WT and MT of the 4 recurrent variants. The RMSD (Å) were calculated among the WT and mutant protein structure and are labeled in each model. (D-E) Surface electrostatic potential of WT and MT models generated by the PyMOL APBS plugin. The color scale for surface electrostatic potential was set from −5 kT/e (red) to 5 kT/e (blue). The location of the variant is represented as a circle. The zoom-up views show the difference in electrostatic potential at the site of mutation.

**Table 1: All variants identified in *HECW2* in this study and published reports.**

| Variant* | ACMG | Inheritance | Recurrent variant | Known/novel variant | CADD score | GERP++ RS score | dbscSNV ADA score | Subject ID in this study^ | Published cases^ |
|---|---|---|---|---|---|---|---|---|---|
| c.412A>G:p.(Ile138Val) | LP | de novo (mosaic [26%])** | | novel | 23.7 | 6.04 | . | 10 | |
| c.2587T>C:p.(Tyr863His) | LP | de novo | | novel | 28.2 | 5.39 | 0.9172 | 16 | |
| c.2818G>A:p.(Ala940Thr) | VUS | unknown*** | | novel | 33 | 4.84 | . | 7 | |
| c.3175C>T:p.(Pro1059Ser) | VUS | unknown*** | | novel | 26.1 | 5.45 | . | 4 | |
| c.3572G>A:p.(Arg1191Gln) | P | de novo, unknown*** | yes | known | 35 | 4.75 | . | 5, 12, 13, 14, 22 | A, C, D |
| c.3577T>G:p.(Phe1193Val) | LP | de novo | | known | 28.4 | 4.75 | . | | E, F^^ |
| c.3583G>C:p.(Ala1195Pro) | LP | de novo | | novel | 32 | 4.75 | . | 19 | |
| c.3597C>A:p.(Asn1199Lys) | LP | de novo | yes | novel | 25.9 | 3.87 | . | 11, 15 | |
| c.3980T>C:p.(Phe1327Ser) | LP | de novo, unknown*** | yes | novel | 33 | 5.08 | . | 1, 3 | |
| c.3988C>T:p.(Arg1330Trp) | P | de novo | yes | known | 34 | 3.32 | . | 9, 17 | B, G, H, J, M |
| c.4323T>G:p.(Phe1441Leu) | LP | de novo | | novel | 28.6 | 1.53 | . | 6 | |
| c.4333G>C:p.(Glu1445Gln) | P | de novo | | novel | 33 | 5.11 | . | 21 | |
| c.4334A>G:p.(Glu1445Gly) | P | de novo | | known | 32 | 5.11 | . | | I |
| c.4355G>T:p.(Gly1452Val) | LP | de novo | | novel | 34 | 5.11 | . | 8 | |
| c.4484G>A:p.(Arg1495Lys) | P | de novo | | known | 34 | 5.36 | . | | K |
| c.4485G>T:p.(Arg1495Ser) | P | de novo | | known | 34 | 3.45 | . | | L |
| c.4507A>G:p.(Thr1503Ala) | LP | de novo | | novel | 28.1 | 5.35 | . | 20 | |
| c.4511C>A:p.(Ser1504Tyr) | LP | de novo | | novel | 33 | 5.35 | . | 2 | |
| c.4514G>C:p.(Ser1505Thr) | LP | de novo | | novel | 33 | 5.35 | . | 18 | |

*Based on NM_020760.4

**Defined as <35% allelic fraction in next generation sequencing reads

***Heterozygous variant presumed to be de novo, however, one or two parent(s) not available to confirm if de novo

^Case numbers correlate with Table 2 and Supplementary Table 2

^^Monozygotic twins

**Table 2: Clinical features identified in novel and published cases with *HECW2*-related neurodevelopmental disease.** NA: Not assessed or unavailable. Individual details are only shown for novel cases.

| Clinical features | Total of clinical features (%) This study | Total of clinical features (%) All reported cases (P and LP)* | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 | Case 7 | Case 8 | Case 9 | Case 10 | Case 11 | Case 12 | Case 13 | Case 14 | Case 15 | Case 16 | Case 17 | Case 18 | Case 19 | Case 20 | Case 21 | Case 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | | | F | M | M | F | F | M | M | F | F | M | F | M | F | M | M | F | M | F | M | F | M | M |
| Variant (Based on NM_020760.4) | | | p.(Phe1327Ser) | p.(Ser1504Tyr) | p.(Phe1327Ser) | p.(Pro1059Ser) | p.(Arg1191Gln) | p.(Phe1441Leu) | p.(Ala940Thr) | p.(Gly1452Val) | p.(Arg1330Trp) | p.(Ile138Val) | p.(Asn1199Lys) | p.(Arg1191Gln) | p.(Arg1191Gln) | p.(Arg1191Gln) | p.(Asn1199Lys) | p.(Tyr863His) | p.(Arg1330Trp) | p.(Ser1505Thr) | p.(Ala1195Pro) | p.(Thr1503Ala) | p.(Glu1445Gln) | p.(Arg1191Gln) |
| Developmental delay | 22/22 (100) | 33/33 (100) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Speech impairment | 22/22 (100) | 30/30 (100) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Muscular disorders | 22/22 (100) | 32/32 (100) | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Intellectual disability | 18/19 (94.7) | 24/24 (100) | + | NA | NA | + | + | + | - | + | + | NA | + | + | + | + | + | + | + | + | + | + | + | + |
| Behavioral problems | 19/21 (90.5) | 24/27 (88.9) | + | NA | - | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Vision problems | 18/22 (81.8) | 26/31 (83.9) | + | + | + | - | - | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + |
| Movement/motor coordination | 14/20 (70) | 21/28 (75) | + | + | + | - | + | - | + | - | - | - | - | + | + | + | NA | + | + | + | + | + | + | NA |
| GI problems | 14/22 (63.6) | 21/30 (70) | + | + | + | + | - | - | - | + | + | - | - | + | + | + | - | - | + | + | - | + | + | + |
| EEG abnormalities | 9/16 (56.3) | 16/23 (69.6) | + | - | + | NA | NA | - | NA | NA | + | NA | + | - | NA | + | + | + | + | - | - | - | - | + |
| Epilepsy/Seizures | 8/21 (38.1) | 19/31 (61.3) | + | NA | + | - | + | - | - | - | + | - | + | - | - | + | + | - | - | - | + | - | - | - |
| Facial dysmorphism | 9/22 (40.9) | 17/30 (56.7) | - | - | - | - | + | - | + | - | - | - | - | - | + | + | - | + | + | + | + | + | - | - |
| MRI Abnormalities | 9/21 (42.9) | 17/32 (53.1) | - | - | - | NA | - | + | - | + | + | + | + | + | - | - | - | - | + | + | - | + | - | + |
| Sleep Abnormalities | 9/22 (40.9) | 12/28 (42.9) | + | - | - | - | - | - | - | - | + | - | - | + | + | + | + | + | - | + | - | + | - | - |
| Skeletal Abnormalities | 4/22 (18.2) | 9/28 (32.1) | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | + | + | - | - |
| Failure to thrive | 6/20 (30) | 7/25 (28) | - | NA | + | - | - | - | - | + | + | - | - | NA | + | - | - | + | + | - | - | - | - | - |
| Short stature | 6/21 (28.6) | 7/28 (25) | - | NA | + | - | - | - | - | + | + | - | - | + | + | - | - | + | - | - | - | - | - | - |
| Cardiac abnormalities | 2/13 (15.4) | 3/15 (20) | NA | + | NA | NA | NA | NA | NA | + | NA | NA | NA | - | - | - | - | - | - | - | + | - | - | - |
| Microcephaly | 4/22 (18.2) | 6/30 (20) | + | - | - | - | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - |

*Individuals with pathogenic (P) and likely pathogenic (LP) variants only

**Supplementary data**

**Supplementary Table 1: Detailed variant annotation of all variants identified in HECW2 in this study and published reports.**

**Supplementary Table 2: Details of all clinical features identified in novel and published cases with *HECW2*-related neurodevelopmental disease.** NA: Not assessed or unavailable.