Haploinsufficiency of the Notch Ligand DLL1 Causes Variable Neurodevelopmental Disorders

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Notch signaling is an established developmental pathway for brain morphogenesis. Given that Delta-like 1 (DLL1) is a ligand for the Notch receptor and that a few individuals with developmental delay, intellectual disability, and brain malformations have microdeletions encompassing *DLL1*, we hypothesized that insufficiency of *DLL1* causes a human neurodevelopmental disorder. We performed exome sequencing in individuals with neurodevelopmental disorders. The cohort was identified using known Matchmaker Exchange nodes such as GeneMatcher. This method identified 15 individuals from 12 unrelated families with heterozygous pathogenic *DLL1* variants (nonsense, missense, splice site, and one whole gene deletion). The most common features in our cohort were intellectual disability, autism spectrum disorder, seizures, variable brain malformations, muscular hypotonia, and scoliosis. We did not identify an obvious genotype-phenotype correlation. Analysis of one splice site variant showed an in-frame insertion of 12 bp. In conclusion, heterozygous *DLL1* pathogenic variants cause a variable neurodevelopmental phenotype and multi-systemic features. The clinical and molecular data support haploinsufficiency as a mechanism for the pathogenesis of this *DLL1*-related disorder and affirm the importance of DLL1 in human brain development.

The evolutionarily conserved Notch signaling pathway operates in many different developmental, homeostatic, and disease processes. Cell-cell contact-mediated Notch receptor-ligand interactions release the Notch intracellular domain (NICD), which enters the nucleus and stimulates transcription of target genes.[1] All mammalian Notch ligands are transmembrane proteins and interact through the extracellular Delta-Serrate-LAG2 (DSL) and amino-terminal (NT) domains with the epidermal growth factor (EGF) repeats 11-12 of the extracellular domain of Notch. Modification of the EGF repeats by β3-*N*-acetylglucosaminyltransferases, encoded by the three mammalian Fringe orthologs Lunatic Fringe (*Lfng*), Manic Fringe (*Mfng*), and Radical Fringe (*Rfng*), enhances or inhibits Notch-ligand activation.[2] Notch signaling is highly sensitive to relative levels of ligands and receptors through feedback loops as well as the mechanism of *cis* inhibition and *trans* activation. Various other regulatory mechanisms like expression profiles of ligands and receptors, post-translational events, and integration with other signaling pathways account for the diversity of Notch signaling outcomes.[1,3]

Several *in vivo* and *in vitro* studies have shown a role for the Notch ligand Delta-like 1 (DLL1) in development of the central nervous system (CNS) as well as in that of somites and lymphocytes.[4–14] *DLL1* (MIM: 606582) is highly expressed in neuronal precursor cells during embryogenesis.[15] It regulates neuronal differentiation through oscillation and lateral inhibition such that differentiating neurons inhibit neighboring cells from undergoing neuronal differentiation by expressing Notch signals in an oscillatory manner.[3,16,17] Consequently, Notch signaling forms cellular fields and influences brain morphogenesis. Supporting its role in neuronal development, loss of Dll1 in mice increases

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Table 1. Clinical Features of Individuals with Heterozygous *DLL1* Pathogenic Variants

Individual	1 (F1/II-1)	2 (F2/II-4)	3 (F2/II-1)	4 (F2/II-2)	5 (F2/I-2)	6 (F3/II-1)	7 (F4/II-1)	8 (F5/II-1)	9 (F6/II-1)	10 (F7/II-1)	11 (F8/II-1)	12 (F9/II-1)	13 (F10/II-1)	14 (F11/II-1)	15 (F12/II-1)	Summary of Clinical Features
Gender	f	f	m	m	f	m	m	f	m	m	f	f	m	f	m	7f/8 m
Age at last visit	3 y	2 y	4 y	5 y	u	8 y	16 y	2 y	2 y	9 y	7 y	35 y	33+2 w	8 y	3 y	birth-35 y
DD/ID (HP:0012758 / HP:0001249)	+	–	–	+	+	+	+	+	+	+	+	+	NA	+	+	12/14
ASD (HP:0000729)	–	–	+	u	u	–	+	–	+	+	+	–	NA	–	+	6/14
ADHD (HP:0007018)	–	u	u	u	u	+	+	–	–	+	–	–	NA	–	–	3/14
Stereotypic behavior (HP:0000733)	–	ND	ND	ND	ND	–	–	+	(+)	–	(+)	–	NA	–	+	4/14
Seizures (HP:0001250)	–	u	u	u	u	+	+	–	–	–	+	+	NA	+	+	6/14
Muscular hypotonia (HP:0001252)	+	ND	ND	ND	ND	+	–	–	(+)	+	+	–	NA	–	+	6/14
Ataxia (HP:0001251)	+	ND	ND	ND	ND	+	–	–	–	–	–	+	NA	–	+	4/14
Abnormal brain MRI (HP:0012443)	+	+	+	ND	ND	+	+	+	+	ND	+	+	+	+	–	11/15
Ventriculomegaly (HP:0002119)	+	–	+	ND	ND	+	–	mild	mild	ND	+	mild	+	–	–	8/15
Hydrocephalus (HP:0000238)	–	+	+	ND	ND	+	–	–	–	ND	–	–	–	–	–	3/15
Abnormal corpus callosum (HP:0001273)	–	–	–	ND	ND	+	+	+	+	ND	+	–	–	+	–	6/15
Cortical dysplasia (HP:0002539)	–	–	–	ND	ND	–	(+)	+	–	ND	–	–	–	(+)	–	3/15
Migration defect (HP:0002269)	–	–	–	ND	ND	–	–	–	–	ND	–	PVNH	–	–	–	1/15
Other brain abnormality	–	–	+	ND	ND	+	–	–	+	ND	–	–	–	+	–	4/15

(*Continued on next page*)

Table 1. ***Continued***

Individual	1 (F1/II-1)	2 (F2/II-4)	3 (F2/II-1)	4 (F2/II-2)	5 (F2/I-2)	6 (F3/II-1)	7 (F4/II-1)	8 (F5/II-1)	9 (F6/II-1)	10 (F7/II-1)	11 (F8/II-1)	12 (F9/II-1)	13 (F10/II-1)	14 (F11/II-1)	15 (F12/II-1)	Summary of Clinical Features
Abnormal prenatal brain imaging	+	+	u	u	u	+	–	+	–	–	+	–	+	–	–	6/15
Abnormal vertebrae (HP:0003468)	ND	ND	ND	ND	ND	sc	sc, ky	sc, ky, sd	ND	ND	sc	ND	–	ND	ND	4/15
Microcephaly (HP:0000252)	–	ND	ND	ND	ND	–	–	–	–	+	–	–	–	ND	+	2/15
Macrocephaly (HP:0000256)	–	ND	ND	ND	ND	+	–	–	–	–	–	–	–	ND	–	1/15
Facial dysmorphism (HP:0000271)	(+)	+	ND	ND	ND	–	+	+	+	+	+	–	+	–	–	8/15
Other (details in Table S1)	+	+	+	+	–	+	+	+	+	+	+	+	–	–	+	12/15
Type of sequence variant	nonsense	nonsense	nonsense	nonsense	nonsense	nonsense	nonsense	nonsense	nonsense	nonsense	nonsense	splice site	splice site	missense	deletion	
Inheritance	*de novo*	affected mother	affected mother	affected mother	u	affected father	u	u	*de novo*	*de novo*	not maternal	*de novo*	*de novo*	*de novo*	*de novo*	

Abbreviations: F, family; f, female; m, male; y, years; w, weeks of gestation; DD, developmental delay; ID, intellectual disability; ASD, autism spectrum disorder; ADHD, attention deficit hyperactivity disorder; NA, not applicable; ND, not determined; u, unknown; PVNH, periventricular nodular heterotopia; (+), subtle/borderline; +, present; –, absent; sc, scoliosis; ky, kyphosis; SD, segmentation defect.

Table 2. Molecular Data of Individuals with Heterozygous *DLL1* Pathogenic Variants (GenBank: NM_005618.3; ENST00000366756.3)

Individual	Sequence Variant (c.DNA)	Amino Acid Change	*DLL1* Deletion	Inheritance
1	c.1492G>T	p.Glu498*	–	*de novo*
2	c.231C>A	p.Cys77*	–	affected mother
3	c.231C>A	p.Cys77*	–	affected mother
4	c.231C>A	p.Cys77*	–	affected mother
5	c.231C>A	p.Cys77*	–	unknown
6	c.1525C>T	p.Arg509*	–	affected father
7	c.2013_2014del	p.Glu673Glyfs*15	–	unknown
8	c.50_51del	p.Cys17Serfs*108	–	unknown
9	c.2013_2014del	p.Glu673Glyfs*15	–	*de novo*
10	c.1401_1405dup	p.Cys469Serfs*70	–	*de novo*
11	c.543_570dup	p.Phe191Thrfs*50	–	not maternal
12	c.54+1G>A	p.Gln18_Val19insIleGlyGlyGln	–	*de novo*
13	c.54_54+1insTAGTCG	p.Val19*	–	*de novo*
14	c.536G>T	p.Cys179Phe	–	*de novo*
15	–	–	arr[GRCh37]6q27 (170591663-170713885)x1; 122 kb deletion of *DLL1* and *FAM120B*	*de novo*

neuronal differentiation and causes CNS hyperplasia and an increased number of neurons,[5,8,18] and Delta-Notch signaling disrupting mutations in the *DLL1* zebrafish ortholog *DeltaA* (*dlA*) cause a wide range of pattering defects in the hindbrain and overproduction of neurons due to the lack of lateral inhibition.[12]

In the past, overlapping microdeletions of the chromosomal region 6q27 with a smallest region of overlap encompassing the genes *DLL1, THBS2* (MIM: 188061), *PHF10* (MIM: 613069), *ERMARD* (MIM: 615532), and others have been identified in individuals with developmental delay (DD), intellectual disability (ID), and brain malformations. These genes have been discussed as candidates for brain malformations,[19] but statistical analysis of large population sequencing data indicates that only *DLL1* is very likely intolerant to loss of function (LoF).[20] Combined with the functions of DLL1 and its homologs and the prior report of a *de novo* nonsense variant in an individual with autism spectrum disorder (ASD), this led us to hypothesize that insufficiency of DLL1 causes a human neurodevelopmental disorder.[19,21–23] We report 15 individuals from 12 unrelated families with DD, ID, ASD, seizures, brain malformations, and other multi-system features with heterozygous pathogenic variants in *DLL1* (Figure 1A). These findings establish *DLL1* as disease-associated gene and delineate the *DLL1*-related phenotypes.

Individuals with *DLL1* pathogenic variants were identified in several genetic centers across the world by exome sequencing (see Supplemental Subjects and Methods). The cohort was identified through GeneMatcher.[24] Referring physicians were asked to provide details on the medical history of the individuals. The referring clinicians obtained informed consent for genetic testing, publication of clinical and genetic data, re-evaluation of MR images, functional analysis of blood cells, and publication of facial photographs (if applicable). The ethics board of the involved institutions approved the study (Charité Berlin: protocol EA2/177/18; Clinical Research Ethics Board at the University of British Columbia [Vancouver, BC, Canada]: protocol H15-00092).

The clinical and molecular data of the 15 individuals with *DLL1* pathogenic variants (GenBank: NM_005618.3) are summarized in Tables 1 and 2. Detailed clinical descriptions of the individuals are provided in the Supplemental Note and Table S1. The most common features in our cohort were DD/ID (12/14), ASD (6/14), other behavioral abnormalities, seizures (6/14), and brain MRI abnormalities (11/15). We reviewed all but three brain MR images of the individuals; Figure 2 shows representative brain MR images. Brain abnormalities were non-specific and included hydrocephalus, ventriculomegaly, anomalies of the corpus callosum, cortical dysplasia, a small cerebellum/pons, and periventricular nodular heterotopias (PVNH) identified in single individuals. Two individuals had microcephaly, and one individual had macrocephaly due to severe hydrocephalus. Three individuals had kyphosis and/or scoliosis and spine radiographs of one individual with lumbar scoliosis (individual 8, F5/II-1 in Figure 1A) showed a segmentation defect of the lumbar spine (L2 half vertebra) and mild asymmetry of the S1 vertebra (Figures 1B). Clinical facial photographs of individuals 1, 6, 7, 9, and 11 (F1/II-1, F3/II-1, F4/II-1, F6/II-1, and F8/II-1 in Figure 1A, respectively) were available for comparison. Some individuals had mild facial

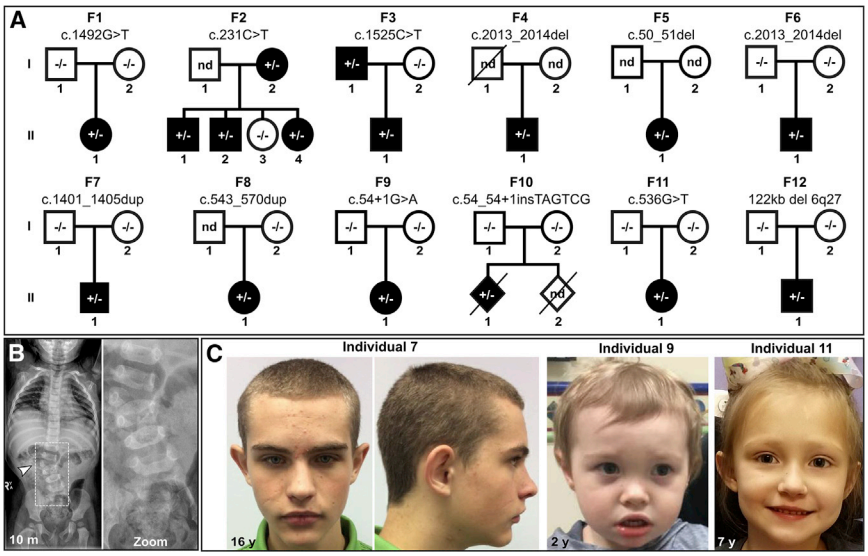

Figure 1. Pedigrees of the Affected Individuals and Phenotypic Presentation

(A) Twelve unrelated families with *DLL1* pathogenic variants. The *DLL1* variants occurred *de novo* in families (F) 1, 6, 7, 9, 10, 11, and 12. The *DLL1* variant co-segregated with the phenotype in F2 and F3. Inheritance is unknown due to lack of parenteral DNA samples for F4, F5, and F8.

(B) Radiographs of individual (I) 8 (F5/II-1 in A) at the age of 10 months demonstrate lumbar scoliosis (34°) due to a segmentation defect of the lumbar spine; the vertebral malformations included an incomplete fusion of the vertebral arch in L1, a right L2 hemivertebra, an abnormally shaped L3, and an asymmetric S1.

(C) Facial photographs of I7 (F4/II-1), I9 (F6/II-1), and I11 (F8/II-1) showing upslanted palpebral fissures and mild retrognathia (I7), a prominent forehead, upslanted palpebral fissures, epicanthal folds, broad and flat nasal bridge, full cheeks, everted upper lip, and full lips (I9) as well as upslanted palpebral fissures (I11).

dysmorphism, but we could not identify a distinctive facial gestalt associated with *DLL1* pathogenic variants. The facial dysmorphism found in more than one individual was upslanted palpebral fissures (Figure 1C).

We identified five different nonsense variants, two splice site variants affecting the canonical splice donor site of intron 2, one missense variant, and one 122 kb microdeletion encompassing *DLL1* and *FAM120B* (Figure 3A). Additional pathogenic variants and rare variants of unknown significance that were identified in some individuals are listed in Table S2. The same nonsense variant c.2013_2014del was present in the unrelated individuals 7 and 9. The variants were absent from public databases including gnomAD[20] (v.2.1). The variants were *de novo* in seven individuals; four individuals inherited the variants from an affected parent and inheritance is unknown in five individuals due to incomplete parental testing. All variants of unknown inheritance were nonsense variants and therefore classified as disease causing. All predicted premature stop codons are located before the penultimate exon and probably cause nonsense-mediated decay (Figure 3A). The only missense variant we found affects Cys179, the third amino acid in the DSL domain of DLL1 (individual 14, F11/II-1 in Figure 1A). This cysteine is invariably conserved in all Notch ligands (Figure 3B). No other amino acid changes affecting Cys179 or other highly conserved cysteines of the DSL domain of DLL1 and other Notch ligands are present in gnomAD or ClinVar, suggesting reduced tolerance to variation in this important domain (Figure 3B).[20,25–27] No LoF variants in *DLL1* are reported in control cohorts, indicating intolerance to LoF (gnomAD pLI score = 1.0). In individual 15 (F12/II-1 in Figure 1A) with the microdeletion of *DLL1*, the only other gene located within the deletion was *FAM120B* (MIM: 612266), a gene not known to be associated with neurodevelopmental issues and is expected to be tolerant to LoF (gnomAD pLI score = 0), suggesting that the deletion of *DLL1* causes the phenotype in this individual. Our preliminary genotype-phenotype analysis of this cohort did not identify significant correlations partly due to the small size of this cohort (Table 1).

For functional analysis of the splice site variant c.54+1G>A, we investigated mRNA extracted from LCLs of individual 12 (F9/II-1 in Figure 1A; see Supplemental Methods). Sanger sequencing of the exon-intron border of exon 2/intron 2 showed a retention of 12 intronic base pairs (bp), resulting in an in-frame insertion of four amino acids (p.Gln18_Val19insIleGlyGlyGln, Figure 3C). This insertion is located immediately downstream of the signal peptide of DLL1 (amino acids 1–17).[25] Analysis of the mutant sequence with Signal Peptide 4.0 predicted a shift of the cleavage site and a mature protein containing four additional amino acids at the N terminus.[28] Individual 13 (F10/II-1 in Figure 1A) was a fetus and material for RNA analysis was not available. Human Splicing Finder[29] predicted that the insertion c.54_54+1insTAGTCG leads to an alternative donor splice site after the insertion, resulting in a premature stop codon (p.Val19*, Figure 3D).

This study identified *de novo* and dominantly inherited heterozygous *DLL1* pathogenic variants (nonsense, missense, splice site, and gene deletion) as a cause of DD, ID, ASD, seizures, variable brain malformations, and scoliosis. The sequence variants identified and previous findings support haploinsufficiency as the mechanism for disease pathogenesis. The phenotype in our cohort shows great overlap with the phenotype of individuals with 6q27 deletions.[19] Multiple studies have delineated the roles of Notch signaling in neuronal development including neuronal migration.[3,30] The presence of neurodevelopmental features (ID/ASD and others) in our cohort further supports the neurodevelopmental role of DLL1, a critical node in this pathway. Brain malformations identified in our cohort included ventriculomegaly, hydrocephalus, abnormalities

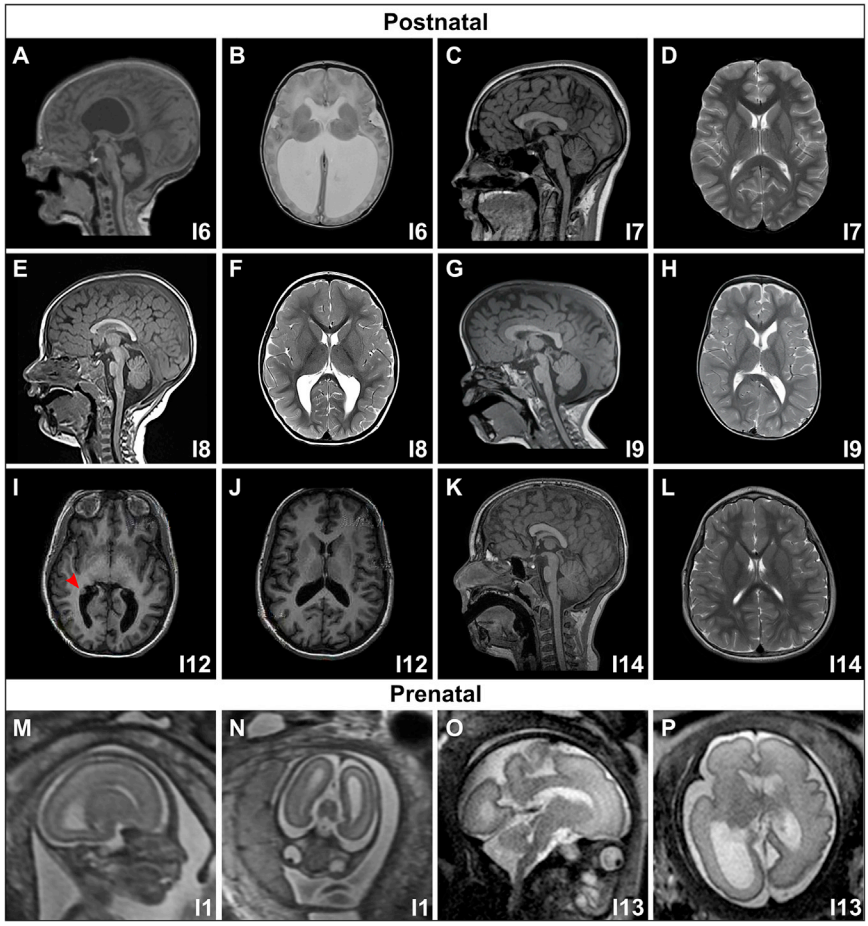

Figure 2. Representative Brain MR Images of the *DLL1* Cohort
(A and B) T1-midsagittal (A) and T2-axial (B) images of I6 showing severe hydrocephalus with a thin/stretched corpus callosum, thin brainstem, small cerebellum, and compression of the overlying cortex.
(C and D) T1-midsagittal (C) and T2-axial (D) images of I7 showing a mildly short and thick corpus callosum, and subtle areas of cortical dysplasia.
(E and F) T1-midsagittal (E) and T2-axial (F) images of I8 showing a mildly dysplastic corpus callosum, mild cortical dysplasia, and mild ventriculomegaly.
(G and H) T1-midsagittal (G) and T2-axial (H) images of I9 showing relatively large brain size with a prominent forehead, mildly dysplastic corpus callosum, and mildly dilated ventricles.
(I and J) T1-axial images of I12 showing PVNH (red arrowhead, I) with mild ventriculomegaly.
(K and L) T1-midsagittal (K) and T2-axial (L) images of I14 showing a mildly short and dysplastic corpus callosum, mildly small pons, and subtle cortical dysplasia.
(M and N) Prenatal T2 sagittal (M) and axial (N) images of I1 showing ventriculomegaly, with no good mid-sagittal views.
(O and P) Prenatal T2 sagittal (O) and axial (P) images of I13 showing asymmetric ventriculomegaly.

of the corpus callosum, mild cortical dysplasia, and a small cerebellum/pons. Notably, one individual had PVNH, which was also reported in patients with 6q27 deletions[21] and that we hypothesize is due to aberrant neuronal migration caused by *DLL1* haploinsufficiency.

It has also been proposed that variants in *DLL1* are associated with holoprosencephaly in humans. This hypothesis was based on the detection of 6q deletions encompassing *DLL1* and most particularly on the detection of an in-frame deletion and a missense variant in probands with holoprosencephaly.[21,31] However, both sequence variants (c.1802_1804del [p.Asp601_Ile602insVal] and c.2117C>T [p.Ser706Leu]) were inherited from a healthy parent. In current databases (gnomAD), c.1802_1804del has an allele frequency of 0.003 and is present in 13 individuals in a homozygous state, and c.2117C>T is present in four individuals in a heterozygous state. Only one of the deletions detected in individuals with holoprosencephaly is restricted to 6q27 (patient 4 in Dupé et al.[21]); the others are larger deletions that contain additional genes. Therefore, haploinsufficiency of other genes located in the deleted region could be causative for holoprosencephaly in these individuals. Further, on careful examination of brain MR imaging in our cohort, we did not detect holoprosencephaly. Taken together, further evidence is needed to confirm an association of *DLL1* haploinsufficiency with holoprosencephaly.

Among the individuals described herein, one had a missense variant (individual 14). The amino acid change p.Cys179Phe affects the third amino acid of the highly conserved DSL domain. Cys179 is predicted to participate in a disulfide bond with Cys188.[25] Disulfide bonds are important for protein folding and stability and are lost if a cysteine is replaced by any other amino acid.[32] Therefore, it is possible that the variant p.Cys179Phe affects correct folding of the DSL domain of DLL1 and interferes with physiological Notch signaling. Amino acid changes at the same position in the DSL domain of JAG1 (GenBank: NM_000214.2; p.Cys187Ser and p.Cys187Tyr) were found in two individuals with Alagille syndrome.[33,34] Combined with the phenotype of individual 14, this information suggests that the *de novo* variant p.Cys179Phe is disease causing and causes LoF.

In contrast to the large number of studies on *in vivo* and *in vitro* knockout (KO) models, there are few reports on *DLL1* haploinsufficiency. Rubio-Aliaga et al. found *Dll1* haploinsufficient mice to be smaller with altered fat tissue and lean mass ratio, higher energy uptake and metabolized energy, probably due to hyperactivity. The authors suspected a neurological phenotype as the cause of the hyperactivity, but this has not been analyzed in detail.[35] These results suggest that this mouse model might reflect the human phenotype at least partially and be suitable for

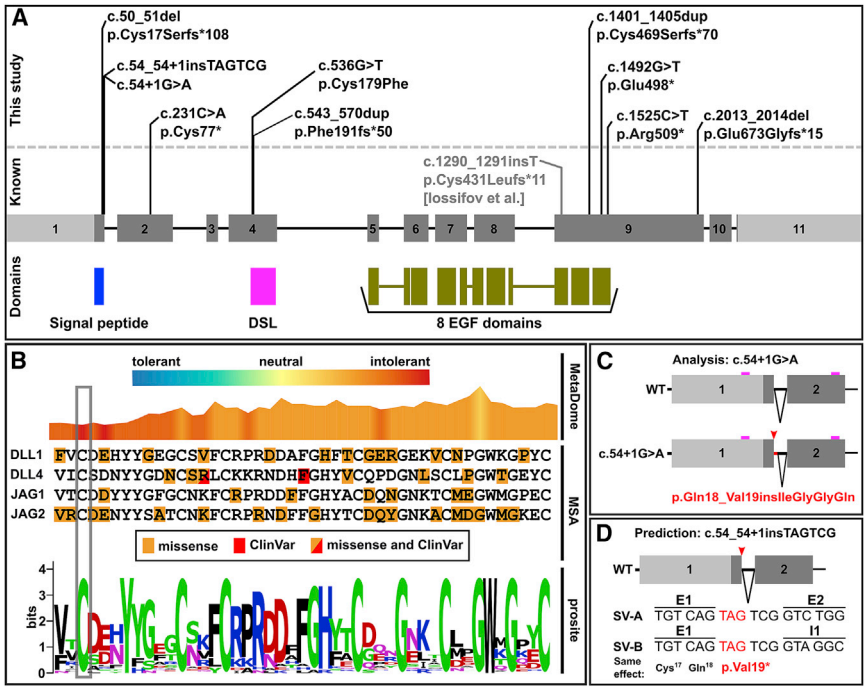

Figure 3. Spectrum of *DLL1* Pathogenic Variants and Functional Interpretations

(A) Schematic of the *DLL1* gene with *DLL1* variants annotated. A previously described LoF variant is indicated in gray.[23] The DLL1 protein domains are listed below.

(B) Sequence and variant characteristics of the DSL domain. The missense variant GenBank: NM_005618.3; p.Cys179Phe of I14 is highlighted in gray. Top: visualization of the MetaDome score.[26] Center: multi sequence alignment (MSA) of the DSL domain of genes annotated with DSL domain by Prosite (DLL1, DLL4, JAG1, JAG4). Positions with missense variants in gnomAD version 2.1 as well as pathogenic ClinVar variants are labeled (no frameshift and nonsense variants are present in gnomAD). Bottom: Conservation of the DSL domain (Prosite entry PS51051). Cysteine is conserved among DSL domains. The MetaDome score indicates the deleterious nature of any amino acid changes at this position.

(C) Scheme of the consequence of the splice site variant identified in I12. Alternative splicing of the allele carrying c.54+1G>A leads to the retention of 12 intronic base pairs (bp), resulting in an in-frame insertion of four amino acids (p.Gln18_Val19insIleGlyGlyGln).

(D) Predicted consequence of the insertion c.54_54+1insTAGTCG identified in I13. Human Splicing Finder[29] predicted that the insertion c.54_54+1insTAGTCG leads to an alternative donor splice site after the insertion, resulting in two different predicted transcript forms. Both lead to a premature stop codon. Boxes present exons, lines present introns; light gray boxes: untranslated regions; dark gray boxes: coding sequence; small boxes in magenta in (C): location of the sequencing primers; red arrows: location of the splice variants; black triangles: intronic sequence removed by splicing; SV-A: splice variant A; SV-B: splice variant B. E1 and E2 represent sequence from exon 1 and 2 respectively, I1 represents intron 1.

future investigations of the pathomechanism. Haploinsufficient mice also had metabolic and immunological abnormalities, and *DLL1* is important for lymphocyte development.[7,35] The individuals in this cohort did not have obvious abnormalities of their metabolism or immune system; only one individual (individual 8) had recurrent infections, which can be due to various factors. However, since the metabolic and immunological function of this cohort was not specifically investigated, a minor effect of the *DLL1* pathogenic variants on the metabolism and the immune system cannot be excluded at present.

Oscillatory expression of *DLL1* is essential for somite segmentation *in vivo*. A complete KO of *Dll1* in mice leads to defects of somite compartmentalization and epithelialization, inhibition of oscillatory *Dll1* expression in mice causes severe segmentation defects, and zebrafish *DeltaD* (*dlD*) mutants show disrupted somitogenesis.[5,16,36,37] KO of *Lfng* in mice, which enhances Notch activation from Dll1, has a similar effect,[38,39] and biallelic mutations in *LFNG* (MIM: 602576) cause spondylocostal dysostosis 3 (MIM: 609813) in humans.[40] In contrast, reduced *Dll1* expression in mice is associated with scoliosis and mild vertebral defects.[35,41,42] Four individuals in our cohort had scoliosis, and one had a segmentation defect of the spine. These results suggest that *DLL1* dosage at critical times during development is needed for correct somite segmentation.

To date, three other Notch ligands have been associated with human monogenic diseases: monoallelic pathogenic variants in *JAG1* (MIM: 601920) causing Alagille syndrome 1 (MIM: 118450)[43,44] and tetralogy of Fallot (MIM: 187500),[45] monoallelic pathogenic variants in *DLL4* (MIM: 605185) causing Adams-Oliver syndrome 6 (MIM: 616589),[46] and biallelic pathogenic variants in *DLL3* (MIM: 602768) causing spondylocostal dysostosis 1 (MIM: 602768).[47] Our data show that *DLL1* pathogenic variants are predominantly associated with a neurodevelopmental phenotype.

In summary, our study confirms heterozygous *DLL1* pathogenic variants as the cause of a variable neurodevelopmental phenotype and other multi-system features. This is consistent with the known role of DLL1 in the CNS and contributes to the understanding of Notch signaling in human development. Future studies are required to delineate fully the *DLL1*-associated phenotype, and functional *in vivo* and *in vitro* studies are needed to uncover the pathomechanism. Of particular interest are the effect of *DLL1* haploinsufficiency on lateral inhibition and oscillatory expression, and why the phenotype is predominantly neurological although DLL1 is essential for somite formation.

Supplemental Data

Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2019.07.002.

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Declaration of Interests

E.T., A.T., Y.S., Y.C., S.L., K.M., and X.W. are employees of GeneDx, Inc., a wholly owned subsidiary of OPKO Health, Inc. All other authors declare no competing interests.

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Web Resources

Bcftools, https://www.htslib.org/doc/bcftools.html
dbSNP, https://www.ncbi.nlm.nih.gov/snp/
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
MGI, http://www.informatics.jax.org/
Mutalyzer, https://mutalyzer.nl/
OMIM, https://www.omim.org/
PubMed, https://www.ncbi.nlm.nih.gov/pubmed/
UCSC Genome Browser, https://genome.ucsc.edu/
Zebrafish Information Network, https://zfin.org

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Supplemental Data

Haploinsufficiency of the Notch Ligand DLL1

Causes Variable Neurodevelopmental Disorders

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SUPPLEMENTAL DATA

Supplemental Note: Case Reports

Individual 1

Individual 1 is a three-year-old female with developmental delays in expressive speech. Prenatal ultrasound indicated a restricted cerebellar growth and fetal magnetic resonance imaging (MRI) showed ventriculomegaly. A small cerebellum was also reported, but was not seen on review of the images. Prenatal triple screen, nuchal translucency and non-invasive prenatal testing (Harmony) did not indicate chromosomal abnormalities. Labor was noticeable for prolonged rupture of membranes. After birth, she had hyperbilirubinemia, which resolved with sunlight. She had mild muscular hypotonia. She was breastfed, but had problems with latching and swallowing. At two years of age, weight was 12.2 kg (+0.14 SD), length was 89.4 cm (+0.82 SD), and head circumference (occipitofrontal circumference, OFC) was 47.5 cm (-0.62 SD). She had childhood apraxia of speech (CAS) with expressive language estimated at 9-23%, but was able to speak in two to three word phrases. Receptive language testing was normal. Motor milestones were reached on time but she had difficulty with balance and tended to fall. She also had mild ataxia as well as auditory and tactile sensory over-responsivity (SOR). She received speech therapy, occupational therapy and physical therapy, which lead to improvement of skills. Both parents were healthy; father was 44 years of age at the time of the birth and mother was 42 years of age. Family history was positive for autism spectrum disorder (ASD) in a first cousin.

Individual 2

Individual 2 is a two years and four months old female. Prenatally, she had obstructive hydrocephalus, and ventriculo-peritoneal shunt was placed for therapy. On examination at two years and three months she presented with synophrys and bilateral single palmar crease. SNP chromosomal microarray (CMA SNP) detected a maternally inherited 231 kb 7p14.1 deletion (arr[hg19] 7p14.1(40,302,290-40,533,666)x1). Both breakpoints lie within *SUGCT* (MIM: 609187). Biallelic pathogenic variants in this gene are associated with autosomal recessive

glutaric aciduria III (MIM: 231690). The deletion was also found in one of her brothers (individual 3) and most likely presents a carrier status for glutaric aciduria III.

Individual 3

Individual 3 is a 4 years old male and an older brother of individual 2. He had ASD and a hydrocephalus with a mega cisterna magna. On examination, a sacral dimple was noted. He carries a 231 kb 7p14.1 deletion, also present in individual 2 and 5.

Individual 4

Individual 4 is a five years and six months old male and an older brother of individual 2. He presented with development delay and apraxia.

Individual 5

Individual 5 is the mother of individual 2, 3 and 4. She has learning disability; she did not graduate from high school. She was overwhelmed and had limited financial resources, and had difficulties coping. Her family history includes a grand aunt and her daughter who were described as slow and having poor speech. She inherited a 231 kb 7p14.1 deletion to individual 2 and 3.

Individual 6

Individual 6 is a 8 years old male. He was born by planned Caesarian section at 39 weeks of gestation to a non-consanguineous couple of First Nations ancestry with a birth weight of 4.065 kg (+1.34 SD), length of 51.5 cm (-0.17 SD) and OFC of 39 cm (+2.85 SD). Apgar scores at 1 and 5 minutes were both 9. He had a large head and small penis (2.4 cm, <3rd centile) but was vigorous and did not need resuscitation. He had a short episode of hypoglycemia at birth but subsequently did not require glucose supplementation and endocrinological evaluation was normal. He developed mild neonatal hyperbilirubinemia that did not require phototherapy. He had normal ophthalmological and neurological examinations. He developed focal seizures at

4 years. These occurred both when awake and when asleep and were characterized by body stiffening with eyes rolling back. The seizures have been reasonably well controlled with carbamazepine. He had delayed acquisition of skills. He rolled by 7 months, sat independently between 9 and 15 months, and crawled by 15 months. He manifested muscular hypotonia in infancy, and by 7.5 years, had increased tone of the lower extremities, spasticity of the gastrocnemius, toe walking, and reduced mobility of the ankles. A developmental assessment at 8 years determined that he had significantly impaired cognitive functioning (IQ = 55), memory, academic achievement, communication, adaptive functioning, executive functioning, attention, social-emotional function, gross motor skills, and fine motor skills. At 8 years, he continued to have difficulty with enuresis and encopresis. When seen at 15 months, individual 6 had obvious macrocephaly, difficulty balancing his large head, low tone, and developmental delay. His height, weight and OFC were 80.5 cm (+0.18 SD), 13.3kg (+1.65 SD), and 53 cm (+4.35 SD), respectively. He had a large anterior fontanelle, frontal bossing, and otherwise normal physical findings. When examined at eight years, he was pleasant, interactive and cooperative. His height, weight and OFC were 132.5 cm (+0.34 SD), 34.4kg (+1.27 SD), and 57 cm (+3.06 SD), respectively. Besides macrocephaly and decreased tone his physical findings were normal. Individual 6 underwent extensive testing that was not diagnostic of his underlying disorder(s). Genetic investigations, which included a chromosomal microarray (Affymetrix CytoScan HD Array; hg19), karyotype, and *FMR1* repeat length, showed no detectable abnormalities. Investigations including transferrin isoelectric focusing, plasma amino acids, urine organic acids, serum acylcarnitines, urine purine and pyrimidines, plasma very long chain fatty acids, and ceruloplasmin, copper, 7-dehydrocholesterol, blood ammonia and lactate levels showed no detectable abnormalities. Investigations including thyroid stimulating hormone, liver function tests, pituitary stimulation (ACTH and growth hormone) tests, plasma electrolytes, and complete blood count also showed no detectable abnormalities. At age of 7.5 years, pelvis radiographs showed coxa valga. At the age of 8 years, spine radiographs showed mild (7°) convex left scoliosis without bony abnormality and elbow radiographs showed no joint or bony abnormality. A brain MRI at age two days showed severe

hydrocephalus with compression of the cortical gyral pattern, a thin and stretched corpus callosum, a thin brain stem and a small cerebellum. A head ultrasound at age 3 weeks showed hydrocephaly with absence of the septum pellucidum, fused thalami, and undetectable corpus callosum. A head CT at 5 years showed massive dilatation of the lateral and third ventricles and normal fourth ventricle; the cerebral aqueduct was not visualized. His mother, older maternal half sister and older maternal half brother had a learning disability. The half brother also had attention deficit and hyperactivity disorder (ADHD), conduct disorder, and oppositional defiant disorder (ODD).

Individual 7

Individual 7 is 16-year-old male with ASD and ADHD, ODD, Tourette syndrome, a mood disorder, and seizure-like activity. He presented with a weight of 52.1 kg (-1.38 SD), a length of 171.8 cm (-0.63 SD) and an OFC of 55.3 cm (-0.69 SD). On genetic assessment, he showed kyphosis, mild scoliosis, upslanted palpebral fissures and mild retrognathia. X-ray of the spine did not show any vertebral malformations. Brain MRI at the age of twelve years showed a mildly short and thick corpus callosum and subtle diffuse cortical dysplasia. Echocardiogram, and renal ultrasound were normal. Prenatal history unknown. His last reported grand mal seizure was at eleven years. He has a voiding dysfunction with urinary incontinence. He has hyperbilirubinemia (7x of upper normal value) and Gilbert syndrome was suspected, but sequencing and copy number analysis of *UGT1A1* did not show a clear pathogenic sequence variant. He is living with his paternal grandmother. His father deceased at age 25 because of Goodpasture syndrome with chronic kidney failure and lung failure. His mother is not involved. She has bipolar disorder and major personality disorder. A maternal half-brother has ADHD and ODD.

Individual 8

Individual 8 is a two-year-old female with developmental delay and abnormal behavior. In 25th weeks of gestation, mild bilateral ventriculomegaly and a hemivertebra were confirmed on fetal ultrasound. She was born at 37 weeks of gestation with a weight of 3141 g (+0.38 SD), a length of 51.5 cm (+0.83 SD) and OFC of 36.0 cm (+1.50 SD). She had cholestatic icterus in the first few weeks of life, which entirely resolved. An abdominal ultrasound at ~five weeks identified dilated bile ducts and a possible stone in the common duct. She had recurrent infections, beginning at nine months of age (ear infections, pneumonias, urinary tract infections). Her motor development was delayed. She sat unsupported at eight months of age, walked at 19 months. Her receptive speech development was normal. At the age of three years she spoke three to four word sentences. At three years, height was 96 cm (+ 0.14 SD) and weight was 15 kg (+0.44 SD). Her OFC at 2 years and eight months was 47.1 cm (-1.76 SD). She had a high nasal bridge, retrognathia, upslanting palpebral fissures, kyphosis and scoliosis. She presented with sensory issues, disrupted sleep, teeth clenching, tantrums and aggressive behavior. Brain MRI at the age of two years demonstrated mild ventriculomegaly, a mild dysplasia of the corpus callosum and mild cortical dysplasia. MRI of her spine at the same age showed mild multifocal syringomyelia without signs of tethering. Spine radiographs at the age of 10 months showed a right convex lumbar scoliosis (34°) due to a segmentation defect of the lumbar spine; the vertebral malformations included an incomplete fusion of the vertebral arch in L1, a right L2 hemivertebra, an abnormally shaped L3, and an asymmetric S1. The results were confirmed at the age of three years.

Individual 9

Individual 9 is a Caucasian boy who was born at full term without complications. His birth weight was 3638 g (+0.39 SD), his length 48 cm (-1.68 SD) and his OFC 35 cm (+0.08 SD). Prenatal exposures included citalopram and clonazepam. His mother developed viral gastroenteritis during pregnancy. He had mild left torticollis which resolved with a brief period of physical therapy. He was hospitalized three times for bronchiolitis between five and twelve months of age. At the age of 16 months, he was initially referred to neurology clinic for

evaluation of intermittent upward gaze deviation which started at 14 months of age. Videos were provided which showed rapid, brief eye deviation in an upward direction lasting less than one second without any other accompanying movement. There was no impaired consciousness or cessation of movement. Episodes occurred on a daily basis but were more commonly seen when he was tired. The movement gradually improved with time and eventually resolved at 26 months of age. His psychomotor development is delayed. He began sitting unsupported at seven months, cruising at eleven months, and started walking at 19 months. He has severely impaired language development. He started saying mama/dada specifically at 24 months with a few other single words around the same time. He knows a few signs but does not put two words together. He can follow simple commands and point to colors and letters. ASD was diagnosed at the age of two and a half years. Neurological exam at the 16 months of age revealed normal mental status and cranial nerve function. Strength was normal and symmetrical. He was mildly hypotonic. Light touch was intact. Reflexes were 2/4 in the upper and lower extremities with toes downgoing. Testing was initiated to assess for paroxysmal tonic upgaze including a routine EEG, which was normal. An MRI of the brain showed a relatively large brain size with a prominent forehead, mildly dysplastic corpus callosum, and mildly dilated ventricles. When last seen at the age of 28 months years, his weight was 13.7 kg (+0.37 SD), height was 90 cm (-0.27 SD) and his OFC was 50 cm (-0.05 SD). He had a prominent forehead, upslanted palpebral fissures, epicanthal folds, a broad and flat nasal bridge, full cheeks, an everted upper lip and full lips. His mother reported that he started grinding his teeth intermittently. She did have multiple miscarriages prior to patient. Family history was noncontributory.

Individual 10

Individual 10 is a nine-year-old Caucasian male who was born at full term without complications. During the pregnancy there were no exposures to drugs, alcohol, tobacco or medications. There were no complications during the pregnancy. The fetal movements were described as normal. There were normal ultrasound exams. At birth, he weighed 3409 g (-0.22

SD) and was 48 cm (-0.75 SD) long. Very early milestones were not particularly delayed, with patient reportedly sitting alone at approximately seven months, crawling at twelve months, and walking alone between 16 and 18 months of age. He spoke his first word between 13 and 14 months of age and put two to three words together at 24 months of age. He was completely toilet trained between four and five years of age. Microarray was performed and was notable for a 1.3 Mb duplication at 15q13.2-q13.3 which is of uncertain clinical significance. He was evaluated at nine years of age and carried a diagnosis of ASD without language impairment and without intellectual disability (FSIQ on WASI-II was 91). His height was 133.5 cm (0.75 SD), 25.4 kg (-0.20 SD), and head circumference (-2.00 SD). He is in a regular classroom, but does report some difficulties with organization, reading, writing, and math. At the time of his evaluation the patient was observed to be dysmorphic, with widely spaced eyes, arched and flared eyebrows with upslanted and narrow palpebral fissures. He also had macrostomia with exaggeration of the nasolabial folds. Upper lip with median pseudocleft and upper vermillion is thin. Mild muscular hypotonia and small joint hypermobility was noted. His back appeared straight without notable scoliosis or dysraphism. The patient had no focal neurologic deficits and was able to carry on a conversation, although with restricted speech content and unusual speech pattern/prosody. Eye contact was poor. Family history was notable for a brother who has a diagnosis of cerebral palsy, which has been attributed to extreme prematurity. His father has ADHD, hypertension, and a mathematics learning disability. Mother has a history of pernicious anemia, but otherwise healthy. A paternal first cousin exhibits similar learning challenges. A maternal aunt has a diagnosis of ADHD, and a maternal first cousin once-removed has a diagnosis of Ehlers-Danlos syndrome, hypermobility type.

Individual 11

Individual 11 is a seven-year-old female with seizures and ASD. She was born at 41 weeks of gestation with a weight of 4082 g (+1.17 SD) and a length of 50.8 cm (-0.64 SD). Prenatal development at 20th week of gestation was normal but at 26th week of gestation ultrasound examination revealed brain abnormalities. Fetal MRI at 40 weeks of gestation showed partial

agenesis of the corpus callosum; enlargement of the lateral ventricular atria and occipital horns. Seizure disorder was diagnosed at her fifth day of life but most likely she had seizures *in utero*. She was treated with phenobarbital during her first year of life. Her development was delayed. She started to walk at 19 months but regressed due to prolonged seizure and restarted walking at 21 months. She was able to speak first word at 18 months. A spinal X-ray showed mild scoliosis but no evidence of vertebral anomaly. She has sleep apnea. Extensive metabolic screening as well as chromosomal analysis, microarray and mitochondrial DNA analysis were normal. At seven years, she presented with upslanted palpebral fissures, stabism (extropia), high arched palate, hyperextensible joints and low muscle tone. During examination, she showed self-stimulating behaviors. Her weight was 25.5 kg (+0.43 SD), her length 126.7 cm (+0.57 SD) and her OFC 51.5 cm (+0.00 SD). A brain MRI at the age of seven years showed large lateral ventricles and unchanged configuration, with persistent disproportionate dilatation of the atrium and occipital horns of the lateral ventricles, bilaterally. Her mother has Lyme disease with associated brain lesions. A maternal half aunt has a son with a possible ASD. Her maternal great grandmother was reported to have significant learning/intellectual disability. There is a significant maternal family history of strokes in the third to fourth decade of life. Her biological father was a sperm donor and not available for genetic testing. He had seizures until his 20's. The identical twin brother of her father is reported to have no medical concerns. Her father has other children, all of which were reported to have a history of seizures. A paternal aunt is reportedly non-verbal. Her paternal grandmother is deceased but reportedly had a history of seizures.

Individual 12

Individual 12 is a 35-year-old female with a borderline mental retardation and seizures. She was born spontaneously at term after an uneventful pregnancy and no abnormalities were detected after birth. Her motor development was delayed (sitting at nine months, independent walking at 18 months). Her speech development was normal. She had learning difficulties, with no available IQ value. First seizures occurred at the age of 33 years (generalized clonic and

absences) and were successfully treated with lamotrigine and lacosamide. A brain MRI at the age of 34 years showed multiple periventricular nodular heterotopias (PVNH) and mild ventriculomegaly. FLNA sequencing did not detect any pathogenic sequence variants. When last seen, her height was 163 cm (-0.8 SD) and her head circumference 55 cm (-0.21 SD). Her parents were unrelated and of Caucasian origin. Her sister has trisomy 21.

Individual 13

Individual 13 was a male fetus born after termination of pregnancy at 33+2 weeks of gestation with a weight of 2280g (+0.29 SD), a length of 48 cm (-0.96 SD) and an OFC of 32 cm (+0.24 SD). Fetal MRI first noticed ventriculomegaly at 25 weeks of gestation. Chromosomal analysis of amniotic fluid was normal. The parents had a normal chromosomal analysis and normal subtelomeric FISH. Congenital infection with human cytomegalovirus was excluded. That fetus had a broad nasal ridge, narrow and long face, high forehead, deeply set ears, and neck edema. The mother has a history of one miscarriage at 16-18 weeks of gestation.

Individual 14

Individual 14 is an eight-year-old female who was born after an uneventful pregnancy at 38 weeks of gestation (birth parameters not available). Her psychomotor development was delayed: She started walking at 20 months. She still wears diapers at night. There are concerns about her language as well as social skills. She repeated first grade. She was getting speech, occupational and physical therapy in the past. Currently she is only receiving speech therapy twice a week. Refractory complex partial seizure left occipital first occurred at the age of 20 months of life. Brain MRI at the age of five and seven years showed a mildly short and dysplastic corpus callosum, a mildly small pons and mild cortical dysplasia. At last exam, her height was 131.1 cm (+0.34 SD) and weight 32.6 kg (+1 SD).

Individual 15

Individual 15 is a three-year-old boy with a history of seizures, global developmental delay,

and ASD. Fetal ultrasound identified a horseshoe kidney. He was delivered full-term with a weight of 3572 g (-0.12 SD) and a length of 49.53 cm (-1.29 SD). He had jaundice shortly after birth because of rhesus incompatibility but no phototherapy was required. Seizures began at approximately one year of age. He showed multiple seizure types consisting predominantly of focal seizures in addition to possible drop attacks. Episodes occurred predominantly at night with two to three episodes occurring on average per night. EEG showed focal epileptiform discharges that were intermittent. Brain MRI did not reveal any evidence of cortical or cerebellar malformations. Array CGH was normal. Seizures are now well controlled with oxcarbazepine. He started walking at 2 years. Examination at three years of age did not reveal any specific dysmorphic facial features. He showed global muscular hypotonia, hyperextensible joints, and one accessory nipple. He presented with a weight of 14.9 kg (+0.34 SD), a length of 96.7 cm (+0.34 SD) and an OFC of 47.4 cm (-2.13 SD). His motor skills and his speech development are delayed. He says approximately 150 words including two to three word sentences. He does make eye contact with family members and non-family members alike, but he exhibits hand flapping behavior when excited. He attends preschool. He is currently getting physical therapy, occupational therapy, and applied behavior analysis. His family members are healthy.

Supplemental Tables

Table S1. Detailed clinical features and molecular data of individuals with heterozygous *DLL1* pathogenic variants (NM_005618.3; ENST00000366756.3). F = family; y = years; m = months; DD = developmental delay; ID = intellectual disability; ASD = autism spectrum disorder; ADHD = attention deficit hyperactivity disorder; na = not applicable; nd = not determined; u = unknown; PVNH = periventricular nodular heterotopia; (+) = subtle/borderline; + = present; - = absent.

Individual	Variant #	Gene	RefSeq ID	c.DNA	GRCh37	aa change	refSNP	Zygosity	Het in gnomAD	Hom in gnomAD
7	**1**	SOS1	NM_005633.3	c.3347-1G>A	chr2:39216456C>T	-	rs141565234	het	37	0
	2	SETD2	NM_014159.6	c.2549A>C	chr3:47163577T>G	p.(Glu850Ala)	rs1222926375	het	1	0
10	**1**	TBC1D20	NM_144628.4	c.105_106del	chr20:428683_428684del	p.(Glu35Aspfs*8)	-	het	0	0
	2	ACY1	NM_000666.3	c.496G>A	chr3:52020490G > A	p.(Ala166Thr)	rs564775955	het	3	0
11	**1**	GRIN2B	NM_000834.4	c.2845T>C	chr12:13717327A>G	p.(Tyr949His)	rs201982602	het	2	0
	2	SCN2A	NM_021007.2	c.6002G>A	chr2:166246318G>A	p.(Arg2001Lys)	rs771968887	het	4	0
	3	DNAH5	NM_001369.2	c.10815del	chr5:13753399del	p.(Pro3606Hisfs*23)	rs397515540	het	47	1
	4	F7	NM_000131.4	c.1151C>T	chr13:113773072C>T	p.(Thr384Met)	rs531225271	het	22	0
12	**1**	TMEM214	NM_017727.5	c.704C>T	chr2:27258904C>T	p.(Thr235Met)	rs759680359	het	15	0
	2	TMEM214	NM_017727.5	c.1709A>G	chr2:27262984A>G	p.(Asn570Ser)	rs374165594	het	83	1

Individual	Variant #	CADD	SIFT	MutationTaster	PolyPhen2	HumanSpliceFinder	ACMG classification	Clinical relevance
7	**1**	32.00	na	disease causing	na	broken WT acceptor site	uncertain significance	
	2	19.34	tolerated_low_confidence	polymorphism	benign	-	uncertain significance	
10	**1**	33.00	na	disease causing	na	-	uncertain significance	
	2	22.00	tolerated	disease causing	benign	-	uncertain significance	
11	**1**	24.20	tolerated	disease causing	possibly_damaging	-	uncertain significance	
	2	23.70	tolerated_low_confidence	disease causing	probably_damaging	-	uncertain significance	
	3	na	na	disease causing	na	-	pathogenic	carrier for ciliary dyskinesia
	4	23.60	deleterious	disease causing	probably_damaging	-	likely pathogenic	likely carrier for factor VII deficiency
12	**1**	24.90	deleterious	disease causing	probably_damaging	-	uncertain significance	
	2	15.43	tolerated	polymorphism	benign	-	uncertain significance	

Table S2. Additional pathogenic variants and rare variants of unknown significance identified by exome sequencing. na = not applicable.

Supplemental Methods

Exome Sequencing

Genomic DNA from the proband (individual 7 and 8), proband and both parents (1, 14 and 15), proband and one parent (individual 11), or proband and other family members (individuals 2, 3, 4, 5) was used to capture the exonic regions and flanking splice junctions through the Clinical Research Exome kit (Agilent Technologies, Santa Clara, CA) (individuals 2, 3, 4, 5) or the IDT xGen Exome Research Panel v1.0 (individuals 1, 7, 8, 11, and 14, 15). Massive parallel (NextGen) sequencing was performed on an Illumina system with 100bp or longer paired-end reads. Reads were aligned to human genome build GRCh37/hg19. Sequence reads were called and analyzed using a custom-developed analysis tool. Additional sequencing technology and variant interpretation protocol were described previously.[1] The general assertion criteria for variant classification are publicly available on the GeneDx ClinVar submission page (http://www.ncbi.nlm.nih.gov/clinvar/submitters/26957/). Individual 6 and his parents had trio-based exome sequencing performed on an Illumina platform at Ambry Genetics (Alisa Viejo, CA, USA) using a hybridization-based targeted capture, followed by transfer of raw read files (fastq format) to the CAUSES Study for alignment and sorting with Bowtie[2] and GATK[3]. Variant detection was performed with Samtools[4] and bcftools (see Web Resources), and were further annotated with the VarSeq software (Golden Helix, Bozeman, MT, USA). Sequence variant filtering was performed as described previously.[5] Trio exome sequencing was performed on genomic DNA of individuals 9 and 10 and parental DNA as described in Thiffault et al.[6] Trio exome sequencing of individual 12 and her parents was performed on a NextSeq 500 Sequencing System (Illumina, San Diego, CA), with a 150bp paired-end high output sequencing kit after a 12-plex enrichment with SeqCap EZ MedExome kit (Roche, Basel, Switzerland), according to manufacturer's specifications. Reads were mapped using BWA-MEM[7] (version 0.7.13), sorted and indexed (samtools 1.4.1), duplicates were flagged (sambamba 0.6.6[8]) and coverage was calculated (picard-tools 2.10.10 [see Web Resources]). Coverage for these samples was 94% at a 20x depth threshold over the enrichment target region. Variant calling was done with GATK 3.7 Haplotype Caller[3] and then annotated with SnpEff 4.3[9], dbNSFP 2.9.3[10], gnomAD[11], ClinVar[12], HGMD[13], Variome

Great Middle East[14], and an internal database. Variants with the following consequences were selected: missense, stop gain, stop loss, start loss, frameshift indel, in-frame indel, and splice acceptor and splice donor region with a prediction >0.7 in dbscSNV[10]. Variants were further filtered by model of inheritance and allele count in gnomAD. For *de novo*, only variants with an allele count less than 4, and for autosomal and X-linked recessive, only homozygous variants in less than 4 individuals were kept. Trio exome sequencing was performed on fetal DNA of individual 13 and parental DNA as described in Mohammad et al.[15] Detected variants were classified according to the guidelines of the American College of Medical Genetics (ACMG).[16]

Splice Site Analysis

For functional analysis of the splice site variant c.54+1G>A, we investigated mRNA extracted from LCLs of individual 12. Peripheral blood was used for isolation of B-lymphocytes and subsequent immortalisation by infection with Epstein Barr virus as previously described.[17] LCLs were also established from two unrelated healthy individuals. Cells were grown at 37°C and 5% CO2 in RPMI with 10% fetal calf serum and antibiotics. Logarithmic growth of cells was ensured by regular cell counting and subculture. Cells were lysed with Trizol (Invitrogen) and total RNA was prepared by a standard RNA extraction protocol. 1 µg RNA was reverse transcribed by RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). RT-PCR was performed and the DLL1 amplicons were extracted from agarose gels and sequenced. All primer sequences are available on request.

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